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1 **Microsatellite analysis of the natterjack toad (*Bufo calamita*) in Denmark: populations**  
2 **are islands in a fragmented landscape**

3

4 Running title: Conservation genetics of *Bufo calamita*

5

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1 **Abstract**

2  
3 The European natterjack toad (*Bufo calamita*) has declined rapidly in recent years, primarily due to  
4 loss of habitat, and in Denmark it is estimated that 50% of the isolated populations are lost each  
5 decade. To efficiently manage and conserve this species and its genetic diversity, knowledge of the  
6 genetic structure is crucial. Based on nine polymorphic microsatellite loci, the genetic diversity,  
7 genetic structure and gene flow were investigated at 12 sites representing 5–10% of the natterjack  
8 toad localities presently known in Denmark. The expected heterozygosity ( $H_E$ ) within each locality  
9 was generally low (range: 0.18–0.43). Further analyses failed to significantly correlate genetic  
10 diversity with population size, degree of isolation and increasing northern latitude, indicating a  
11 more complex combination of factors in determining the present genetic profile. Genetic  
12 differentiation was high (overall  $\theta = 0.29$ ) and analyses based on a Bayesian clustering method  
13 revealed that the dataset constituted 11 genetic clusters, defining nearly all sampling sites as distinct  
14 populations. Contemporary gene flow among populations was undetectable in nearly all cases, and  
15 the failure to detect a pattern of isolation by distance within major regions supported this apparent  
16 lack of a gene flow continuum. Indications of a genetic bottleneck were found in three populations.  
17 The analyses suggest that the remaining *Bufo calamita* populations in Denmark are genetically  
18 isolated, and represent independent units in a highly fragmented gene pool. Future conservation  
19 management of this species is discussed in light of these results.

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## 1 Introduction

2  
3 It is widely accepted that genetic diversity is crucial in ensuring long term survival of a species or  
4 population (e.g. Frankham et al. 2002), and genetic biodiversity is recognized by the World  
5 Conservation Union (IUCN) as one of three levels of biodiversity that deserves attention (McNeely  
6 et al. 1990). Small isolated populations are prone to losing genetic diversity due to genetic drift, and  
7 this may lead to inbreeding depression and loss of evolutionary potential. Many amphibian species  
8 have very small effective population sizes, typically less than 100 individuals (Beebee & Griffiths  
9 2005; Funk et al. 1999), and exchange of genetic material among populations is therefore necessary  
10 to counteract loss of genetic diversity. Amphibians in general exhibit low dispersal capabilities  
11 (Larson et al. 1984; Blaustein et al. 1994; Beebee 2005; Kraaijeveld et al. 2005) and in fragmented  
12 landscapes, migration is only further impeded (e.g. Hels and Buchwald 2001; Vos and Chardon  
13 1998; Johansson et al. 2005; Lesbarrères et al. 2006). This implies that amphibians, in particular,  
14 may be at risk in terms of genetic depletion.

15 The natterjack toad (*Bufo calamita*) is restricted to central and western Europe; a region  
16 characterized by severe habitat loss and fragmentation throughout the past century. The IUCN  
17 Global Amphibian Assessment (IUCN 2006) reports that over 40% of the world's amphibian  
18 species are declining (Stuart et al. 2004), including *Bufo calamita* (IUCN 2006). In Denmark about  
19 50% of the populations are lost each decade (Briggs and Adrados 2005). Natterjack toads breed  
20 primarily in shallow temporary water bodies found in nutrient-poor environments such as coastal  
21 meadows, marshes and dune systems (Fog et al. 1997). A profound loss of breeding ponds is  
22 typically regarded as the main cause of decline in this species, but a recent study of Estonian  
23 natterjack toad populations suggested that suitable habitats for all life stages are equally important  
24 for population persistence (Rannap et al. 2007). The rapid rate of population decline has prompted  
25 habitat management such as pond digging and removal of vegetation in certain areas of Denmark,  
26 but the information necessary to define an overall systematic management strategy does not exist.  
27 *Bufo calamita* is listed in the EU Habitat Directive, Annex IV with an "unfavourable" national  
28 conservation status, implying that the overall number is decreasing in Denmark and that populations  
29 in general are considered too small and isolated to be self-sustaining (Pihl et al. 2000). However,  
30 Pihl et al. (2000) argue that the natterjack toad is not threatened by extinction on a national scale,  
31 since it is suggested that western parts of Jutland (Figure 1) support a large single population (Pihl  
32 et al. 2000). However, prior to this study there was no genetic evidence to test this claim.

33  
34 In natural populations a positive correlation between population size and genetic diversity has been  
35 described (Frankham 1996). Given that a substantial fraction of the remaining Danish populations  
36 are small and isolated on small islands or by distance in a landscape fragmented by agricultural  
37 fields and a highly developed infrastructure, one could expect low genetic diversity. Moreover,  
38 Beebee and Rowe (2000) showed that genetic variation within natterjack toad populations declined  
39 with geographic distance to the southern distribution limit of the species in Spain, probably due to  
40 post glacial colonisation patterns (Beebee and Rowe 2000). The Danish populations represent the  
41 north-western edge of the species' distribution, suggesting that prehistoric levels of genetic  
42 diversity could already have been low. This prediction was supported in a recent study investigating  
43 populations from the entire species distribution range, including one population from Denmark. The  
44 observed genetic variation in the Danish population, proved to be amongst the lowest values  
45 reported so far for the natterjack (Rowe et al. 2006).

46 Another correlation linking neutral genetic diversity and fitness was demonstrated empirically by  
47 Reed and Frankham (2003) in a meta-analysis of 34 datasets. This correlation has been reported for

1 *B. calamita* (Rowe et al. 1999), emphasizing the relevance of examining genetic diversity among  
2 Danish natterjack toad populations in a conservation context.

3  
4 In this study we characterise the overall patterns of genetic diversity, differentiation and gene flow  
5 associated with an endangered species, in an area where its natural habitat is highly fragmented and  
6 degraded. Initial hypotheses of low genetic diversity, limited gene flow and a high degree of  
7 differentiation, were tested with a range of genetic methods based on microsatellite data. We  
8 estimated levels of genetic variation within populations representing different geographic regions  
9 and various demographic histories to reveal contemporary genetic structure. Measures of genetic  
10 diversity were tested for a correlation with population size, degree of isolation and latitude. We  
11 quantified differentiation among populations and regions, estimated rates of contemporary gene  
12 flow and tested for a pattern of isolation-by-distance. In recognition of the importance of  
13 demographic stability and rapid loss of genetic diversity as important issues when assessing the  
14 conservation status (Pihl et al. 2000), possible traces of population bottlenecks were also  
15 investigated. Finally, the overall phylogenetic relationship of the Danish populations was examined,  
16 contributing to a discussion of conservation units.

## 17 **Materials and methods**

### 18 *Sampling*

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20 *Sampling*  
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22 Sampling was conducted in Summer 2004 and 2005 at 12 localities, representing three major  
23 geographic regions of Denmark separated by open sea: Jutland, Funen and Zealand (Figure 1). Six  
24 sites in Jutland were sampled, of which Grærup (GR), Harboøre (HA) and Bygholm Vejle (BV) are  
25 located within the grey area of Figure 1; previously hypothesized to support a single large  
26 population (Pihl et al. 2000). Råbjerg Mile (RM) is a large population located in the dunes of  
27 northern Jutland, whereas Honum (HO) and Hedensted (HE) are sites in old gravel pits. Four sites  
28 were sampled from Funen: Espe (ES) is an old gravel pit, and Fyns Hoved East and West (FHE,  
29 FHW) are two closely situated meadow ponds approximately 1.5 km apart. Avernakø (AV) is a  
30 small island south of Funen. From Zealand two localities were sampled: Dybsø (DY) is a small  
31 island (<100 meters from the mainland), and Munke Bjerghby (MB) is an active gravel quarry. We  
32 endeavoured to collect 40 tail clips of tadpoles from each locality (Table 2), and this tissue was  
33 stored in a 20% DMSO and saturated NaCl solution. To achieve a representative sample of each  
34 cohort and to minimize the number of siblings in the samples, individuals were collected from  
35 various sites around each breeding pond. When several breeding ponds were present at a locality,  
36 all were sampled. The sample from Dybsø (DY) included larvae from eight egg strings reared in  
37 captivity. The sample from Råbjerg Mile (RM) contained an equal proportion of tail clips and  
38 whole eggs. Collection of these eggs was the only example of invasive sampling in this study.

