



LIFE-Project:



„Management von Rotbauchunken-Populationen im Ostseeraum“

“Management of fire-bellied toads in the Baltic region”

LIFE04NAT/DE/00028

Genetic report



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1. Introduction

1.1. Project background

Many populations of the fire-bellied toad, *Bombina orientalis*, are threatened with extinction. Therefore this species has been included in the annexes II and IV of the EU Habitats Directive. (Habitats Directive (92/43/EEC) Council Directive 92/43/EEC of 21 May 1992 on the conservation of natural habitats and of wild fauna and flora *Official Journal L 206*, 22/07/1992 P. 0007 – 0050)

The aim of the LIFE-Bombina project is the improvement of the status of nature conservation of populations of the fire-bellied toad (*Bombina orientalis*) in Latvia, Denmark, Sweden and Germany (Schleswig-Holstein) with the long term aim of 500 calling males per site or population. For further information: www.life-bombina.de

In the framework of this specie-targeting project, mainly focussing on activities on the ground, the aspect of genetic has been tackled. A rather unusual approach, but it became obvious that other than pure habitat measures are responsible for the vitality of small populations of *B. orientalis*.

The genetic diversity within populations is an important feature for the vitality of a population. If the genetic diversity is reduced e. g. by the loss of habitat, changes of the environment, etc. populations might end in a vicious circle (Fig. 1). If populations are already small habitat improvement per se will not be sufficient to improve the state of populations or to avoid extinction. A population management is needed.

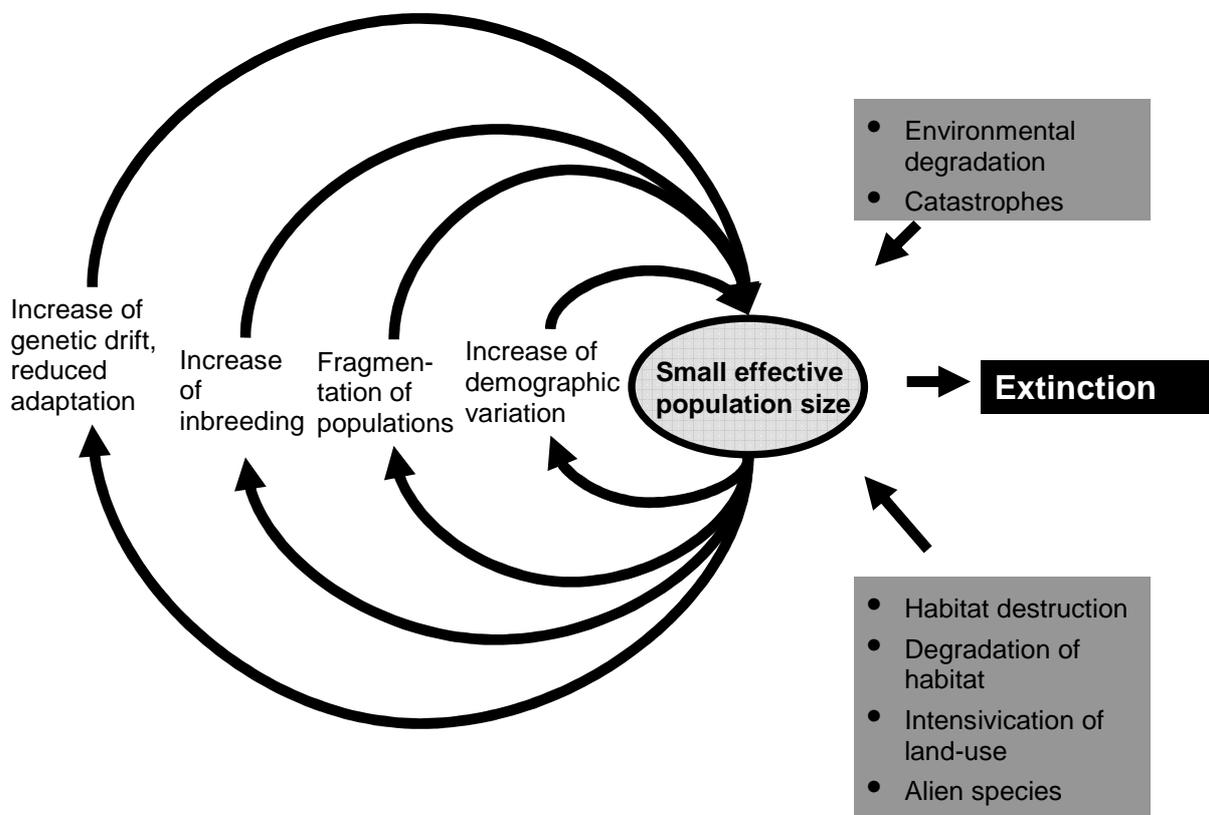


Fig. 1: Vicious circle of extinction: The stepwise reduction of the effective population size can end in the extinction of species (According to Primack 1993)

The present international LIFE project (Management von Rotbauchunken-Populationen im Ostseeraum/"Management of fire-bellied toads in the Baltic region" LIFE04NAT/DE/00028) is based on experiences of a previous national Danish LIFE project, which dealt with consolidation of the fire-bellied toad, *Bombina bombina*, in Denmark (LIFE99NAT/DK/006454). During that project (1999-2003) six remaining *Bombina* populations in Denmark were secured by a number of measures, including establishment of so-called "mirror populations" as a genetic reservoir of a threatened, isolated population from e.g. small Danish islands.

This means that from each population offspring was reared in captivity and then released at a new site to form a population that genetically is as close as possible to the original population. This gives an extra security to safeguard their gene pool. In case the original population would go extinct by some accident animals from the mirror population could be brought back to the original locality to rebuild a population there with an authentic genetic composition.

The project was carried out as planned and useful experience was gathered. This gave the impetus to continue with a LIFE-Nature project on an international level where:

- the experience gained will be better disseminated to other countries in northern Europe (Germany, Sweden and Latvia) within a wider platform and active involvement;
- habitat conditions for *Bombina bombina* will be improved in remaining localities by establishing breeding ponds, foraging habitats and hibernation sites;
- implementation of extensive grazing regimes in the land habitat between the ponds has proven helpful for a sustainable habitat regime, Experience and knowledge in running and managing these grazing schemes shall be disseminated and spread out involving local farmers and land owners;
- mirror populations shall be made also of the remaining seventh Danish *Bombina bombina* population where a mirror population has not yet been made;
- re-establishment of *Bombina bombina* at sites in Germany where the species got extinct in the last decades;
- assumed incomplete transfer of genetic variation to the first mirror populations could be remedied if there are indications from genetic survey verifying this speculation and
- artificially re-established populations in South Sweden could be improved genetically in the case that genetic deficits are found.

