

The genetic structure of natterjack toad (*Bufo calamita*)
populations and the role of the river

Diploma thesis by

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ZUSAMMENFASSUNG	3
ABSTRACT	3
INTRODUCTION	4
METHODS	6
Study area and study sites.....	6
Sampling	7
Molecular analysis	8
Analysis of genetic diversity	8
Hardy-Weinberg equilibrium	9
Linkage equilibrium.....	9
Founder effects and genetic drift	9
Admixture of subpopulations	10
Sibling effect.....	10
Nonrandom mating.....	11
Genetic structure	11
Population structure.....	12
The role of the river as a barrier to gene flow.....	13
Quantification of the barrier effect.....	14
RESULTS	16
Analysis of genetic diversity.....	16
Hardy-Weinberg equilibrium	16
Linkage equilibrium.....	17
Small populations and founder effects.....	17
Admixture of subpopulations	17
Sibling bias.....	17
Genetic structure	18
Population structure.....	18
The role of the river as a barrier to gene flow.....	18
Quantification of the barrier effect.....	19
DISCUSSION	20
Population structure.....	20
Genetic diversity	22
Some methodological remarks.....	22
Conclusions.....	23
ACKNOWLEDGMENTS	24
REFERENCES	25
TABLES	28
FIGURES	35
APPENDIX	41

ZUSAMMENFASSUNG

Mit der fortschreitenden Habitatfragmentierung durch menschliche Aktivitäten ist im Naturschutz die Problematik von zerteilten Populationen immer mehr in den Blickpunkt gerückt. Probleme wie Isolation und Inzucht, die daraus für kleine Populationen entstehen, geben zu Besorgnis Anlass. Anhand genetischer Methoden ist es möglich festzustellen, wieweit einzelne Populationen isoliert sind, und wie die Gruppierungen von Populationen durch geographische Strukturen beeinflusst werden.

In dieser Studie habe ich zehn Kreuzkrötenpopulationen im unteren Reusstal untersucht, die von der Reuss in zwei Gruppen geteilt werden. Im Mittelpunkt der Studie stand die genetische Strukturierung der Populationen und der Einfluss, den die Reuss auf diese Strukturierung hat. Acht Primer für Mikrosatelliten standen für die genetische Untersuchung zur Verfügung. Die angewandte F-Statistik (mit den Parametern F_{st} und R_{st}) betrachtete die genetische Struktur innerhalb und zwischen den Populationen und untersuchte den Effekt des Flusses als Barriere für Migration und Genfluss. Einige statistische Probleme müssen noch geklärt werden, bevor endgültige Schlüsse aus dieser Arbeit gezogen werden können.

Die Populationen unterschieden sich in ihrer genetischen Zusammensetzung signifikant sowohl innerhalb als auch zwischen den beiden Flussufern. Auch die phylogeographische Stammbäume zeigen auf, dass der Fluss als Migrationsbarriere wirkt.

Diese Erkenntnisse haben Einfluss auf weitere naturschützerische Projekte. Die kleinräumlichen Strukturierungen von Populationen sollten in Betracht gezogen werden, falls zur Unterstützung von Populationsgrössen Translokationen in Betracht gezogen werden. Alle untersuchten Populationen sind klein bis sehr klein und sollten durch Habitatverbesserungen gestärkt werden.

Die genetische Vielfalt dieser schweizer Populationen ist trotz der Kleinheit der Populationen grösser als die englischer Populationen. Dies könnte durch die biogeographische Vergangenheit dieser Art und ihre Ausbreitung in Europa nach der letzten Eiszeit erklärbar sein.

ABSTRACT

Because of ongoing habitat fragmentation through human activity, population subdivision has become of a major interest for conservation biologists. While larger scale studies try to define the evolutionary significant units (ESU) of endangered species, more local studies are concerned about the degree of isolation and inbreeding of small populations. In this study I examined the genetic structure of ten local natterjack populations in the lower valley of the river Reuss in Switzerland, which are divided in two groups by the river. Genetic data was obtained from eight microsatellite marker loci. Hierarchical F-statistics (R_{st} and F_{st}) were used to determine the genetic structure within and among breeding sites and to examine the effect of the river as a barrier to gene flow. I found significant differentiation between populations and between the two river banks. Furthermore, phylogenetic trees were generated which suggest that the river might be a barrier to gene flow. Resulting consequences for conservation plans of *B. calamita* are discussed. The genetic diversity of the populations is compared to British populations, and discussed in the context of the natural history of *B. calamita*. Implications of linkage disequilibrium between loci on statistical power of measures of population structure are mentioned.

INTRODUCTION

The natterjack toad (*Bufo calamita*) is an endangered amphibian species in Switzerland (Grossenbacher, 1994) and legally protected since 1967 (BNH, 1966; VNH, 1966). Its primary habitat in Central Europe - mainly flooded plains along rivers and shallow riverbanks - was destroyed as a consequence of human activities like agriculture, correction of rivers, and urban development. Extant populations live in secondary habitat such as gravel pits and nature reserves which often are isolated by hostile land (Golay, 1996; Grossenbacher, 1988). As many other amphibians, adult natterjack toads are mainly sedentary and show some degree of site fidelity. The main dispersers are the juveniles. Sinsch (1997b) observed that postmetamorphic juveniles migrate several 100 m in a few days after they left their home pond. However, no direct estimates of maximal dispersal distances from recaptures are available so far. Observations of first breeders at sites with no successful reproduction allow indirect estimates of minimal migration distances of 3 to 5 km from the next site (Sinsch, 1997b; Sinsch and Seidel, 1995). Among adults, females seem in general more likely to switch between breeding areas over years than males (Seidel, 1994; Sinsch, 1998). Site fidelity and dispersal ranges of only a few kilometres predestine natterjack toads and amphibians in general as study organisms for the effects of population differentiation and restrictions to gene flow (Rowe et al., 1998).

Several methods have been applied to analyse the genetic structure of natterjack populations. Schröder (1993) used enzyme electrophoresis and showed genetic differentiation between populations in Germany. Hitchings and Beebee (1996) on the other hand did not find enough allozyme polymorphism in British populations for phylogeographic analyses. Rowe et al. (1997) designed microsatellite markers for natterjack toads. Using these markers, they examined the genetic structure of British natterjack populations on a large geographic scale where they found strong genetic differentiation among populations separated by geographic distances from less than 1 to more than 200 km (Rowe et al., 1998). Microsatellites are a group of tandem repeat DNA markers of usually non-coding DNA and a high mutation rate of 10^{-3} to

10⁻⁵ which manifests in a high genetic diversity (Bowcock et al., 1994; Estoup et al., 1998).

This variability makes them a good measure to study intraspecific genetic variation on ecological time scales (Bowcock et al., 1994; Rank, 1992).

In this study I analyse the genetic structure of ten local natterjack populations in Switzerland.

Although population density in the study area is high compared to other regions of Switzerland (Grossenbacher, 1988), many populations are very small and probably geographically isolated due to lack of suitable habitat. This combination of small size and isolation can cause inbreeding depression and loss of genetic variation in populations by random drift and thus increase their probability of extinction (Meffe and Carroll, 1997). First, I analyse two crucial aspects of these mechanisms, namely the genetic structure and the degree of isolation between populations (fixation index), and the state of inbreeding within populations (inbreeding coefficient).

Second, I analyse if the river as a large habitat structure, which could be a barrier to gene flow, does enhance population differentiation. So far it is assumed that rivers might restrict migration in *B. calamita* and therefore increase population differentiation, but no tests were made (Golay et al., 1995). Although natterjack toads were found to breed at shallow edges of lakes and rivers, in the study area no populations are known to breed at the river bank at least since the river correction in the last century. Additionally I quantified the effect of the river in terms of extra geographic or genetic distances between across-river population pairs. If the river has a strong effect, it would be recommended to take this population structure into account when planning conservation management actions such as translocations for increasing small population sizes.

METHODS

Study area and study sites

The study area lies in the eastern part of Switzerland in the lower valley of the river Reuss (between 8°16' and 8°28' longitude and 46°37' and 46°54' latitude; fig. 1a, 1b). The average altitude of the valley is 385 m above sea and it is enclosed by hill sides which rise up to 450 and 600 m above sea. The river Reuss that runs through the valley is approximately 20 m in width. It has been dammed since the middle of the 19th century (Schelbert et al., 1995).

In this study I examined ten populations of *B. calamita*, which were all found in secondary habitat as nature reserves or gravel pits. Four sites lie within the plane of the valley, one site north of the plain in the river valley, and five sites on the hill sides. Five sites lie on each side of the river (fig. 1c). Although each site consists of several ponds and puddles, individuals at each site are considered one population. These ponds and puddles within a site all are less than 100 m apart whereas the distances between the sites are between 756 m and 28 km. Night censuses did not meet the minimal standard recommended by Golay *et al.* (1996; at least three counts in nights of good conditions), but still give evidence about the actual population size together with information of the last ten to fifteen years about the rough population size at the sites.

Population size was determined in two ways. First by night counts in two or three night during the breeding season 1998. Only the night with the highest number of individuals was used.

Second, data from surveys after 1980/81 were used. The latter were grouped in size classes.

The classes are: 1 = 1-5 individuals counted, 2 = 6 - 30 individuals, 3 = 31 - 100 individuals, 4 $\geq$ 100 individuals. These size classes are minimal estimates, because they usually rely on calling males, which represent at most between 40 to 50% of all males (Golay, 1993a; Golay et al., 1995).

Sampling

B. calamita is legally protected in Switzerland. Sampling adults would have meant toe clipping which could harm the individuals when infections occur (Golay, 1996). Because losses at the adult stage have a higher impact on the demography of a population than losses at an early life stage I only sampled tadpoles with the permission of the environmental departments of all three cantons. Due to the prolonged breeding season each site was visited between five and eight times during the breeding season from 10th of May to 4th of August 1998. At each site all ponds and puddles with tadpoles or spawn were sampled. Two kinds of samples were taken at each visit: From each spawn string some eggs were collected and raised in the lab. From each pond a sample of already freeswimming tadpoles ("mixed samples") was collected and kept separately. Because females only exceptionally lay more than one spawn string per year (Banks et al., 1993), tadpoles from different spawn strings could be half sibs if they share the same father, but it is more likely that they have different fathers and are not related. Also, tadpoles sampled from the same spawn string were considered full sibs, whereas tadpoles from mixed samples were considered individuals from an unknown number of families (i.e. spawn strings). Tadpoles from 44 mixed samples and 47 single spawn strings were collected from all the populations (tab. 2). Generally in the first half of the sampling season more samples in total and more spawn strings were collected than in the second half (tab. 2).

For the genetic analysis 40 tadpoles from each site were analysed. Those tadpoles were preserved in 70% ethanole. All other tadpoles that I had raised in the lab were released to their original pond if possible or, if the ponds had dried out, at least within their original site. To solve the problem of overrepresentation of the full sibs (spawn strings) in the final sample of 40 tadpoles per site, I gave the full sib and the mixed samples a different weight: It is very likely that the mixed samples contain tadpoles from more than one family, because different developmental stages were observed among freeswimming tadpoles during sampling. Also because the adults counted at night and especially the females were not very abundant it is not very likely that many females spawned in one pond between two sampling events. According to these observations I decided that the tadpoles in the mixed samples originated in average from

three spawn strings. Therefore for the final sample of forty tadpoles three times more individuals were taken from mixed samples than from spawn strings. Like this each (hypothetical) family was represented by the same proportions. In cases where it was not possible to divide the proportions evenly among the families I took more tadpoles from the mixed sample.

Molecular analysis

For each population 40 individuals were analysed. DNA was extracted with Chelex-100 (Walsh et al., 1991) from tissue of the tail tips, and amplified in a PCR using eight polymorphic microsatellite loci *Bcal1* - *Bcal8* (GenBank accession numbers X99281 -X99288). The DNA fragments were labelled with [³³P] and alleles were separated by electrophoresis on 6% polyacrylamide gels and scored with M13 size markers as reference (Rowe et al., 1997). For further details on primer sequences, PCR conditions and modification of DNA extraction with Chelex-100 see Rowe et al. (1997; 1998).

