

# Habitat fragmentation causes bottlenecks and inbreeding in the European tree frog (*Hyla arborea*)

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A genetic study of the European tree frog, *Hyla arborea*, in Denmark was undertaken to examine the population structure on mainland Jutland and the island of Lolland after a period of reduction in suitable habitat and population sizes. The two regions have experienced the same rate of habitat loss but fragmentation has been more severe on Lolland. Genetic variation based on 12 polymorphic DNA microsatellites was analysed in 494 tree frogs sampled from two ponds in Jutland and 10 ponds on Lolland. A significant overall deviation from Hardy–Weinberg expectations could be attributed to three ponds, all on Lolland. This was most probably caused by an inbreeding effect reducing fitness, which was supported by the observed significant negative correlation between larva survival and mean  $F_{IS}$  value and mean individual inbreeding coefficient. A significant reduction in genetic variation (bottleneck) was detected in most of the ponds on Lolland. Population-structure analysis suggested the existence of at least 11 genetically different populations, corresponding to most of the sampled population units. The results indicated that the populations were unique genetic units and could be used to illustrate the migration pattern between newly established ponds arisen either by natural colonization of tree frogs or by artificial introduction. A high degree of pond fidelity in the tree frogs was suggested. A severe fragmentation process reducing population size and fitness within some of the populations probably caused the significant reduction in genetic variation of tree frog populations on Lolland.

**Keywords:** *Hyla arborea*; genetic variation; inbreeding; microsatellite DNA; bottleneck; fragmentation

## 1. INTRODUCTION

The intensive land use during recent centuries in Denmark as well as in other European countries has caused a decline in undisturbed natural habitats. Subsequently, the European tree frog, *Hyla arborea*, as well as other amphibians, has experienced a fragmentation of suitable habitats, i.e. warm low-watered non-eutrophicated ponds without fish by draining and road-building projects, pond fillings and changes in vegetation apt for the migration of amphibians between ponds (Fog 1993; deMaynadier & Hunter 2000).

The expected effects of habitat reduction and fragmentation are a reduction in size of remaining populations. This reduction will change the populations genetically owing to loss of connectivity between subpopulations and genetic drift and increase the possibility of inbreeding. Together, genetic drift and inbreeding could result in predicted low levels of genetic variation, which may be detected by significant deviations from Hardy–Weinberg expectations (HWEs) in terms of positive  $F_{IS}$  estimates (Frankham 1995; Hedrick 2000; Frankham *et al.* 2002; Reed & Frankham 2003). Consequently, a correlation is expected between genetic variation and population size, which has been validated empirically (Frankham 1996). Furthermore, the amount of genetic variation is predicted to be correlated with the fitness and evolutionary potential of the population (Frankham 1995; Lande 1995). The

correlation between levels of genetic variation and fitness may, however, be weak or non-existent (Reed & Frankham 2003). The controversy on whether genetic variation is expected to be correlated with long-term population fitness, and what genetic measures are most relevant for predicting fitness if the population size is important for fitness, were addressed by Reed & Frankham (2003). On the basis of a meta-analysis they conclude that fitness and adaptability are reduced in small populations owing to drift and inbreeding depression and that heritability, heterozygosity and population size correlate significantly with fitness. This result emphasizes the importance of using genetic markers in the evaluation of the conservation status of species requiring specific attention due to low or declining numbers such as some of the species on annex II and IV in the EF-habitat directive (Anon. 1992).

The present study focuses on the genetic consequences of habitat fragmentation causing a decline in population sizes owing to loss of suitable ponds in tree frog populations from two parts of Denmark, namely eastern Jutland and Lolland (figure 1). The tree frog populations in both areas went through bottlenecks during the 1980s but the Vejle population in eastern Jutland recovered quickly and expanded the distribution range from 11 to 76 ponds with a total population size of 800 calling males (Skriver 2001). On Lolland the tree frog population size was halved from 500 to 250 calling males (Fog 1992), and in several isolated ponds, the number of calling males was as low as two and five. In 1991 a rescue plan was instigated (Hels & Fog 1995) and as a result the tree frog survived in many of the small populations that had become isolated

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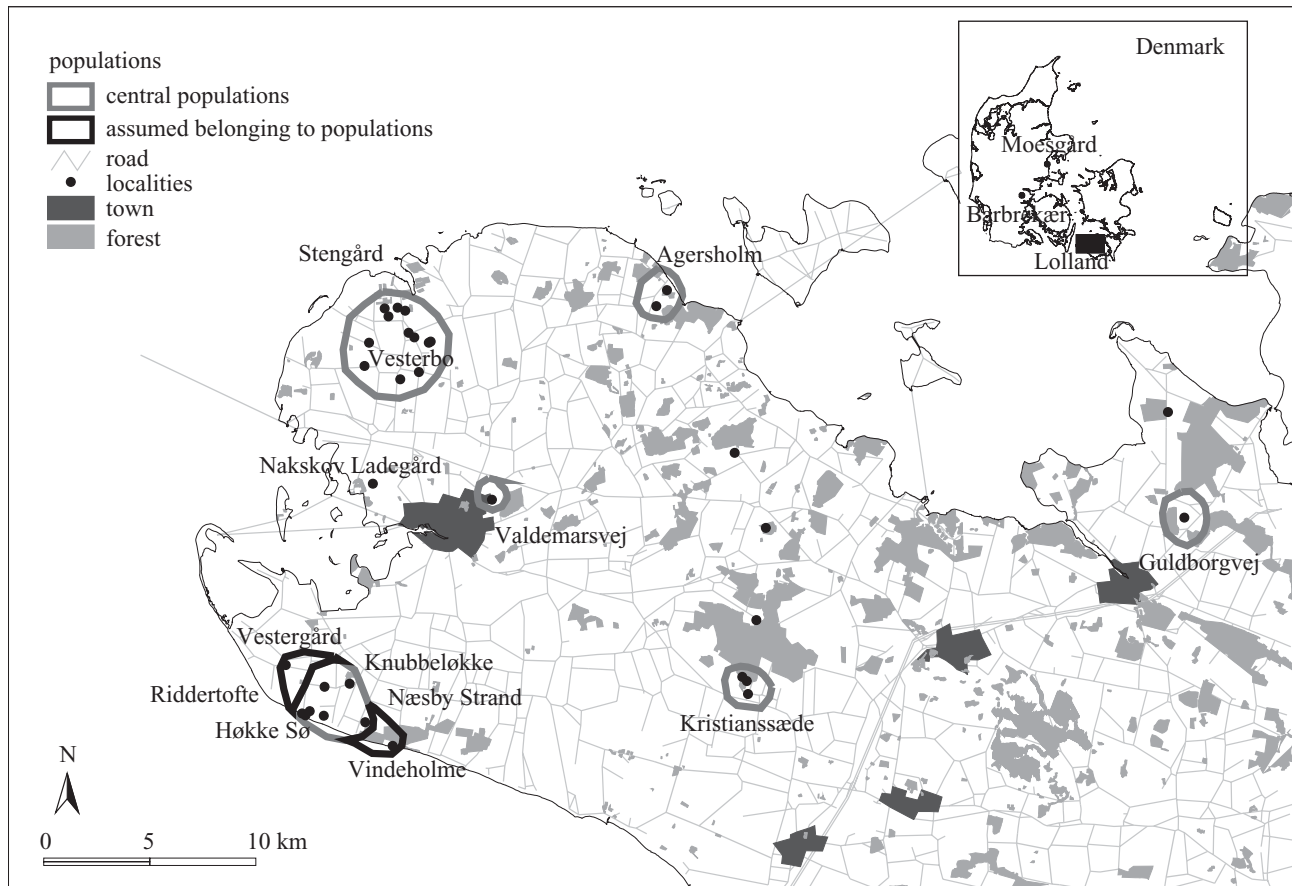