These activities were prepared during a LIFE starter project, and subsequently carried out during the present LIFE project "Management von Rotbauchunkenpopulationen im Ostseeraum" ("Management of fire-bellied toads in the Baltic region"), which runs from 2004 to 2009.

An important new item in the present project is the action of a broadscale genetic analysis of several international *B. bombina* populations carried out within the project and for the first time at all funded under a LIFE-Nature Program. The results of this analysis will be the basis for decisions on population management for the project sites.

1.2. Reasons for carrying out genetic analyses

The genetic analyses should help to make proper decisions on population management.

They were meant to give answers to the following general questions:

- Which populations contain sufficient genetic variation so that no special population management is necessary?
- Which of the small relict populations are so severely threatened by inbreeding depression that specific measures to improve the genetic diversity are necessary in order to secure their lasting persistence?
- Which populations are of local (autochthonous) origin and thus suitable as source populations for introductions at other localities in the vicinity?
- Which populations are of dubious origin (e.g. unintentional introduction with fish brood) and therefore less suitable as source populations for propagation?

The analyses should also serve to answer the following questions concerning specific regions:

- How large are the genetic differences between different subpopulations within the relatively sharply demarcated area of distribution in the Holsteinian lake district? Which consequences does that have for a proper long-term management of the populations?
- Has the establishment of mirror populations in Denmark been successful, or will it be necessary to carry out additional release to transfer alleles and genetic variation that are present in the source populations, but still missing in the mirror populations?
- What is the genetic composition of the mixed populations on Ærø and Birkholm in Denmark?
- Has the establishment of artificial populations in Sweden by introductions of Danish animals of mixed origin led to populations with an elevated genetic variation that reflects all sources, or have genes from specific source populations failed to become established in the new Swedish populations?
- What is the genetic composition of the population of fire-bellied toads that is kept in Riga Zoo in Latvia? Could this captive population be used as a source for supportive breeding and release in specific Latvian localities?

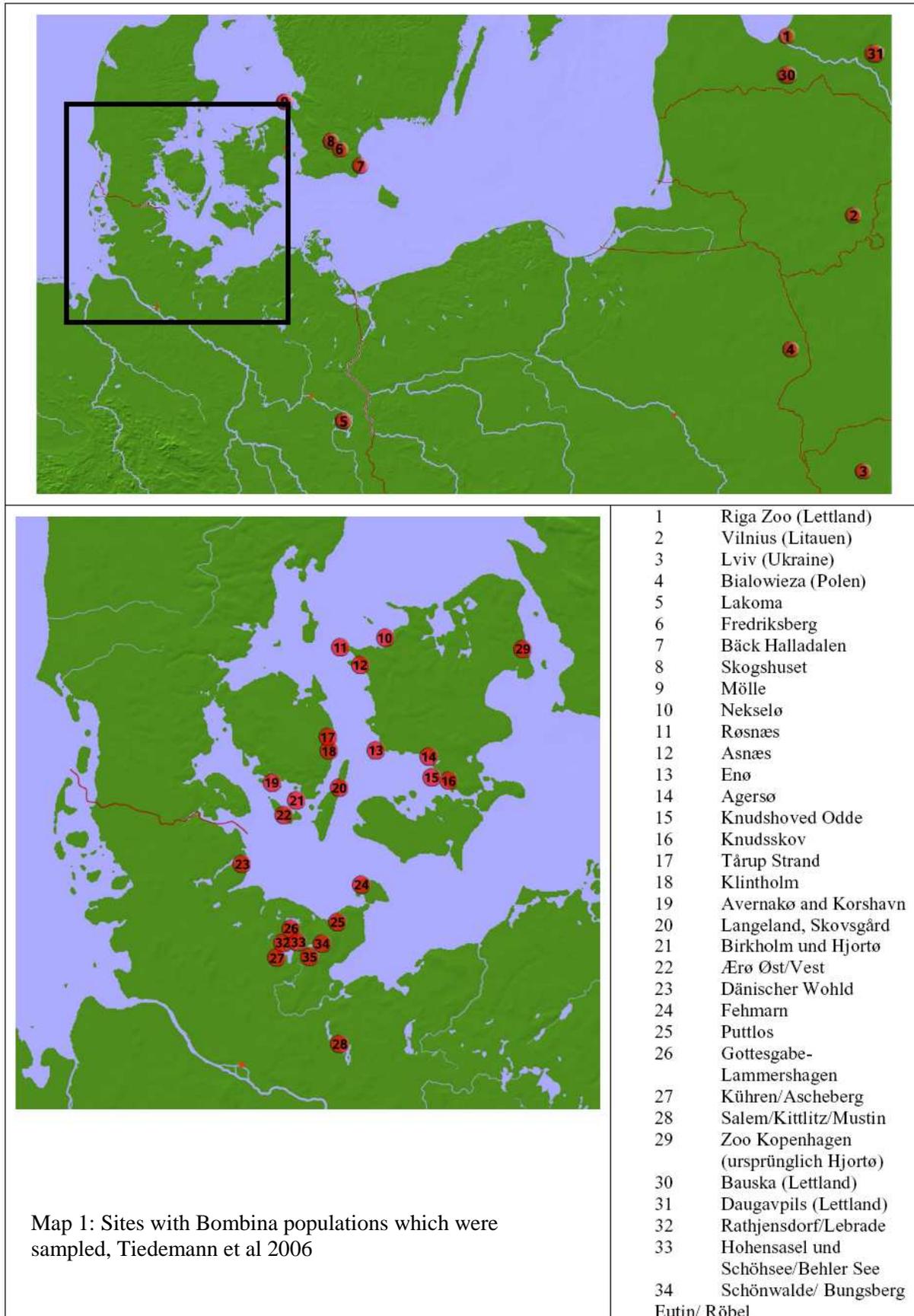
1.3. Sampling and analysis

Fire-bellied toads were caught in their ponds by field herpetologists. One tissue sample was taken from each animal and stored in a small vial with preservative liquid (DMSO-Buffer), whereupon the animal was set back in the pond. It was attempted to use a non-interfering method, namely by having the animals depositing saliva samples on cotton sticks. Unfortunately, this method often failed by yielding too little material for genetic analysis, and therefore a more intrusive method, the cutting of toe tips, was used in some cases. Thirty individuals were sampled per population, if possible.