Analysis of genetic diversity

Genetic diversity of the populations was analysed based on mean number of alleles per locus (mean a/l), mean polymorphism of the loci at the 95% criterium (95%), mean expected and mean observed heterozygosity (H_E and H_O , respectively). All calculations were carried out with BIOSYS-1 (Swofford and Selander, 1981). H_E was calculated from the allele frequencies per site under Hardy-Weinberg equilibrium (HWE), H_O from direct count of the heterozygous individuals in the samples.

It was tested if a correlation exists between age of the population and the different measures of genetic diversity like mean a/l , 95%, H_O or H_E . Also the correlation between the two measures of population size and the diversity measures was examined. For all three correlation sets Spearman's rank correlation was used.

Hardy-Weinberg equilibrium

All loci at all sites were tested for random association of alleles with GENEPOP v3.1d (Raymond and Rousset, 1995). Either a complete enumeration method (Louis and Dempster, 1987) was applied for loci with less than five alleles or a Markov chain method to estimate an unbiased p-value (Guo and Thompson, 1992) (1000 dememorizations, 100 batches and 1000 iterations per batch) for more than five alleles. Probability of multiple tests was corrected with sequential Bonferroni (Rice, 1989). Disequilibrium could arise for several reasons: selection, mutation, migration, or non-random mating (Weir, 1996). The loci were then tested for alternative hypotheses of heterozygote excess or deficit. Heterozygote deficit may be caused by inbreeding, the presence of null alleles, or by the admixture of subpopulations in one sample (Wahlund's effect). Heterozygote excess on the other hand would be caused by balancing selection, which is not assumed in microsatellites.

Linkage equilibrium

Linkage equilibrium, the random association of gametes (Hartl, 1988), was analysed with Fisher's exact test in GENEPOP 3.1d (Raymond and Rousset, 1995). Each pair of loci in each population was tested for linkage equilibrium. Bonferroni corrections were used to assess probability values (Rice, 1989). Because a considerable amount of linkage disequilibrium was found in the population I further examined the mechanisms that could cause disequilibrium. Some mechanisms like selection or physical linkage on the same chromosome can be neglected because the microsatellites are supposed to be selectively neutral and were sampled randomly from the whole genome (Rowe et al., 1997). But others seem probable for microsatellites and should be examined.

Founder effects and genetic drift

When only few individuals are founding a new population, they can introduce linkage disequilibrium, which might still be present at the time of sampling, when populations are still young (Hartl, 1988). Purely by chance some chromosome may drift to high frequency due to

finite population size (Hartl, 1988; Maynard Smith, 1998). In this study most populations are young and still rather small. Hence, any of those mechanism could likely play a role in these populations. The correlation between the two measures of population size and the number of locus pairs that are found to be in linkage disequilibrium as well as the correlation between the age of the populations and locus pairs in linkage disequilibrium was analysed with Spearman's rank correlation.

Admixture of subpopulations

Admixture of populations with differing gametic frequencies cause linkage disequilibrium (Hartl, 1988). It is possible that the populations in this study are structured into subpopulations. Most likely these substructures would be the ponds within a site. Thus the spawn and mixed samples collected from each pond were pooled within ponds and tested for linkage disequilibrium. The number of locus pairs in linkage disequilibrium should be reduced when each pond is analysed by itself. The number of locus pairs in disequilibrium within each pond was compared to the sample size of this pond (Spearman's correlation coefficient) because sample sizes were very small (appendix 3) and I suspected an effect of sample sizes. Only ponds with >2 individuals sampled were included.

Sibling effect

Siblings only represent a subset of all possible genotypes of a population. If one sibship is overrepresented in the samples this would cause linkage disequilibrium among their parental alleles. To avoid any disproportion of siblings in the samples despite of the weighing regime mentioned above, I reduced the number of siblings in my data set in two steps. First, only one individual was analysed from each spawn string but all individuals from the mixed samples were kept. This removes all known siblings. The new data set was tested for linkage equilibrium. In a second step the conditions were more stringent: To rule out any sibship also in the mixed samples I analysed only one individual from each spawn string and mixed sample. Like this any possibility for sibships was removed. However, it should be noted that the sample sizes drop below ten individuals for most sites and to 20 and 22 in two populations when only one individual is analysed from each sample. Another test for linkage disequilibrium

was carried out with this data set and the number of locus pairs in disequilibrium compared to the complete samples.

Nonrandom mating

Nonrandom mating can lead to linkage disequilibrium, because as a consequence there is correlation between alleles at different loci in the same gamete (Falconer and Mackay, 1996; Futuyma, 1998). Because no direct demographic data is available about the study populations there is no possibility to test for this.

Genetic structure

Hierarchical population structure can be estimated with Wright's F-statistics (Wright, 1951) which measures the proportion of total genetic diversity (expressed as expected heterozygosity) due to divergence among subpopulations (Mills and Allendorf, 1996). I used two different estimates of hierarchical F-statistics. One was Weir and Cockerham's unbiased analogous of Wright's F-statistics, which corrects for multiple alleles and loci, and unequal sample sizes (Weir and Cockerham, 1984). The other estimate was Slatkin's unbiased R_{st} (Slatkin, 1995). F-statistics are based on an infinite allele model and assume that every mutation gives rise to a new allele. R_{st} on the other hand additionally considers the particular stepwise mutation mechanism of microsatellites where each mutation is an addition or deletion of (generally) one repeat unit. Estimates of R_{st} rely on the assumption of these single step mutations, which is still under debate (Di Rienzo et al., 1994). It includes information about allele sizes and accounts for homoplasies. There seems to be a bias between deletion and addition of repeat units and allele size is limited by maximum sizes (Di Rienzo et al., 1994). When the stepwise mutation model is correct, R_{st} gives more appropriate measures of population differentiation. However, if these assumptions are not met, F_{st} turns out to be more robust (Slatkin, 1995). Thus, where possible, both F_{st} and R_{st} estimates are given. F_{st} was calculated with TFPGA (Miller, 1997) except for differentiation within loci across all populations and for differentiation among ponds within sites. For those two tests GENEPOP v3.1d (Raymond and Rousset, 1995) was used. 95% confidence intervals were computed with 1000 permutations, standard errors with 1000

bootstraps on TFPGA, whereas GENEPOP does not give estimates of significance. Slatkin's R_{st} was calculated with R_{st} Calc v2.2 (Goodman, 1996). Significance level of R_{st} was determined with 1000 permutations, 95% confidence intervals and standard errors were assessed with 1000 bootstraps.

The population structure was examined in two parts. Firstly, the genetic structure within and among populations was analysed. Then the role of the river as a barrier to gene flow was examined. The notation I used for both F-statistics refers to the hierarchical levels analysed. For example F_{ps} (or R_{ps}) describes the differentiation between ponds (p) compared to the site (s), or F_{rt} (R_{rt}) describes the differentiation between the river banks (r) compared to the total of all populations (t). The hierarchical levels of population (sub)structures are described in table 1.

Population structure

First the genetic structure between populations was analysed across all loci, and then also separately calculated for each single locus.

The genetic differentiation between ponds within sites was calculated.

I also analysed the possibility of temporal populations. In Germany genetically different temporal populations were found (Schröder, 1993; Sinsch and Seidel, 1995). Sinsch (1998) argues that this is possible because males and females do not breed throughout the whole season but some breed earlier and some later. Therefore toads breeding at the same time but at different sites could be more closely related than toads breeding at the same site but at different times of the breeding season. Temporal subdivision of populations seems to be a regional phenomenon, though, which was found in Germany, but not in England (Sinsch, 1998) or the Alsace close to Basel (N. Golay, pers. comm.). For this analysis the sampling period was halved in an early (10th of May - 20th of June) and a late (21st of June - 4th of August) generation. Only ponds where both samples from the early and late generation were collected were included in this analysis. The individuals from those samples were pooled across all ponds into two groups of early and late within each site.

The role of the river as a barrier to gene flow

The river is expected to prevent migration between populations and should therefore cause additional differentiation between populations. Five populations on the east and west bank of the river were analysed (fig. 1c). I tried several approaches to analyse the effect of the river, namely F-statistics, isolation by distance patterns, and phylogenetic trees.

First, the populations were pooled within the river sides east and west, and the genetic differentiation within and among the river sides was computed with Weir & Cockerham's F_{st} and Slatkin's R_{st} .

Isolation by distance, which was first introduced by Wright (1943), describes the accumulation of local genetic differences under geographically restricted dispersal (Slatkin, 1993). If no other migration barriers than the geographic distance between populations occur, a pattern of isolation by distance should be found. However, if migration barriers are present, genetic divergence should not correlate with geographic distance. Pairwise genetic differentiation estimates of Slatkin's R_{st} were computed with R_{st} Calc 2.2 (Goodman, 1996), and geographic distances between population pairs were calculated from their coordinates. Both are summarised in a semimatrix (Appendix 4). The significance of the correlation was calculated with the Mantel test (Mantel 1967) with 10'000 permutations using GENEPOP v3.1d. R_{st} was normally distributed but the geographic distances could not be transformed to reach normal distribution. This might not be so much of a problem because in contrast to Mantel's original calculation of the statistic Z , GENEPOP v3.1d uses Spearman's rank correlation and not a parametric test. The Mantel estimate of the correlation was very robust to any transformation of the data (results not shown). From the ten populations all 45 resulting pairs were tested. Because the populations on the east bank of the river are clustered further north than the populations on the west bank (fig. 1c), the largest distances are also between populations across the river (fig. 2). The effect of isolation by distance and the presumed barrier effect of the river are thus very much entangled and it is impossible to tell them apart with this approach. To get an idea to what extent the three largest distances between the populations *AG8* and *LU12*, *LU118*, *LU234* affect the result of isolation by distance, I also calculated the Spearman's correlation between the geographic and

genetic distances. Although this test does not correct for multiple replicates as the Mantel's test does, it is sufficient to point out the influence these largest three distances have on the overall correlation. Isolation by distance was also computed separately among the populations of each river bank.

In the third approach two unrooted phylogenetic trees were built using the PHYLIP 3.5c program package (Felsenstein, 1993). This analysis is independent of geographic distances. The trees are based on 1000 times bootstrapped allele frequencies made with the program SEQBOOT. Bootstraps give probability values of the branching. The two methods are different in their underlying model. UPGMA (Unweighed Pair Group Method with Arithmetic means) uses a clustering algorithm and genetic distance data to build a tree. Here I used the Cavalli-Sforza chord distance estimates (Cavalli-Sforza and Edwards, 1967) as genetic distance. The calculations of the tree from each bootstrapped data set was carried out with the program NEIGHBOR. The second tree was built with an optimality criterion (maximum likelihood) which uses nucleotide data. The trees were calculated in the program CONTML. Consensus trees of the bootstrap trees were calculated with CENSUS for both methods and drawn in DRAWTREE. For better illustrations the consensus trees were further processed in a drawing program.

Quantification of the barrier effect

The barrier effect of the river on gene flow and population differentiation can be seen as equivalent to the effect of a larger geographic distance and its corresponding increase in population differentiation. To quantify the strength of the barrier effect in terms of geographic distance several geographic distances from 1 to 30 km - called "barrier distances" - were added in the distance matrix to the 25 population pairs that lie across the river. Isolation by distance was calculated with the Mantel test and also the corresponding Spearman's correlation coefficient r_s value was calculated for better illustration. For each barrier distance the mean of ten replicates was computed. The barrier distance which yielded the most significant isolation by distance was assumed to best represent the real strength of the barrier effect. As a control

that the obtained result is not random, I carried out a randomization test where the "course" of the river was artificially changed. The beforehand obtained best barrier distance was added to 25 randomly chosen population pairs in the distance matrix either across the river or on the same side of the river. 100 such random distance matrices were created and the correlation estimate of isolation by distance of each matrix with the genetic distance matrix calculated. A probability estimate of the statistics of the real course of the river was obtained by summarising p-values from the simulation matrices that are smaller or equal to the real statistics and dividing them by 100.

Another way to approach the same problem is by taking the genetic distance into account instead of the geographic distance. As a standard for isolation by distance without the river effect the function of the regression lines $Rst = b(distance) + a$, obtained from the two river banks (tab. 13), were used to extrapolate the the genetic distances expected between population pairs across the river according to their geographic distance. I also calculated a mean regression line by averaging the constants a and b of the regression function. These "weighted" genetic distances were included in the distance matrix and the resulting correlation with the geographic distances was calculated and compared to the unweighed value.