Figure 1. Map showing the two sampling regions in Denmark, Jutland and Lolland. The populations units defined by coherent groups of ponds are indicated with larger grey circles, whereas the black circles indicate possible connections between ponds or populations but the actual connections are unknown.

in 1991. During 1985–1987 a new population of tree frogs was established from the reintroduction of 6000 froglets from the Vejle population to the east central Jutland (Århus area; Skriver 1988; P. Skriver, personal communication).

The aim of the present study was to analyse the genetic variation in the previously mentioned populations of the European tree frog in Jutland and on Lolland, using 12 polymorphic microsatellite loci. The aims were as follows.

- (i) To test the hypothesis of a correlation between the degree of genetic variation (heterozygosity) and the known population history described as the number of calling males observed in the ponds. To analyse if the reduction in population sizes could be detected as a bottleneck effect.
- (ii) To identify if inbreeding as a result of small population size has affected fitness by correlating the fitness components (percentage of hatching and brood survival among spawn brought to aquaria for rearing) with genetic variation.
- (iii) To analyse if previous assessments of conservation status (favourable/unfavourable; Pihl *et al.* 2000) were correlated with the level of genetic variation in terms of  $F_{IS}$ . Differences could be a reflection of fragmentation effects.
- (iv) To analyse the population structure and genetic composition to determine the origin of rather recently established or colonized ponds.

## 2. MATERIAL AND METHODS

### (a) *Sampling collection*

Tissue was sampled from a total of 494 European tree frog larvae representing 13 ponds from Lolland and two ponds from Jutland (figure 1, table 1). In Jutland, one pond (Moesgård) represented an artificially introduced population at Moesgård, Århus, and one pond (Barbrekær) from Viuf Skov, south of Vejle, represented the source population. At the two localities in Jutland and from five ponds on Lolland (Riddertoft, Høkke Sø, Knubbeløkke, Vestergård and Næsby Strand), tadpoles were caught, tail clipped and released. In the other populations on Lolland, tadpoles had been reared from eggs found in the ponds and transferred to a hatchery in captivity from 1998 to 2001. Attempts were made to find all eggs in the small populations. When the population was large each pond was visited only two or three times and a random selection of the egg clumps found were sampled. In the hatchery, each sample or 'clutch' (i.e. the eggs from one pond on one date) was kept separate. Tissue samples were collected from a small number of all the reared, live tadpoles, typically about four from each sample. It was checked whether the egg clumps found on one day were the outcome of only one mating using the individual's multilocus genotypes. This was the case in only one clutch out of 37 clutches sampled. All tail clips were stored in saturated salt–20% dimethyl sulfoxide solution until use.

Three ponds with the smallest sample size, Vestergård, Knubbeløkke and Næsby Strand were included only in the analysis of population structure and admixture conducted in STRUCTURE

Table 1. The overall expected heterozygosity ( $H_c$ ) across the 12 microsatellite markers and tests for goodness of fit to the HWEs ( $F_{IS}$  total) in tree frog samples from 10 different ponds on Lolland and two ponds in Jutland, Denmark. (Results of tests for population bottlenecks [ $M$ ,  $p(M)$ ], where the mutation model used included 80% one-step mutations and 3.5 mean size non-one-step mutations (Garza & Williamson 2001). The  $N_{em}$  used was the harmonic means of calling males for the populations. The mean  $F_{IS}$ , mean IDB (Ruzzante *et al.* 2001) and IR (Amos *et al.* 2001) are given at the population level for the eight ponds where the fitness components were estimated.)

	$H_c$ mean	s.e.m.	$M$	$p(M)$	$F_{IS}$ mean	IDB mean	IR mean	$N$	$N_{em}$	population
Lolland										
Vesterbo <sup>a</sup>	0.435	0.260	0.714	0.024	—	—	—	39	—	C1
Stengård <sup>a</sup>	0.474	0.284	0.736	0.04	0.088 <sup>d</sup>	0.353 <sup>d</sup>	0.330 <sup>d</sup>	40	223.87 <sup>d</sup>	C2
Nakskov Ladegård <sup>a</sup>	0.461	0.237	0.716	0.004 <sup>c</sup>	0.203	0.454	0.364	28	3.19	E
Valdemarsvej <sup>a</sup>	0.497	0.293	0.671	0.001 <sup>c</sup>	0.225	0.376	0.350	29	31.45	F
Agersholm <sup>a</sup>	0.420	0.240	0.692	0.001 <sup>c</sup>	0.198 <sup>c</sup>	0.458	0.436	38	6.76	H
Kristianssæde <sup>a</sup>	0.395	0.284	0.634	0.0001 <sup>c</sup>	0.091	0.375	0.396	35	7.18	M
Riddertoft <sup>a</sup>	0.443	0.201	0.660	0.0003 <sup>c</sup>	—	—	—	32	—	P1
Høkke Sø <sup>a</sup>	0.471	0.236	0.670	0.001 <sup>c</sup>	0.120 <sup>d</sup>	0.437 <sup>d</sup>	0.345 <sup>d</sup>	30	28.64 <sup>d</sup>	P2
Vindeholme <sup>a</sup>	0.370	0.254	0.680	0.002 <sup>c</sup>	0.214 <sup>c</sup>	0.515	0.528	48	7.97	Q
Guldborgvej <sup>a</sup>	0.351	0.183	0.702	0.003 <sup>c</sup>	0.265 <sup>c</sup>	0.549	0.545	52	9.91	S
Vestergård <sup>a</sup>	—	—	—	—	—	—	—	18	—	P3
Næsby Strand <sup>a</sup>	—	—	—	—	—	—	—	16	—	P4
Knubbeløkke <sup>a</sup>	—	—	—	—	—	—	—	11	—	KN
Jutland										
Barbrekær <sup>b</sup> , Vejle	0.534	0.212	0.747	0.1218	0.131	—	—	38	413.26	JUT1
Moesgård <sup>b</sup> , Århus	0.513	0.197	0.753	0.0317	0.138	—	—	40	78.58	JUT2
over all loci and ponds	—	—	—	—	0.105 <sup>c</sup>	—	—	—	—	—

<sup>a</sup> Collected by Kåre Fog in 1999, 2000 and 2001.