The numbered samples were brought to the DNA laboratory at Potsdam University in Germany (Lehrstuhl für Evolutionsbiologie, Institut für Biochemie und Biologie, Universität Potsdam, Prof. Ralph Tiedemann). Here, they were analysed for three types of DNA:

- a) The D-loop region of mitochondrial DNA.
- b) Microsatellites in the nuclear DNA. Seventeen variable loci were analysed.
- c) The major histo-compatibility complex (MHC) of the nuclear DNA.

The results have been presented in a technical/scientific report from the DNA laboratory (Tiedemann et al 2006).



2. Overview of results from the genetic survey

2.1. Mitochondrial DNA

2.1.1. Indigenous and introduced types

Twelve different haplotypes of mitochondrial DNA were found. Eleven of these were closely related, and each of these could be derived from one of the others by just a single mutation. One type, designated DEG, differed greatly from all others and could only be derived from them by at least 9 mutational steps.

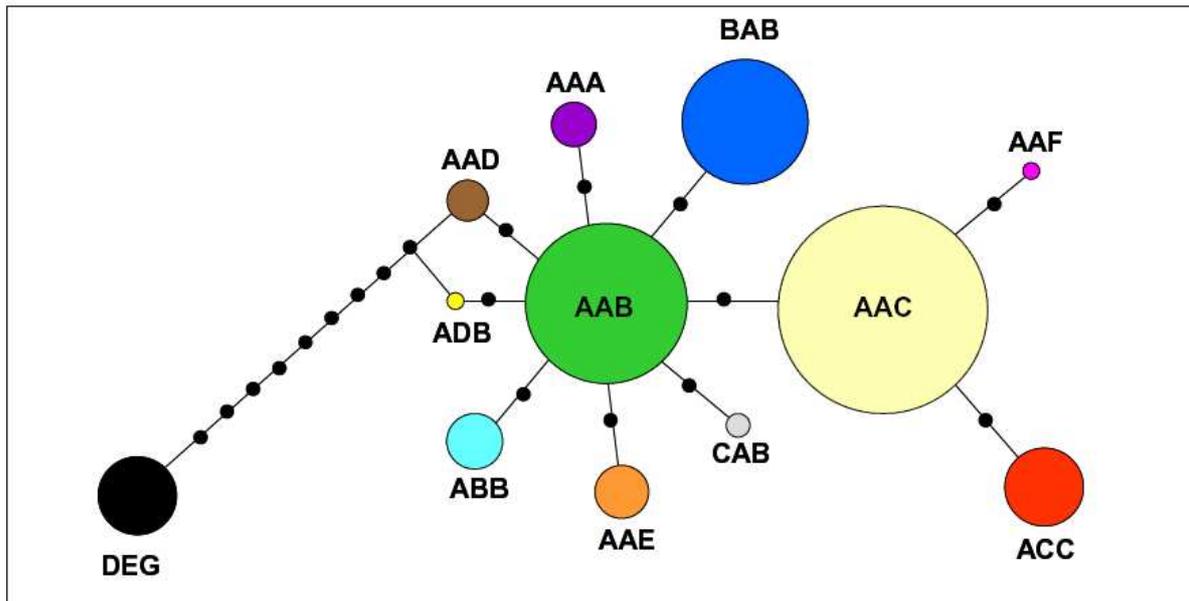


Fig 2: Minimum spanning network: The number of dots on the connecting lines indicates the number of nucleotides differing between the haplotypes, Tiedemann et al 2006.

The eleven closely related types are evidently original north European types. The aberrant DEG type, on the other hand, occurs only in populations which seem to have been influenced by introduction of animals of foreign origin. We do not know where the DEG haplotype occurs naturally, but for reasons indicated in the following we suggest that it is searched for in eastern Austria; populations here, and elsewhere in the Pannonian Basin west of the Carpathians, may be those European populations who are genetically most distant. They belong to a different line of descent than the populations which presumably have colonised north Europe via a route east of the Carpathians.

2.1.2. Reconstruction of the immigration route into northwest Europe

Mitochondrial genes are inherited clonally without recombination, which means that any mutation will be inherited to all descendants. This allows us to follow a pedigree back in time.

In the case of *Bombina bombina* in northwest Europe, we may safely assume that immigration after the ice age came from the south or southeast. Therefore, it seems obvious that the main part of the pedigree originates from the haplotype AAB, which is widespread south and east of the Baltic Sea. The AAB type differs by one mutation from AAD, which is present in low frequency in Poland, in Lauenburg and in one of the release sites in Sweden. It is not possible to say if AAB originated from AAD, or vice versa, but today AAB is by far the commonest. The presence of AAD at one site in Sweden (Skogshuset) indicates that it must be or have been present also in the Sjælland region in Denmark, although it has not been detected there in the present samples. The presence of AAB at Mölle in Sweden also suggests that this was present in Sjælland in the 1970s or the 1980s, when animals from there were introduced to Mölle.

By one mutation, AAB has changed into AAC, which is found in low frequency at Lakoma in eastern Germany, and in higher frequencies in Holstein and Denmark. AAB and AAC are the dominating haplotypes in most of the studied area. But here and there either of these has transmuted into other forms, which may in some places have become the only or most frequent type due to local genetic drift.

We may conclude that one branch of the pedigree colonised Dänischer Wohld, at the westernmost limit of the species range, and was later separated from the rest. This branch must have contained the two main haplotypes, AAB and AAC, and a mutation from AAB to AAA has then occurred locally. As an alternative hypothesis, one might suspect that the AAA haplotype here could be due to some introduction from elsewhere, but as long as this haplotype is not known from anywhere else, there is no support for such a hypothesis.