RESULTS

Analysis of genetic diversity

A summary of allele frequencies of all loci in all populations is given in Appendix 1a. All loci were polymorphic. Loci *Bcal2* and *Bcal8* were fixed for the same allele in two populations each (Appendix 1a). Nine private alleles were found in five populations. Five of those private alleles were found in population *AG8*, which is the most northern population and large compared to the other populations. Four additional alleles were shared between populations within river banks, one on the east bank and three on the west bank (Appendix 2). Mean number of alleles per locus, percentage of loci polymorphic, and mean observed and expected heterozygosity (H_O and H_E) for each site are summarised in table 4. In both, mean allele number and H_E , site *ZH45* has the lowest values. Also at this site only 75% of the loci are polymorphic.

Compared to the British populations (tab. 4) more genetic diversity was found within populations although the Swiss populations in most cases are much smaller (Rowe et al., 1998). No correlation was found between population size (night counts of males 1998 or size class) or age (tab. 3) and measures of genetic diversity. Only the correlation between age of the population and mean number of alleles per population showed some positive tendency ($r_s = 0.648$, $p = 0.0517$).

Hardy-Weinberg equilibrium

15 out of 80 possible locus by site pairs (18.75%) were in HW disequilibrium, and after sequential Bonferroni correction (Rice, 1989) it were still 10 of 80 (12.5%) (tab. 5). When tested for heterozygote excess or deficit, one locus in *ZH47* showed an excess and two loci in *ZH45* a deficit of heterozygotes. HW disequilibrium was evenly distributed across populations ($\chi^2 = 11$, $df = 9$, $p < 0.05$) and loci ($\chi^2 = 1.53$ $df = 7$, $p < 0.05$). Thus there is no specific locus that shows systematic HW disequilibrium.

Linkage equilibrium

After sequential Bonferroni correction 10% (28 of 280) of all locus pairs were in linkage disequilibrium, and the populations show between zero and 15 loci pairs in linkage disequilibrium (tab. 6). More than half of those loci pairs were found in population ZH45 which is significantly different from an even distribution ($\chi^2 = 64.14$, $df = 9$, $p < 0.05$). After removal of ZH45 the distribution is even ($\chi^2 = 9.88$, $df = 8$, $p > 0.05$). Also there is no systematic linkage between particular loci pairs ($\chi^2 = 23.0$, $df = 27$, $p > 0.05$). This supports the assumption that the loci are not physically linked on the same chromosome but the linkage disequilibrium must have other causes.

Small populations and founder effects

There was no significant correlation between the number of loci pairs in linkage disequilibrium and any of the measures of size or age of the populations. Only the correlation between the number of males counted in 1998 and amount of disequilibrium shows a slight trend to a negative correlation ($r_s = -0.503$, $p = 0.1313$).

Admixture of subpopulations

No decrease but a slight increase from 15 to 17 loci pairs in linkage disequilibrium was found when tested within ponds (Appendix 3). Still more than half of the loci pairs were found in ZH45 in one pond. The correlation of the number of loci pairs in linkage disequilibrium and the sample size of the ponds is significant with ($r_s = 0.602$, $p = 0.0008$) or without ($r_s = 0.564$, $p = 0.0024$) the ponds from site ZH45. This indicates decreasing power of the test to detect linkage disequilibrium.

Sibling bias

The number of linked loci pairs decreases from 10% to 7.9% with the removal of known siblings and to zero percent with the removal of all potential siblings from the samples. The decrease of linked loci pairs also goes along with a decrease in sample size.

Genetic structure

Population structure

The differentiation between populations is significantly different from zero in both F_{st} and R_{st} . The overall differentiation among sites is 0.1722 and 0.1950 for F_{st} and R_{st} , respectively (tab. 7). The inbreeding coefficient within populations (F_{is}) is not significantly different from zero. When single loci are analysed across all populations the following pattern is found: Five of the loci (*Bcal1* - 5) show low positive inbreeding coefficients within sites, but most differentiation among sites (tab. 8). At the loci *Bcal6* and *Bcal7* a high heterozygote deficiency is found ($F_{is} = 0.1054$ and $F_{is} = 0.1118$, respectively). *Bcal2* and *Bcal8* show heterozygote excess within the sites ($F_{is} = -0.0774$ and $F_{is} = -0.1085$, respectively).

A summary of genetic differentiation among ponds within sites is given in table 9. Site *ZH47* has a highly negative inbreeding coefficient within ponds ($F_{ip} = -0.1789$). Differentiation between ponds within sites (F_{ps}) is quite high in some populations (tab. 9). Especially site *ZH45* shows three times more differentiation between the two ponds ($F_{ps} = 0.4634$) than the site *AG27*, which has the second largest differentiation between ponds. The overall inbreeding coefficient F_{is} within sites is positive for most sites. Only site *AG8* shows almost no heterozygote deficiency whereas *ZH47* shows a clear heterozygote excess.

No significant differentiation between the generations early and late are found (tab. 10) when analysed by F_{st} . R_{st} between the two generations is small but significantly different from zero.

The role of the river as a barrier to gene flow

When analysed separately, both river banks show very similar hierarchical genetic structure (tab. 11). The genetic differentiation mostly among sites is $F_{sr} = 0.1452$, and 0.1362 for the east and west bank. The differentiation between the river banks - F_{rt} and R_{rt} - is small but different from zero (tab. 12). In relation to the total differentiation F_{it} , it is about 40%.

Significant isolation by distance was found with the Mantel test ($p = 0.007$). The Spearman correlation between genetic and geographic distance is highly significant when all population pairs are included ($r_s = 0.509$, $p = 0.0007$), but is lower ($r_s = 0.466$, $p = 0.0028$) after removal of those largest distances between *AG8* and *LU12*, *LU118*, *LU 34* (fig. 2). Among the

populations within each river side isolation by distance is less strong. On the west bank no isolation by distance is found ($p = 0.252$, Mantel test), while on the east bank the effect was significant ($p = 0.017$, Mantel test). The smaller sample sizes of within-river side tests than across-river should be mentioned. Similar to these results the Spearman's correlation is only significant across all population pairs ($r_s = 0.458$, $p = 0.0247$) but not within river banks ($r_s = 0.600$, $p = 0.0719$ for east, $r_s = 0.406$, $p = 0.2232$ for west).

The two unrooted trees show an almost identical pattern (fig. 3a,b). In both trees the populations of each river bank are grouped in two clusters. Only within the east bank population ZH45 is placed differently in the two trees. Thus this pattern is quite consistent although the bootstrap values for the branching nodes are not very high in most cases.

Quantification of the barrier effect

A barrier distance of additional 4 - 6 km gives the best correlations of geographic and genetic distance between population pairs across the river (fig. 4a, 4b). I decided to use the barrier distance of 5 km as a mean representative. When this distance is added to the across-river population pairs, the significance of the isolation by distance correlation increased one order of magnitude ($p = 0.0007$). Further, the randomization test was significant for the 5-km barrier distance ($p = 0.03$).

The "weighted" R_{st} values inferred from the regression lines within river banks, show much higher correlation in isolation by distance ($p = 0.0004$) than the real genetic distances ($p = 0.007$). The mean R_{st} values resulting from averaging the regression variables give the lowest p-value in the corresponding Mantel test (tab. 13).

DISCUSSION

Population structure

This study demonstrates that on a local scale natterjack populations are clearly differentiated between sites. Averaged across all populations, no significant inbreeding coefficient within populations is found which indicates random mating within populations, although some loci show heterozygote deficit or excess (tab. 8). However, when analysed separately, most sites express positive inbreeding coefficients F_{is} (tab. 9). These inbreeding coefficients seem to reflect somehow the size of the breeding population, because the two sites that show no inbreeding (AG8) or negative inbreeding coefficient (AG47) are also the sites with most samples collected and thus with the highest (minimal) number of breeding females in the year of the study (tab. 2). Although I cannot estimate the effective population size, the counted population sizes seem to be very small and most of the populations might have a problem of inbreeding. This is also confirmed by the Hardy-Weinberg and linkage disequilibrium found in the populations.

Substructures between ponds within sites were found. Ponds show a general heterozygote excess and rather high differentiation among ponds (tab. 9). But because most sample sizes are small (Appendix 3) these results should be taken with caution. The same goes for the reduced sibling bias. Final sample sizes are so small that any effect found might as well be a sample size effect. Therefore no definite conclusions about the mechanism causing the linkage disequilibrium can be drawn.

Temporal populations as they were found in Germany could exist, but the results are somewhat ambiguous, with R_{st} indicating slight differentiation but F_{st} does not indicate significant differentiation. First of all it should be mentioned that the division into an early and a late generation by halving the sampling period is very crude, because I do not have any information about the immigration waves of males and females. The immigration waves are dependent on rainfall and temperature and can vary between years (Sinsch, 1998). There is also the possibility that the pattern found with temporal populations could be caused by other reasons

like genetic differentiation between age classes in a population (Beebee, pers. comm; Scribner et al, 1997).

In this study I present good evidence that a river of medium size can increase population differentiation. Although the degree of differentiation caused by the river is rather small compared to the "background" differentiation between all population pairs, it is very consistent for different measurements. From the results of the F-statistics it could be argued that also other combinations of ten populations divided in two groups of five each could by coincidence give the same results. But evidence gained from the phylogenetic trees contradicts this.

Genetic differentiation was measured with neutral markers. Thus no inferences can be drawn about the local adaptation of the populations from their degree of differentiation. But it is recommended that conservationists should not only consider the population genetic structure if managment plans for *B. calamita* are set up, but also the larger-scale structure that the river imposes.

The study populations are very heterogenous in age and size but have mostly decreased in size over the years. One exception is population AG8, which has increased in size from a very small population of maximal 5 individuals counted in 1978 up to 59 individuals counted in 1998.

(Effective) size is an important factor for the viability of populations. As a very general rule of thumb populations below 50 individuals are prone to loss of genetic variability and stochastic events, and only populations above 1000 to 10,000 individuals can ensure long-term persistence (Meffe and Carrol, 1997). All of the study populations have clearly less than 1000 individuals, and most single populations have sizes below 50 individuals. For the survival of the populations it will be essential that they increase in size and that their genetic variability is maintained. An important factor for the survival of small populations is their connection by migration and gene flow with other populations . However, increased gene flow might also cause outbreeding depression (Storfer, 1999). This should be considered when managment of the populations is planned.

The issue of gene flow was not directly addressed in this study. Although estimates of population differentiation can give an estimate of gene flow (Slatkin, 1985) the mechanisms that can cause differentiation are not necessarily migration and gene flow.

Genetic diversity

Compared to British populations of *B. calamita* (Rowe et al., 1998) the populations in this study show more genetic diversity within populations despite the smaller population sizes. Swiss populations show the same range of mean H_E as four other continental populations (Rowe et al., 1999), but smaller average number of alleles per population than three Spanish populations (Gomez-Mestre, pers. comm.; see appendix 1b). This cline in genetic diversity from the Iberian peninsula across Central Europe to Northern Europe could probably be explained by the natural history of *B. calamita*. During the ice age of the pleistocene until 10,000 years BP natterjack toads were restricted to the Iberian peninsula. After the retreat of the glaciers they dispersed again to the east and north across France and Germany, and supposedly crossed the channel before the land bridge between the continent and the British Isles disappeared between 9000 - 1000 years BP (Sinsch, 1998). Genetic diversity within populations is assumed to decrease successively from south to north and from west to east because of the continuous bottlenecking and founder effects during the expansion of the species (Rowe et al., 1998).

Some methodological remarks

In this study I included full sibs and tadpoles of unknown origin and gave them a different weight in the sample. This method leaves some uncertainties. It could be possible that full sibs were overrepresented in the sample. The safest way would probably be to analyse only one individual per spawn string. This reduces the sample size somewhat but perhaps cancels a possible bias as well. On the other hand, chances are increased that the spawn strings are then underrepresented. If mixed samples have any sibling bias, this bias is more likely to be evenly

distributed among all samples across all sites and thus could be neglected (Rowe et al., 1998).

