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The annual number of breeding adults and the effective population size of syntopic newts (*Triturus cristatus*, *T. marmoratus*)

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Abstract

Pond-breeding amphibians are deme-structured organisms with a population genetic structure particularly susceptible to demographic threats. We estimated the effective number of breeding adults (N_b) and the effective population size (N_e) of the European urodele amphibians *Triturus cristatus* (the crested newt) and *T. marmoratus* (the marbled newt), using temporal shifts in microsatellite allele frequencies. Eight microsatellite loci isolated from a *T. cristatus* library were used, five of which proved polymorphic in *T. marmoratus*, albeit with high frequencies of null alleles at two loci. Three ponds in western France were sampled, situated 4–10 kilometres apart and inhabited by both species. Parent–offspring cohort comparisons were used to measure N_b; samples collected at time intervals of nine or 12 years, respectively, were used to measure N_e. The adult population census size (N) was determined by mark–recapture techniques. With one exception, genetic distances (F_{ST}) between temporal samples were lower than among populations. N_b ranged between 10.6 and 101.8 individuals, N_e ranged between 9.6 and 13.4 individuals. For the pond where both parameters were available, N_b/N (overall range: 0.10–0.19) was marginally larger than N_e/N (overall range: 0.09–0.16), which is reflected in the temporal stability of N. In line with the observed differences in reproductive life-histories between the species, N_b/N ratios for newts were about one order of magnitude higher than for the anuran amphibian *Bufo bufo*. Despite of the colonization of the study area by *T. cristatus* only some decades ago, no significant genetic bottleneck could be detected. Our findings give rise to concerns about the long-term demographic viability of amphibian populations in situations typical for European landscapes.

Keywords: effective number of breeders, effective population size, microsatellites, temporal method, *Triturus cristatus*, *Triturus marmoratus*

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Introduction

The concept of effective population size (N_e) is at the heart of population genetics. N_e is the most important determinant for the amount of genetic drift, a fundamental parameter influencing genetic population structure. For conservation biological issues, N_e is regarded as important because it is the main reason behind loss of genetic diversity (Frankham 1995a), with low N_e increasing the probability for population extinction (Newman & Pilson 1997). However, measuring N_e is notoriously difficult, and not all analytical methods lead to comparable results. Of particular practical importance in the study of free-living organisms is the ratio of N_e to the adult population census size (N). Demographic expectations of N_e/N range from 0.25 to 0.75, assuming a stable N (Nunney 1993), whereas empirical studies revealed on average much lower values (0.1, Frankham 1995b).

Genetic estimates of N_e in wild populations are often obtained with the 'temporal method', which is based on temporal variance in marker allele frequencies (Waples

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1989a; Schwartz *et al*. 1998; Williamson & Slatkin 1999; see also Crandall *et al*. 1999). Microsatellites are currently having a large impact in many studies of evolutionary and conservation biology, as they have enabled genetic variability to be measured and compared with a higher resolution than other markers such as allozymes. However, when measuring the degree of differentiation between populations, the high mutation rate and distinct mutational behaviour of microsatellites requires a careful interpretation of the observed variation (Hedrick 1999), and/or new analytical tools (Luikart & England 1999). In sampling regimes typical for the temporal method the probability impact of mutation events can be regarded as negligible. Nevertheless, few studies have so far measured N_e from microsatellite genetic variation (but see Miller & Kapuscinski 1997; Lehmann *et al*. 1998; Fiumera *et al*. 2000).

Many temperate amphibians reproduce in small breeding ponds and are particularly amenable to population studies. N_e, or the related parameter N_b (the effective number of breeding adults), has been estimated as mostly below 100 individuals (Merrell 1968; Gill 1978; Easteal 1985; Berven & Grudzien 1990; Scribner *et al*. 1997; Driscoll 1999; Funk *et al*. 1999; Seppä & Laurila 1999), but the underlying data sets and the biological significance of the results are based on different methods and, therefore, not easy to interpret. N_e and N_b are expected to be low when variance in female reproductive success is high, multiple matings are rare, and the sex ratio is skewed (Nunney 1993; Nunney 1996). Scribner *et al*. (1997) used temporal shifts at DNA (minisatellite) markers between adults and their offspring for estimating the effective number of breeding anurans *Bufo bufo* (common toad), a species characterized by 'explosive' breeding at uneven sex ratio. In fact, they revealed N_b/N ratios as low as around 0.01.

European urodeles of the genus *Triturus* usually have a sex ratio close to 1:1, with females producing fewer eggs than *B. bufo* and receiving spermatophores from several males (Halliday 1998). In western France, the two large-bodied newts *Triturus cristatus* (crested newt) and *T. marmoratus* (marbled newt) are largely separated by their occurrence in different landscapes, and locally form a hybrid zone where they breed in identical ponds. Due to a recent expansion of the distribution range, *T. cristatus* currently inhabits areas which were some decades ago exclusively occupied by *T. marmoratus* (Arntzen & Wallis 1991). The objectives of this study are to: (i) compare the degree of spatial and temporal genetic differentiation of syntopic *T. cristatus* and *T. marmoratus* populations; (ii) measure N_b and N_b/N in both species by comparing microsatellite allele frequencies of adults and their progeny; (iii) measure N_e and N_e/N with samples collected at time intervals of nine and 12 years, respectively; (iv) test the hypothesis that newts have a higher N_b/N ratio than common toads; and (v) test the hypothesis that *T. cristatus* populations experienced a genetic bottleneck during the recent colonization of the new ponds.