The study of other mutations indicates unequivocally the route of immigration into Denmark after the ice age: from the regions southeast of the Baltic Sea to north Germany (where AAC arose) and from there further into Denmark. The earliest Danish populations must have contained three haplotypes (AAB, AAC and AAD), and within Denmark, AAB mutated locally to BAB, which is common in parts of the Sjælland region and also present in Fyn, whereas AAC mutated locally to ACC, which is known only from Fyn county. The facts that AAB mutated to BAB within Denmark, that AAB subsequently disappeared nearly totally from Denmark; and that BAB has been found both in the Fyn region and the Sjælland region, suggests that the Danish populations were interconnected in the beginning, and only later separated. On the other hand, the fact that ACC is common to Fyn and an isolated island south of Fyn, but absent from the Sjælland region, suggests that the Fyn and the Sjælland regions have been separated for a long time, longer than the separation of the south Fyn archipelago from mainland Fyn.

The scenario sketched here makes sense because warm temperatures, sufficient for *Bombina bombina*, had come about 9,000 years b.p., whereas the separation of the large Danish islands from North Germany and from each other happened somewhat later, shortly before 8,000 b.p. The connection between Fyn and the land areas south of Fyn may have become disrupted at about 7,500 b.p. by a narrow sea strait. But the final fragmentation of the land areas south of Fyn into an archipelago has happened only within the last 2,000 years.

2.1.3. Genetic diversity at the mitochondrial level

Today, the frequencies of the different haplotypes differ very much between populations.

Within Holstein, the differences are moderate: all populations are dominated by one of the two most common types, AAB and AAC, with a trend for the proportion of AAC to increase in a cline from the east to the west. Some populations have retained only one type, which must be due to genetic drift. Dänischer Wohld has three haplotypes, which is a high diversity for such a small, isolated population.

Denmark has harboured a relatively high diversity, with five haplotypes, of which two are inferred only from their presence in Swedish specimens that descend from Danish animals. But the recent population bottlenecks have led to recent fixation or near-fixation in many populations. Samples from eight localities were monotypic; only two populations contained two haplotypes and only one population (Klintholm) contained three haplotypes. The Klintholm population arose by release from Tårup Strand during the years 1990-1995, wherefore we may assume that the source population still contained three haplotypes in the 1990s. When one of these has apparently been lost now, this may either be due to incomplete sampling, or to genetic drift during the last c. 10 years (another, less likely explanation is that a few old females, with BAB mitochondrial haplotype, survived in Klintholm until they could mate with new animals that were released there from Tårup).

The Nekselø population, which contains two haplotypes, was source population for that at Røsnæs, where only one haplotype is found. This is probably due to a founder effect and/or genetic drift. The presence of two further haplotypes in Swedish animals that are believed to have descended from animals introduced from the Sjælland region indicates that these haplotypes may have been lost in the source populations by recent genetic drift, or may have been overlooked because of insufficient sampling. As explained below (2.2.4, Skogshuset), there is a fair chance that AAD actually occurs on north Nekselø, which has not been well sampled.

Altogether, the data from Denmark point to a rather high diversity originally, which has been much reduced due to genetic drift in the small remaining populations in recent years.

Both populations in Latvia contain only one haplotype, as far as is known up to now. This suggests that these populations that are at the northern frontier of the species range, have been small in the past, or have originated from few founders.

2.1.4. Indications of unreported introductions

2.1.4.1. Mölle

The DEG type is found in the majority of all samples from Mölle in Sweden, where introduction of animals from central Europe is suspected, most probably from eastern Austria.

The original population here had died out by 1960, but in 1977 a few *Bombina* individuals (7 males) were heard here again. According to an anonymous letter sent to the local authority at that time, there had been an illegal introduction of 14 adults and 10 larvae from "Central Europe". This happened probably in 1974 and in any case at a time when eastern Austria was the main accessible place to sample *Bombina* west of the iron curtain in central Europe. Neusiedler See in easternmost Austria was known to have been visited during an excursion of a group of young Swedish naturalists and would be a likely place for the origin of such animals. Therefore it would be relevant to analyse specimens from eastern Austria to see if the DEG type actually occurs there. Unfortunately, such analyses have not been made up to now.

A second anonymous source of information is that people from Scania had taken about 30 *Bombina* individuals from Knudshoved in Denmark and released them at Mölle. This may have happened in 1977.

When the animals at Mölle were inspected by Claes Andrén and Göran Nilson in 1979, they concluded from their analysis of the colour patterns that there were some signs of

introgression of genes from *Bombina variegata*. This was based e.g. on the fact that the yellow colour on the underside of the forelimbs stretched right to the fingertips. No animals appearing like Danish Bombina were seen. The animals should not be considered as hybrids, but as descendants from a *Bombina bombina* population where a considerable degree of introgression of *variegata* genes has occurred. An independent verification of this by other, e.g. genetic methods would be useful.

The indication that the origin should be a place near a hybridization zone between *B. bombina* and *B. variegata* likewise points to eastern Austria as a likely source area.

The years around 1977 were very dry, and for some years the very shallow ponds at Mölle were dried out. In the early 1980s, very few specimens of Bombina were observed, and it was believed that the illegal introductions had failed. At the time when legal introduction from Denmark was initiated (in 1983), the ponds were inspected without finding any animals. But there is information that a few adults were recorded in 1984. From then on, animals of illegal and legal origin must have had the possibility to interbreed. It was believed that the "Austrian" genes would be so much diluted by the several later introductions from Denmark that the re-established population would essentially be of Danish origin.

The analysis of the samples taken there in 2004, however give a very different result. Out of 15 specimens, 13 (87 %) had the Central European haplotype, DEG. Thus the proportion of the DEG type seems to be larger than what would be expected from the ratio of Central European relative to Danish specimens released. This suggests that animals of Central European origin may have had a competitive advantage or superior fitness.

2.1.4.2. Frederiksberg

This locality is situated in central Scania, a few km from the locality "Skogshuset"; but it seems that no migrating animals have yet crossed from one locality to the other. The population at Frederiksberg was established by release of reared animals from Fyn County in Denmark. 90 juveniles from east Fyn were released in 1998, 380 juveniles from Ærø and Avernakø. Since then, the population has grown very much. Unexpectedly, the genetic analyses showed that 6 out of 11 specimens (55 %) had the DEG mitochondrial haplotype. In accordance with this, inspection of the very numerous newly metamorphosed froglets in September 2006 showed that some of these (5 - 10 %) had distinct *variegata* characters, in the spot pattern on the dorsal as well as the ventral side. The most likely explanation is that an unrecorded/illegal introduction of animals from Mölle has taken place.