At least for the results of pairwise genetic differentiation between populations (R_{st}) obtained from such a reduced data set correlate highly with the results from the original data set ($r_s = 0.939$, $p = 0001$). Thus I assume that the sibling bias is not very extreme in this case.

Several locus pairs show linkage disequilibrium. The cause could not be defined with certainty because most tests were limited by small sample sizes. But irrespective of the underlying cause, nonrandom association between loci violates the assumption of independence for F-statistics, which average over loci. Thus, it is not clear to what extent the power of the F-statistics is reduced by linkage disequilibrium. Because linkage disequilibrium was randomly distributed between locus pairs, it could not be removed from the data set. As a substitute for F-statistics genetic distances like Nei's D (Nei, 1978) could be used. These take into account allele identity and do not assume independence of loci (Cornuet et al., 1999 ; J. Dallas, pers.comm.).

However, this approach would not allow the investigation of hierarchical population structures.

Conclusions

With several methods it was shown that populations of *B. calamita* show high differentiation between populations as well as between river banks. This should be considered in future management plans of this species. The effects of linked loci on measures of population structure have to be resolved before definite conclusions can be drawn.

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TABLES

TABLE 1: Hierarchical levels of the population structure.

<i>hierarchical level</i>	<i>abbreviation</i>
individual	i
pond/puddle	p
site	s
river bank	r
total	t
<u>generation (early-late)</u>	<u>g (e-l)</u>

The level "generation" lies outside the nested spatial hierarchy with its two subgroups early and late.

TABLE 2: Collection of samples during the breeding season.

<i>site</i>	<i>early</i>			<i>late</i>			<i>all</i>
	<i>spawn</i>	<i>mixed</i>	<i>total</i>	<i>spawn</i>	<i>mixed</i>	<i>total</i>	<i>total</i>
AG8	17	2	19	0	3	3	22
AG27	0	2	2	0	3	3	5
AG32	4	2	6	0	1	1	7
ZH45	0	2	2	0	1	1	3
ZH46	2	3	5	0	2	2	7
ZH47	12	1	13	4	3	7	20
ZH48	2	1	3	0	1	1	4
LU12	2	3	5	0	5	5	10
LU118	1	3	4	0	2	2	6
LU 234	2	3	5	0	2	2	7
<i>total</i>	43	21	64	4	23	27	91

Early = sampling season from 8th of May to 20th of June, late = sampling season from 21st of June to 4th of August. Spawn = collected spawn strings, mixed = number of samples with freeswimming tadpoles.

TABLE 3: Size and age of the populations.

<i>site</i>	<i>population size</i>				<i>age</i>
	<i>m</i>	<i>f</i>	<i>size class</i>	<i>year of survey</i>	
<i>AG8</i>	53	5	1	1991/92	20
<i>AG27</i>	25	1	1	1998	2
<i>AG32</i>	15	0	1	1991/92	6
<i>ZH45</i>	15	0	1	1997	4
<i>ZH46</i>	6	1	3	1995	18
<i>ZH47</i>	24	1	1	1998	2
<i>ZH48</i>	5	1	2	1993	5
<i>LU12</i>	25	?	4	1990/91	18
<i>LU118</i>	2	0	3	1990/91	> 18
<i>LU234</i>	11	0	2	1990/91	> 50

m = maximal number of males counted at best night during 1998; *f* = maximal number of females counted at best night during 1998; size class = population size assessed in last official census, number given in classes: 1 = 1-5 ind., 2 = 6 - 30 ind., 3 = 31 - 100 ind., 4 $\geq$ 100 ind. Age in years is calculated after the first sighting of toads or since the time the site was mentioned first.

TABLE 4: Summary of genetic variability at all sites.

site	mean <i>N</i>	mean <i>a/l</i>	<i>SE</i>	95%	mean heterozygosity			
					<i>H_O</i>	<i>SE</i>	<i>H_E</i>	<i>SE</i>
<i>AG8</i>	40.0	3.8	0.3	100.0	0.525	0.075	0.517	0.06
<i>AG27</i>	40.0	3.0	0.2	100.0	0.444	0.046	0.484	0.06
<i>AG32</i>	39.6	2.9	0.2	100.0	0.512	0.055	0.505	0.06
<i>ZH45</i>	40.0	2.5	0.4	75.0	0.334	0.093	0.441	0.1
<i>ZH46</i>	39.5	3.0	0.3	100.0	0.473	0.054	0.504	0.07
<i>ZH47</i>	38.1	3.1	0.4	87.5	0.577	0.083	0.512	0.08
<i>ZH48</i>	40.0	3.1	0.2	100.0	0.500	0.051	0.524	0.04
<i>LU12</i>	39.3	3.8	0.3	100.0	0.478	0.052	0.488	0.06
<i>LU118</i>	40.0	3.6	0.3	100.0	0.419	0.087	0.450	0.09
<i>LU234</i>	39.9	3.4	0.5	87.5	0.512	0.089	0.527	0.08
<i>mean</i>	39.64	3.22		95	0.4774		0.4952	
<i>GB</i>		1.25-3.50		25.0-87.5	0.099-		0.088-	
					0.439		0.359	

N = sample size, *a/l* = allele number per locus, 95% = loci polymorphic at the 95% criterium, *H_O* = frequency of heterozygotes counted in the samples (averaged over loci), *H_E* = frequency of heterozygotes expected when population is in HW equilibrium (averaged over loci). *GB* = results from British populations (Rowe *et al.*, 1998).

TABLE 5: Number of loci in Hardy-Weinberg disequilibrium.

<i>site</i>	<i>HW</i>	<i>excess</i>	<i>deficit</i>
<i>AG8</i>			
<i>AG27</i>	Bcal6		
<i>AG32</i>			
<i>ZH45</i>	Bcal1, 3, 5, 7		Bcal3, 7
<i>ZH46</i>	Bcal 6		
<i>ZH47</i>	Bcal2	Bcal2	
<i>ZH48</i>	Bcal 8		
<i>LU12</i>			
<i>LU118</i>	Bcal5, 7		
<i>LU234</i>			

HW calculated with a two-tailed test. Excess and deficit calculated with a one-tailed test.

TABLE 6: Loci in linkage disequilibrium.

<i>site</i>	<i>AG8</i>	<i>AG27</i>	<i>AG32</i>	<i>ZH45</i>	<i>ZH46</i>	<i>ZH47</i>	<i>ZH48</i>	<i>LU12</i>	<i>LU118</i>	<i>LU234</i>	<i>total</i>
<i>linkage</i>	0	2	2	15	2	0	4	0	2	1	28 (10%)

Number of loci pairs significantly in linkage disequilibrium in each population after sequential Bonferroni correction. When site *ZH45* is removed from the analysis, 5.2% of all loci pairs are in linkage disequilibrium.

TABLE 7: Genetic divergence between sites.

	<i>95% CI</i>			<i>95% CI</i>		<i>p</i>
Fis	0.0353	-0.0098 - 0.0737				
Fst	0.1721*	0.1345 - 0.2088	Rst	0.1950*	0.1766 - 0.2142	<0.0001
Fit	0.2014*	0.1491 - 0.2453				

Fst and Rst with 95% confidence intervals, Rst with p-value from 1000 bootstraps.

* = significantly different from zero.

TABLE 8: Population differentiation for loci.

<i>locus</i>	<i>F_{is}</i>	<i>F_{st}</i>	<i>F_{it}</i>
<i>Bcal1</i>	0.0029	0.0710	0.0737
<i>Bcal2</i>	-0.0774	0.2396	0.1808
<i>Bcal3</i>	0.0483	0.2434	0.2800
<i>Bcal4</i>	0.0266	0.1514	0.1739
<i>Bcal5</i>	0.0521	0.1873	0.2297
<i>Bcal6</i>	0.1054	0.1840	0.2700
<i>Bcal7</i>	0.1118	0.1375	0.2339
<i>Bcal8</i>	-0.1085	0.1921	0.1045

Summary of population differentiation measured with F-statistics for each locus separately, averaged over all sites.

TABLE 9: Genetic differentiation among ponds within the single sites.

<i>site</i>	<i>F_{ip}</i>	<i>F_{ps}</i>	<i>F_{is}</i>
<i>AG8</i>	-0.0636	0.0599	0.0001
<i>AG27</i>	-0.0359	0.1675	0.1376
<i>AG32</i>	-0.0748	0.0826	0.0140
<i>ZH45</i>	-0.0607	0.4634	0.4308
<i>ZH46</i>	0.0217	0.0800	0.1000
<i>ZH47</i>	-0.1789	0.0596	-0.1086
<i>ZH48</i>	-0.0529	0.1463	0.1011
<i>LU12</i>	0.0060	0.0252	0.0310
<i>LU118</i>	0.0333	0.0533	0.0848
<i>LU234</i>	-0.0558	0.0802	0.0289

For each site a hierarchical analysis of population differentiation was carried out. *F_{ip}* = genetic differentiation between individuals within a pond, *F_{ps}* = differentiation between the ponds within a site, *F_{is}* = total differentiation between individuals within a site across ponds.

TABLE 10: Hierarchical F-statistics of the generations early and late.

	<i>Fis</i>	<i>Fsg</i>	<i>Fgt</i>	<i>Fit</i>	<i>Rgt</i>
	-0.0558	0.2	-0.198*	0.1551	0.01766
<i>SE</i>	0.0185	0.0209	0.0046	0.0193	0.001
<i>95%</i>	-0.0959 - -0.022	0.1656 - 0.2364	-0.0277 - -0.011	0.1228 - 0.1917	<i>p</i> = 0.001

Fis = inbreeding coefficient within sites; *Fsg* = differentiation between sites within generation early or late; *Fgt*, *Rgt* = differentiation between the generations early and late from Weir & Cockerham's *Fst* and Slakin's *Rst*; *Fit* = overall differentiation. *SE* = standard error, 95% = confidence intervals. * = not significantly different from zero. For the calculation of *Rgt* the populations within generations were pooled across all populations.

TABLE 11: Genetic differentiation within the river sides east and west.

	<i>east</i>	<i>west</i>
<i>Fis</i>	0.0336	0.0370
<i>Fsr</i>	0.1452	0.1362
<i>Fir</i>	0.1740	0.1682

Fis = inbreeding coefficient between individuals within sites, *Fsr* = differentiation between sites within river sides, *Fir* = total differentiation within river sides.

TABLE 12: Genetic differentiation between river sides compared to the total differentiation.

	<i>95% CI</i>		<i>Rrt</i>	<i>95% CI</i>	<i>p</i>
<i>Fir</i>	0.1475	0.0964-0.1930			
<i>Frt</i>	0.0899	0.0448-0.1454	0.0845	0.0654-0.1057	<i>p</i> < 0.00001
<i>Fit</i>	0.2241	0.1647-0.2809			

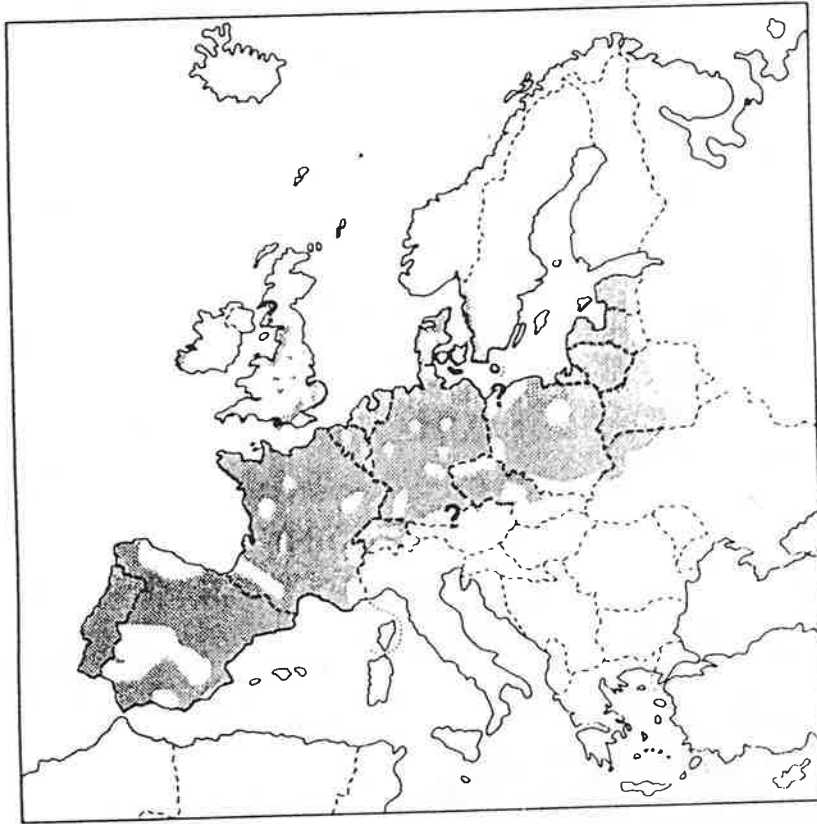
Populations are pooled within river banks. *Fir* = differentiation within river sides, *Frt* = differentiation between river sides, *Fit* = total differentiation over the whole study area. *Rrt* = differentiation between river sides, bootstrapped mean averaged over variance components.