Materials and methods

Sample collections

Samples were collected at three cattle ponds inhabited by both study species, situated in the 'Départment de Mayenne'. For detailed descriptions, locations, and schematic drawings of Ponds 1 and 3 see Jehle *et al*. (2000). Pond 2 (about 30 m^2 in area and 1 m deep) is identical with Pond 278 in Arntzen & Wallis (1991). The straight-line distance is 3.8 km between Ponds 1 and 2, 10.5 km between Ponds 1 and 3, and 7.3 km between Ponds 2 and 3.

Newts were captured from all pond regions by dipnetting or underwater funnel traps (Jehle *et al.* 2000). Individuals were marked by removing toes. Estimates of $N \pm$ SE were obtained using mark–recapture calculations (Begon's weighted mean, Begon 1979), which are based on comparisons between numbers of recaptured and newly encountered individuals in successive sampling sessions. In 1998, the toes were stored in 96% ethanol and used as tissue samples for microsatellite analysis. Other samples were obtained from sacrificed larvae collected in 1986 (Pond 2) and 1989 (Pond 1), and nondestructively by removing a gill from larvae captured in 1998 (Ponds 1 and 3).

Microsatellite genotyping and scoring

DNA extractions were performed using standard phenol–chloroform procedures (Sambrook *et al.* 1989). Primers were used for eight microsatellite loci designed from a *Triturus cristatus* library (Krupa *et al*. in preparation). Polymerase chain reaction (PCR) amplifications were carried out in 10 μL reaction volumes under the following conditions: 1 or 1.5 mM $MgCl_2$, 0.1 mM dNTPs, 0.5 U polymerase (Thermoprime Plus, Advanced Biotechnologies, Epsom, UK) and 0.1 mM of each primer in the manufacturer's buffer (buffer IV). Thermal profiles for *Tcri*27, *Tcri*35, and *Tcri*43 were 39 cycles of 30 s at 93 °C, 30 s at the primer-specific annealing temperature, 45 s at 72 °C; for *Tcri*13, *Tcri*29, *Tcri*32 and *Tcri*46b we used a 'touchdown' thermal profile (for more details see Krupa *et al*. in preparation). *Tcri*46b amplifies the same microsatellite as *Tcri*46 in Krupa *et al*. (in preparation), but with a different primer sequence (forward: GTTTGGGTAGCCATGCACTT, reverse: ATCCAAGCATTGGGATTTCA). Primers were labelled with fluorochromes TET, HEX, and FAM, and PCR products were run on 5% denaturing polyacrylamide gels (Long Ranger) on an automated ABI 377 DNA sequencer. PCR products were compiled and analysed with ABI GENESCAN software using standard Tamra 2500 (*Tcri*32) or

Tamra 500 (all other primers). Samples for which more than two primers failed to amplify were not included in the analysis.

For scoring the PCR products we used ABI GENOTYPER and visual inspections of gels. Allele sizes were rounded to the nearest integer. Called and actual allele size can differ by about one base pair (Haberl & Tautz 1999), but the tetranucleotide nature of our microsatellites allowed unambiguous designation within and between gels.

Data analysis

Observed (H_O) and expected (H_E) heterozygosity, probability tests for genotypic linkage disequilibrium and measures of genetic differentiation (F_{ST}) were computed using GENEPOP 3.1d (Raymond & Rousset 1995). Temporal genetic differences within populations should not be described with conventional contingency tests (Waples 1989b), and we used F_{ST} for direct comparisons between temporal and spatial degrees of differentiation (cf. Nielsen *et al*. 1999). Departures from Hardy–Weinberg equilibrium at each locus were also computed using GENEPOP, with the Markov chain method (Guo & Thompson 1992) to obtain unbiased estimates of Fisher's exact test through 1000 iterations. Pairwise Wilcoxon tests were used to compare observed heterozygosities and numbers of alleles per locus between temporal samples. Frequencies of null alleles were estimated using the algorithm provided in CERVUS 1.0 (Marshall *et al*. 1998), with data pooled across populations. Tests for recent bottlenecks were conducted with BOTTLENECK 1.2.02 (Cornuet & Luikart 1996), using the Wilcoxon test with 1000 iterations, and assuming both the infinite allele (IAM) and the stepwise mutation model (SMM). Tests for bottlenecks were conducted for the 1998 samples (in Ponds 1 and 3 with adults and larvae pooled) for loci in Hardy–Weinberg equilibrium.

On average 4% of adult newts from ponds where *T. cristatus* and *T. marmoratus* both occur are F_1-hybrids (Arntzen & Wallis 1991). In this study, adult hybrids were identified morphologically and larval hybrids were identified genetically with diagnostic microsatellites (Jehle *et al*. 2000), and excluded from the analysis. The frequency of introgressed protein loci is approximately 0.3% (Arntzen & Wallis 1991). For microsatellites, 0.7% of all individuals morphologically designated as either *cristatus* or *marmoratus* had an 'alien' allele in the species-diagnostic locus (see below). Such alleles were excluded from N_b and N_e calculations.