### 39 *DNA extraction and PCR conditions*

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42 DNA was extracted with chloroform using a standard CTAB-buffer and proteinase K procedure  
43 (Milligan, 1992). Twelve species specific genetic markers were screened. Nine proved polymorphic  
44 and were used in the analyses (Table 1). However, the majority of previously published data using  
45 *B. calamita* microsatellite primers have been based on a certain combination of eight markers. To  
46 allow for direct comparison with previous natterjack studies, we also estimated the genetic diversity  
47 for those eight markers alone (Table 1, 2). A standard PCR mix using Ampliqon™ products (0.5 µl  
48 1.25 mM dNTP solution, 0.5 µl of each 5 µM primer solution, 1.5 µl standard buffer, 0.01 µl 25mM

1 MgCl<sub>2</sub>, 0.04 µl Taq DNA polymerase (5u/µl), 1.2 µl DNA solution and topped up with distilled  
2 H<sub>2</sub>O to 10 µl) was prepared for each reaction unless noted in Table 1. For Buca 6 the reaction  
3 worked better using an ammonium buffer. PCR's were initiated with 95° for 3 min, followed by one  
4 of two primer-specific amplification conditions: A) 40 cycles of 94° for 45 sec, annealing  
5 temperature for 45 sec, 72° for 20 sec or B) 7 cycles of 94° for 45 sec, annealing temperature I for  
6 45 sec, 72° for 20 sec followed by 30 cycles of 94° for 45 sec, annealing temperature II for 45 sec,  
7 72° for 20 sec (see Table 1). Finally, each reaction was terminated with 72° for 15 min. The  
8 samples were subsequently genotyped using an ABI PRISM 377 automated sequencer.

### 11 *Genetic variation, tests for Hardy-Weinberg proportions and genotypic linkage equilibrium*

13 Allele frequencies and genetic diversity expressed as allelic richness ( $AR$ ), observed ( $H_O$ ) and  
14 expected heterozygosity ( $H_E$ ) (Nei 1987) were calculated in the Microsatellite Toolkit (Park 2001).  
15 To investigate possible deviations from Hardy Weinberg proportions Weir and Cockerham's (1984)  
16 estimator of  $F_{IS}$  ( $f$ ) was calculated for each locality and locus using FSTAT ver 2.9.3 (Goudet 2001).  
17 The significance of any deviation was assessed with GENEPOP ver 3.4 (Raymond and Rousset 1995)  
18 employing the Markov chain algorithm of Gou and Thompson (1992) in combination with the score  
19 test for heterozygote excess or deficiency (Rousset and Raymond 1995)

21 To test for independent segregation of loci, the concordance between observed genotype  
22 frequencies and the expected frequencies under genotypic linkage equilibrium was investigated for  
23 all pairwise locus combinations at each locality. This was done by a permutation test implemented  
24 in FSTAT ver 2.9.3 (Goudet 2001). Overall deviations from genotypic linkage equilibrium were  
25 assessed using combined  $P$ -values for all pairwise combinations of loci (across all localities) and for  
26 each locality (across all pairwise loci combinations). The  $P$ -values were combined with the  
27 weighted  $Z$ -method (Whitlock 2005).  $Z$ -values (standard normal deviates of  $P$ ), were weighted  
28 according to sample size ( $n$ ) and amount of genetic information (expected heterozygosity) present at  
29 the two loci ( $i, j$ ) in the given sample. The  $P$ -value in a sample with two nearly monomorphic loci  
30 will often be very close to 1, since randomizations cannot create less linkage disequilibrium than  
31 that observed. A  $P$ -value (or  $Z$ -value in this case) representing such pair should not be given the  
32 same weight as a value representing the association of two very polymorphic loci, allowing for a  
33 thorough test of the significance. Hence, each weight ( $w$ ) was calculated as:  $w = n \times H_E(i) \times H_E(j)$

### 35 *Number of calling males*

37 Demographic data from the localities were included in this study for two reasons. The observed  
38 number of calling natterjack toad males ( $N_C$ ) is assumed to be proportional to the population size,  
39 with a  $N/N_C$  ratio of approximately four (Briggs and Damm 1996). Hence, a positive correlation of  
40  $N_C$  and genetic diversity was expected and could be investigated. Secondly, a comparison of genetic  
41 and demographic data was necessary, when evaluating the consequences of possible bottlenecks in  
42 the various populations. Calling males were counted in optimal weather conditions at night time, by  
43 a combination of aural and visual detection with a flashlight. Sites were visited several times each  
44 season by experts in amphibian monitoring, and the highest count was recorded as  $N_C$ . The time  
45 span for which monitoring was performed varied markedly from site to site.  $N_{CM}$  was calculated  
46 using data from the following years: AV: 1988, 1990-96, 2000-01, FHE and FHW: 1977, 1981,  
47 1983, 1987-88, 1991-96, 2000-02, HO and HE: 1999-2005, DY: 1996, 1998, 2002, GR: 2003, HA:  
48 2005 and MB: 2006 (data provided by Amphi Consult). Reliable data did not exist for RM, ES and

1 BV, so  $N_C$  estimates, were based partly on male counts and partly on the assumed carrying capacity  
2 of the localities (Lars Briggs and Lars Christian Adrados, unpubl. data).

### 3 4 *Correlations of genetic diversity*

5  
6 Three possible associations were investigated:

7 1) To correlate genetic diversity and population size, mean  $N_C$  and the harmonic mean of the  
8 number of calling males ( $N_{CM}$ ) was calculated for each study site. Observed genetic diversity is  
9 influenced by genetic drift through many generations, and in a population with fluctuating  
10 demography, years with few breeders have a disproportionately large effect. Hence, when  
11 calculating the harmonic mean, small  $N_C$  values are weighted above large values. For all localities,  
12 the associations of  $H_E$  and  $AR$  with mean  $N_C$  and the harmonic mean  $N_{CM}$  was investigated with  
13 Pearson's product-moment correlation coefficient. Both indicators of population size were log  
14 transformed to meet the requirements for this parametric test. Due to uncertainty in  $N_C$  and  $N_{CM}$   
15 estimates from GR, HA, MB, RM, ES and BV, possible correlations were also investigated after  
16 excluding these localities.

17 2) The association of  $H_E$  and allelic richness with degree of isolation was also assessed.  
18 Isolation was quantified in a simple rank order system with each population receiving points  
19 between 1 and 10 in three categories: i) degree of present physical isolation, ii) density of natterjack  
20 toad sites in the particular region and iii) assumed extent of time isolated (if isolated). Summed  
21 values were ranked and tested against genetic diversity with Spearman's correlation coefficient.

22 3) Finally, we tested whether the previously demonstrated linear relationship of genetic  
23 diversity in natterjack toad populations and increasing northern latitude (across Europe) also applied  
24 within Denmark. Distances (km) were measured from the latitude of Avernakø (the southernmost  
25 population investigated here) to the latitude of the other localities. Correlations of this distance with  
26  $H_E$  and  $AR$  were tested with Pearson's product-moment correlation coefficient.