TABLE 13: Weighting of the isolation by distance regression.

	<i>a</i>	<i>b</i>	<i>p</i> (Mantel)
<i>east</i>	0.1298767	0.00000332	0.0006
<i>west</i>	0.1106447	0.00000517	0.0007
<i>mean</i>	0.1201607	0.000004245	0.0004

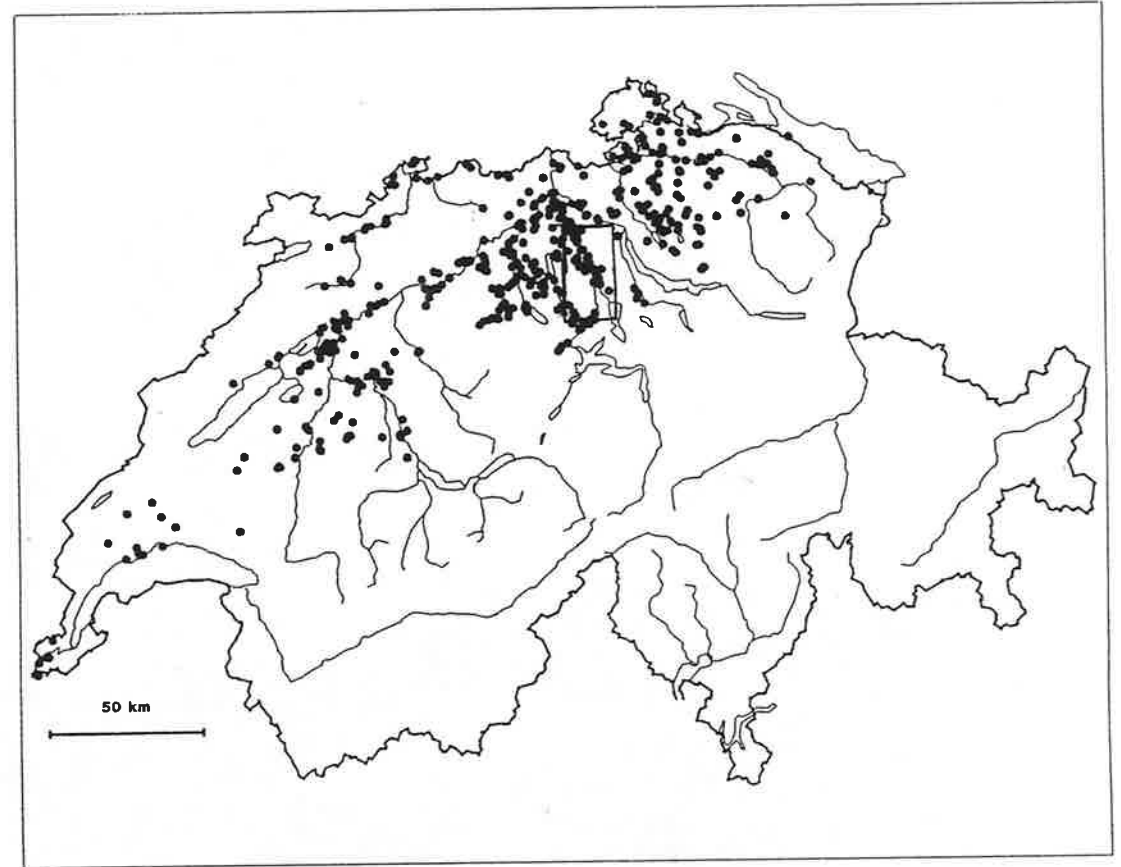
Constants *a* and *b* of the regression equation $Rst = b(distance) + a$ of isolation by distance within each river bank east and west and a mean calculated from the two constants. Rst values inferred from these equations were included in the isolation by distance test. The p-values of the Mantel test are given.

FIGURE 1a: Distribution of *B. calamita* across Europe.



From Nöllert, A. and Nöllert, C. (1992).

FIGURE 1b: Distribution of *B. calamita* in Switzerland.



Framed area is the sampling region (see fig. 1.c). From Grossenbacher, 1988.

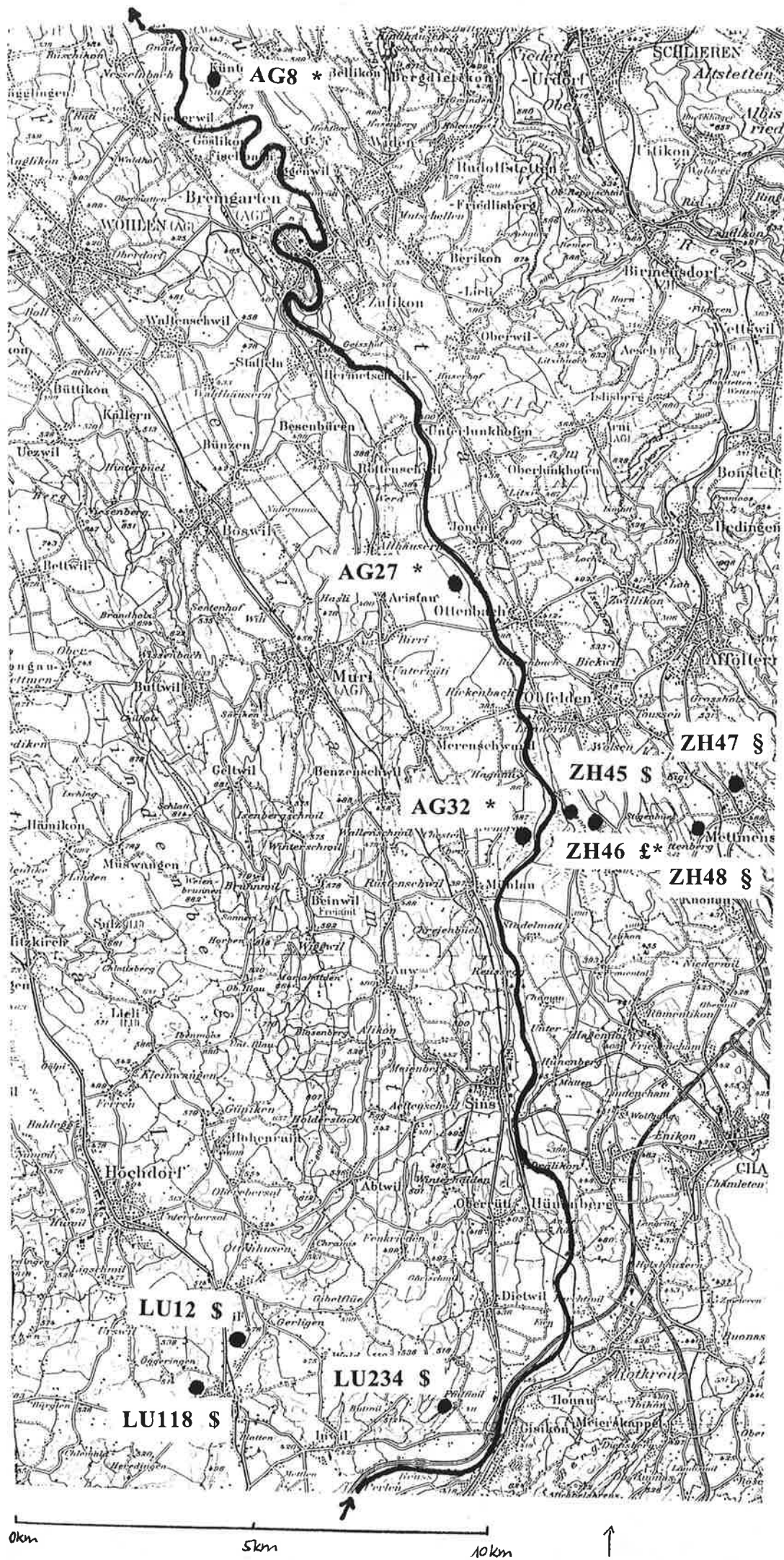
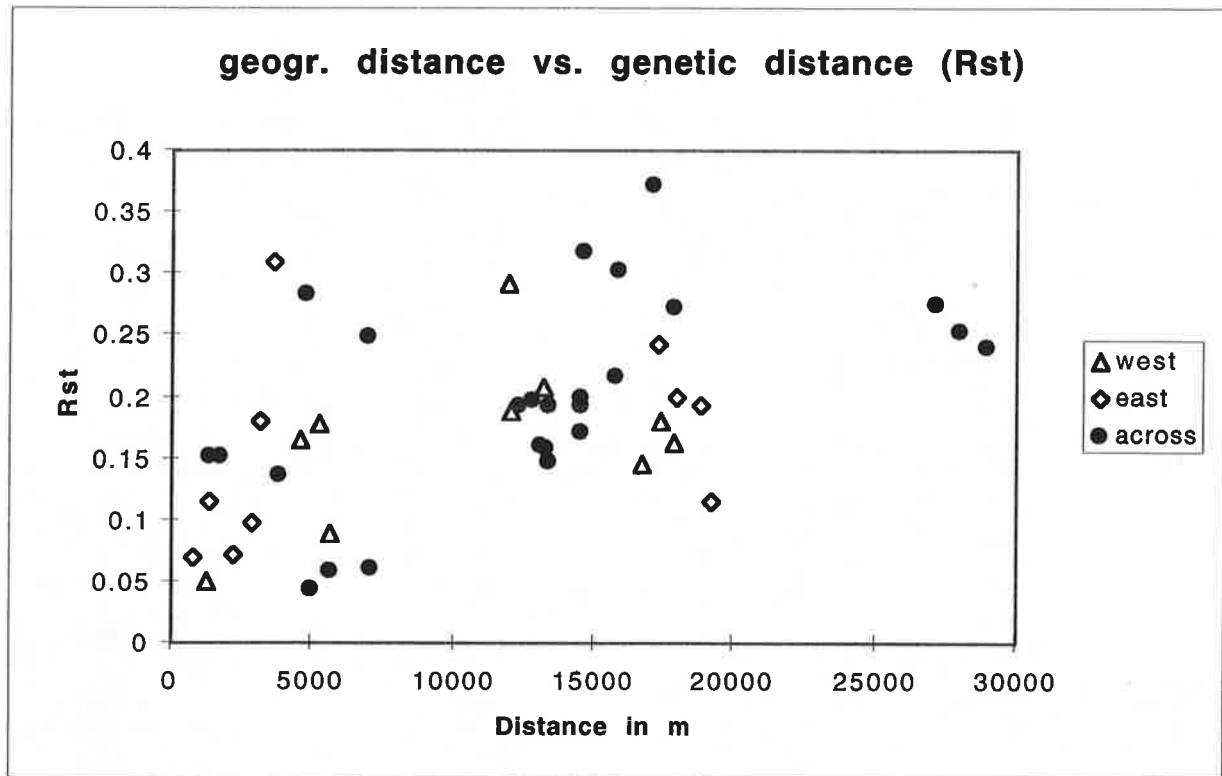


FIGURE 1c: Sampling sites.

Sampling sites in the Reuss valley in Switzerland. Five populations lie on each side of the river. AG = canton Aargau, LU = canton Luzern, ZH = canton Zürich. The dark line indicates the river, and the arrows the direction of the current. * nature reserve, \$ gravel pit under work, £ closed down gravel pit (turned into nature reserve in ZH46), § building site.