Calculations of N_b *and* N_e

Variation in allele frequencies between adults and their larval progeny was used for calculating N_b (Ponds 1 and 3). On the assumption that our sampling was nondestructive, we followed Plan I from Waples (1989a). Larval allele frequencies from 1986 and 1989 were compared with adult allele frequencies from 1998 for calculating N_e (Ponds 1 and 2), in this case following Plan II as larvae were removed (Waples 1989a). The standard variance of allele frequency change (F_c) was calculated after Nei & Tajima (1981):

$$F_c = 1/K\, \Sigma(x_i - y_i)/[(x_i + y_i)^2/(2 - x_i y_i)] \qquad (1)$$

where K is the number of alleles, and i is the frequency of the respective allele at times x and y, respectively. F_c was then corrected for sample size and actual population size with Equations 12 (N_b) and 11 (N_e) from Waples (1989a):

$$N_b = t/2[F_c - 1/(2S_o) - 1/(2S_t) + 1/N] \qquad (2)$$

$$N_e = t/2[F_c - 1/(2S_o) - 1/(2S_t)] \qquad (3)$$

where t is the generation time, S_o and S_t represent sample sizes at times zero and time t, and N represents the adult population census size at time zero. In the study area, the mean number of reproductive years is 2.5 for *T. cristatus* and 4.0 for *T. marmoratus*, following the onset of sexual maturity at ages of three and four years, respectively (Francillon-Vieillot *et al*. 1989; Arntzen & Hedlund 1990). In the absence of data on age-dependent reproductive success, the generation time t was estimated as 4.3 years (= 3 + 2.5/2) for *T. cristatus* and as 6.0 years (= 4 + 4/2) for *T. marmoratus*. For N_b estimates, t was set at one (cf. Scribner *et al*. 1997). Due to the presence of null alleles (see below), and further missing data for example due to the omission of alien alleles, sample size varied among loci, and we used the harmonic mean of S_o and S_t. Under the assumption that N_e follows a χ^2-distribution, we used equation 16 from Waples (1989a) in order to calculate 95% confidence intervals:

$$(1 - \alpha) \text{ confidence interval for } F_c = nF_c/(\chi^2\alpha/2[n]),\; nF_c/(\chi^2 1 - \alpha/2[n]) \qquad \mathbf{(4)}$$

where n is the number of degrees of freedom associated with $F_c[n = \Sigma(\text{number of alleles} - 1)]$ and $\chi^2\alpha/2[n]$ is the critical $\alpha/2$ chi-square value for n degrees of freedom. The confidence limits obtained were used in place of F_c to determine the confidence interval of N_b and N_e.

The assumptions underlying the calculation are that sampling is at random, selection and mutation are negligible, and that there is no migration between populations and no population substructure. *Tcri*36 failed to reliably amplify the small 1989 sample for *T. cristatus* in Pond 1 and was excluded from the analysis. N_b and N_e calculations were performed with all loci together, and separately only for loci in Hardy–Weinberg equilibrium. Pond 2 contained only few *T. cristatus* and this species was not analysed.

Table 1 Characterization of the polymorphic microsatellite loci observed in the newts *Triturus cristatus* and *T. marmoratus*. *n*, number of alleles; H_E, expected heterozygosity; H_O, observed heterozygosity; *P*, probability level of test for Hardy–Weinberg equilibrium; n.s., non significant

		Pond 1			Pond 2		Pond 3	
Locus	Allele	1989 larvae	1998 adult	1998 larvae	1986 larvae	1998 adult	1998 adult	1998 larvae
T. cristatus		8	35	40	—	—	168	87
*Tcri*13	*n*	3	3	3			4	2
	H_O	0.500	0.429	0.550	—	—	0.196	0.184
	H_E	0.577	0.455	0.486	—	—	0.223	0.169
	P	n.s.	n.s.	n.s.	—	—	n.s.	n.s.
*Tcri*27	*n*	4	8	6			6	6
	H_O	0.375	0.571	0.600	—	—	0.613	0.448
	H_E	0.325	0.619	0.537	—	—	0.553	0.531
	P	n.s.	n.s.	n.s.	—	—	n.s.	n.s.
*Tcri*29	*n*	3	3	3			5	4
	H_O	0.375	0.629	0.450	—	—	0.536	0.460
	H_E	0.667	0.567	0.537	—	—	0.588	0.547
	P	n.s.	n.s.	n.s.	—	—	n.s.	n.s.
*Tcri*32	*n*	2	4	5			7	9
	H_O	0.125	0.400	0.600	—	—	0.476	0.701
	H_E	0.125	0.410	0.628	—	—	0.513	0.713
	P	—	n.s.	n.s.	—	—	n.s.	n.s.
*Tcri*35	*n*	3	4	3			5	5
	H_O	0.500	0.571	0.500	—	—	0.571	0.540
	H_E	0.548	0.635	0.467	—	—	0.552	0.551
	P	n.s.	n.s.	n.s.	—	—	n.s.	n.s.
*Tcri*36	*n*		2	3			3	4
	H_O	—	0.457	0.600	—	—	0.470	0.563
	H_E	—	0.464	0.506	—	—	0.463	0.502
	P	—	n.s.	n.s.	—	—	n.s.	n.s.
*Tcri*46b	*n*	5	5	7			6	5
	H_O	0.375	0.314	0.500	—	—	0.238	0.230
	H_E	0.725	0.612	0.689	—	—	0.518	0.495
	P	*	**	**	—	—	**	**
*Tcri*43	*n*	3	6	6			9	8
	H_O	0.250	0.486	0.775	—	—	0.655	0.724
	H_E	0.414	0.570	0.646	—	—	0.684	0.762
	P	n.s.	n.s.	n.s.	—	—	*	n.s.
T. marmoratus		23	66	79	90	43	27	7
*Tcri*27	*n*	2	5	5	6	4	3	3
	H_O	0.304	0.515	0.430	0.222	0.209	0.259	0.286
	H_E	0.324	0.476	0.401	0.211	0.229	0.259	0.272
	P	n.s.	*	n.s.	n.s.	n.s.	n.s.	n.s.
*Tcri*32	*n*	4	4	4	6	6	4	2
	H_O	0.130	0.379	0.367	0.500	0.372	0.407	0.439
	H_E	0.126	0.326	0.375	0.470	0.386	0.514	0.363
	P	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
*Tcri*35	*n*	4	5	5	6	5	4	4
	H_O	0.522	0.530	0.430	0.467	0.349	0.407	0.429
	H_E	0.436	0.561	0.427	0.688	0.437	0.507	0.571
	P	n.s.	n.s.	n.s.	**	n.s.	n.s.	n.s.
*Tcri*46b	*n*	3	6	6	5	6	5	2
	H_O	0.043	0.318	0.076	0.256	0.326	0.148	0.000
	H_E	0.211	0.427	0.241	0.288	0.348	0.079	0.254
	P	**	**	**	n.s.	n.s.	**	n.s.
*Tcri*43	*n*	3	9	5	6	2	6	3
	H_O	0.000	0.364	0.291	0.133	0.023	0.296	0.000
	H_E	0.174	0.546	0.540	0.317	0.074	0.515	0.408
	P	**	**	**	**	n.s.	**	n.s.