### 27 28 29 *Genetic structure and gene flow*

30  
31 The Bayesian clustering method implemented in STRUCTURE ver 2.1 (Pritchard et al. 2000) was  
32 used to determine the number of distinct genetic units ( $K$ ) in the dataset. Without using prior  
33 knowledge of sampling localities STRUCTURE assigns individuals into groups, minimizing  
34 deviations from Hardy-Weinberg proportions and genotypic linkage equilibrium. This method has  
35 been used previously to identify migrants and their origins (e.g. Eldridge et al. 2001; Berry et al.  
36 2004; Kim et al. 2006). However, since tadpoles are largely non-migratory and combined with the  
37 fact that unsampled populations exist between the ones included in this study, the relevance of  
38 detecting migrants is limited here. Instead, we applied the method to illustrate genetic  
39 differentiation and to clarify whether each sampling locality should be treated as a distinct  
40 population in further investigations. We selected the "no admixture model" with uncorrelated allele  
41 frequencies, which is most appropriate when the populations are believed to be rather discrete  
42 (Pritchard and Wen 2004). The use of this model was justified since it generated significantly  
43 higher posterior probabilities compared to runs under the "admixture model" (data not shown). The  
44 output under the "no admixture model" does not provide admixture proportions but instead  
45 individual posterior probabilities of belonging to each defined cluster (Pritchard and Wen 2004).  
46 The length of the burn-in period was set to 100,000 iterations followed by 500,000 MCMC  
47 repetitions. Simulations were run 20 times for each proposed  $K$ -value in the realistic range from 7 to  
48 15 and likelihood values  $\Pr(X|K)$  for each run were recorded. Identification of the number of

1 distinct genetic clusters ( $K$ ), was performed following the procedure described by Evanno et al.  
2 (2005). The average posterior probabilities for each  $K$  were used to create a likelihood curve. The  
3 true number of clusters was recognized as the  $K$ -value representing the highest second order rate of  
4 change (i.e. where the curve breaks the most) corrected for larger variance in probability for  
5 increasing  $K$ -values (Evanno et al. 2005).

6  
7 The significance of genetic differentiation among localities was examined by testing allele  
8 frequency differences using GENEPOP ver 3.4 (Raymond and Rousset 1995), and the degree of  
9 differentiation was quantified with Weir and Cockerham's (1984) unbiased  $F_{ST}$  estimator  $\theta$ .  $\theta$ .  $\theta$   
10 and 95% confidence intervals were calculated in FSTAT ver 2.9.3. (Goudet 2001) at three levels: i)  
11 the entire dataset, ii) within major geographic regions defined as: Jutland (GR, HA, BV, RM, HO,  
12 HE), Funen (AV, FHE, FHW, ES) and Zealand (MB, DY) and iii) for all locality pairs. A  
13 hierarchical analysis of the molecular variance (AMOVA) among regions was performed in  
14 ARLEQUIN ver 2.0 (Schneider et al. 2000) and the significance level was assessed based on 10,000  
15 permutations.

16  
17 Migration rates were estimated between all localities with a Bayesian model implemented in the  
18 program BAYESASSNM (Jehle et al. 2005). Compared to indirect estimators of long-term gene flow  
19 (such as  $Nm$  calculated from  $F_{ST}$ ) this approach is relatively assumption free since genotype  
20 frequencies are allowed to deviate from Hardy-Weinberg proportions within populations.  
21 Furthermore, BAYESASSNM estimates the contemporary migration rate (over the most recent  
22 generations), which is the timescale of most relevance in the context of conservation. BAYESASSNM  
23 (Jehle et al. 2005) accounts for a situation, where the sampled individuals are not considered to be  
24 migrants, but at most descendants of migrants. Default settings were used since they provided the  
25 analysis with an acceptable proportion of change in parameter values (40-60%) when exploring the  
26 parameter space through MCMC repetitions (Wilson and Rannala 2003).

### 27 28 *Isolation by distance*

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30 To evaluate a possible association of geographic and genetic distance, several approaches were  
31 employed. Pairwise genetic distances were plotted against shortest geographic distance (km) and  
32 against the logarithm ( $\ln(d)$ ) of the same distance.  $\ln(d)$  is considered most appropriate when  
33 populations are distributed in two dimensions (Rousset 1997). The significance of correlations  
34 between geographic and genetic distance matrices was tested by a Mantel test (10,000  
35 permutations), implemented in the program IBD (Bohonak 2002). Three different measures of  
36 genetic distance were applied: i) pairwise  $\theta/(1-\theta)$  distances (Slatkin 1993; Rousset 1997),  
37 correcting for the theoretical fact that  $\theta$  reaches a plateau with long geographic distances in a  
38 stepping stone model. ii) pairwise  $R_{st}$  counterparts,  $R_{st}/(1-R_{st})$ .  $R_{st}$ -values were calculated according  
39 to Rousset (1996) using FSTAT ver 2.9.3 (Goudet 2001), thereby taking the presumed stepwise  
40 mutation processes of microsatellites into consideration, and iii) Pairwise Cavalli-Sforza & Edwards  
41 chord distances ( $D_c$ ), assuming mutations are insignificant to genetic drift in evolutionary processes  
42 (Cavalli-Sforza and Edwards 1967).

43  
44 Correlations of genetic and geographic distance were only evaluated within the major regions –  
45 being Jutland and Funen respectively. We did not apply this analysis to the entire dataset, since a  
46 detected pattern of IBD could then simply have reflected ancient barriers between major regions,  
47 instead of potential contemporary gene flow restricted by distance.



## 1 *Bottleneck*

2 The occurrence of recent demographic bottlenecks was investigated using the program  
3 BOTTLENECK ver 1.2 (Piry et al. 1999). During a bottleneck allelic diversity is lost faster than  
4 heterozygosity, resulting in the observed number of alleles being less than the number expected  
5 from the observed Hardy-Weinberg heterozygosity (Cornuet and Luikart 1996). Assuming  
6 mutation-drift equilibrium conditions, the significance of heterozygosity excess was evaluated for  
7 each of the 12 localities. A Wilcoxon's test is implemented in BOTTLENECK to test for concordance  
8 of expected heterozygosity ( $H_E$ ) and expected equilibrium heterozygosity ( $H_{EQ}$ ). As recommended  
9 for microsatellite data, a two phase mutation model was applied with 95% single-step and 5%  
10 multiple-step mutations (Di Rienzo et al. 1994; Piry et al. 1999).

## 11 *Genetic relationship*

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14 Cavalli-Sforza and Edwards chord distances ( $D_C$ ) were chosen as the basis for phylogenetic  
15 inference using neighbour joining (NJ) analysis. The reliability of this genetic distance, which  
16 assumes drift as the major differentiation force, as expected in small isolated populations, has  
17 previously been demonstrated on a simulated microsatellite dataset – even under various mutation  
18 models (Takezaki and Nei 1996). Distance calculations, bootstrapping (based on 10,000 replicates),  
19 and visualization of the tree were performed using GENDIST, NEIGHBOR, SEQBOOT, CONSENSE and  
20 DRAWTREE in the PHYLIP package ver 3.6 (Felsenstein 2004).

## 21 **Results**

### 22 *Genetic variation, tests for Hardy-Weinberg proportions and genotypic linkage equilibrium*

23  
24 For the 9 microsatellite markers examined, the number of alleles varied from 2 to 10 and 42 alleles  
25 were present in total. Only 6 alleles were present at every locality, whereas 9 alleles (21%) were  
26 confined to a single locality, two of these at Dybsø (DY), Zealand (see Online Appendix). Overall  
27 mean  $H_E$  was 0.319 ranging from 0.183 at Munke Bjerghby (MB) to 0.429 at Fyns Hoved West  
28 (FHW) (Table 2). Variation at the eight loci, used only for comparison with previous studies from  
29 other regions, was considerably lower ranging from 0.082 (MB) to 0.258 (FHW) (Table 2). Of these  
30 eight markers Bcal $\mu$  4 and Bcal $\mu$  6 proved monomorphic in our dataset (Table 1).