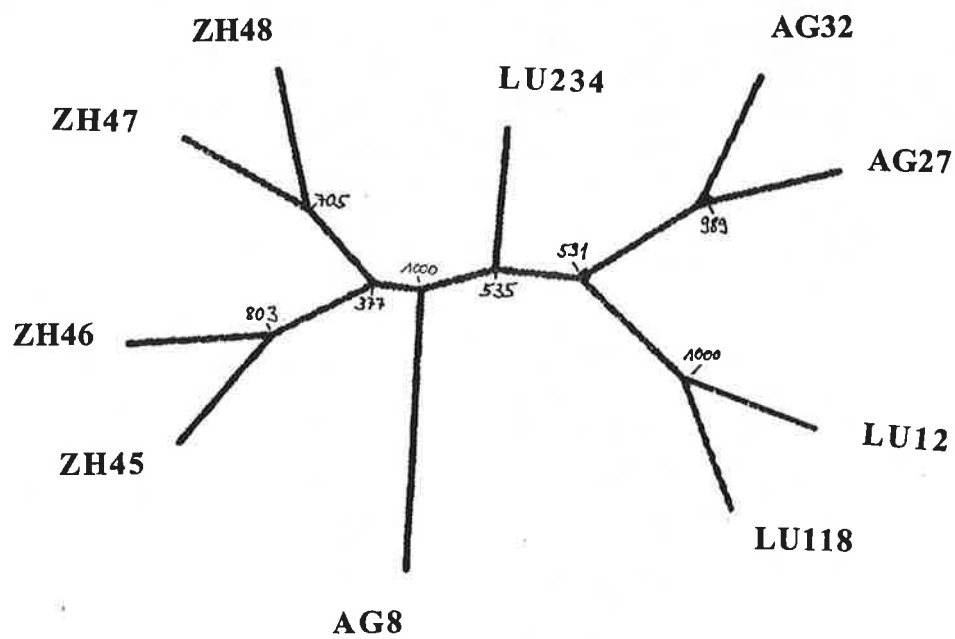
FIGURE 2: Correlation of the pairwise geographic and genetic distances.



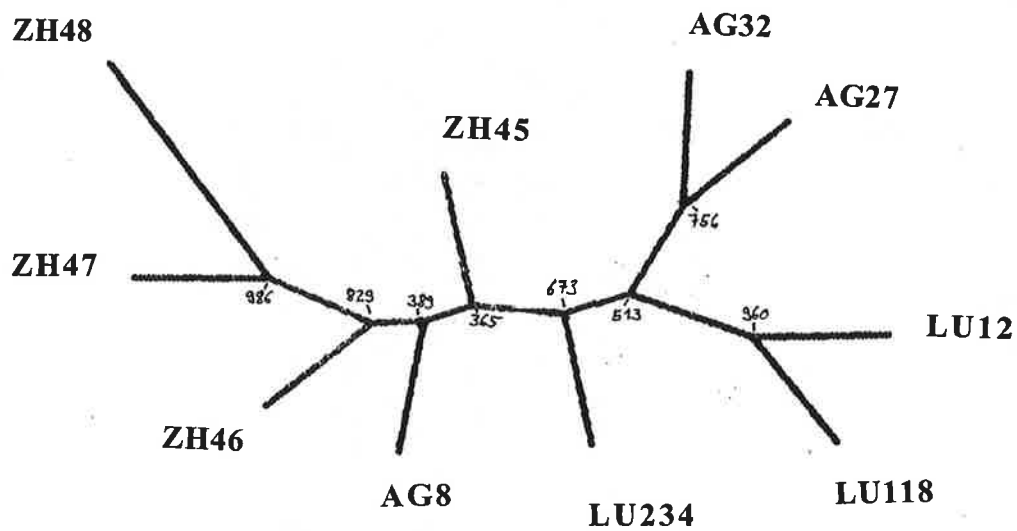
Geographic distances in m, genetic differentiation between population pairs in Rst. Filled circles are the distances between population pairs across the river ($n = 25$), the open squares and triangles between pairs on the east and west bank, respectively ($n = 10$ each).

FIGURE 3: Pylogenetic trees

A



B



A. Consensus UPGMA tree with Cavalli-Sforza genetic distance. B. Consensus maximum likelihood tree. Numbers at the nodes are the amount of times this branching occurred among the 1000 bootstrapped trees.

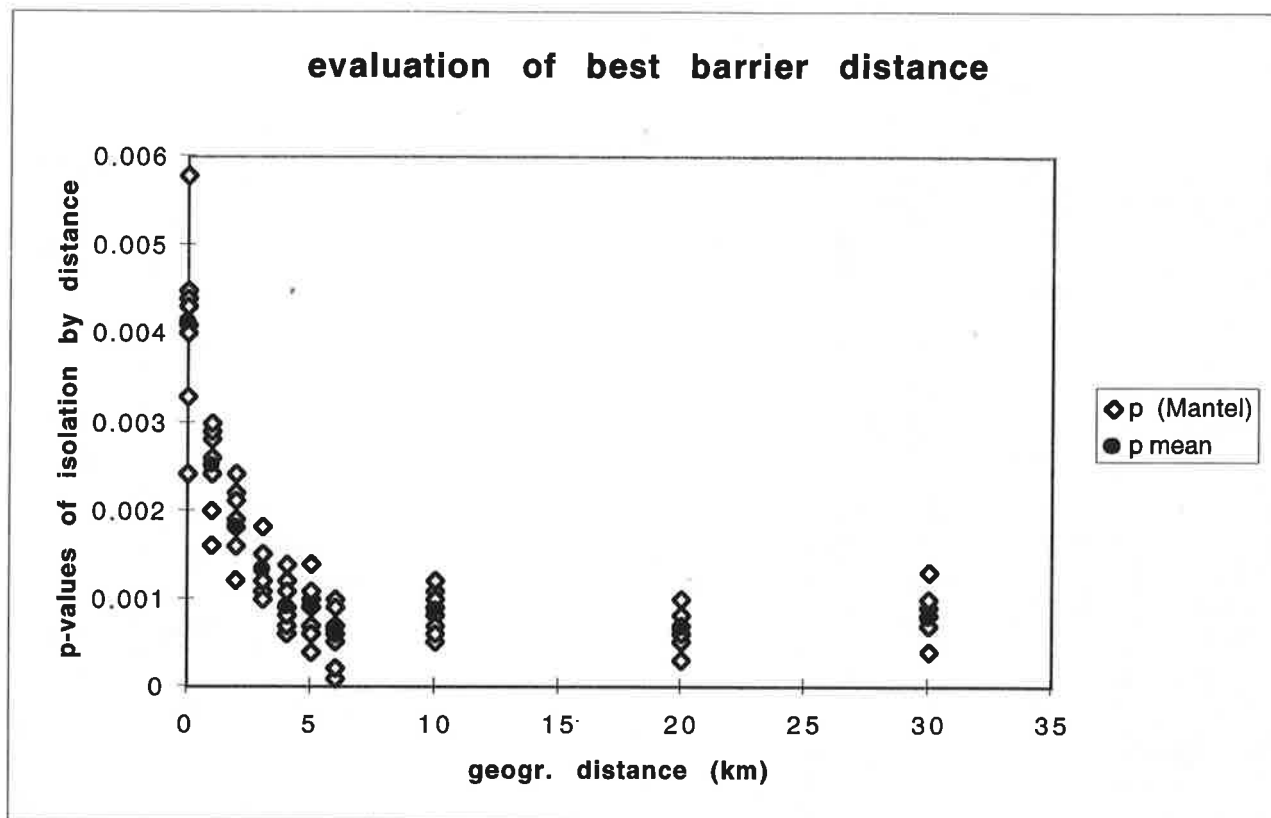


FIGURE 4a: Different barrier distances were added to the geographic distances between the population pairs that lie across the river. Ten replicates of each barrier distance were calculated and the probability values of the isolation by distance estimates (Mantel test) are plotted against the barrier distance (diamonds). The black dots are the means of the replicates.

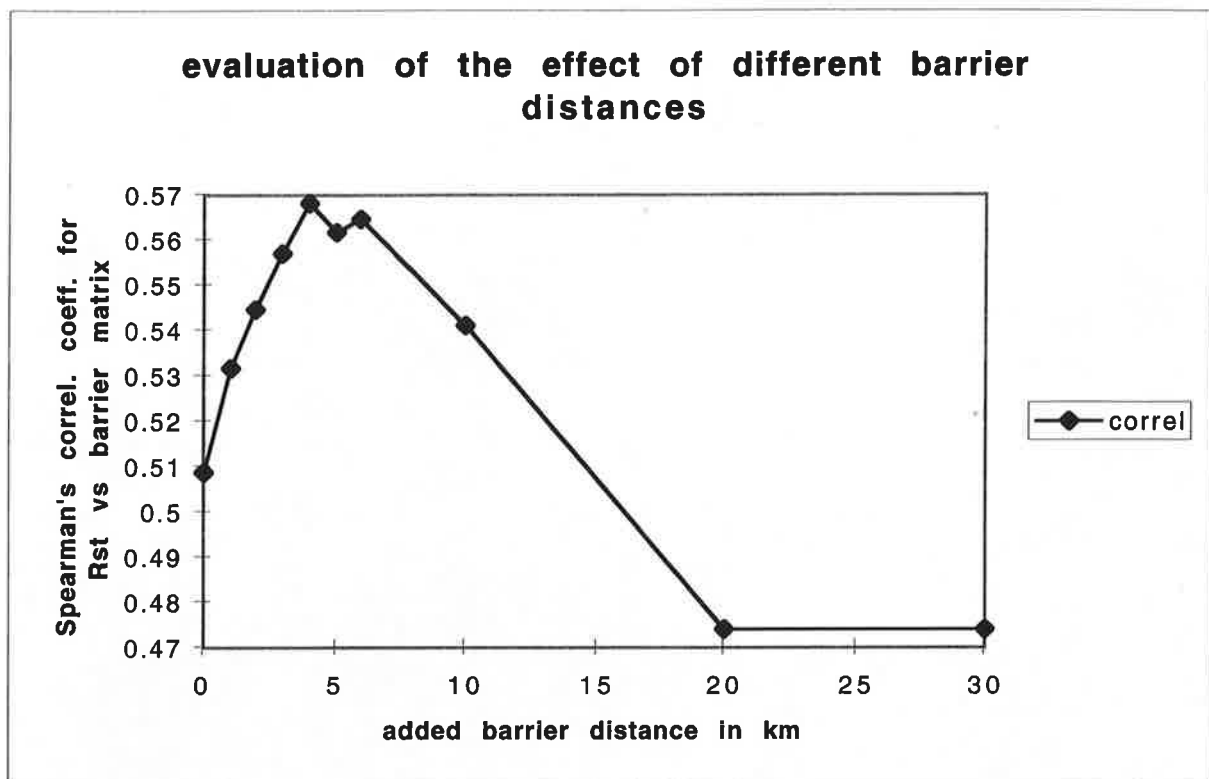


FIGURE 4b: Best barrier distance, Spearman's correlation. Evaluation of the barrier distance that gives the isolation by distance value with the best correlation between geographic and genetic distance. The barrier distances are added to all geographic distances between across-river population pairs .