<sup>b</sup> Collected by Peter Skriver in 2001.

<sup>c</sup> Significant at the 5% level after application of the sequential Bonferroni procedure.

<sup>d</sup> Indicates the mean for Vesterbo + Stengård, Høkke Sø + Riddertoft. Samples sizes for all 15 ponds are given ( $N$ ).

(Pritchard *et al.* 2000) and ARLEQUIN v. 2.0 (Schneider *et al.* 2000).

### (b) Number of calling males

As an estimate of the average number of calling males from each of the populations, the 9 years were used (see below for details on how calling males were counted and the numbers). These years represent the period of the bottleneck, and by calculating the harmonic mean an index of the narrowness of the bottleneck was obtained. In the Vejle population reliable data were present for only 6 years.

#### (i) Counting of males

The number of calling males in each pond was counted once (rarely twice) per season in optimal weather conditions at night at the peak of the season. Control counts have shown that these population estimates are highly reproducible concerning totals for isolated populations. The frogs were located by a combination of listening and detection by torchlight. In small populations counts may give the actual number present, but with increasing chorus size the true numbers may be underestimated by a factor of two (Stumpel 1987). On Lolland the calling males were counted intensively from 1991 to 2001. At some point during the past 10 years the number of calling males fell below 10 in all the ponds on Lolland (table 2) (K. Fog, personal communication; Fog 1992). In Barbrekær the number of males was counted in 1985–2001 and in Moesgård from 1987 to 2001 (P. Skriver, personal communication).

### (c) Population definition

As tree frogs rarely migrate more than 4 km (Stumpel & Hanekamp 1986; Fog 1993), clusters of ponds separated by less than this distance are considered as populations that exchange individuals constituting genetic units. We use the term 'population' to define these genetic units. The number of calling males in the population was defined as the total number of calling males in all the connected ponds within the cluster and not only from the sampled pond (figure 1). The Vesterbo and Stengård ponds and the ponds at Riddertoft, Høkke Sø and Vestergård represented the northwest Lolland and southwest Lolland populations, respectively.

### (d) Definition of favourable conservation status

The conservation status of the tree frog populations was evaluated according to Lehmkuhl (1984) and Soulé (1980). Given the debate about minimum viable population (MVP) estimates, it was decided that the conservation status for the tree frog populations was considered favourable when  $N_c > 50$  and increasing or when  $N_c > 500$  (Pihl *et al.* 2000). The relation between  $N_c$  and  $N$  (census population size) for the tree frog was not known, but it was assumed that if the number of adult males was 50 or 500 ( $N_c$ ) then the adult population was 100 or 1000 ( $N$ ), respectively (Pihl *et al.* 2000).

### (e) DNA extraction

Genomic DNA was extracted using a standard CTAB buffer and proteinase K procedure (Milligan 1992). Eleven of the 12

Table 2. Number of calling males counted in the populations.

populations	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001	$N_{em}^a$
<b>Lolland</b>												
Northwest Lolland <sup>b</sup> C	97	177	178	205	210	310	370	650	—	—	650	223.87
Nakskov Ladegård <sup>b</sup> E	—	3	3	1	4	2	—	22	—	70	30	3.19
Valdemarsvej <sup>b</sup> F	25	30	41	21	—	35	—	30	—	—	65	31.45
Agersholm <sup>b</sup> H	9	5	9	9	8	—	3	6	7	—	33	6.76
Kristianssæde <sup>b</sup> M	3	3	6	9	8	29	20	—	30	—	15	7.18
Southwest Lolland <sup>b</sup> P	18	13	17	33	40	64	—	80	—	—	210	28.64
Vindeholme <sup>b</sup> Q	40	7	11	8	14	7	0	13	—	—	3	7.93
Guldborgvej <sup>b</sup> S	8	6	8	11	20	7	10	18	—	—	19	9.91
<b>Jutland</b>												
Vejle <sup>c</sup>	—	—	—	—	—	200	261	577	610	820	913	413.26
Århus <sup>c</sup>	—	22	76	90	106	180	245	150	87	—	132	78.58

<sup>a</sup>  $N_{em}$ , harmonic mean.

<sup>b</sup> K. Fog and T. Hels (personal communication).

<sup>c</sup> P. Skriver (personal communication).

used microsatellites were species specific (Arens *et al.* 2000) whereas one marker was originally developed for the Pacific tree frog (*Hyla regilla*) (Call & Hallett 1998). The PCR amplification conditions were 95 °C for 3 min, 35 × 94 °C for 45 s, annealing temperature for 45 s, 72 °C for 15 min and performed on a GeneAmp PCR system 2700 (Perkin Elmer) machine (see Arens *et al.* (2000) for annealing temperatures for the species-specific primers, the annealing temperature for *Hyre64* was 48/50 °C). The individuals were run and genotyped on an ABI PRISM 377 automated DNA sequencer.

#### (f) Genetic data analyses

Genetic variation expressed as expected heterozygosity (Nei 1987) and tests for goodness of fit to the Hardy–Weinberg proportions were performed in FSTAT (Goudet 1995). The correlation between the genetic variation and the harmonic mean ( $N_{em}$ ) of calling males (table 1) was analysed by log transforming the mean numbers. The relationship between population size and genetic variation (expected heterozygosity) was examined by non-parametric Spearman's rank correlation coefficient (SAS v. 8.02 2001).

Population bottlenecks were inferred using the  $M$  ratio (Garza & Williamson 2001), which is a relation between the number of alleles,  $k$ , and the distance between alleles and the overall range in allele size,  $r$ . Owing to the observation of large gaps in the allele range in two of the 12 loci, the mutation model used was a modification of the one recommended by Garza & Williamson (2001). In the present study,  $p_s$  (per cent one-step mutations) was set to 80%, whereas  $\Delta_g$  (mean size of non-one-step mutations) was the recommended 3.5. The  $N_e$  value used was the harmonic means of calling males for the populations (table 1).