Some photographs of specimens taken at Frederiksberg at the LIFE-Bombina workshop excursion on 12th of September 2006 give an idea about morphological variation in *B. bombina* from Frederiksberg. Photo 1 shows the belly pattern of two adults, one of which has indications of *variegata* influence, especially concerning the connection of the yellow colour from the loins to the hind part of the belly. Two experts from Ukraine and Austria who are familiar with Bombina hybrids both conclude from the picture that this individual is unusual for pure *B. bombina* and could have some *B. variegata* influence in its genes.

Photo 2 shows five newly metamorphosed toads in a rut near the Frederiksberg locality. Four of them are typical for *B. bombina*, with green "duck weed" spots on the neck and, adjoining these duck weed spots, one or two parallel oblong dark spots. The fifth individual, however, does not have the duck weed spots, and, more importantly, does not have the oblong spots next to them.



Photo 1: Belly pattern of two individuals of *B. bombina* from Frederiksberg, Sweden, left individual with some indications of *B. variegata* influence. (For details see text)



Photo 2: Young fire bellied toads at Frederiksberg with different patterns on the back, remarkable is the right individual without green pattern and no oblong, dark spots at the back. (For details see text)

2.1.4.3. Bungsberg

The DEG haplotype has also been found in a sample from eastern Holstein. This sample is called "Schönwalde/Bungsberg" in the report from Potsdam, but it will henceforth be called only "Bungsberg", because the ponds are rather far from the town Schönwalde, but much closer to the hill Bungsberg. The sample actually consists of samples from two localities, Neuhamrsdorf and Neutestorf, separated by a distance of about 4 km. The DEG haplotype was present in 3 specimens from Neuhamrsdorf (out of a total of 8 specimens), but in none from Neutestorf (n = 6).

The hypothesis that some of the animals here arise from an unreported introduction from Central Europe is not quite unlikely. The place at Neuhamrsdorf belongs to a hobby herpetologist, and there are also unexplained occurrences of *Hyla arborea* (which were otherwise believed to have been extinct in the region for 40 years) and possibly of *Rana ridibunda* (these have not been determined to species with certainty, but seem in any case to have been introduced).

2.2. Microsatellite DNA

2.2.1. What microsatellite DNA can tell

The microsatellites are found in the nuclear DNA that is it is subject to recombination. It is not normally neutral in relation to selective forces, and it has a high rate of mutation. Thus it is ideal for studying the relatively recent phases of genetic differentiation.

One way to utilise the information on microsatellites is to calculate the genetic distance between samples. The matrix of pair wise genetic distances allows the construction of a dendrogram which indicates groups of more or less closely related samples. When this is done for the Bombina samples, we see clear divisions that coincide with geographic regions. Latvia and Lithuania form a loose group. Ukraine, Poland and eastern Germany also form a loose group, connected to the former. Holstein with Fehmarn and Dänischer Wohld forms a tight group; Fyn forms a group with the south Fyn archipelago, and the samples from the Sjælland region form a tight group.

Within each group, there is a finer resolution which is also informative. However, this is not analysed very well with the genetic distance. Instead, assignment tests using the data programme STRUCTURE can be used to recognise certain combinations of alleles that characterise distinct samples or regions. This yields a better resolution of fine structure for instance within the east Holsteinian lake district. It also allows analysing mixed populations and obtaining a crude impression of how much of the total genetic variation has been contributed by each source population.

Other kinds of useful information obtained from the microsatellite data are

- - the number of alleles (allele richness),
- - the number of private alleles, i.e. alleles not found elsewhere and
- - the amount of genetic variation within each sample, expressed as the "expected heterozygosity", H_e .¹

¹ H means heterozygosity, and H_e (with the e as a small lowered letter) means expected heterozygosity.

2.2.2. Geographic differentiation according to genetic distances

2.2.2.1. Latvia

The two populations at Islice south of Riga and at Daugavpils are very different. That at Daugavpils deviates much not only from Islice, but also from Lithuania and Poland. It seems to represent a more eastern branch of the pedigree. The animals kept at Riga Zoo are clearly of the type from Daugavpils, not from Islice.

2.2.2.2. Northwest Germany

The genetic differentiation shows a close fit with geography. The sample from Lauenburg is closest to the common root, and is differentiated from the rest. The group of rather closely situated localities in eastern Holstein is not well resolved by the dendrogram. Dänischer Wohld is differentiated from the rest, but not very strongly so. It is clearly related to the rest of Holstein. Fehmarn is most closely related to Putlos, but relatively differentiated, which may be due to the low population size and the resulting genetic drift. The most deviating sample is that at Bungsberg. This confirms the results of the mitochondrial analysis, i.e. that this sample must be affected by introduction of animals of foreign origin.

2.2.2.3. Fyn County in Denmark

All samples from Fyn county group together, but within this group, there is a sharp separation of mainland Fyn (Tårup Strand and Klintholm) from the south Fyn archipelago. The samples from the small island Hjortø are relatively close to the common root, whereas all populations where animals reared from Avernakø have been released, group together. The Korshavn population that was founded with animals from Avernakø about 20 years ago, is already much differentiated. Ærø is much more closely related to Avernakø than to Hjortø.

2.2.2.4. The region around Sjælland in Denmark

Enø and Knudshoved group closely together. The geographic distance between the two localities - over the sea - is only 9 km as the crow flies, but 46 km if a toad should migrate over land. The most likely explanation for the short genetic distance is therefore not natural migration, but transport with man. Fishermen from Enø fish every day at Knudshoved, where some of their gear is stored, and it is quite likely that they have some time in the past transported Bombinas to or from Enø on purpose.

The two isolated island populations at west Sjælland, Agersø and Neksøl, are fairly well differentiated from each other and from Enø-Knudshoved, although all *Bombina* populations in the Sjælland region are obviously related.

2.2.2.5. Sweden

Four of the Swedish introduced populations have been analysed.

Skogshuset in central Scania was stocked in 1987-91 with animals originating from three localities on Sjælland. In accordance with this, it very clearly groups together with other samples from the Sjælland region.

The nearby locality Frederiksberg was stocked with animals originating from Fyn County in the years 1998-99. In accordance with this, it groups clearly together with samples from Fyn County. The indication from analysis of mitochondria and from phenology that there is a substantial influence from Central European genes is not reflected in the genetic distance.