31  
32 Significant deviations from Hardy-Weinberg proportions ( $\alpha = 0.05$ ) were observed in 11 of 74 tests  
33 representing every polymorphic locus/locality combination (see Online Appendix). This was  
34 somewhat greater than expected ( $74 \times 0.05 = 3.7$ ). The dominance of negative  $f$ -values (Table 1, 2)  
35 suggests this was largely due to heterozygote excess. Following sequential Bonferroni correction,  
36 an overall excess of heterozygotes was observed at the Bcal $\mu$  3 and Bcal $\mu$  11 loci (Table 1) and  
37 across all loci at the Espe (ES) and Honum (HO) sites (Table 2), while no localities or loci exhibited  
38 overall heterozygote deficiency (Table 1,2).

39  
40 Significant deviations from genotypic linkage equilibrium were detected in 27 of 432 tests (data not  
41 shown). Only between Bcal $\mu$  3 and Bcal $\mu$  10 ( $P = 2.1 \times 10^{-8}$ ) was an overall deviation detected  
42 across all localities (after sequential Bonferroni correction,  $\alpha = 0.05$ ) when combining  $P$ -values  
43 with the weighted  $Z$ -method. However, when combining  $P$ -values across pairs of loci *within*  
44 localities, overall significant deviations were found at FHE ( $P = 0.001$ ), FHW ( $P = 0.0009$ ), ES ( $P$   
45  $= 2.8 \times 10^{-5}$ ), HO ( $P = 1.23 \times 10^{-6}$ ) and HA ( $P = 1.16 \times 10^{-5}$ ) following sequential Bonferroni  
46 correction.  
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## *Correlating genetic diversity*

Expected heterozygosity was not positively correlated with population size.  $r$ -coefficients proved negative and non-significant when correlating against  $\log N_C$  ( $r = -0.29$ ,  $P = 0.36$ ) and  $\log N_{CM}$  ( $r = -0.32$ ,  $P = 0.31$ ). Similar results were obtained when using  $AR$  instead of  $H_E$ , and when populations with potentially less reliable  $N_C$  values were excluded (data not shown). Weak, but non-significant, associations were observed when correlating  $H_E$  with the degree of isolation ( $r = -0.06$ ,  $P = 0.83$ ) and latitude ( $r = -0.38$ ,  $P = 0.22$ ). Using allelic richness instead of  $H_E$  yielded even weaker correlations (data not shown).

## *Genetic structure and gene flow*

The clustering method implemented in STRUCTURE (Pritchard et al. 2000) revealed that  $\Delta K_{max}$  was 3.02, peaking at  $K = 11$  (data not shown), indicating that the dataset included 11 distinct genetic units. Results from the output providing the highest posterior probability ( $\ln \Pr(X|K) = -4443.6$ ) of the 20 runs for  $K = 11$  are presented, with the probabilities of belonging to a cluster for all individuals in Figure 1. The mean posterior probabilities for each sampling site are listed in Table 3. In total 307 of 442 individuals (69.5%) were assigned to a cluster with a probability  $\geq 0.90$ . Only 19 of these 307 (6.2%) were assigned to a different cluster than the majority of individuals of similar origin (Table 3). The highly non-random association of colours in Figure 1 demonstrates the obvious genetic structure associated with sampling localities. Only the two localities from Fyns Hoved (FHE and FHW) were indistinguishable using this method. It was also notable that a substantial fraction of the sampled HA gene pool resembled the other two sites defined within the West Coast region (GR and BV).

Overall  $\theta$  was 0.286, implying that 28.6% of the genetic variation present in the dataset was ascribed to variation among localities. Mean values within the three major regions were as follows: Jutland ( $\theta = 0.229$ ), Funen ( $\theta = 0.107$ ) and Zealand ( $\theta = 0.214$ ). For all pairs of localities  $\theta$  ranged from 0.017 between FHE and FHW to 0.624 between BV and MB (Table 4). BV and MB display the two lowest values of  $H_E$  (Table 2) and the large differentiation was due to fixation (or near fixation) of different alleles. Only between FHE and FHW did the 95% confidence interval include 0 (data not shown). All tests for differences in allele frequencies between localities were highly significant even after applying Bonferroni correction (data not shown). Contrary to the results from STRUCTURE, FHE and FHW also proved to be divergent ( $P = 2 \times 10^{-5}$ ). As a consequence, all sampling localities were treated as separate populations in subsequent analyses. A hierarchical AMOVA revealed a significant substructure, with 18.3% of the variation defined by major regions (Jutland, Funen and Zealand) and 14.7% ascribed to variation among localities within regions.

Immigration rates between all sites were estimated in BAYESASSNM (Jehle et al. 2005) and were in general very low. In almost all cases the standard deviation proved to be greater than the reported mean of the distribution. However, four estimated immigration rates (of 132 possible directions of gene flow) were above 1%, and were considerably higher than the remaining estimates (data not shown). Migration between the two localities of Fyns Hoved was asymmetric with a rate of 1.5% from FHW to FHE and 20% from FHE to FHW. From AV to FHE and FHW, estimated rates were 1.2% and 1.1% respectively

1 No isolation by distance pattern was detected. Testing the association of all possible combinations  
2 of geographic and genetic distances provided  $r^2$  values ranging from 0.000 to 0.561 and  
3 corresponding  $P$ -values ranging from 0.079 to 0.613.

4  
5 The neighbour joining analysis yielded a phylogenetic tree with high bootstrap values on three  
6 internal branches (80%, 99% and 88%) (Figure 2), defining three distinct genetic clusters  
7 corresponding to Funen (AV, FHE, FHW, ES), Zealand (MB, DY) and West Coast of Jutland (GR,  
8 HA, BV). Jutland in its entirety was not well defined, with a bootstrap value of only 42%.

#### 9 *Bottleneck*

10  
11  
12 Testing for genetic bottlenecks, a Wilcoxon test revealed an excess of heterozygosity compared to  
13 the expected equilibrium at three localities: AV ( $P = 0.039$ ), FHE ( $P = 0.004$ ) and HO ( $P = 0.039$ )  
14 indicating the presence of a recent demographic bottleneck at these sites. After applying sequential  
15 Bonferroni correction only FHE deviated significantly.

## 16 **Discussion**

### 17 *Deviations from Hardy-Weinberg proportions and genotypic linkage equilibrium*

18  
19  
20  
21 Deviations from Hardy-Weinberg proportions in terms of heterozygote excess could be explained  
22 by differences in allele frequencies between male and female parents, which is likely to occur when  
23 the effective population size is small (Robertson 1965; Rasmussen 1979). Heterozygote excess has  
24 previously been described in studies analysing samples of tadpoles (e.g. Funk et al. 2005; Rowe et  
25 al. 2006). Genotypic linkage disequilibrium could be ascribed to tight physical linkage between loci  
26 that would impose a systematic bias on the entire dataset, or simply be due to non-random  
27 association of alleles in the gamete pool (Hedrick 2000). Overall, deviations from genotypic linkage  
28 equilibrium were predominantly observed within populations rather than across populations. This  
29 indicates that deviations can be attributed to population characteristics (such as siblings being  
30 present in some of the samples) rather than linked marker loci. Hence, the most likely cause for the  
31 described deviations from Hardy-Weinberg proportions and genotypic linkage equilibrium was poor  
32 breeding success at several localities.