Genetic variation and fitness were correlated by using the mean  $F_{IS}$  values for eight ponds on Lolland (table 1), where the percentage of surviving tadpoles was obtained from eggs sampled in the ponds and kept in aquaria. Upon hatching the numbers of dead eggs and live hatchlings were counted. Hatchlings were released back into the ponds of origin (supportive breeding) as mature tadpoles at stage 39/40 (Gosner 1960), i.e. one week before metamorphosis. The number of surviving tadpoles released was counted. The number hatched (egg survival) and the number released (larvae survival) were calculated as

percentages of the number of eggs originally sampled. These percentages were averaged over all samples from each pond. The percentages of eggs hatched and of larvae that survived were obtained from the combined Vesterbo-Stengård ponds (northwest Lolland, C), and from one pond in each of the populations E, F, H, M, P, Q and S. The genotypes were obtained from a subsample of the released live larvae.

The relations between the fitness components and genetic variation in terms of mean  $F_{IS}$ , mean individual inbreeding coefficient estimated (IDB) according to Ruzzante *et al.* (2001) and mean internal relatedness (IR) estimated according to Amos *et al.* (2001) for the eight ponds in question were analysed by using Spearman's rank correlation coefficient (SAS v. 8.02). IR is a measure of parental relatedness. A high mean  $F_{IS}$ , IDB and IR were expected to reflect inbred individuals in the ponds.

#### (g) Population structure and admixture

The number of populations represented in the samples was estimated using a Markov chain Monte Carlo method that clusters individuals to minimize Hardy–Weinberg disequilibrium and gametic phase disequilibrium between loci as implemented in STRUCTURE v. 2 (Pritchard *et al.* 2000). All samples were pooled and assumed to have originated from one to 15 populations without using the information of sample origin. The suggested structure (the number of populations) is revealed by the increasing likelihood of the data. Based on the estimated proportion of the individual's genotype originating from one of the populations, the clusters of individuals forming the number of populations with the highest likelihood were assigned to the sampling localities.

The degree of population differentiation was analysed as implemented in ARLEQUIN v. 2.0 (Schneider *et al.* 2000) after 10 000 permutations over loci. Both  $F_{ST}$  statistics (IAM, infinite allele mutation model) and  $R_{ST}$  statistics (SMM, stepwise mutation model) were employed (Weir & Cockerham 1984; Weir 1990; Slatkin 1995; Michalakis & Excoffier 1996).

Isolation by distance (IBD) was analysed for the 10 ponds on Lolland in GENEPOP v. 3 (Raymond & Rousset 1995), where the correlation between geographical and genetic distance was estimated using the Mantel test. The Spearman's rank correlation coefficient and the regression suggested by Rousset (1997) were obtained after 10 000 permutations. The pairwise distance

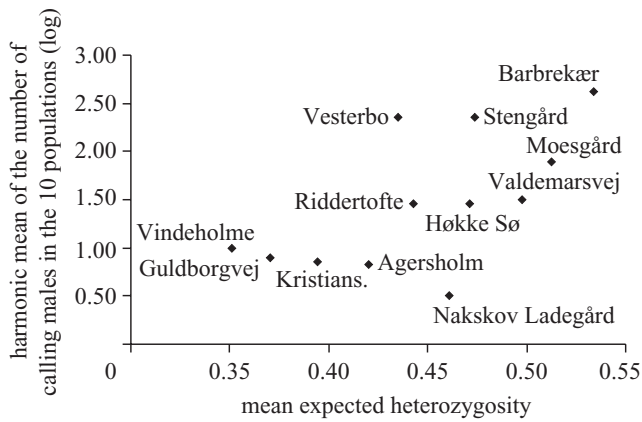


Figure 2. Genetic variation (mean expected heterozygosity) and population size expressed as harmonic mean of counted calling males over 9 years for the 10 populations represented by 12 ponds. Spearman's  $\rho = 0.6386$ ,  $p = 0.0254$  (significant at the 5% level after application of the sequential Bonferroni procedure significant at the 5% level).

between the ponds was arbitrarily estimated in a straight line without contemplating whether the surrounding habitat promoted tree frog movements.

A sequential Bonferroni procedure (Rice 1989) was applied to adjust the significance level ( $\alpha < 0.05$ ) whenever multiple comparisons were performed.

### 3. RESULTS

#### (a) *Microsatellite analyses*

In total for the 12 microsatellite markers the number of alleles varied from 6 to 21. Most loci were polymorphic, but a few loci were monomorphic in some ponds. Genetic variation in terms of mean expected heterozygosity ranged from 0.351 in Guldborgvej, Lolland, to 0.534 in Barbrekær, Jutland (table 1, only the overall figures are given). Deviations from HWEs were observed in 12 out of 144 tests at the single locus level (data not shown) after applying sequential Bonferroni corrections ( $\alpha = 5\%$ ) and in all cases were caused by heterozygote deficiency. Test for HWE across ponds and loci (table 1) indicated significant deviations in Agersholm, Vindeholme and Guldborgvej on Lolland. The observed heterozygote deficiency may be a result of non-random mating, the presence of null alleles or the Wahlund effect. As none of the loci exhibited deviations from HWEs in all the ponds, the deviations were observed in seven out of the 12 loci and the populations were rather small, we concluded that the heterozygote deficiency is most probably caused by inbreeding. However, a Wahlund effect cannot be excluded in some of the ponds.

#### (b) *Genetic variation and population size*

The mean number of calling males estimated for the 10 populations correlated significantly with mean expected heterozygosity obtained from the 12 ponds (figure 2, Spearman's  $\rho = 0.6386$ ,  $p = 0.0254$  (significant at the 5% level after application of the sequential Bonferroni procedure significant at the 5% level)). A significant population bottleneck was observed in the eight ponds, E, F, H, M, P1, P2, Q and S, whereas this was not detected in