*$P < 0.05$, **$P < 0.01$.

Table 2 *P*-values obtained from Wilcoxon tests for the significance of differences in number of alleles (above diagonal) and observed heterozygosity (H_O, below diagonal) between temporal samples within populations (in bold) and between populations. Pond number is given first, followed by study year; A, adults; L, larvae. *Triturus cristatus* samples from Pond 1 and *T. marmoratus* larvae from Pond 3 were omitted due to $n < 10$ ($n > 23$ for included samples)

T. cristatus	1, 1998A	1, 1998L	3, 1998A	3, 1998L
1, 1998A	—	**0.78**	0.08	0.30
1, 1998L	**0.12**	—	0.07	0.23
3, 1998A	0.80	0.09	—	**0.59**
3, 1998L	0.89	0.21	**1.00**	—

T. marmoratus	1, 1989	1, 1998A	1, 1998L	2, 1986A	2, 1998A	3, 1998A
1, 1989A	—	**0.07**	**0.07**	0.04*	0.03*	0.11
1, 1998A	**0.04***	—	**0.18**	0.85	0.91	0.07
1, 1998L	**0.14**	**0.50**	—	0.18	0.23	0.48
2, 1996A	0.23	0.46	0.92	—	**0.13**	0.07
2, 1998A	0.50	0.14	0.50	**0.08**	—	0.49
3, 1998A	0.35	0.12	0.60	0.35	0.25	—

*significant at $P < 0.05$.

Results

Estimated adult population census sizes (*N*) were remarkably stable between samples. Pond 1 was inhabited by 76.5 ± 25.6 (1989), or 72.5 ± 18.8 (1998) *Triturus cristatus*, and 146.0 ± 55.4 (1989), or 147.4 ± 28.4 (1998) *T. marmoratus*. Pond 2 was inhabited by 101 ± 14.1 (1986), or 108.0 ± 31.0 (1998) *T. marmoratus*. Pond 3 in 1998 contained 585.6 ± 78.3 *T. cristatus* and 57.0 ± 32.6 *T. marmoratus*.

The analysed microsatellites yielded from two to nine alleles per locus and population (Table 1, see the Appendix I for the allelic data). Five of the eight loci were polymorphic in *T. marmoratus*, with one (*Tcri*32) having alleles of species-diagnostic length. In *T. cristatus*, *Tcri*46b showed significant heterozygote deficiencies (Table 1), with an estimated frequency of null alleles of 0.29. In *T. marmoratus*, heterozygote deficiencies were observed at two of the five loci (*Tcri*46b and *Tcri*43), with null allele frequencies of 0.21 and 0.23, respectively. No significant linkage disequilibrum between loci was observed (pairwise comparisons, *P*-values > 0.05). Assuming that the mutation rate of microsatellites is 10^{-5} (Ellegren 1995) and using the mean size of the two temporal samples, the probability of a single mutation was 0.0024 for *T. cristatus* and 0.0021 for *T. marmoratus* in Pond 1, and 0.0044 in Pond 2. Under the assumption of the IAM a bottleneck in *T. cristatus* was suggested in both ponds ($P < 0.01$ in both cases), whereas under the SMM the deviation from mutation-drift equilibrium was not significant ($P > 0.05$ in both cases). For *T. marmoratus*, the Wilcoxon test was not significant for Ponds 1 and 2 (IAM and SMM: $P > 0.05$), but significant under the IAM for Pond 3 ($P < 0.01$, SMM: $P > 0.05$).

Allele frequencies were subject to change between temporal samples. Common alleles maintained their frequency ranks, but some alleles that were rare in one sample were not observed in the other sample. *T. marmoratus* in 1989 (Pond 1) had a lower genetic diversity than the other samples, which is associated with the significant differences in allele numbers or H_O (Table 2). No significant differences were observed for other pairwise temporal or spatial comparisons. F_{ST} for parent–offspring cohort comparisons was at least one order of magnitude smaller than between samples from the same populations involving longer time intervals (Table 3). With the exception of *T. marmoratus* in Pond 2, spatial F_{ST} was higher than temporal F_{ST}.