### 33 *Genetic diversity*

34  
35  
36 When comparing genetic diversities among populations from various European regions (Rowe et al.  
37 1998; Beebee and Rowe 2000), the Danish populations appeared in the lower end of the  $H_E$   
38 spectrum. Published values of  $H_E$  span from 0.082 at Munke Bjerby (MB) documented in this  
39 study to 0.689 at Velez, Southern Spain (Beebee and Rowe 2000). The observed low diversity in  
40 Denmark is most likely due to a combination of factors but our analyses did not generate any clear  
41 answers. Genetic diversity was not correlated with the number of calling males. Founder effects and  
42 bottlenecks may have blurred such correlations. Using the harmonic mean of  $N_C$  in the correlations  
43 should partly account for such discrepancies, but this data transformation would have no effect if  
44 the bottleneck or founder effect happened before monitoring was initiated. For example, Råbjerg  
45 Mile (RM) is a large population but represents one of the very northernmost outposts for this  
46 species, possibly founded by a few persevering pioneers. This could explain the low  $H_E$  (Table 2).  
47 Moreover, the areas south of RM are presently unsuitable for foraging and migration (Briggs and  
48 Adrados 2005), thereby eliminating gene flow as a counteracting force against genetic erosion due

1 to drift. Though not significant, a gradient was apparent nonetheless when genetic diversity was  
2 plotted against latitude ( $r = -0.38$ ). Since this study only covered 320 latitudinal kilometres, a highly  
3 significant correlation was not really expected. The study of Beebee and Rowe (2000) included  
4 populations from Spain to Sweden, to successfully demonstrate this effect of early post glacial  
5 dispersal patterns.

6 We have shown that degree of isolation was only very weakly associated with genetic diversity  
7 among the Danish populations. Again, a turbulent phylogeographic history in a region with many  
8 islands and a varying degree of fragmentation of the landmasses through time could have masked  
9 these otherwise expected correlations. Finally, poor breeding success in the year of sampling could  
10 easily bias such analyses. Perhaps exemplifying this, the large BV population was rated as being the  
11 least isolated of the 12 studied populations. However, it displays low genetic diversity and it is  
12 possible that the 20 collected individuals do not properly represent the gene pool of the entire  
13 population. If Bygholm Vejle (BV) is excluded from the isolation analysis, a much stronger but still  
14 non-significant, correlation was revealed ( $r = -0.28$ ,  $P = 0.39$  compared to  $r = -0.06$ ,  $P = 0.83$   
15 when BV was included).

16 Overall, none of the investigated parameters appeared to be solely responsible for the observed  
17 pattern, but latitude and degree of isolation were likely components, among other factors,  
18 determining the present genetic profile. In this study we were not able to show reduced genetic  
19 diversity due to small population sizes or recent demographic fluctuations, but further investigations  
20 are needed to reject these factors as determinants of the genetic profile.

21  
22 Whatever the causes, the observed low genetic diversity is perhaps concerning for the small Danish  
23 populations. Correlations of various fitness traits and measures of genetic diversity have recently  
24 been demonstrated for tree frogs (*Hyla arborea*) in Denmark (Andersen et al. 2004) and for  
25 common frogs (*Rana temporaria*) in Sweden (Johansson et al. 2007). Moreover, the *R. temporaria*  
26 study revealed that variation in quantitative traits ( $Q_{ST}$ ) was higher among populations living in a  
27 continuous habitat compared to populations in a fragmented landscape, suggesting a lowered  
28 adaptive potential in the latter. A correlation of larval growth rate and  $H_E$  has been described for *B.*  
29 *calamita* (Rowe et al. 1999) where larvae from a population exhibiting  $H_E = 0.19$  developed at  
30 about half the rate compared to larvae from a population with  $H_E = 0.34$ . Using the same  
31 combination of genetic markers, five of the populations included in our study exhibited  $H_E$  below  
32 0.19 (Table 2). Rapid larval development and metamorphosis is crucial for population survival in  
33 the natterjack toad, since shallow temporary ponds are preferred breeding sites. If natterjack toads  
34 experience degradation in this capability, desiccation mortality will increase (Rowe et al. 1999).  
35 Additionally, a recent study showed that small isolated populations of Swedish natterjack toads  
36 exhibited strikingly low sperm viability (Freiburghaus 2006).

37 However, it is important to distinguish between low genetic diversity as an outcome of recent  
38 habitat fragmentation or an echo of ancient colonization patterns. Ficetola et al. (2007) showed that  
39 genetic diversity within populations of the frog *Rana latastei* was jointly affected by postglacial  
40 colonization and current isolation, but hatching success was only affected by the latter. Whether  
41 Danish natterjack toad populations are experiencing reduced fitness, is not known at present.

#### 42 43 *Genetic structure and gene flow*

44  
45 Andersen et al. (2004) investigated the population genetics of the European tree frog (*Hyla*  
46 *arborea*) at two localities from Jutland and 14 from eastern parts of Denmark. Genetic  
47 differentiation was quantified with an overall  $\theta$  of 0.225, which was slightly less than the 0.286  
48 demonstrated here for *Bufo calamita*. In the UK, where the natterjack toad has an even more

1 fragmented distribution, differentiation among populations from mutually isolated regions in  
2 England ranged between 0.23-0.53 (Rowe et al. 1998), somewhat higher than the 0.183 found here.  
3 In studies of toad populations from areas with few barriers to gene flow, differentiation is markedly  
4 lower than that described in this study. For example, an overall  $\theta$  value of 0.060 was reported within  
5 an assumed natterjack toad metapopulation in the UK (Rowe et al. 2000) and, in an allozyme study  
6 of a metapopulation of common toad (*Bufo bufo*) in Finland, an overall  $\theta$  of 0.019 was revealed  
7 (Seppä and Laurila 1999).

8  
9 The initial hypothesis of greatly limited gene flow was supported in several analyses. Bearing in  
10 mind that a substantial fraction of the populations shown in Figure 1 no longer exist, the large  
11 geographic distances between many localities demonstrates the improbability of individuals  
12 migrating between populations. In this study, exchange of individuals seemed possible between  
13 FHE and FHW (1.5 km apart), and between HE and HO (13 km apart). Two studies tracking  
14 natterjack toads over a season showed a maximum dispersal distance from breeding areas of 980  
15 meters (Miaud et al. 2000) and 2600 meters (Sinsch 1992) respectively, but the migratory potential  
16 over an entire lifespan is not known. However, distance alone does not necessarily prevent gene  
17 flow. Although direct evidence is lacking, it has been suggested that amphibian eggs can be  
18 dispersed by birds (entangled in feathers or feet), and storms or floating branches have also been  
19 proposed as mediators of long range dispersal (see Measey et al. 2007). Human-induced migration  
20 can occur at all three amphibian life stages. This kind of dispersal was suggested, and later  
21 confirmed, as an explanation of a peculiar colonization pattern revealed in the tree frog study of  
22 Andersen et al. (2004). Moreover, unsampled natterjack toad localities exist in some areas at least,  
23 and genes can travel over long distances as in a stepping-stone model, when the landscape, species  
24 distribution and behaviour allow it. Gene flow and migration over substantial distances has  
25 previously been detected for amphibians in some instances (Austin et al. 2004; Funk et al. 2005;  
26 Smith and Green 2005). A low but detectable migration rate was apparent from the AV population  
27 to the two FH localities approximately 70 km away (of which 5 km is across open sea). Since not all  
28 populations have been sampled in this study, a more probable interpretation of the results might  
29 suggest that part of the sampled gene pool at northern Funen originates from southern Funen. This  
30 could reflect long range gene flow in recent times, mediated by one of the vectors mentioned above,  
31 but given recently demonstrated limitations of the employed Bayesian method (Faubet et al. 2007),  
32 this is perhaps an over-interpretation. Apart from this exception, gene flow across considerable  
33 distances in the Danish landscape was not detected, even between the rather closely-spaced HO and  
34 HE populations. Furthermore, the significantly differentiated allele frequency distributions, with a  
35 high fraction of private alleles, indicated that gene flow has been disrupted for a considerable period  
36 of time.