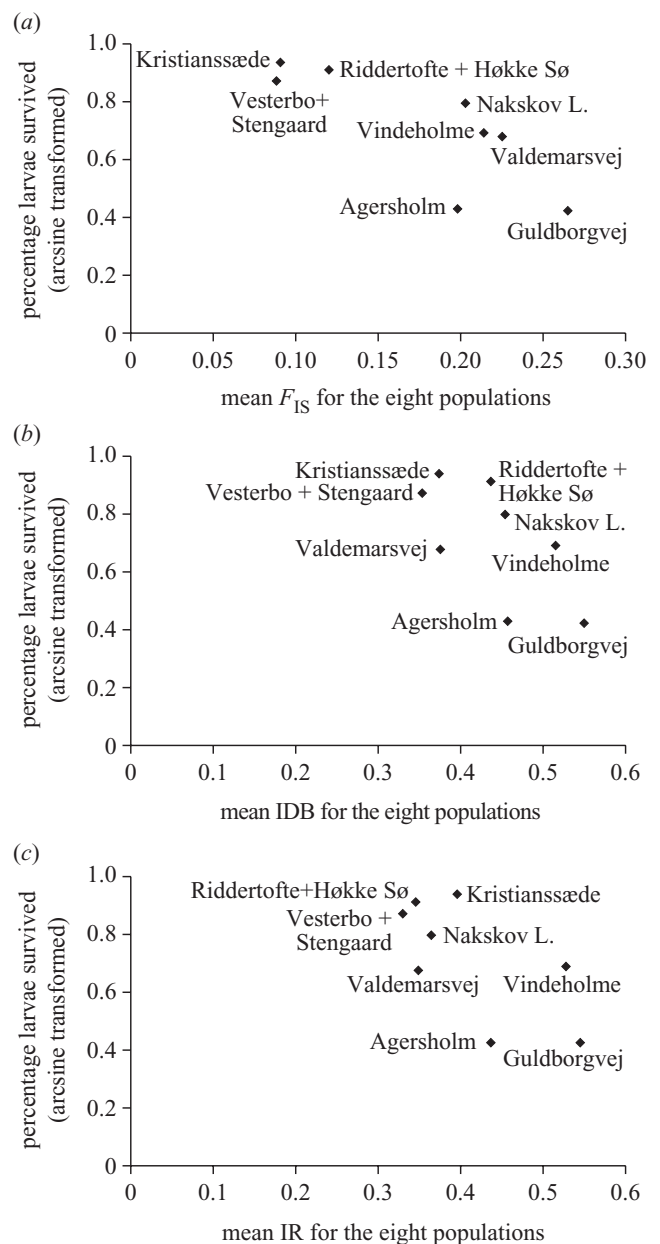


Figure 3. Significant correlation between inbreeding effect in terms of mean  $F_{IS}$ . (a) Spearman's  $\rho = -0.7857$ ,  $p = 0.0208$  (significant at the 5% level after application of the sequential Bonferroni procedure significant at the 5% level), mean IDB. (b) Spearman's  $\rho = -0.7143$ ,  $p = 0.0465$  (significant at the 5% level after application of the sequential Bonferroni procedure significant at the 5% level) and IR. (c) Spearman's  $\rho = -0.5952$ ,  $p = 0.1195$  and percentage larvae survived (arcsine transformed).

the two ponds in Jutland and the two ponds constituting the population on northwest Lolland (table 1, M).

#### (c) *Genetic variation and fitness*

Using the population means of the three different measures  $F_{IS}$ , IR and IDB for the eight ponds (table 1) a significant correlation was observed between the percentage of surviving larvae (arcsine transformed) and mean  $F_{IS}$  and mean IDB (Spearman's  $\rho (F_{IS}) = -0.7857$ ,  $p = 0.0208$  (significant at the 5% level after application of the sequential Bonferroni procedure significant at the 5% level), Spearman's  $\rho (IDB) = -0.7143$ ,  $p = 0.0465$  (significant at

Table 3. Identification of the 12 tree frog populations based on the highest loglikelihood estimate suggested by STRUCTURE (Pritchard *et al.* 2000) without using prior population information. (The number of individuals assigned to the 12 suggested populations and the mean individual admixture proportion [ $q(\text{mean})$ ] are given and the populations identified (suggested populations). Knubbeløkke (KN) and Næsby Strand (P4) were more recently colonized ponds.)

suggested populations	C1	C2	E	F	H	M	P1	P2	P3	P4	Q	S	KN	JUT1	JUT2
Vesterbo (C1)	25 (0.6564)	3 (0.4683)							1			1	4 (0.6498)		
Stengård (C2)	5	35 (0.8033)							1			1	2 (0.6705)		
Nakskov (E)	6 (0.6227)		26 (0.8757)							1					
Valdemarsvej (F)		1		27 (0.8791)								1			
Agersholm (H)		1			36 (0.8790)										
Kristianssæde (M)					1	34 (0.9071)	1				3	1			
Riddertofte + Høkke (P1, P2) + Næsby Strand (P4)	1						28 (0.8381)	15 (0.8199)		10 (0.7241)	1	1	1		
Høkke + Vestergård (P2, P3)							2	12 (0.7595)	14 (0.8135)	4 (0.8388)	1				
Vindeholme (Q)							1		1		41 (0.8523)		2		
Guldborgvej (S)												44 (0.9034)	1		
Barbrekær + (JUT1) Moesgård (JUT2)											1			38 (0.8878)	40 (0.9120)
Twelve unidentified	2		2	2	1	1	3	3	1	1	1	3	1		

the 5% level after application of the sequential Bonferroni procedure significant at the 5% level), Spearman's  $\rho$  (IR) = -0.5952,  $p = 0.1195$ ; figure 3). Hence, increased inbreeding was related to increased larval mortality. Likewise, inbreeding, measured by mean  $F_{IS}$ , was found to be significantly less severe in the populations that were classified as having a favourable conservation status (populations C and P on northwest and southwest Lolland, respectively) compared with populations classified as having an unfavourable conservation status (E, F, H, M, Q, S) (one-way ANOVA, Wilcoxon test:  $Z = -1.667$ ,  $p = 0.0478$ ).

#### (d) Admixture and population structure

The number of populations ( $k$ ) was estimated by using STRUCTURE (Pritchard *et al.* 2000). The likelihood curve suggested that the hypothetical population contained at least 12 genetically distinct groups ( $k = 10$ ,  $\text{Ln} = -10\,955.2$ ;  $k = 11$ ,  $\text{Ln} = -10\,847.4$ ;  $k = 12$ ,  $\text{Ln} = -10\,783.3$ ;  $k = 13$ ,  $\text{Ln} = -10\,772.5$ ). Based on the individual admixture proportions ( $q$ ; i.e. the individual was assigned to the population from which the largest proportion of its admixed genotype came) the 12 defined populations were identified (table 3). (The mean  $q$  estimates for the highest number of individuals in the suggested populations ranged between 0.4683 and 0.9120, some with rather wide 95% confidence intervals (0–1).) Eighteen individuals constituting the suggested population No. 12 did not belong to a certain geographical area but had an admixed genotype that was not characteristic of any of the sampled ponds.