Table 3 F_{ST} values between temporal and spatial samples of *Triturus cristatus* and *T. marmoratus*. Due to the small sample size ($n < 8$) *T. cristatus* adults collected in 1989 from Pond 1 and *T. marmoratus* larvae from Pond 3 are not considered

	T. cristatus	*T. marmoratus*
Temporal samples		
Pond 1, adult-larvae 1998	0.0006	0.0139
Pond 1, 1989–98	—	0.0063
Pond 2, 1986–98	—	0.0704
Pond 3, adult-larvae 1998	0.0005	—
Spatial samples (adults only)		
Pond 1-Pond 2	—	0.1758
Pond 1-Pond 3	0.0455	0.0816
Pond 2-Pond 3	—	0.1313

*F*c, reflecting the variation in allele frequency changes, ranged between 0.04 and 0.18 (Table 4), and was larger when measured over longer time intervals (data from Pond 1). N_b and N_e represented a fraction of *N*, and the upper confidence limits reached *N* only for *T. marmoratus* in Pond 3 (estimated as infinity). N_b ranged between 9.2 and 101.8 newts, N_e ranged between 9.6 and 13.4 newts. N_b/N ranged between 0.10 and 0.19, and N_e/N ranged between 0.09 and 0.16. N_b/N and N_e/N in *T. cristatus* were always equal to or higher than in *T. marmoratus*. For

Table 4 Variables used for the calculations of effective population size [F_c, standard variance in allele frequency change according to Nei & Tajima (1981); t, number of generations between samples for N_e; S_0 and S_1, sample size at time zero and 1, respectively]; number of breeding adults (N_b) and effective population size (N_e) with associated 95% confidence interval; N_b/N, respectively N_e/N values of syntopic *Triturus cristatus* and *T. marmoratus* populations. Data from Pond 2 refer to *T. marmoratus*. *alternative measures of N_e and N_b calculated only from loci which are in Hardy–Weinberg equilibrium

	F_c	t	S_0	S_1	N_b, N_e	N_b/N, N_e/N	N_b, N_e*
Pond 1							
T. cristatus							
N_b	0.050	1	162.2	72.4	14.1 (8.7–27.7)	0.19	9.3 (4.5–17.7)
N_e	0.189	2.18	61.4	73	12.2 (4.7–47.9)	0.16	11.1 (4.0–49.3)
T. marmoratus							
N_b	0.043	1	106	128	15.2 (7.8–27.9)	0.10	62.8 (15.9–1737.1)
N_e	0.104	1.50	26	110.8	13.4 (5.3–43.0)	0.09	50.6 (7.7–∞)
Pond 2						—	
N_e	0.107	2.00	148.3	65	9.6 (6.0–19.5)	0.09	10.5 (4.5–21.4)
Pond 3						—	
T. cristatus							
N_b	0.0133	1	294	161.5	101.8 (41.0–506.4)	0.17	89.3 (35.5–408.9)
T. marmoratus							
N_b	0.144	1	44	11.6	10.6 (3.6–∞)	0.17	∞ (3.5–∞)

T. cristatus, removing *Tcri*46b had little effect on N_b or N_e. When removing such loci for *T. marmoratus*, however, in Pond 1 N_b and N_e increased about fourfold, and in Pond 3 N_e could not be defined.

Discussion

Temporal population genetic studies are scarce compared to studies on spatial population structure. Microsatellites have the technical advantage that PCR amplifications are possible from samples taken decades ago for purposes other than DNA analysis (for example from fish scales, Miller & Kapuscinski 1997; Nielsen *et al.* 1999), and have proven to be useful for assessing the temporal stability of populations (e.g. Taylor *et al.* 1994; Tessier & Bernatchez 1999). For the temporal method of calculating N_e, the mutation rates of microsatellites are such that they provide a high level of within-population polymorphism, while keeping the probability of mutations at an insignificant level. The codominant nature of microsatellite markers provides approximately twice the precision compared to markers displaying dominant gene expression (Jorde *et al.* 1999). However, multiallelic microsatellite data sets, such as ours, at present render a calculation of N_b or N_e using likelihood approaches computationally prohibitive (Williamson & Slatkin 1999), and do not increase the power of N_e calculations based on linkage disequilibrium (Hill 1981; Schwartz *et al.* 1998).

Microsatellites have to be treated with some caution, as the assumption of Mendelian inheritance may not always be met (Ardren *et al.* 1999). Deviations from Hardy–Weinberg equilibrium were relatively frequent, especially in *Triturus marmoratus*. As observed for some of the loci, it is unlikely that deviations were caused by population substructuring. Moreover, heterozygotes were typed in both sexes, indicating that none of the loci are sex-linked. Deviations from Hardy–Weinberg equilibrium were associated with missing PCR products, and we concluded that null alleles were the cause. The frequent occurrence of null alleles in species for which the PCR primers were not originally designed has been reported previously (Paetkau & Strobeck 1995). Allele frequencies, and therefore the temporal method, should not be seriously affected when the relative number of amplification failures is similar across samples. The estimates of N_e for *T. marmoratus* with the loci that were out of Hardy–Weinberg proportions excluded, inevitably yielded a larger confidence interval.

Spatial vs. temporal genetic differentiation

As recommended for microsatellite data and moderate sample sizes (Gaggiotti *et al.* 1999), we used F_{ST} based on Weir & Cockerham (1984) to measure genetic differentiation. Between Ponds 1 and 3 F_{ST} was twice as large for *T. marmoratus* as for *T. cristatus*, but data from additional populations would be required to test whether this is a general pattern, and the possibility has to be considered that F_{ST} might be affected by the different number and performance of the used loci. The high temporal F_{ST} of *T. marmoratus* in Pond 1 cannot be explained by genetic drift, as Pond 2 had a similar N_e but a much lower F_{ST}, and was associated with a low degree of genetic diversity in 1989.

That spatial F_{ST} exceeds temporal F_{ST} in a microsatellite data set was also observed by Nielsen *et al*. (1999) for salmonid fish, with, however, different sampling regimes and study objectives.