Bäckhalladalen in eastern Scania was stocked in 1983-85 with animals from Sjælland, and later, in 1998 and 2002, with animals from Fyn County. The samples for genetic analyses

were taken from a pond where animals from Fyn had been released, and in accordance with this, the sample groups together with one of those localities on Fyn from which the latest introduction (2002) had happened.

The samples from Mölle do not group together with any Danish samples. Instead, it shows a weak relationship with Ukraine. This confirms the results from analysis of mitochondria, namely that the population mainly descends from Central Europe.

2.2.3. Differences between small and large populations

2.2.3.1. F_{ST} values

The fixation index, F_{ST} , indicates what proportion of the genetic variation is variation between populations. If D is some measure of the genetic difference between two randomly chosen individuals, then F_{ST} is calculated by a formula of the type

$F_{ST} = (D_{\text{between}} - D_{\text{within}}) / D_{\text{within}}$, where D_{between} indicates differences between populations and D_{within} the average of the differences within each of the two populations.

The results concerning F_{ST} are different for small and large populations. For example, let us compare two of the largest populations, Lakoma and Lviv, which are separated by a wide geographic distance. We get $F_{ST} = 0.13$. This is a fairly small value. We may also compare two small populations, Hjortø and Riga Zoo, which are also separated by a wide geographic distance. Then we get $F_{ST} = 0.44$, which is the highest value found in this study. The point is that when we look at small populations with a reduced genetic variation, then D_{within} is small, and when the same difference between populations is divided by a smaller denominator, then the result will be a larger figure.

This is a part of the reason why we find remarkably high F_{ST} values when comparing some of the small populations. The animals at Riga Zoo descend essentially from Daugavpils. In spite of this, comparison of the two gives $F_{ST} = 0.13$. The population at Korshavn was made by release of offspring from Avernakø in the years 1983-85. Although this is little more than 20 years ago, we already find the comparison of Korshavn with Avernakø to yield $F_{ST} = 0.18$. That is, these two populations should now be more different than Lakoma and Lviv.

2.2.3.2. Nei genetic distances

We may instead compare populations by looking at the Nei genetic distance. But here, too, we find remarkably high differences between small populations. If, again, we compare Korshavn and Avernakø, then we find a genetic distance of 0.28. This is approximately the same as between Lakoma and Bialowieza (which are separated by a geographic distance of 700 km) or between Avernakø and Lauenburg (which are separated by a divergence time of more than 8,000 years).

The genetic distance between populations on the mainland of Fyn and in the archipelago south of Fyn is large (Tårup strand vs. Avernakø: 0,56). This is practically the same as the distance between Tårup Strand and Bialowieza (0,57).

So, even when we use Nei's genetic distance as the parameter, we still find that small populations are genetically widely separated from others. The main explanation must be genetic drift in small populations. As shown by the genetic distance between Korshavn and Avernakø, which have only been (partially) separated for about 20 years, this process is extremely fast in populations as small as Korshavn. And on longer time scales, the Danish populations that have become isolated on each their island, have become just as different from each other as they are from the larger more coherent populations on the continent. The local differentiation on the Danish islands is thus very considerable. Much of this differentiation may have happened in rather recent time.

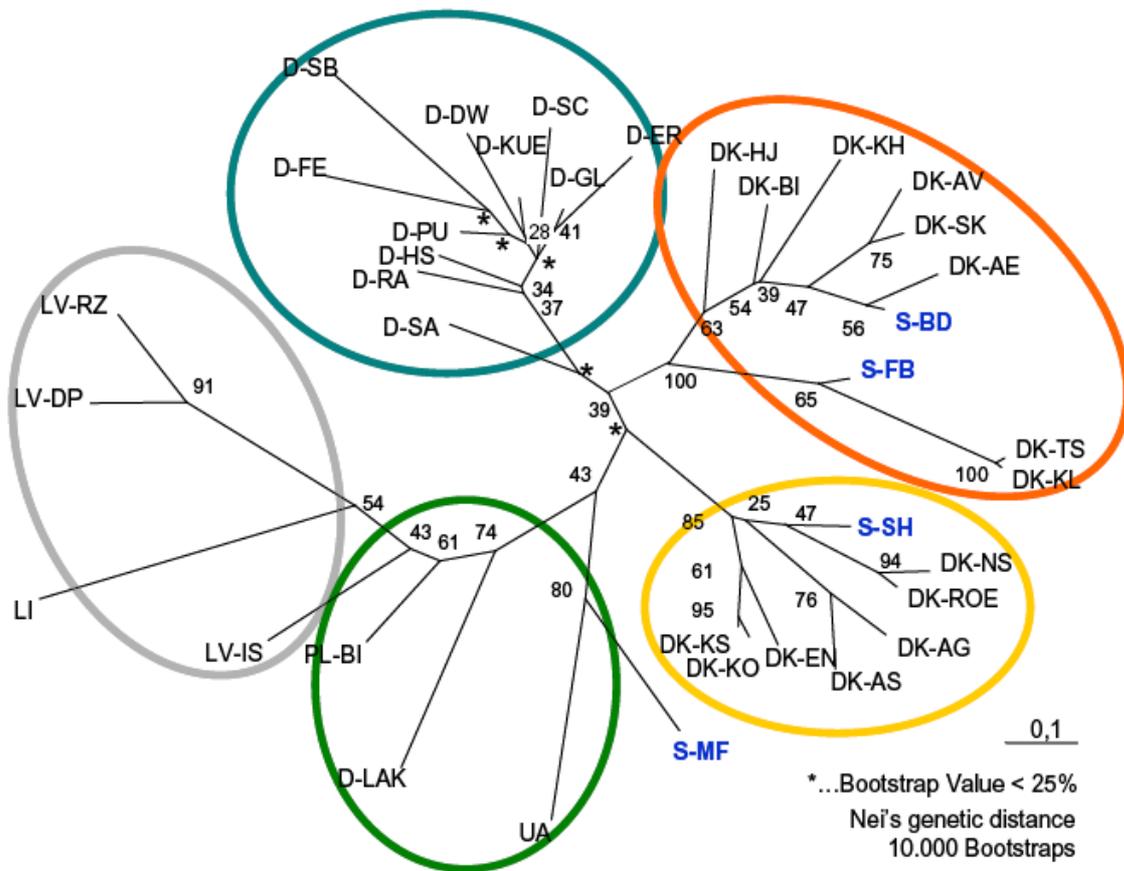


Fig 4: Genetic similarity (Nei's genetic distance) between populations according to the results of the Microsatellite analysis:

DK-TS=Tårup,
DK-KL=Klintholm,
DK-AV=Avernakø,
DK-KH=Korshavn,
DK-SK=Langeland Skovsgård,
DK-AE=Æ-East an West,
DK-HJ=Hjortø (KIM & Zoo),
DK-BI=Birkholm,
DK-NS=Nekselø,
DK-ROE=Røsnås,
DK-AG=Agersø,
DK-AS=Asnæs,
DK-EN=Enø north
DK-KO=Knudshoved Odde,
DK-KS=Knudsskov,
LV-IS=Bauska; Latvia
LV-DP=Daugavpils; Latvia
LV-RZ=Riga Zoo; Latvia
UA=Lviv, Ukraine

S-MF=Mölle,
S-SH=Skogshuset,
S-FB=Frederiksberg,
S-BD=Bäckhalladalen,
D-DW=Dänischer Wohld,
D-FE=Fehmarn,
D-KUE=Kühren/Ascheberg,
D-RA=Rathjensdorf/Lebrade,
D-SC=Schöhsee/Behler See,
D-HS=Hohensasel,
D-GL=Gottesgabe/Lammershagen,
D-SB=Schönwalde/Bungsberg,
D-ER=Eutin/Röbel,
D-PU=Putlos,
D-SA=Salem/Kittlitz/Mustin,
D-LAK=Lakoma, Brandenburg,
PL-BI=Bialowieza; Poland,
LI=Wilnius; Lithuania,

Colours for regions: orange=Fyn; yellow=Sjælland; blue=Sweden; turquoise=Germany, Schleswig-Holstein; grey=Latvia, green=reference populations (out-group), Tiedemann et al 2006

An important lesson from these considerations is that when populations stay small, like the one on Korshavn, then genetic drift is very fast, and erosion of genetic variation is considerable.

In this light, it is no wonder that most Danish populations have preserved only one mitochondrial haplotype, and it is no wonder that in Sweden we find two haplotypes which most likely have come from Denmark, but which have not been found there now. Such haplotypes could easily disappear out of the small Danish populations within the time span that has elapsed since the first transfers of genetic material to Sweden in 1983. To preserve the genetic variation that is still present, it is obviously of great concern that the populations be very soon brought to much higher size levels.

2.2.3.3. Assignment tests using STRUCTURE

The data program STRUCTURE is a tool for assigning an analysed sample to a certain origin. This is especially relevant for populations that originate from mixed introductions.

Analysis with STRUCTURE was performed either with all samples together or with samples for specific regions only. In some cases, the detailed analysis for specific regions gives the most information, but in some cases, the most realistic division into groups has been obtained with the analysis for all samples together. In one version, the programme has divided these into 13 groups, in another version into 15 groups. Especially the latter version has been used here. On the basis of the graphic representation of the results, a crude estimate has been made of how mixed populations are composed of genetic material from different sources; these estimates cannot be considered as facts, but rather as indications of likely situations.

2.2.3.3.1. Denmark: Ærø

The genes in the present population on Ærø may originate from three sources: 1) from the last surviving male on Ærø, which was mated with a female from Hjortø in 1987; 2) from the population on Hjortø, which was still fairly large in 1987-1991 when offspring from there was released in Ærø east; 3) from the population on Avernakø, from where offspring was released on Ærø west in 1991 and on Ærø west and east in 2000 and 2002.

The genetic composition in the areas Ærø west and Ærø east are rather similar. This may reflect that in both areas there have been released animals from both Hjortø and Avernakø, although in different proportions. Another interpretation could be that there is an efficient exchange of genes between east and west, but this is partially contradicted by the analysis of private alleles (see below).

The presence of genes originating from Avernakø may easily be recognized, because the source population has survived well. But the recognizing of genes from the two other sources is hampered by the fact that the Ærø population is extinct, and the Hjortø population has gone through an extremely narrow bottleneck after 1991, so that many original Hjortø alleles may have disappeared there before samples were taken for analysis.

14 specimens from Ærø are included in the analysis made with STRUCTURE. On average, about 8 % of the genes are referred to Hjortø, and the rest to Avernakø. This should not be taken for a well-founded result, and there are reasons to believe that this overestimates the influence from Avernakø. One of these reasons is that in total, 71 alleles are known from Avernakø and populations originating from Avernakø, whereas only 34 are known from Hjortø. The low number for Hjortø is obviously due to recent inbreeding, and we can be certain that in the years around 1990, there would have been more alleles present on Hjortø. When these are now present in specimens on Ærø, they could just as well have originated from Hjortø as from Avernakø, but the data programme will recognize them only if they are known from Avernakø, and for this reason it will overestimate the contribution from there.

More will be said about this question in the paragraph on private alleles.

It is unfortunate that the mitochondria from Ærø east and Birkholm have not been analysed. This might have given a better impression of what percentage of the genetic material originates from Hjortø resp. from Avernakø.

2.2.3.3.2. Denmark: Birkholm

The present Bombina population on the small island Birkholm was established by the introduction in 1998 of 36 specimens originating from Ærø. There have later been introduced additional animals from Hjortø, but it is believed that the animals sampled are mainly from the first introduction. This was before the large-scale release of Avernakø-offspring on Ærø, and therefore we would expect that there would be a smaller fraction of Avernakø alleles on Birkholm than there is on Ærø at present.

This assumption is verified. When analysing the results from STRUCTURE, we arrive at a result that 25 % of the genetic material on Birkholm originates from Hjortø, compared to 8 % as estimated for Ærø. The ratio between these figures may reflect the true situation, but the absolute values of these figures are not reliable, as indicated in the preceding paragraph.

Another source of information is the analysis of fixed loci. In Avernakø, the locus D2 is fixed for allele 232, whereas in Hjortø, we have both allele 226 and 232 on this locus. All individuals from Ærø that have been analysed have only allele 232, which points to an Avernakø origin. But the individuals from Birkholm are different. They have 43 % allele 226 and 57 % allele 232. This proves that a high proportion of the alleles there - at least more than 43 % - do not originate from Avernakø. As these individuals were offspring from Ærø, there must have been a high proportion of Hjortø alleles there at the time when they were reared. This proportion obviously has declined since then, which means that the additional releases of many Avernakø animals during 1999-2003 has diluted the original contribution from Hjortø so much that the present population is much more similar to Avernakø than before.