All the  $F_{ST}$  estimates revealed evidence of significant pairwise genetic differentiation among the 13 ponds (including the Vestergård pond; data not shown). The magnitude of  $F_{ST}$  over all loci ranged between 0.029 for Barbrekær and Moesgård and 0.331 for Barbrekær and Kristianssæde and 0.332 for Guldborgvej and Vindeholme, i.e. the differentiation between Jutland and Lolland was not greater than within Lolland. The overall  $F_{ST}$  estimate indicated that 0.2245 ( $p < 0.0001$ ) of the genetic variance was distributed among the ponds. When the degree of genetic differentiation was measured by the pairwise  $R_{ST}$  (data not shown) generally the same picture was found. However, 19 pairwise estimates (including the two ponds in Jutland and the ponds in western Lolland) did not show significant genetic differentiation. The overall  $R_{ST}$  estimate indicated that 0.1788 ( $p < 0.0001$ ) of the genetic variance was distributed among the ponds.

A significant correlation was observed between geographical and genetic distance for the 10 ponds with the highest number of individuals sampled on Lolland (fitting  $F_{ST}/(1 - F_{ST})$  to  $a + b \text{Ln}(\text{distance})$   $a = -0.054\,81$ ,  $b = 0.116\,95$ ,  $p = 0.000\,30$  (significant at the 5% level after application of the sequential Bonferroni procedure significant at the 5% level)), thus indicating an isolation by distance effect.

## 4. DISCUSSION

### (a) Genetic variation

The genetic variation ( $H_e$ ) observed for the Danish European tree frog was found to be comparable to the mean  $H_e$  observed in the common and widespread frog *Rana sylvatica* (0.44–0.50, five microsatellite loci, number of

alleles: 2–18; Newman & Squire 2001) and slightly higher compared with the small British relict populations of *Bufo calamita* (natterjack toad, 0.242–0.386, eight microsatellite loci, number of alleles: two to eight; Rowe *et al.* 1998). In former studies of the genetic variation in European tree frogs based on allozyme variability, lower levels of heterozygosity have been found. For example, in the Swedish tree frog populations, virtually no genetic variation was found (Edenhamn *et al.* 2000).

The known history of the number of calling males in the ponds indicated that many of the ponds in the populations have passed through short narrow bottlenecks or through longer periods of rather low population sizes. A low population size over a longer period will be reflected in a reduction in the genetic variation and increase in homozygosity resulting from drift (Avice 1994; Frankham 1998; Frankham *et al.* 2002) provided there is no immigration. The admixture analysis (see later) demonstrated that the populations F, H, M, Q and S on Lolland were isolated. Hence, the significant heterozygote deficiency observed in the Agersholm (H), Vindeholme (Q) and Guldborgvej (S) ponds could indicate an inbreeding effect resulting from drift, which was supported by the observation of the varying egg viability from year to year in H and Q.

If sampling caused the inbreeding-drift effect we would expect a similar effect in all the ponds experiencing identical sampling procedure and egg-clump numbers. This was not observed. In Vesterbo, Stengård, Kristianssæde, Nakskov Ladegård and Valdemarsvej the sampling procedure was identical to the one from Agersholm, Vindeholme and Guldborgvej, collecting almost the same number of egg clumps (four to seven), supporting the inbreeding hypothesis.

### (b) Genetic variation and population size

Theory predicts a decline in genetic variation with declining population size (Frankham 1996). In the tree frog the level of genetic variation was positively correlated with an increasing harmonic mean number of calling males in the populations (figure 2). This relationship has also been observed in other amphibian species. Rowe *et al.* (1999) observed significant correlation between heterozygosity and population size, degree of isolation and distance from range edge in the natterjack toad, *B. calamita*.

The results of the bottleneck tests demonstrated a significant depletion in the genetic variation owing to low population sizes observed in most of the ponds on Lolland. This observation supported the assessed favourable conservation status for the population in northwest Lolland and Jutland (Pihl *et al.* 2000).

The higher genetic variation observed in the tree frog populations in Jutland relative to Lolland could probably be attributable to differences in the levels of recent habitat fragmentation. Whereas the rate of habitat loss up to around 1990 has been the same in Jutland and Lolland (Fog 1997), the pattern of fragmentation has been different. The tree frog populations in Jutland have been concentrated in still smaller core areas, whereas the tree frog populations on Lolland have been divided into many small fragments, causing bottleneck effects in most of the populations.

**(c) Genetic variation and fitness**

Recently, Keller & Waller (2002) have shown that an increased number of studies on wild plant and animal populations actually do detect inbreeding by using molecular techniques such as DNA microsatellites. In amphibians like the natterjack toad, Rowe *et al.* (1999) observed that a low larval growth rate was related to a reduction in mean  $H_e$  in the smallest isolated natterjack population in the study. Rowe *et al.* (1999) did not detect a correlation between growth rate and mean  $F_{IS}$ . In a later study Rowe & Beebee (2001) were not able to detect a significant correlation between the fitness-related traits such as larval growth and developmental rates and genetic variation in two outbred anuran species, the natterjack toad and common frog (*Rana temporaria*).

The present study demonstrated that the tree frogs in some of the populations on Lolland showed signs of inbreeding depression as suggested by Fog (1994; figure 3). The significant larger mean inbreeding coefficient observed in the ponds with unfavourable conservation status confirmed an expected correlation between a low population size and a high inbreeding coefficient (Frankham *et al.* 2002; Reed & Frankham 2003), which supported the indication of a reduced reproductive success caused by inbred individuals in some of the ponds. Another possibility is that the high genetic drift in the small populations has led to a fixation of unfavourable alleles.

A possible way to improve the conservation status and reduce the effect of genetic drift might consequently be to increase the migration possibilities between ponds.

In the brown trout (*Salmo trutta*), Ruzzante *et al.* (2001) observed that the median IDB coefficients were significantly larger than the population inbreeding value,  $F$ , in some river systems, which they ascribed to be caused by a Wahlund effect rather than inbred individuals. Amos *et al.* (2001) showed a significant negative relationship between IR (as a measure of parental similarity) and reproductive success for three long-lived vertebrates, i.e. the higher the parental dissimilarity, the more offspring the individuals tend to get compared with the average numbers of offspring. Recently, Acevedo-Whitehouse *et al.* (2003) showed that individuals of California sea lions (*Zalophus californianus*) with higher than normal parental relatedness (IR) were infested with a higher number of different pathogens. A significant correlation was not observed between IR and any of the fitness components in the present study (figure 2c).

In comparison, the fitness in the egg and early larval stages was better in the tree frogs in Scania, where the populations were larger and more continuous than on Lolland (Edenhamn 1996; Edenhamn *et al.* 2000).

Older unpublished records (by F. H. Møller to The Zoological Museum of Copenhagen 1942) indicate that populations on Lolland could have diverged from a common ancestral population a few generations (more than 25) ago as the distribution of the tree frogs was still nearly continuous before 1950. Hence, the suggested inbreeding must be a recent phenomenon. The inbreeding signs coincided with the formerly described population bottleneck and habitat fragmentation process on Lolland, where the numbers of suitable ponds have been reduced severely.