N_b *and* N_e *of* T. cristatus *and* T. marmoratus

The habit of breeding in small ponds makes large-bodied newts amenable to measure N_b and N_e. Although part of our study is based on whole larvae collected prior to the routine use of PCR, tissue can be sampled nondestructively and in a straightforward way. To fit the assumption that sampling is at random with respect to family structure, adults and larvae were captured over the whole pond area. In another amphibian, larval growth but not survival was significantly correlated with microsatellite heterozygosity (Rowe *et al*. 1999). As larval sampling was not size-selective, there was no evidence for biased N_b and N_e due to selection at early life stages. At present there is no method available that calculates N_e taking migration into account. Ignoring constant migration leads to N_e estimates that are too high (Lehmann *et al*. 1998), whereas an episodic migration from sources with different allele frequencies would probably bias the estimate downward. Radio-tracking of 44 adult *T. cristatus* and *T. marmoratus* at Ponds 1 and 3 did not result in any indications for newts to disperse (Jehle & Arntzen 2000; Jehle 2000). Juveniles are frequently speculated to be the most important life stage for amphibian dispersal, but recent studies on *T. cristatus* suggest that their migration behaviour is similar to that of adults (by following their directional cues, Hayward *et al*. 2000), and indicate a high degree of pond fidelity (Cummins & Swan 2000). Nevertheless, over a sampling period of more than one decade, some bias of N_e due to migration cannot be completely ruled out.

The bias in N_e estimates caused by overlapping generations can be regarded as small when a time period covering more than one generation is sampled (Jorde & Ryman 1995). This is the case in our study, but the lack of age-specific reproductive rates led to an only rough estimate of generation time. However, body size and fecundity, and not body size and age, are usually correlated in amphibians (Duellman & Trueb 1986), suggesting that reproductive rates do not markedly differ between age classes. Hybridization is another potential source of bias. In the analysis of a deer hybrid zone with 11 diagnostic microsatellites, without any F_1-hybrids in the data set, up to 40% of all individuals carried introgressed alleles (Goodman *et al*. 1999). We excluded alien alleles for N_b and N_e calculations, but introgressed alleles from nondiagnostic loci with species-specific frequencies might have biased the N_b and N_e estimates slightly upwards, similar to the effect of constant migration. For discussions of further possible violations of assumptions of the temporal method see: Waples (1989a); Husband & Barrett (1992); Scribner *et al*. (1997); Ingvarsson & Olsson (1997); Miller & Kapuscinski (1997); and Lehmann *et al*. (1998).

As fewer loci were used in *T. marmoratus*, the confidence limits for N_e estimates are mostly wider than for *T. cristatus*. Caution should be taken for N_e estimates based on a small number of samples (Waples 1989a), and the indeterminable N_b for *T. marmoratus* from Pond 3 (loci out of Hardy–Weinberg equilibrium excluded) was indeed associated with a low sample size and small number of loci. Pooling adults and larvae from 1998 would have increased S_t in Pond 1, but proved unnecessary and would have introduced a bias due to the merging of generational cohorts. It has to be noted, however, that the substantial confidence intervals of N, N_b and N_e become multiplied in the N_b/N and N_e/N ratios, and lower their accuracy. Pooling rare alleles (frequencies < 0.05) only marginally changed the estimated N_e and N_b values (results not shown).

N_b/N and N_e/N were relatively stable across populations, and in the same range as the mean N_e/N of 0.10 measured across 102 taxa (ranging from plants to humans, Frankham 1995b). That behavioural mechanisms and/or sperm competition cause only few males to reproduce successfully is probably more likely than frequent reproductive failures of females. In Pond 1, but not in Pond 3, N_b/N and N_e/N are twice as large for *T. cristatus* than for *T. marmoratus*. *T. marmoratus* has a higher fecundity and longevity than *T. cristatus* (Arntzen & Hedlund 1990), but the promiscuous mating system of newts in combination with internal fertilizations and the lack of parental care render it impossible to obtain precise measures of variance in reproductive success from life-history data. It has to be also noted that species-specific differences might be caused by the different performance and number of loci used. Vucetich *et al*. (1997) showed that a fluctuation in population size can lead to the discrepancy of N_e/N being higher in theoretical than in empirical estimates. The population sizes in Ponds 1 and 2 were stable between the sampling dates, and also within the sampling period there were no excessive fluctuations (J. W. Arntzen, unpublished data). N_b as measured from one reproductive period is identical to N_e only when the study organism has a single breeding cycle. However, large-bodied newts can visit their breeding ponds for up to at least five seasons (Ellinger & Jehle 1997), implying that the true N_e is probably larger than the measured N_b. From this view, our data confirm that N_e decreases with increasing sampling interval (Vucetich *et al*. 1997), despite a stable census size.

A comparison between newts (this study) and toads (Scribner et al. *1997)*

That life-history parameters influence N_e/N is well documented (e.g. Nunney 1993, 1996), but empirical comparisons between related taxa with different reproductive strategies have so far been lacking. Scribner *et al.* (1997) used other marker loci but the identical calculation method for calculating N_b/N in *Bufo bufo*, and revealed values in the order of 20 times lower (0.005–0.012) than this study on newts. It is unknown how multiple matings, which are common in newts (Halliday 1998) but rare or absent in toads, influence N_b and N_e. Two factors, however, probably account for a lower variance in reproductive success in newts. First, female toads are outnumbered by males (Arntzen 1999), whereas newts reproduce at an even sex ratio (Faber 1995). Second, female toads deposit whole egg strings in a very restricted pond area, exposing them to catastrophic events such as predation or desiccation, whereas female newts singly distribute their eggs over vast ponds areas, reducing the risk of complete reproductive failures. Assuming that N in *T. cristatus* is typically one order of magnitude smaller than for *B. bufo* (Halley *et al.* 1996) leads to the conclusion that their effective population sizes are of the same order. *B. bufo* has the ability to migrate over larger distances than large-bodied newts, enabling the colonization of more distant breeding ponds (Halley *et al.* 1996). Human alterations to the landscape mostly result in pond loss and increased between-pond distances, and large-bodied newts seem to be more affected than *B. bufo*.