APPENDIX

APPENDIX 1a: Summary table of the allele frequencies of all loci in all populations.

west							east				
<i>Allele</i>	<i>AG27</i>	<i>AG32</i>	<i>LU12</i>	<i>LU118</i>	<i>LU234</i>		<i>AG8</i>	<i>ZH45</i>	<i>ZH46</i>	<i>ZH47</i>	<i>ZH48</i>
<i>Bcal1</i>	(<i>N</i>)	40	40	39	40	40	40	40	40	38	40
1	0	0	0	0	0	0	0.013	0	0	0	0
2	0.15	0.325	0.154	0.087	0.063		0.162	0.287	0.313	0.395	0.213
3	0.613	0.563	0.308	0.4	0.463		0.625	0.538	0.512	0.539	0.712
4	0.237	0.112	0.474	0.412	0.475		0.2	0.175	0.175	0.066	0.075
5	0	0	0.064	0.1	0		0	0	0	0	0
<i>Bcal2</i>	(<i>N</i>)	40	40	38	40	40	40	40	40	38	40
1	0.9	0.75	0.829	0.925	1		0.775	1	0.825	0.329	0.675
2	0	0.063	0.092	0.05	0		0.138	0	0.175	0.671	0.325
3	0.1	0.188	0.079	0.025	0		0.087	0	0	0	0
<i>Bcal3</i>	(<i>N</i>)	40	40	40	40	40	40	40	39	39	40
1	0	0	0.125	0.075	0.287		0	0	0	0	0
2	0.275	0.387	0.313	0.338	0.225		0.038	0.613	0.256	0.013	0.4
3	0	0	0	0	0		0	0	0.013	0	0
4	0.475	0.512	0.538	0.463	0.063		0.025	0	0	0.077	0
5	0.25	0.1	0.013	0.075	0.3		0.913	0.387	0.603	0.564	0.475
6	0	0	0.013	0.05	0.125		0.025	0	0.128	0.346	0.125
<i>Bcal4</i>	(<i>N</i>)	40	37	39	40	40	40	40	37	37	40
1	0	0	0	0	0		0.313	0	0	0	0
2	0.063	0.108	0.128	0.25	0.225		0.35	0.125	0.216	0.514	0.475
3	0.325	0.473	0.462	0.488	0.162		0	0	0	0.108	0.013
4	0.475	0.284	0.256	0.188	0.512		0.162	0.613	0.243	0.095	0.262
5	0.138	0.135	0.154	0.075	0.1		0.175	0.262	0.541	0.284	0.25

west							east				
	Allel	AG27	AG32	LU12	LU118	LU234	AG8	ZH45	ZH46	ZH47	ZH48
Bcal5	(N)	40	40	40	40	40	40	40	40	38	40
	1	0.112	0	0.038	0	0.575	0.075	0.1	0.125	0.329	0.175
	2	0	0	0	0	0	0	0	0	0.053	0
	3	0	0	0.538	0.5	0.087	0	0.287	0.125	0	0
	4	0.762	0.913	0.338	0.412	0.213	0.625	0.538	0.637	0.303	0.475
	5	0	0	0	0	0.05	0	0	0	0.145	0.175
	6	0.125	0.087	0.087	0.087	0.075	0.3	0.075	0.112	0.171	0.175
Bcal6	(N)	40	40	39	40	40	40	40	40	38	40
	1	0	0	0.077	0.025	0	0	0	0	0	0
	2	0.063	0	0.128	0.013	0.05	0.387	0.338	0.188	0.25	0.175
	3	0.587	0.35	0.128	0.125	0.475	0.112	0.138	0.35	0.553	0.762
	4	0.35	0.65	0.667	0.837	0.475	0.5	0.525	0.463	0.197	0.063
Bcal7	(N)	40	40	40	40	39	40	40	40	39	40
	1	0	0	0	0	0	0	0	0	0	0.013
	2	0	0	0	0	0.103	0	0	0	0	0
	3	0	0	0	0	0	0.075	0	0	0	0
	4	0.563	0.488	0.013	0.15	0.051	0.563	0.338	0.262	0.282	0.5
	5	0.162	0.275	0.125	0.175	0.333	0.05	0.313	0.213	0.09	0
	6	0.275	0.237	0.863	0.675	0.513	0.313	0.35	0.525	0.628	0.488
Bcal8	(N)	40	40	39	40	40	40	40	40	38	40
	1	0.138	0.45	0.154	0.038	0	0.063	0	0	0	0.038
	2	0.813	0.512	0.833	0.95	0.688	0.575	1	0.925	1	0.7
	3	0	0	0	0	0	0	0	0.075	0	0.262
	4	0.05	0.038	0.013	0.013	0.313	0.112	0	0	0	0
	5	0	0	0	0	0	0.025	0	0	0	0
	6	0	0	0	0	0	0.225	0	0	0	0

N = Number of individuals analysed for each locus and population.

APPENDIX 1b: Allele sizes

<i>locus</i>	<i>allele size in bp</i>						<i>Spanish pops</i>
<i>allele no.</i>	1	2	3	4	5	6	<i>n° of alleles</i>
<i>Bcal1</i>	106	116	120	128	138		14
<i>Bcal2</i>	167	179	183				5
<i>Bcal3</i>	109	113	115	117	119	121	12
<i>Bcal4</i>	182	192	198	202	208		16
<i>Bcal5</i>	211	221	225	227	229	231	11
<i>Bcal6</i>	148	150	152	154			11
<i>Bcal7</i>	121	137	139	141	143	145	16
<i>Bcal8</i>	141	145	147	149	151	157	22

Number of alleles and allele sizes in base pairs for each locus. Additionally the number of alleles summarized across three Spanish populations are given.

APPENDIX 2: Private alleles

	<i>AG8</i>	<i>ZH46</i>	<i>ZH47</i>	<i>ZH48</i>	<i>LU234</i>	<i>east</i>	<i>west</i>
<i>Bcal1</i>	106						138
<i>Bcal2</i>							
<i>Bcal3</i>		115					109
<i>Bcal4</i>	182						
<i>Bcal5</i>			221				
<i>Bcal6</i>							148
<i>Bcal7</i>	139			121	137		
<i>Bcal8</i>	151, 157					147	

Private alleles of each site, and of the river banks east and west. Allele sizes in base pair length.

APPENDIX 3: Linkage disequilibrium within ponds.

<i>site</i>	<i>pond</i>	<i>n° loci in LD</i>	<i>n° ind.</i>
<i>AG8</i>	1	0	6
	2	0	11
	3	1	16
	4	0	6
	5	-	1
<i>AG27</i>	1	0	16
	2	0	8
	3	0	16
<i>AG32</i>	1	1	12
	2	0	10
	3	1	18
<i>ZH45</i>	1	0	14
	2	9	26
<i>ZH46</i>	1	1	26
	2	0	7
	3	0	7
<i>ZH47</i>	1	0	21
	2	0	7
	3	0	6
	4	-	1
	5	0	4
<i>ZH48</i>	1	0	20
	2	0	5
	3	2	15
<i>LU12</i>	1	0	10
	2	0	20
	3	0	10
<i>LU118</i>	1	-	2
	2	0	8
	3	0	15
	4	2	15
<i>LU234</i>	1	0	15
	2	-	2
	3	-	2
	4	0	7
	5	0	7
	6	0	7
<i>total</i>		17	

Number of sampled ponds per site, their sample sizes and number of loci pairs in linkage disequilibrium. - = no statment about linkage disequilibrium can be made because either only one individual was sampled or all individuals sampled were fixed for the same allele. These samples were left out of the correlation analysis. LD = linkage disequilibrium.

APPENDIX 4: Pairwise geographic and genetic distance.

<i>Rst</i>	<i>AG8</i>	<i>AG27</i>	<i>AG32</i>	<i>ZH45</i>	<i>ZH46</i>	<i>ZH47</i>	<i>ZH48</i>	<i>LU12</i>	<i>LU118</i>
<i>AG27</i>	0.1934								
<i>AG32</i>	0.2718	0.0881							
<i>ZH45</i>	0.2412	0.0432	0.1511						
<i>ZH46</i>	0.2	0.0577	0.1514	0.0695					
<i>ZH47</i>	0.1933	0.2492	0.2829	0.3086	0.1792				
<i>ZH48</i>	0.1144	0.0613	0.1365	0.0968	0.0721	0.114			
<i>LU12</i>	0.2738	0.1447	0.1883	0.1466	0.1581	0.3027	0.1714		
<i>LU118</i>	0.2539	0.1619	0.2079	0.1914	0.1994	0.3713	0.2168	0.05	
<i>LU234</i>	0.2402	0.1801	0.2917	0.1596	0.1958	0.3171	0.1917	0.1647	0.1763

<i>distance</i>	<i>AG8</i>	<i>AG27</i>	<i>AG32</i>	<i>ZH45</i>	<i>ZH46</i>	<i>ZH47</i>	<i>ZH48</i>	<i>LU12</i>	<i>LU118</i>
<i>AG27</i>	12354								
<i>AG32</i>	17836	5629							
<i>ZH45</i>	17308	4957	1379						
<i>ZH46</i>	17968	5615	1677	756					
<i>ZH47</i>	18830	6987	4823	3678	3149				
<i>ZH48</i>	19215	7055	3824	2907	2230	1300			
<i>LU12</i>	27088	16731	12017	13356	13327	15861	14562		
<i>LU118</i>	27975	17843	13219	14565	14548	17098	15800	1238	
<i>LU234</i>	28926	17429	11985	13117	12831	14647	13387	4586	5247

Semi matrices of the genetic (*Rst*) and the geographic distances between population pairs.
Geographic distances in m.

Die genetische Struktur von Kreuzkrötenpopulationen und der Effekt des Flusses (deutsche Kurzfassung)

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März 2000

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Zusammenfassung

Mit der fortschreitenden Habitatfragmentierung durch menschliche Aktivitäten ist im Naturschutz die Problematik von zerteilten Populationen immer mehr in den Blickpunkt gerückt. Probleme wie Isolation und Inzucht, die daraus für kleine Populationen entstehen, geben zu Besorgnis Anlass. Anhand genetischer Methoden ist es möglich festzustellen, wieweit einzelne Populationen isoliert sind, und wie die Gruppierungen von Populationen durch geographische Strukturen beeinflusst werden.

In dieser Studie habe ich zehn Kreuzkrötenpopulationen im unteren Reusstal untersucht, die von der Reuss in zwei Gruppen geteilt werden. Im Mittelpunkt der Studie stand die genetische Strukturierung der Populationen und der Einfluss, den die Reuss auf diese Strukturierung hat. Acht Primer für Mikrosatelliten standen für die genetische Untersuchung zur Verfügung. Die angewandte F-Statistik (mit den Parametern F_{st} und R_{st}) betrachtete die genetische Struktur innerhalb und zwischen den Populationen und untersuchte den Effekt des Flusses als Barriere für Migration und Genfluss. Einige statistische Probleme müssen noch geklärt werden, bevor endgültige Schlüsse aus dieser Arbeit gezogen werden können.

Die Populationen unterschieden sich in ihrer genetischen Zusammensetzung signifikant sowohl innerhalb als auch zwischen den beiden Flussufern. Auch die phylogeographischen Stammbäume zeigen auf, dass der Fluss als Migrationsbarriere wirkt.

Diese Erkenntnisse haben Einfluss auf weitere naturschützerische Projekte. Die kleinräumlichen Strukturierungen von Populationen sollten in Betracht gezogen werden, falls zur Unterstützung von Populationsgrössen Translokationen in Betracht gezogen werden. Alle untersuchten Populationen sind klein bis sehr klein und sollten durch Habitatverbesserungen gestärkt werden.

Die genetische Vielfalt dieser schweizer Populationen ist trotz der Kleinheit der Populationen grösser als die englischer Populationen. Dies könnte durch die biogeographische Vergangenheit dieser Art und ihre Ausbreitung in Europa nach der letzten Eiszeit erklärbar sein.

Einleitung

Kreuzkröten sind eine gefährdete Amphibienart in der Schweiz und seit 1967 gesetzlich geschützt. Ihre Primärhabitats wie überschwemmte Auenlandschaften sind fast vollständig verschwunden. Sekundärhabitats wie Kiesgruben, Industriebrachen oder Naturschutzgebiete werden jedoch gerne genutzt. Diese Habitats sind oft lokal begrenzt und isoliert. Adulte Tiere sind meistens ortstreu, und die Hauptverbreitung geschieht durch Juvenile, welche grosse Strecken zurücklegen können. Die Populationsdichte im Untersuchungsgebiet ist zwar hoch, die einzelnen Populationen sind wahrscheinlich aber durch die intensive Nutzung des Landwirtschaftsgebietes isoliert. Zudem sind die Populationen meist klein, was zu Inzucht führen kann. Isolation und Inzucht können zu einem Verlust an genetischer Vielfalt führen, was die Aussterbewahrscheinlichkeit der Populationen erhöht.

In dieser Studie habe ich zwei Aspekte der Strukturen kleiner und isolierter Populationen analysiert: Einerseits die genetische Struktur und der Grad der Isolation zwischen den Populationen (Fixationsindex) und der Grad der Inzucht innerhalb von Populationen (Inzuchtskoeffizient). Andererseits analysierte ich den Fluss als Habitatstruktur, und seinen Effekt auf Differenzierung zwischen Populationen. Ich nahm an, dass Flüsse die Migration zwischen Kreuzkrötenpopulationen einschränken können und dadurch die Differenzierung zwischen Populationen verstärken.