**(d) Population structure**

Eleven out of the 12 genetically different populations suggested by STRUCTURE (Pritchard *et al.* 2000; table 3) were identical to most geographically defined populations. The last suggested population was not related to any of the sampled ponds but consisted of individuals representing 11 of the ponds on Lolland. This could probably be attributed to the occurrence of multilocus genotypes consisting of alleles with high and therefore not very informative frequencies.

The results indicated the connections of tree frogs between ponds as individuals not sampled in the geographically suggested pond mainly came from neighbouring ponds within migration distance. Furthermore, migration directions were also indicated as tree frogs from the pond at Vesterbo contained individuals with admixed genotypes characteristic of Nakskov Ladegård pond, suggesting that Nakskov Ladegård belongs to the northwest Lolland population.

Høkke Sø, a recently colonized pond, was not identified as a separate genetic unit but consisted of tree frogs with admixed genotypes characteristic of the nearest possible source ponds, Riddertofte and Vestergård. This result was supported by the significant heterozygote deficiency observed in three of the loci in Høkke Sø, suggesting the recent mixing from other populations. Likewise, the individuals sampled in the Næsby Strand pond in the year that the pond was colonized were migrants with the main proportion of the admixed genotypes characteristic of Riddertofte/Høkke Sø and Vestergård.

The clustering analysis also revealed a suspected artificial introduction in Knubbeløkke pond on southwest Lolland. The observed genotypes were characteristic of Vesterbo, Stengård and Vindeholme, the two former belonging to the population C on northwest Lolland more than 20 km away. The probability of migration between the two areas was extremely low, corroborating information that the landowner at Knubbeløkke introduced tree frog eggs from elsewhere.

In the pairwise analysis all ponds on Lolland were mutually genetically differentiated based on  $F_{ST}$  statistics. The  $F_{ST}$  values obtained were in the higher range compared with other amphibian populations (Newman & Squire 2001) given the distance range (1.1–43.2 km) between ponds on Lolland. Høkke Sø was not identified as a separate genetic unit by STRUCTURE (Pritchard *et al.* 2000), which probably could be attributed to the recent colonization of this pond, implying that genetic drift has been acting over a very short time span. The  $R_{ST}$  statistics indicated that the ponds constituting the northwest Lolland and southwest Lolland populations, respectively, were not differentiated.

The conflicting results of the  $R_{ST}$  and  $F_{ST}$  estimates may reflect a combination of the associated larger variance of  $R_{ST}$  (Slatkin 1995) and the differences in assumptions behind the two estimates. In the latter, mutation together with drift are responsible for the variation, hence indicating that the ponds on western Lolland could have belonged to one larger coherent population recently, which is the case in Jutland, where one population is recently founded from the other. This was furthermore supported by the significant IBD effect observed in the tree frogs on Lolland, which indicated that the geographical



pattern of genetic variation on Lolland was shaped mainly by the limited dispersal range of tree frogs. In the moor frog (*Rana arvalis*), Vos *et al.* (2001) also observed a significant IBD effect. They found that the landscape mosaic in terms of the number of barriers was the most important factor reducing the exchange between populations.

The results suggested that the tree frogs show rather high site fidelity. Such site fidelity has also been demonstrated in mark-recapture studies (Stumpel & Hanekamp 1986; Friedl & Klump 2002). In The Netherlands, Stumpel & Hanekamp (1986) found that out of more than 1000 recaptured frogs, 6% had changed pond between the first and second capture.

The findings of the present study show that habitat fragmentation has caused a loss in genetic variation in most of the analysed tree frog populations on the island Lolland and consequently a rise in the inbreeding level, which again has led to a reduced survival in some populations.