Tests for recent bottlenecks in T. cristatus *populations*

Due to the rapid reduction in the number of alleles, gene diversity in bottlenecked populations is higher than expected under the mutation-drift equilibrium of constant population size (Cornuet & Luikart 1996). Under the IAM, but not under the SMM, we found evidence that bottlenecks occurred in *T. cristatus* populations. However, microsatellites exhibit an intermediate mutation mechanism, and to be statistically conservative only results under the SMM should be considered (Luikart & Cornuet 1998). Perhaps the local colonization took place too many generations ago, or the bottleneck is overshadowed by the expansion of the local *T. cristatus* populations as a whole, or by migration between ponds. Still, across all studied ponds, in concordance with our hypothesis the statistical evidence for bottlenecks in *T. cristatus* is slightly stronger than for *T. marmoratus*, although the species-specific difference is somewhat difficult to interpret as the performance and number of loci was not identical.

Implications for conservation

The relative stability of N_b and N_e in the present study should not be regarded as typical, as the populations were chosen due to their large size, and, a posteriori, due to their long-term persistence. Low N_e leads to a rapid loss of allelic diversity, but the small number of studied populations precludes a correlation of N_e and heterozygosity (see also Funk *et al.* 1999). The effective population sizes of newts might be especially sensitive to suboptimal habitat quality, for example through the disruption of spermatophore transfer at turbid ponds with muddy substrates. Given that microsatellite heterozygosity may be correlated with fitness parameters (Coltman *et al.* 1998; Rowe *et al.* 1999), experimental studies to compare N_b or N_e under regimes of varying pond quality might provide important insights into whether there is greater genetic threat to amphibian populations under suboptimal conditions than in good-quality habitats, and whether such mechanisms could have contributed to the observed global amphibian population decline.

The typical amphibian population structure is conducive to high levels of inbreeding. Our study reveals N_e values for large-bodied newts that are larger than for other amphibians, but still one order of magnitude smaller than the actual census size. In metapopulations with relatively constant gene flow between subpopulations, the overall N_e is higher relative to a panmictic population living in the same resource, but falls below this level when demic extinction and recolonization rates are high (Whitlock & Barton 1997). European pond breeding amphibians often consist of populations with a census size of < 100 individuals. Stable metapopulations have been described for situations where ponds are to some degree interconnected (Rowe *et al.* 2000), but due to landscape fragmentation many populations are now restricted to completely isolated habitat remnants. The effective size of amphibian populations at the landscape scale could be alarmingly low, with loss of alleles and random genetic drift dominating the structural dynamics. Future studies could combine N_b or N_e measures with interdemic migration rates, and extrapolate such data sets over the landscape level in order to assess whether from a genetic point of view amphibian populations in fragmented habitats are viable in the long term, and to what extent they require active management strategies.

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Appendix I

Allele frequency data for eight polymorphic microsatellite data observed in the newts *Triturus cristatus* and *T. marmoratus*. *Tcri*32 is diagnostic between the species. For *Tcri*27, *Tcri*35, and *Tcri*46b, one or more alleles occur in one species but not in the other. *n* = per-locus sample size

		Pond 1			Pond 2		Pond 3	
Locus	Allele	1989 larvae	1998 adult	1998 larvae	1986 larvae	1998 adult	1998 adult	1998 larvae
T. cristatus								
*Tcri*13	A	0.429	0.227	0.308	—	—	0.129	0.093
	B	0.143	0.091	0.051	—	—	0.003	0.000
	C	0.429	0.682	0.641	—	—	0.858	0.907
	D	0.000	0.000	0.000	—	—	0.010	0.000
	n	14	66	78	—	—	302	172
*Tcri*27	A	0.333	0.015	0.194	—	—	0.000	0.000
	B	0.000	0.030	0.000	—	—	0.034	0.021
	C	0.333	0.545	0.597	—	—	0.541	0.486
	D	0.000	0.015	0.000	—	—	0.021	0.039
	E	0.000	0.152	0.097	—	—	0.219	0.277
	F	0.167	0.015	0.014	—	—	0.014	0.007
	G	0.000	0.167	0.083	—	—	0.000	0.000
	H	0.167	0.061	0.014	—	—	0.171	0.170
	n	16	66	72	—	—	282	146
*Tcri*29	A	0.250	0.132	0.243	—	—	0.169	0.205
	B	0.000	0.000	0.000	—	—	0.003	0.000
	C	0.250	0.309	0.176	—	—	0.325	0.295
	D	0.500	0.559	0.581	—	—	0.460	0.473
	E	0.000	0.000	0.000	—	—	0.043	0.027
	n	16	68	74	—	—	302	146
*Tcri*32	A	0.000	0.000	0.000	—	—	0.004	0.030
	B	0.000	0.222	0.229	—	—	0.453	0.375
	C	0.000	0.056	0.010	—	—	0.020	0.024
	D	0.000	0.000	0.019	—	—	0.059	0.083
	E	0.000	0.000	0.000	—	—	0.000	0.006
	F	0.000	0.000	0.000	—	—	0.000	0.006
	G	0.75	0.500	0.514	—	—	0.313	0.298
	H	0.25	0.222	0.229	—	—	0.148	0.167
	I	0.000	0.000	0.000	—	—	0.004	0.006
	n	14	44	80	—	—	256	168
*Tcri*35	A	0.000	0.000	0.000	—	—	0.007	0.026
	B	0.214	0.333	0.444	—	—	0.217	0.208
	C	0.000	0.030	0.074	—	—	0.086	0.110
	D	0.571	0.424	0.278	—	—	0.569	0.565
	E	0.214	0.212	0.000	—	—	0.121	0.101
	n	14	66	54	—	—	304	154
*Tcri*36	A	—	0.000	0.000	—	—	0.007	0.012
	B	—	0.500	0.513	—	—	0.493	0.476
	C	—	0.500	0.474	—	—	0.500	0.494
	D	—	0.000	0.013	—	—	0.000	0.018
	n		64	78	—	—	306	164
*Tcri*46b	A	0.000	0.000	0.014	—	—	0.021	0.012
	B	0.063	0.000	0.054	—	—	0.080	0.102
	C	0.483	0.065	0.054	—	—	0.000	0.000
	D	0.25	0.194	0.122	—	—	0.003	0.012
	E	0.25	0.468	0.378	—	—	0.626	0.651
	F	0.000	0.242	0.311	—	—	0.264	0.223
	G	0.063	0.032	0.068	—	—	0.006	0.000
	n	16	62	74	—	—	326	166