### 37 38 *Isolation by distance*

39  
40 We failed to identify any correlation between genetic and geographic distance within regions. A  
41 pattern of isolation by distance (IBD) is a consequence of a continuous distribution but restricted  
42 dispersal, creating a balance between local genetic drift and geographically mediated gene flow  
43 (Wright 1943; Slatkin 1993). The absence of IBD was most likely a result of genetic isolation  
44 through several generations. When gene flow is completely absent, allele frequencies in each  
45 population evolve independently, as on isolated islands (Wright 1943; Strand et al. 1996). However,  
46 several scenarios could lead to an apparent absence of IBD. Firstly, a panmictic population structure  
47 would eliminate such a pattern, but this was clearly rejected in the present study. Inappropriate  
48 measures of genetic and geographic distances could obviously mask a correlation, but here we

1 employed several genetic and geographic distance measures. Using the shortest geographic  
2 distances without correcting for potential barriers to gene flow could also induce a bias. In the  
3 Danish landscape, distance alone is the overriding barrier, and when attempting to detect IBD over  
4 relatively large distances, no obvious alternative exists than the shortest geographic distance  
5 between localities within major regions.  
6 In the context of the sampling scheme used here, the populations exhibited genetic isolation but  
7 further studies are needed to analyse local dynamics (i.e. possible metapopulation structure) in the  
8 few regions where migration between localities seem more likely, e.g. in certain areas along the  
9 West Coast of Jutland or southern Funen. However, the degree of genetic differentiation, absence of  
10 gene flow, general fragmentation of the Danish landscape and limited dispersal capability of the  
11 natterjack toad are all highly relevant factors when discussing conservation of genetic biodiversity.  
12 A conservative interpretation suggests that the majority of populations in Denmark behave as  
13 isolated islands with genetic drift altering allele frequencies in each population independently.  
14 Moreover, the results suggest that the majority of Danish natterjack localities represent distinct  
15 populations, though this might not be the case in all regions.

16  
17 The highly fragmented gene pool of Danish natterjack toads is presumably caused by a combination  
18 of natural processes and human induced changes of the landscape. *Bufo calamita* colonized Europe  
19 in the last interstadial, which commenced about 14000 years before present (BP) (Beebee and Rowe  
20 2000). About 8000 years BP the sea level rose and turned Denmark into a kingdom of islands  
21 (Aaris-Sørensen 1988). This early isolation of regions is reflected in the AMOVA analysis and  
22 Figure 2. Fragmentation within major regions probably began during Atlanticum (8000-5000 BP)  
23 with the emergence of huge forests isolating the meadows where the natterjack toads lived. The fact  
24 that within region differentiation (14.7%) in the AMOVA analysis proved almost as high as  
25 differentiation among regions (18.3%) supports this theory of an early intra-regional fragmentation.  
26 Alternatively, it is simply a result of  $\theta$ -values reaching a plateau, since differentiation cannot exceed  
27 the level of average within-population homozygosity (Hedrick 1999). Human activities are also  
28 responsible for the present isolation of populations. Highly developed infrastructure and intensive  
29 agricultural exploitation of the land area has fragmented the landscape. Also, many breeding sites  
30 have disappeared through drainage, pond filling and eutrophication due to agricultural activities,  
31 creating large distances between the remaining populations. In this study it was impossible to  
32 discriminate between genetic consequences inflicted by human changes of the landscape, and long  
33 term natural processes.

#### 34 35 *A recent bottleneck on Northern Funen*

36  
37 An estimation of migration rates revealed a significant amount of contemporary gene flow between  
38 the two localities on northern Funen (FHE and FHW). The asymmetric migratory pattern, showing  
39 that dispersal predominantly occurs from FHE to FHW is interesting in the light of available  
40 demographic data. The natterjack toads in this area experienced a bottleneck at the beginning of the  
41 1990s culminating in no toads being detected at FHW, and only four calling males at FHE in 1991.  
42 Following a conservation management effort including restoration of old ponds and introduction of  
43 grazing cattle, the numbers started rising in FHE and subsequently in FHW. Though the  
44 STRUCTURE analysis defined FHE and FHW as one population, a significant deviation in allele  
45 frequencies among the two sites was detected nonetheless. This is possibly the outcome of a severe  
46 decrease in population size, eliminating migration between the two localities for several  
47 generations. At present, FHE individuals are obviously in the process of recolonizing the FHW  
48 locality. As populations rebound, migration increases correspondingly, implying that with time

1 genetic differentiation will fade. The bottleneck test was significant at FHE ( $P = 0.004$ ), but not at  
2 FHW ( $P = 0.098$ ), partly confirming that the demographic bottleneck has affected the gene pools by  
3 eliminating rare alleles. Surprisingly, FHE and FHW still display the highest genetic diversity in the  
4 study (Table 2). This may be because the bottleneck was not long-lasting, that undetected  
5 individuals may have remained in the area, and/or that management has indeed been very  
6 successful. Alternatively, FHW and FHE may not have recovered through habitat restoration alone.  
7 Three suspicious features were apparent: i) a low but detectable incoming gene flow from southern  
8 Funen as discussed above, ii) the non-significant bottleneck test at FHW, and relatively high genetic  
9 diversity despite a severe demographic bottleneck in the 1990s, and iii) the presence of a private  
10 allele (Bcal $\mu$  2, 201bp) only found at FHW, where it proved quite common (13.8%) (see Online  
11 Appendix). All though only suggestive, these findings could perhaps point towards unregistered  
12 reintroduction by humans in this area.

### 13 14 *Perspectives for conservation*

15  
16 In terms of defining genetic units worth preserving, it has been argued that units should be  
17 reproductively isolated, demonstrate significant divergence of allele frequencies and represent an  
18 important component of the evolutionary legacy (for review see Fraser and Bernatchez 2001). Our  
19 analyses suggest that many Danish populations fulfil the first two criteria, but whether each of them  
20 represents an important component of the evolutionary legacy is debatable. The natterjack toad is in  
21 acute danger of becoming extinct on Zealand, with only four to five small populations remaining  
22 (Pihl et al. 2000). The neighbour joining analysis (Figure 2) defined populations from Zealand as a  
23 distinct genetic group with a bootstrap value of 99%. Since this entire branch of the gene pool is  
24 likely to have been isolated for 8000 years, we argue that it fulfils the last criteria concerning an  
25 important evolutionary legacy, at least on a national scale. Hence, immediate actions need to be  
26 taken to preserve the few remaining populations on Zealand.

27  
28 To avoid inbreeding and retain fitness in the short term an effective population size above 50 is  
29 required, but to permanently retain evolutionary potential and robustness against demographic and  
30 environmental stochasticity a  $N_E$  of 500 – 5000 is believed more adequate (Frankham 2002; Nunney  
31 and Campbell 1993). The genetic structure demonstrated in this study show that populations are  
32 isolated and hence, most of them will never attain this size. It is questionable whether these  
33 proposed threshold values are easily applied to amphibians due to the metapopulation dynamics of  
34 many amphibian populations (Smith and Green 2005) and the small and fluctuating effective  
35 population sizes that naturally occur. As an example,  $N_E/N_C$  ratios were assessed in three British  
36 natterjack toad populations and estimates varied between 0.02 and 0.56 depending on analytical  
37 method and population (Rowe & Beebee 2004). Regardless of threshold values, it is a fact that the  
38 majority of remaining Danish populations are small and isolated. To minimize extinction risks and  
39 reduce genetic drift and potential inbreeding, it is essential to establish migration corridors between  
40 populations in areas where practical. Such management strategies were recently suggested in a long  
41 term conservation plan of the West Coast areas in Jutland, partly with the aim of favouring the  
42 natterjack toad (Briggs and Adrados 2005). It has been suggested that western Jutland constitutes a  
43 single large population (Pihl et al. 2000), but this theory is clearly rejected in our analyses.  
44 However, it is evident that genetic differentiation in this region is less distinct (Figure 1, Table 4),  
45 with a mean  $\theta$  of 0.134 among GR, HA and BV, especially when considering the relatively large  
46 distances between the three sampled sites. It therefore seems reasonable to aim to reconnect  
47 populations in this area, where isolation is likely to have happened most recently. By doing so a

1 large metapopulation would be established, ensuring the presence of *Bufo calamita* in its north  
2 western biogeographic range into the future.