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## REFERENCES

- Acevedo-Whitehouse, K., Gulland, F., Grieg, D. & Amos, W. 2003 Disease susceptibility in California sea lions. *Nature* **422**, 35.
- Amos, W., Worthington Wilmer, J., Fullard, K., Burg, T. M., Croxall, J. P., Bloch, D. & Coulson, T. 2001 The influence of parental relatedness on reproductive success. *Proc. R. Soc. Lond. B* **268**, 2021–2027. (DOI 10.1098/rspb.2001.1751.)
- Anonymous 1992 Council directive 92/43/EEC of 21 May 1992 on the conservation of natural habitats and of wild fauna and flora. See <http://europa.eu.int/comm/environment/nature/habdir.htm>.
- Arens, P., Van't Westende, W., Bugter, R., Smulders, M. J. M. & Vosman, B. 2000 Microsatellite markers for the European tree frog *Hyla arborea*. *Mol. Ecol.* **9**, 1944–1946.
- Avise, J. C. 1994 *Molecular markers, natural history and evolution*. New York: Chapman & Hall.
- Call, D. R. & Hallett, J. 1998 PCR primers for microsatellite loci in the anurans *Rana luteiventris* and *Hyla regilla*. *Mol. Ecol.* **7**, 1083–1090.
- deMaynadier, P. G. & Hunter, M. L. 2000 Road effects on amphibian movements in a forested landscape. *Nat. Areas J.* **20**, 56–65.
- Edenham, P. 1996 Spatial dynamics of the European tree frog (*Hyla arborea* L.) in a heterogenous landscape. PhD thesis, Uppsala University, Uppsala, Sweden.
- Edenham, P., Höggren, M. & Carlson, A. 2000 Genetic diversity and fitness in peripheral and central populations of the European tree frog *Hyla arborea*. *Hereditas* **133**, 115–122.
- Fog, K. 1992 Løvfrøer og andre padder på Lolland 1991. Rapport om registrering og vandhulspøje. (European tree frogs and other amphibians on Lolland. Report on monitoring and pond restoration.) 35 pp. Technical Administration, Storstrøms County, Denmark. [In Danish.]
- Fog, K. 1993 Migration in the tree frog *Hyla arborea*. In *Ecology and conservation of the European tree frog* (ed. H. P. Stumpel & U. Tester). Wageningen, The Netherlands: DLO Institute for Forestry and Nature Research.
- Fog, K. 1994 Vi handler i blinde. Padderne er en truet dygrøppe... (We act blindly. Amphibians are threatened.) *Jord og Viden* **9**, 14–16. [In Danish.]
- Fog, K. 1997 A survey of the results of pond projects for rare amphibians in Denmark. *Memoranda Soc. Fauna Flora Fennica* **73**, 91–100.
- Frankham, R. 1995 Effective population size/adult population size ratios in wildlife: a review. *Genet. Res.* **66**, 95–107.
- Frankham, R. 1996 Relationship of genetic variation to population size in wildlife. *Conserv. Biol.* **10**, 1500–1508.
- Frankham, R. 1998 Inbreeding and extinction: island populations. *Conserv. Biol.* **12**, 665–675.
- Frankham, R., Ballou, J. D. & Briscoe, D. A. 2002 *Introduction to conservation genetics*. Cambridge University Press.
- Friedl, T. W. P. & Klump, G. M. 2002 The vocal behaviour of male European tree frogs (*Hyla arborea*): implications for inter- and intrasexual selection. *Behaviour* **139**, 113–136.
- Garza, J. C. & Williamson, E. G. 2001 Detection of reduction in population size using data from microsatellite loci. *Mol. Ecol.* **10**, 305–318.
- Gosner, K. L. 1960 A simplified table for staging anuran embryos and larvae with notes on identification. *Herpetologica* **16**, 183–190.
- Goudet, J. 1995 FSTAT v. 2.9.3.1: a computer program to calculate F statistics. *J. Heredity* **86**, 485–486.
- Hedrick, P. W. 2000 *Genetics of populations*, 2nd edn. Sudbury, MA: Jones and Bartlett.
- Hels, T. & Fog, K. 1995 Does it help to restore ponds? A case of the Tree Frog (*Hyla arborea*). *Memoranda Soc. Fauna Flora Fennica* **71**, 93–95.
- Keller, L. F. & Waller, D. M. 2002 Inbreeding effects in wild populations. *Trends Ecol. Evol.* **17**, 230–241.
- Lande, R. 1995 Breeding plans for small populations based on the dynamics of quantitative genetic variance. In *Population management for survival and recovery* (ed. J. D. Ballou, M. Gilpin & T. J. Foose), pp. 318–340. New York: Columbia University Press.
- Lehmkühl, J. F. 1984 Determining size and dispersion of minimum viable populations for land management planning and species conservation. *Environ. Mngmt* **8**, 167–176.
- Michalakis, Y. & Excoffier, L. 1996 A generic estimation of population subdivision using distances between alleles with special reference to microsatellite loci. *Genetics* **142**, 1061–1064.
- Milligan, B. 1992 Plant DNA isolation. In *Molecular genetic analysis of populations: a practical approach* (ed. A. R. Hoelzel), pp. 59–88. Oxford: IRL Press.
- Nei, M. 1987 *Molecular evolutionary genetics*. New York: Columbia University Press.
- Newman, R. A. & Squire, T. 2001 Microsatellite variation and fine-scale population structure in the wood frog (*Rana sylvatica*). *Mol. Ecol.* **10**, 1087–1100.
- Pihl, S., Søgaard, B., Ejrnæs, R., Aude, E., Nielsen, K. E., Dahl, K. & Laursen, J. S. 2000 Habitat and species covered by the EEC habitats directive. A preliminary assessment of distribution and conservation status in Denmark. NERI Technical Report No. 365. Roskilde, Denmark: NERI.
- Pritchard, J. K., Stephens, M. & Donnelly, P. 2000 Inference of population structure using multilocus genotype data. *Genetics* **155**, 945–959.
- Raymond, M. & Rousset, F. 1995 GENEPOP v. 3.0. Population genetics software for exact tests and ecumenicism. *J. Heredity* **86**, 248–249.
- Reed, D. H. & Frankham, R. 2003 Correlation between fitness and genetic diversity. *Conserv. Biol.* **17**, 230–237.

- Rice, W. R. 1989 Analyzing tables of statistical tests. *Evolution* **43**, 223–225.
- Rousset, F. 1997 Genetic differentiation of gene flow from  $F$ -statistics under isolation by distance. *Genetics* **140**, 1413–1419.
- Rowe, G. & Beebee, T. J. C. 2001 Fitness and microsatellite diversity estimates were not correlated in two outbred anuran populations. *Heredity* **87**, 558–565.
- Rowe, G., Beebee, T. J. C. & Burke, T. 1998 Phylogeography of the natterjack toad *Bufo calamita* in Britain: genetic differentiation of native and translocated populations. *Mol. Ecol.* **7**, 751–760.
- Rowe, G., Beebee, T. J. C. & Burke, T. 1999 Microsatellite heterozygosity, fitness and demography in natterjack toads *Bufo calamita*. *Anim. Conserv.* **2**, 85–92.
- Ruzzante, D., Hansen, M. M. & Meldrup, D. 2001 Distribution of individual inbreeding coefficients, relatedness and influence of stocking on native anadromous brown trout (*Salmo trutta*) population structure. *Mol. Ecol.* **10**, 2107–2128.
- SAS 2001 v. 8.02. SAS Inc, Cary, NC, USA.
- Schneider, S., Roessli, D. & Excoffier, L. 2000 *ARLEQUIN, v. 2.0: a software for population genetics data analysis*. Genetics and Biometry Laboratory, University of Geneva.
- Skriver, P. 1988 A pond restoration project and a tree-frog (*Hyla arborea*) project in the municipality of Aarhus. *Memoranda Soc. Fauna Flora Fennica* **64**, 146–147.
- Skriver, P. 2001 Overvågning af løvfrølokaliteter mellem Vejle og Kolding. (Monitoring European tree frog localities between Vejle and Kolding in Jutland, Denmark.) Rapport til Vejle Amt. (Report to Vejle County, Denmark.) [In Danish.]
- Slatkin, M. 1995 A measure of population subdivision based on microsatellite allele frequencies. *Genetics* **139**, 457–462.
- Soulé, M. E. 1980 Thresholds for survival: maintaining fitness and the evolutionary potential. In *Conservation biology: an evolutionary–ecological perspective* (ed. M. E. Soulé & B. A. Wilcox), pp. 111–124. Sunderland, MA: Sinauer.
- Stumpel, A. H. P. 1987 Distribution and present numbers of the tree frog *Hyla arborea* in Zeeland Flanders, The Netherlands (Amphibia, Hylidae). *Bijdragen de Dierkunde* **57**, 151–163.
- Stumpel, A. H. P. & Hanekamp, G. 1986 Habitat ecology of *Hyla arborea* in The Netherlands. In *Studies in herpetology, Proc. of 3rd meeting of S. E. H., Prague 1985* (ed. Z. Roček), pp. 409–411. Prague: Charles University.
- Vos, C. C., Antonisse-de Jong, A. G., Goedhart, P. W. & Smulders, M. J. M. 2001 Genetic similarity as measure for connectivity of the moor frog (*Rana arvalis*). *Heredity* **86**, 598–608.
- Weir, B. S. 1990 *Genetic data analysis*. Sunderland, MA: Sinauer.
- Weir, B. S. & Cockerham, C. C. 1984 Estimating  $F$ -statistics for the analysis of population structure. *Evolution* **38**, 1358–1370.

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