Appendix I *continued*

		Pond 1			Pond 2		Pond 3	
Locus	Allele	1989 larvae	1998 adult	1998 larvae	1986 larvae	1998 adult	1998 adult	1998 larvae
*Tcri*43	A	0.000	0.000	0.000	—	—	0.019	0.065
	B	0.071	0.034	0.013	—	—	0.036	0.018
	C	0.000	0.121	0.118	—	—	0.062	0.101
	D	0.000	0.000	0.000	—	—	0.023	0.048
	E	0.714	0.431	0.382	—	—	0.179	0.185
	F	0.214	0.345	0.408	—	—	0.308	0.238
	G	0.000	0.017	0.039	—	—	0.351	0.327
	H	0.000	0.052	0.039	—	—	0.016	0.000
	I	0.000	0.000	0.000	—	—	0.006	0.018
	n	14	58	76	—	—	308	168
T. marmoratus								
*Tcri*27	B	0.000	0.009	0.000	0.000	0.000	0.000	0.000
	C	0.000	0.018	0.009	0.014	0.030	0.000	0.083
	D	0.000	0.000	0.000	0.007	0.000	0.000	0.000
	E	0.656	0.554	0.491	0.076	0.091	0.140	0.083
	F	0.344	0.366	0.466	0.854	0.833	0.840	0.833
	G	0.000	0.054	0.026	0.042	0.045	0.020	0.000
	H	0.000	0.000	0.009	0.007	0.000	0.000	0.000
	n	32	112	116	144	66	50	12
*Tcri*32	J	0.026	0.000	0.000	0.011	0.036	0.019	0.000
	K	0.053	0.032	0.026	0.052	0.036	0.000	0.000
	L	0.921	0.079	0.083	0.155	0.036	0.185	0.000
	M	0.000	0.802	0.776	0.695	0.774	0.667	0.786
	N	0.026	0.087	0.115	0.080	0.071	0.130	0.214
	O	0.000	0.000	0.000	0.006	0.000	0.000	0.000
	P	0.000	0.000	0.000	0.000	0.048	0.000	0.000
	n	38	126	156	174	84	54	14
*Tcri*35	A	0.000	0.000	0.000	0.185	0.000	0.000	0.000
	B	0.028	0.016	0.008	0.012	0.025	0.000	0.000
	C	0.000	0.000	0.008	0.238	0.013	0.019	0.071
	D	0.278	0.306	0.249	0.393	0.025	0.222	0.214
	E	0.611	0.564	0.660	0.155	0.688	0.667	0.643
	F	0.083	0.105	0.076	0.000	0.250	0.093	0.071
	G	0.000	0.008	0.000	0.018	0.000	0.000	0.000
	n	36	124	132	168	80	54	14
*Tcri*46b	B	0.000	0.012	0.021	0.007	0.027	0.206	0.200
	C	0.357	0.023	0.042	0.074	0.041	0.088	0.000
	D	0.214	0.233	0.042	0.797	0.770	0.059	0.000
	E	0.429	0.512	0.760	0.101	0.108	0.471	0.800
	F	0.000	0.186	0.115	0.020	0.027	0.176	0.000
	G	0.000	0.035	0.021	0.000	0.027	0.000	0.000
	n	14	86	96	148	74	34	10
*Tcri*43	A	0.000	0.028	0.029	0.000	0.000	0.000	0.000
	B	0.200	0.009	0.000	0.043	0.000	0.025	0.000
	C	0.200	0.019	0.065	0.054	0.000	0.100	0.000
	D	0.000	0.123	0.065	0.098	0.000	0.075	0.000
	E	0.400	0.500	0.529	0.565	0.417	0.500	0.500
	F	0.200	0.236	0.312	0.228	0.583	0.225	0.250
	G	0.000	0.009	0.000	0.011	0.000	0.075	0.250
	H	0.000	0.066	0.000	0.000	0.000	0.000	0.000
	I	0.000	0.009	0.000	0.000	0.000	0.000	0.000
	n	10	106	138	92	12	40	8