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13

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1 **Figures and tables**

2

3 **Figure 1:**

4 *Colour scheme:* Graphical output from STRUCTURE (Pritchard et al. 2000) for  $K = 11$  (modified in  
5 DISTRUCT (Rosenberg 2002)). Each vertical line represents an individual, and the colour  
6 composition displays the probability of belonging to each of the 11 clusters defined by STRUCTURE.  
7 *Map:* Distribution of *B. calamita* localities in Denmark (black dots) observed after 1990 (modified  
8 from Pihl et al. 2000). More recent monitoring suggests that a significant proportion of the indicated  
9 natterjack toad localities are now extinct. Grey scaled area along the west coast of Jutland  
10 represents a region, previously hypothesized to constitute a single large population. The 12 sampled  
11 localities have colours corresponding to the genetic cluster for which the majority of their respective  
12 individuals were assigned.

13

14 **Figure 2:**

15 Unrooted neighbor joining tree displaying the genetic relationship of the 12 sampled localities,  
16 based on Cavalli-Sforza and Edwards chord distances. Numbers are bootstrap values from 10,000  
17 replicates. Boxes frame regions with high bootstrap support (solid lines): Funen (AV, FHE, FHW,  
18 ES), Zealand (MB, DY) and Western Jutland (GR, HA, BV). Dashed line encompasses sampling  
19 locations within Jutland, which does not represent a genetically-distinct cluster.

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Figure 1

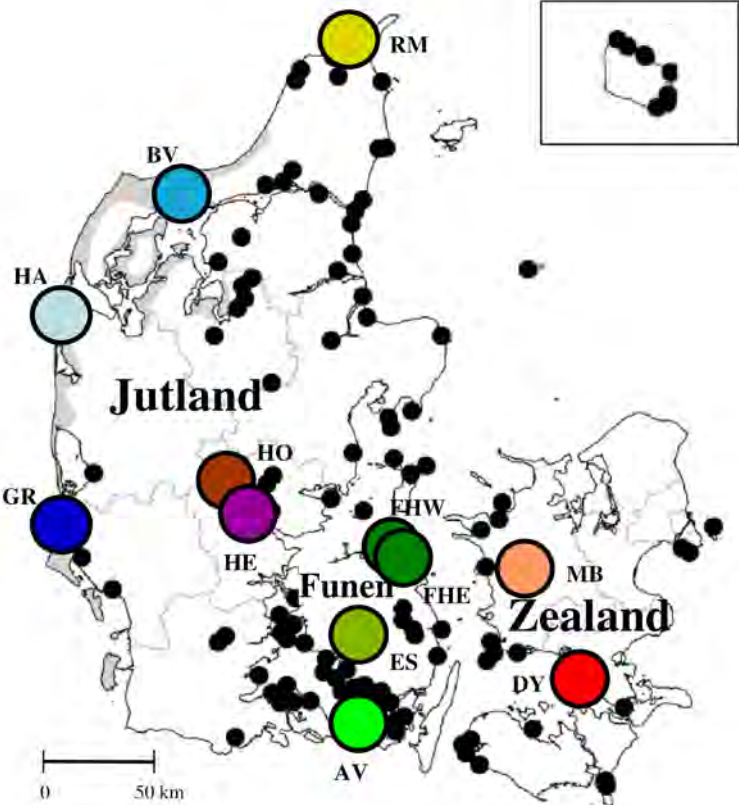
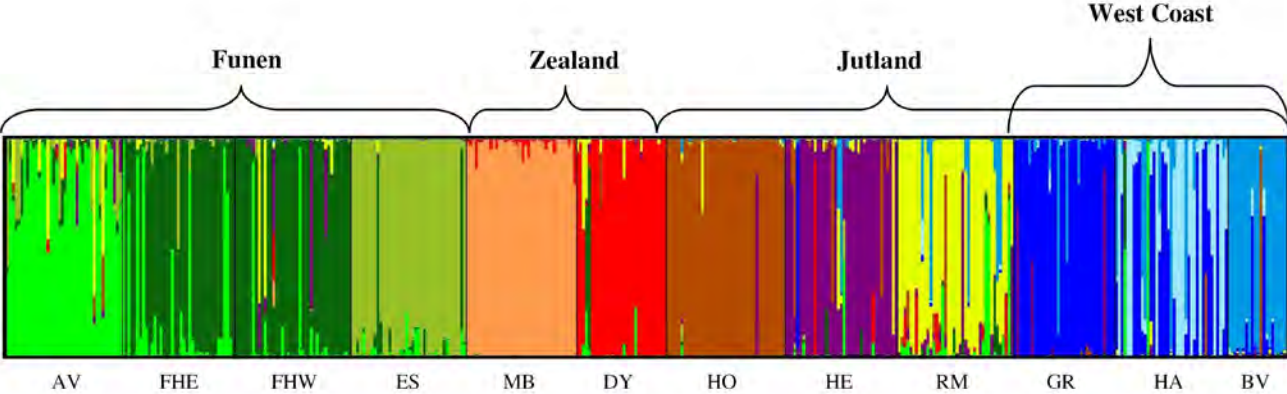
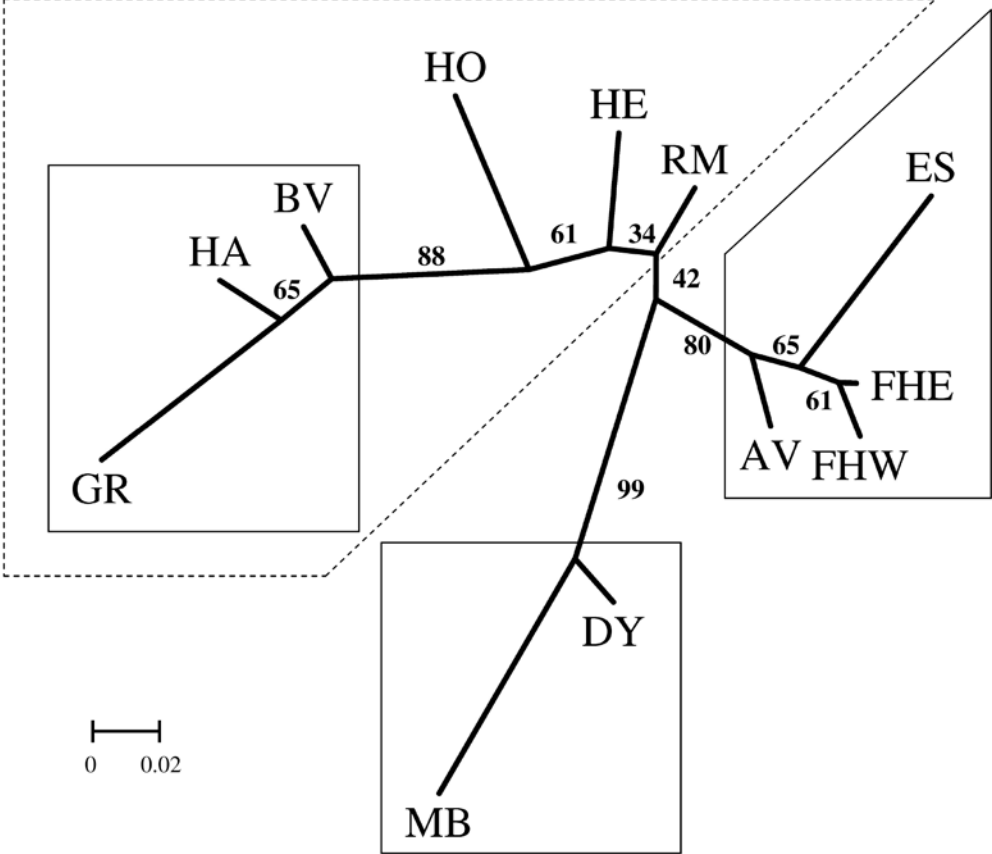