Wenn der Fluss einen starken Effekt zeigt, wäre es sinnvoll, diese Erkenntnisse in zukünftige Pflegepläne der Kreuzkrötenpopulationen einzubeziehen, vor allem falls Translokationen zur Vergrösserung und Stärkung der Populationen in Betracht gezogen würden.

Material und Methoden

Studiengebiet und Laichplätze.

Das Studiengebiet umfasst das untere Reusstal und seine weitere Umgebung zwischen Bremgarten (AG) und Eschenbach (LU) und erstreckt sich über die Kantone Aargau, Luzern und Zürich (Abb.1c). Die Populationen liegen im Tal und den angrenzenden Hügelketten zwischen 370 und 480 m.ü.M. Die Reuss wurde Mitte des 19. Jahrhunderts eingedämmt. Alle zehn untersuchten Kreuzkrötenpopulationen befinden sich in Sekundärhabitat. Jeweils fünf Populationen liegen am Ost- bzw. Westufer der Reuss. Jeder Laichplatz besteht aus mehreren Teichen oder Pfützen, welche aber als Laichplätze einer Population angesehen werden, da sie weniger als 100 m voneinander entfernt sind, während verschiedene Populationen zwischen 756m und 28 km auseinander liegen.

Probennahme

Jede Population wurde zwischen dem 10. Mai und dem 4. August 1998 fünf bis acht Mal besucht. Bei jedem Besuch habe ich frischen Laich und Kaulquappen aus allen Laichgewässern entnommen, so dass ich von jeder Population eine repräsentative Stichprobe von 40 Tieren hatte. Insgesamt wurden 47 Laichschnüre und 44 Proben mit Kaulquappen gesammelt.

Analyse der genetischen Diversität

Die molekulargenetischen Analysen wurden mit Hilfe von Mikrosatelliten durchgeführt. Verschiedene Parameter der genetischen Diversität wurden untersucht: Die durchschnittliche

Anzahl von Allelen pro Locus (Mikrosatellit), der durchschnittliche Polymorphismus pro Locus (kein einzelnes Allel ist häufiger als 95%), die durchschnittlich nach dem Hardy-Weinberg Gleichgewicht erwartete sowie die beobachtete Heterozygotität.

Mit einer Spearman's Rangkorrelation wurde getestet, ob ein Zusammenhang zwischen dem Alter und der Grösse der Population und den verschiedenen Parametern der genetischen Diversität besteht.

Hardy-Weinberg Gleichgewicht und Kopplungsgleichgewicht.

Eine "ideale" Population hat ein genetisches Gleichgewicht zwischen den Allel- und Genotyphäufigkeiten an einzelnen Genen (Hardy-Weinberg Gleichgewicht). Ausserdem werden verschiedene Gene mit gleicher Wahrscheinlichkeit kombiniert (Kopplungsgleichgewicht).

Diese idealisierte Population im Hardy-Weinberg-Gleichgewicht dient als Referenz für die Analyse natürlicher Populationen, in denen Faktoren wie Mutation, Selektion, Immigration und Emigration oder genetische Drift (zufällige Änderungen der Allelfrequenzen im Genpool aufgrund kleiner Populationsgrössen) wirken. Auch der Wahlund's Effekt (die Vermischung von Individuen verschiedener Subpopulationen in einer Probe) kann zu einer künstlichen Störung dieser Gleichgewichte führen. Das Hardy-Weinberg- und Kopplungsgleichgewicht wurde für alle Populationen getestet.

Die genetische Struktur der Populationen

In einem ersten Teil wurde die genetische Struktur der Populationen über das gesamte Studiengebiet sowie innerhalb der einzelnen Populationen untersucht. Die F-Statistik von Wright misst den Anteil an der totalen genetischen Diversität (gemessen als die erwartete Heterozygotität), der aufgrund der Abweichungen in Allelfrequenzen zwischen (Sub)Populationen entsteht. F_{st} ist das Mass für die F-Statistik von Wright, die hier angewendet wurde. Als zweites Mass wird R_{st} benutzt, das die speziellen Mutationseigenschaften der Mikrosatelliten miteinbezieht. Wo technisch möglich wurden beide Masse verwendet.

Der Effekt des Flusses

In einem zweiten Teil versuchte ich mit drei verschiedenen Ansätzen, den Effekt des Flusses auf die genetische Struktur der Populationen in der Region zu beschreiben:

1. Zunächst wurden die Populationen auf beiden Seiten des Flusses jeweils zu einer grossen "Population" zusammengefasst, und mit der F_{st} - und R_{st} -Statistik konnte ich auf signifikante Unterschiede zwischen diesen beiden Übergruppen testen.

2. Die "Isolation durch Distanz"-Theorie besagt, dass (bei einer ungehinderten Ausbreitung) benachbarte Populationen einander genetisch ähnlicher sind, als weiter entfernte Populationen. Dadurch entsteht eine Korrelation zwischen genetischer und geographischer Distanz zweier Populationen. Dieses Muster wird gestört, wenn Migrationsbarrieren eine gleichmässige Ausbreitung verhindern. Die "Isolation durch Distanz"-Theorie wurde über alle Populationen, aber auch zwischen den Populationen innerhalb der Flusssufer getestet.

Da die Populationsgruppen der beiden Flusseiten in Nord-Süd-Richtung gegeneinander verschoben sind (Abb. 1c), wird der Effekt der grössten Distanzen mit dem Effekt des Flusses vermischt, und es ist schwer, eine eindeutige Aussage zu erhalten.

3. Die phylogeographischen Stammbäume sind eine weitere Methode, den Effekt des Flusses aufzuzeigen. Anhand der genetischen Daten werden die Populationen in einem "Stammbaum" gruppiert, der die genetische Ähnlichkeit der Populationen darstellt. Ich wählte zwei verschiedene Ansätze zur Berechnung der Stammbäume.

Ergebnisse

Genetische Diversität

Alle Loci waren polymorph und zeigten einen relativ hohen Grad an genetischer Diversität (Zusammenfassung der Allelfrequenzen in Appendix 1a). Die Population AG8 zeigt einen hohen Anteil an "privaten" Allelen, die nur in dieser Population vorkommen. Diese Population ist auch vergleichsweise gross. Tabelle 4 zeigt die Werte für die genetische Diversität. Die Population ZH45 hat die niedrigsten Werte.

Im Vergleich mit britischen Populationen sind die schweizer Populationen genetisch diverser, obwohl sie meistens kleiner sind.

Ich konnte keine Korrelation zwischen dem Alter oder der Grösse der Populationen und ihrer genetischen Diversität feststellen.

Hardy-Weinberg Gleichgewicht und Kopplungsgleichgewicht

Es wurden in erstaunlich hohem Masse Loci und Populationen mit Hardy-Weinberg Ungleichgewicht und Kopplungsungleichgewicht gefunden (Tab. 5,6). Gleichzeitig konnte ich aber keine systematischen Ungleichgewichte an einem bestimmten Locus feststellen, womit es sehr schwierig ist, eine genaue Ursache für die Ungleichgewichte zu bestimmen.

Genetische Struktur

Zwischen den einzelnen Populationen wurden signifikante Unterschiede in der genetischen Zusammensetzung gefunden. Dagegen waren die Inzuchtskoeffizienten innerhalb der Populationen nicht signifikant verschieden von Null (Tabelle 7). Werden die Loci einzeln betrachtet, so zeigen die meisten einen Ueberschuss an homozygoten Individuen, was auf Inzucht hindeutet (Tab. 8). Die genetischen Unterschiede zwischen Tümpeln innerhalb einer Population sind in Tabelle 9 dargestellt. Die meisten Populationen zeigen innerhalb von Tümpeln keinen Inzuchtseffekt, allerdings sind Probengrössen klein bis sehr klein.

Der Effekt des Flusses

Beide Flusseiten zeigen eine ähnliche Verteilung der genetischen Diversität: Der grösste Anteil genetischer Unterschiede liegt zwischen den Populationen innerhalb einer Flusseite (Tabelle 11). Die genetischen Unterschiede zwischen den beiden Flusseiten sind kleiner, aber signifikant (Tabelle 12).

Die "Isolation durch Distanz" ist signifikant, wobei die drei Populationspaare mit den grössten geographischen Distanzen viel zu der Signifikanz beitragen. Die "Isolation durch Distanz" ist viel weniger stark, wenn sie zwischen Populationen innerhalb einer Flusseite gemessen wird. Dabei kann auch mitwirken, dass bei diesen Tests die Probengrösse viel kleiner geworden ist.

Die zwei phylogeographischen Stammbäume zeigen beide wie sich die Populationen der Flusseiten jeweils gruppieren (Abb. 3). Einzig auf der Ostseite wird die Population ZH45 anders plaziert. Dies spricht für ein robustes Muster, auch wenn die Wahrscheinlichkeitswerte an den Verzweigungsstellen relativ niedrig sind.

Diskussion

Im allgemeinen kann gesagt werden, dass die Populationen stark voneinander unterschieden sind. Wenn die Populationen einzeln angeschaut werden, zeigen die meisten einen gewissen Inzuchtseffekt. Dies könnte auf die kleinen Populationsgrößen zurückzuführen sein, da in den einzigen Populationen, die keinen Inzuchtseffekt aufweisen gleichzeitig auch die meisten Laichschnüre und gemischte Proben gesammelt wurden.

Ein Fluss von der Grösse der Reuss kann die genetischen Unterschiede zwischen Kreuzkrötenpopulationen verstärken. Der Unterschied, der durch den Fluss verursacht wird, ist zwar relativ klein verglichen mit den Unterschieden, die sowieso zwischen Populationen gefunden werden, ist aber dennoch konstant.

Mikrosatelliten stammen aus nicht-codierender DNA. Sie unterliegen deshalb keinem Selektionsdruck. Somit können aus den vorliegenden genetischen Daten keine Rückschlüsse auf die lokale Anpassung der Populationen gezogen werden. Dennoch ist es empfehlenswert, wenn bei Pflegemassnahmen und sogar eventuellen Plänen für Translokationen zur Aufstockung der Populationsgrößen, die genetischen Unterschiede aufgrund geographischer Strukturen wie einem Fluss miteinbezogen werden.

Mit Ausnahme der Population AG8 haben alle Populationen seit den letzten Zählungen an Grösse abgenommen. Die (effektive) Populationsgrösse ist aber ein wichtiger Faktor für die Überlebenswahrscheinlichkeit von Populationen. Als eine grobe Regel mag gelten, dass Populationen unter 50 reproduzierenden Individuen genetische Diversität verlieren, und zufälligen Ereignissen viel stärker ausgesetzt sind. Dies trifft auf fast alle der untersuchten Populationen zu. Für das Ueberleben der Populationen ist es wichtig, die Populationsgrösse zu erhöhen und zu stabilisieren. Wichtige Faktoren sind dabei die Verbindung durch Migration und der Genfluss zwischen den Populationen. Dabei sollte aber auch bedacht werden, dass Genfluss auch zu Auszuchtdepressionen führen kann.

Genetische Diversität

Die schweizer Populationen zeigen trotz ihrer geringeren Grösse eine höhere genetische Diversität als britische Populationen. Im Vergleich mit spanischen Populationen sind sie dagegen weniger divers. Dieser Gradient von Süden nach Norden kann mit der "natural history" der Kreuzkröten zusammenhängen. Während der letzten Eiszeit war diese Art auf die Iberische Halbinsel zurückgedrängt, und breitete sich in den letzten 10'000 Jahren immer weiter nach Norden aus. Durch wiederholte genetische Flaschenhälse bei der Gründung neuer Populationen könnte bei der Ausbreitung nach Norden ein grosser Teil der genetischen Vielfalt verlorengegangen sein.

Methodische Bemerkungen

Das Hardy-Weinberg- und Kopplungsungleichgewicht, das in den Proben gefunden wurde, erschwert die Interpretation der Ergebnisse. Ungleichgewichte verletzen die Annahmen der F-Statistik, und kann deren Aussagekraft schwächen. Dieser Punkt muss noch genauer geklärt werden.