Figure 2



**Table 1**

Marker	PCR procedure	Annealing temp. I, II	Flourescent dye label	PCR mix	Number of alleles	Size range (bp)	$H_E$	$f$	Used in analyses	Used in comparison
Bcal $\mu$ 1*	A	56°	Tet	Standard	6	147-157	0.397	-0.104	x	x
Bcal $\mu$ 2*	B	50°, 52°	6-Fam	Standard	2	199-201	0.020	-0.146	x	x
Bcal $\mu$ 3*	A	50°	Tet	Standard	10	126-148	0.546	-0.078 $\alpha$	x	x
Bcal $\mu$ 4*	A	59°	6-Fam	Standard	1	208	0.000			x
Bcal $\mu$ 5*	A	50°	Hex	Standard	2	230-234	0.032	-0.259	x	x
Bcal $\mu$ 6*	B	44°, 46°	Tet	Standard	1	165	0.000			x
Bcal $\mu$ 7*	B	48°, 52°	6-Fam	Standard	2	154-158	0.219	-0.179	x	x
Bcal $\mu$ 8*	B	44°, 46°	Hex	2x (Taq, MgCl <sub>2</sub> , DNA)	7	171-195	0.316	0.051	x	x
Bcal $\mu$ 10**	A	52°	6-Fam	Standard	5	151-163	0.594	-0.078	x	
Bcal $\mu$ 11**	B	60°, 62°	Tet	Standard	3	196-200	0.368	-0.125 $\alpha$	x	
Buca 5***	A	50°	Tet	Standard	1	133	0.000			
Buca 6***	A	57°	Tet	2x (Taq, MgCl <sub>2</sub> )	5	229-239	0.378	0.002	x	

$\alpha$   $f$  values representing significant deviations from H-W proportions after sequential Bonferroni correction

\* Rowe et al. 1997

\*\* Rowe et al. 2001

\*\*\* Rogell et al. 2005

**Table 2**

Locality	$n$	$N_{CM}$	Allelic richness ( $AR$ )	$H_E$	$f$	$H_E$ Bcal $\mu$ 1-8
Avernakø (AV)	40	171	2.107	0.345	0.022	0.167
Fyns Hoved East (FHE)	39	22	2.431	0.408	-0.083	0.241
Fyns Hoved West (FHW)	40	18	2.473	0.429	-0.049	0.258
Espe (ES)	40	10	2.515	0.382	-0.201 $\alpha$	0.240
Munke Bjergby (MB)	38	57	2.601	0.183	-0.169	0.082
Dybsø (DY) *	31	35	2.060	0.308	-0.084	0.187
Honum (HO) **	41	24	2.162	0.318	-0.205 $\alpha$	0.200
Hedensted (HE) **	39	84	2.772	0.356	-0.015	0.238
Råbjerg Mile (RM)	40	250	2.055	0.297	-0.019	0.126
Grærup (GR)	35	35	1.812	0.304	-0.125	0.228
Harboøre (HA)	39	5	2.237	0.270	0.071	0.206
Bygholm Vejle (BV)	20	250	1.889	0.229	0.070	0.125

$\alpha$   $f$  values representing significant deviations from H-W proportions after sequential Bonferroni correction

\* Collected by Kåre Fog.

\*\* Collected by Lars Christian Adrados.

**Table 3**

Sampled sites	Genetic groups defined by STRUCTURE											<i>P</i> < 0.90
	1	2	3	4	5	6	7	8	9	10	11	
AV	<b>16</b> / 0.769	0.049	0.077	0.000	0.007	0.012	0.000	0.063	0.000	0.000	0.000	24
FHE	<b>4</b> / 0.204	<b>23</b> / 0.766	0.026	0.000	0.000	0.000	0.000	0.003	0.000	0.000	0.000	13
FHW	<b>1</b> / 0.064	<b>29</b> / 0.829	0.004	0.003	0.005	0.000	0.050	0.044	0.000	0.000	0.000	10
ES	0.025	<b>1</b> / 0.062	<b>31</b> / 0.910	0.000	0.000	0.000	0.001	0.002	0.000	0.000	0.000	8
MB	0.000	0.000	0.000	<b>35</b> / 0.972	0.027	0.000	0.000	0.000	0.000	0.000	0.000	3
DY	0.018	0.043	0.000	0.001	<b>25</b> / 0.890	0.008	0.005	0.036	0.000	0.000	0.000	6
HO	0.001	0.001	0.002	0.000	0.000	<b>38</b> / 0.961	0.021	0.011	0.000	0.000	0.003	3
HE	0.015	0.010	0.006	0.000	<b>1</b> / 0.029	0.069	<b>25</b> / 0.771	<b>1</b> / 0.046	<b>1</b> / 0.025	0.000	0.028	11
RM	0.072	0.023	0.001	0.000	0.027	0.030	0.054	<b>14</b> / 0.699	0.002	0.005	0.087	26
GR	0.000	0.000	0.000	0.000	0.000	0.002	0.059	0.000	<b>27</b> / 0.873	0.006	<b>1</b> / 0.060	7
HA	<b>1</b> / 0.038	0.012	0.000	0.000	0.000	0.010	0.003	0.004	<b>6</b> / 0.378	<b>11</b> / 0.428	<b>1</b> / 0.128	20
BV	0.000	0.001	0.000	0.000	0.000	<b>1</b> / 0.048	0.003	0.004	0.089	0.018	<b>14</b> / 0.836	5

**Table 4**

	AV	FHE	FHW	ES	MB	DY	HO	HE	RM	GR	HA
	<b>Funen</b>										
FHE	0.095										
FHW	0.095	0.017									
ES	0.162	0.110	0.147								
MB	0.374	0.327	0.323	0.417	<b>Zealand</b>						
DY	0.253	0.229	0.228	0.286	0.214						
HO	0.234	0.267	0.232	0.263	0.412	0.236	<b>Jutland</b>				
HE	0.224	0.209	0.167	0.269	0.369	0.221	0.182				
RM	0.173	0.205	0.171	0.288	0.402	0.193	0.199	0.122			
GR	0.409	0.370	0.297	0.411	0.598	0.450	0.282	0.232	0.322	<b>West Coast</b>	
HA	0.374	0.368	0.308	0.384	0.610	0.450	0.264	0.264	0.266	0.136	
BV	0.393	0.326	0.260	0.364	0.624	0.448	0.297	0.205	0.260	0.134	0.131

## Table captions

### Table 1

PCR conditions and selected characteristics of the 12 microsatellite markers screened in this study.  $H_E$  = expected heterozygosity. A combination of nine polymorphic markers were used for analyses in this study, but another combination of markers (Bcal $\mu$  1-8) were used when comparing genetic diversity across natterjack toad studies.

### Table 2

The sample sizes ( $n$ ), harmonic mean of number of calling males ( $N_{CM}$ ) and expected heterozygosity ( $H_E$ ) for each sampling site across the 9 loci.  $f$ -values indicate accordance of observed genotypes to expected Hardy-Weinberg proportions. The final column displays values of  $H_E$  using only the markers Bcal1-8, relevant when comparing with previous genetic studies of natterjack toads from other regions.

### Table 3

Results from STRUCTURE (Pritchard et al. 2000) for  $K = 11$  with mean posterior probabilities of belonging to a cluster for each locality. Numbers in bold refer to the number of individuals assigned to one of the 11 clusters with a probability  $\geq 0.90$ . Numbers in the last column refer to individuals below this assignment threshold.

### Table 4

Genetic differentiation among localities measured as pairwise  $\theta$  values. Boxes frame values within each of the three major regions: Funen (AV, FHE, FHW, ES), Zealand (MB, DY) and Jutland (HO, HE, RM, GR, HA, BV). The three sites located within the grey scaled area of Western Jutland (GR, HA, BV) (Figure 1) are also framed.

### Online Appendix:

Allele frequencies for each of the 9 variable microsatellite loci within each population. Observed and expected heterozygosity ( $H_O$  and  $H_E$ ) and level of significance in H-W tests are listed for each locus/population combination and overall. Numbers of successfully genotyped individuals are also shown for each locus/population combination.