If we accept that the analysis of locus D2 gives a representative impression, then we may roughly guess that the true proportion of non-Avernakø alleles on Birkholm is about 50 %. This is twice the proportion estimated from STRUCTURE. On this basis we may guess that also on Ærø the true proportion of non-Avernakø alleles is about twice that estimated by STRUCTURE.

2.2.3.3.3. Sweden: Mölle

The legal introduction of Bombina to Mölle was established by release of animals reared in Göteborg. They originated from three localities in the Sjælland region: Knudshoved, Agersø and Nekselø.

The result of the assignment test indicates that probably about 3 % of the present genes originate from Knudshoved, and 10 % from Agersø and/or Nekselø. This leaves 87 % with some other origin - which is believed to be eastern Austria. The agreement with the mitochondrial data, which also indicate a proportion of Austrian genes of 87 %, is excellent, but this agreement is probably accidental and does not imply that the estimate from STRUCTURE is very reliable.

2.2.3.3.4. Sweden: Skogshuset

The population at Skogshuset was established by release of animals reared in Göteborg. They originated from three localities in the Sjælland region: Knudshoved, Agersø and Nekselø. It is unknown at what proportions the three localities contributed. However, the assignment test indicates that the animals at Skogshuset originate practically 100 % from Nekselø.

This result is somewhat at variance with the mitochondrial data. The BAB haplotype is

found in 87 % of the samples from Nekselø. But the mitochondria from Skogshuset were only 1 BAB (= 6 %), 1 AAD and 14 AAC. The discrepancy must be due to two founder effects: 1) Nearly all samples from Nekselø are from the population at south Nekselø that was founded by very few animals around 1990; 2) there is also a founder effect in the establishment of the population at Skogshuset.

The presence of one animal with a AAD mitochondrial haplotype is interesting. This haplotype is otherwise known only from Lauenburg and Poland. The data suggests that it may occur on Nekselø also. If further samples were taken on north Nekselø, it might be found here.

2.2.3.3.5. Sweden: Frederiksberg

The population at Frederiksberg was established in 1998-1999 by release of toads reared from Fyn county (Tårup Strand, Klintholm, Avernakø, Ærø).

The result of the assignment test indicates that probably about 7 % of the present genes originate from Tårup Strand or Klintholm, and 10 % from Avernakø or Ærø. That leaves 83 % that must be of some other origin. This supports the conclusion from the mitochondrial analysis that there must have been an illegal introduction from elsewhere, most likely from Mölle. And knowing that a large majority of the genes at Mölle are of foreign origin, we may infer that a slightly smaller majority the genes at Frederiksberg are also of foreign origin. For comparison, the mitochondrial analysis indicates that about 55 % of the genetic material is of non-nordic origin.

One of the specimens at Frederiksberg is by assignment test referred to Skogshuset rather than to Frederiksberg. The most likely explanation for this is that it is a migrant from a nearby locality. This could be Skogshuset (distance 3 km) but it could also be Ållskog or Svinahøjden (distance 2 km), because the populations here - which have not been sampled - have the same origin as Skogshuset. The important point is that migrations between these localities do occur.

2.2.3.3.6. Sweden: Bäckhalladalen

The 13 samples taken here were from a pond where offspring from Fyn county had been released in 1998 and 2002, i.e. few years before. The two source localities were Tårup Strand and Avernakø. The assignment test indicates that on average about 10 % of the genes are from Tårup Strand and 90 % from Avernakø. Animals originating from Sjælland were released previously at another locality nearby; there is no sign that these have contributed to the sampled population.

2.2.3.3.7. Germany: Bungsberg

The mitochondrial analyses suggested that some samples from Neuhammsdorf near Bungsberg in eastern Holstein contained foreign genes, probably from Central Europe.

The output from the STRUCTURE analysis confirms this. The analysed samples from Bungsberg are 8 samples from Neuhammsdorf, and 6 samples from Neutestorf. All 8 from Neuhammsdorf contain alleles that are related to alleles found at Mölle and Frederiksberg in Sweden. Actually, the number of alleles that are just found in the three populations and nowhere else is so remarkably high that one is led to assume that the introductions are possibly made from the very same locality. As the introductions in Sweden may possibly have come from Neusiedler See in Austria, it will be relevant to suspect an introduction from here also to Neuhammsdorf.

STRUCTURE indicates that the proportion of Central European genes may be c. 40 %. Accidentally, this agrees very well with the mitochondrial data that 3 out of 8 specimens had the foreign haplotype, i.e. 37.5 %. The fact that foreign nuclear genes are present in all analysed individuals indicates that the introduction must have happened some time ago, since there has been time for the indigenous and the introduced genes to mix quite

well.

When we disregard the 40 % of the genetic variance of foreign origin, the rest shows a high similarity to the neighbouring locality at Neutestorf, 4 km away. This indicates that the populations have been in contact fairly recently, but not after the introduction of foreign genes. The relation of the indigenous part of the genetic variation to other Holsteinian samples is dealt with in the following paragraph.

2.2.3.3.8. Fine structure within Holstein

The results from STRUCTURE show that the samples in Holstein fall into three groups:

- A) Bungsberg (excluding introduced genes) and Putlos, with relations to Fehmarn.
- B) Eutin/Röbel, Hohensasel and Gottesgabe-Lammershagen
- C) Kühren/Ascheberg and Dänischer Wohld, with Schönsee/Behler See and Rathjensdorf/Lebrade associated mostly to this group, but also to group B.

It should be mentioned that the difference between the three groups is quite small, and that they evidently have been in contact genetically. But the gene flow between the groups has not been so strong that all differences have been levelled out.

At first sight, the grouping into A, B and C is not very understandable when compared with geography. One would have anticipated that the populations in the east Holsteinian lake district formed one group, because the known *Bombina* ponds cluster fairly closely, without any obvious geographic subdivision. And one would expect that Dänischer Wohld and Putlos, which are separated from the central area by gaps more or less without *Bombina* localities, would be genetically separate. But the genetic pattern does not follow this expectation. Instead, it may be interpreted as illustrated in Figure 5, in which each group is interpreted as a route of dispersion.

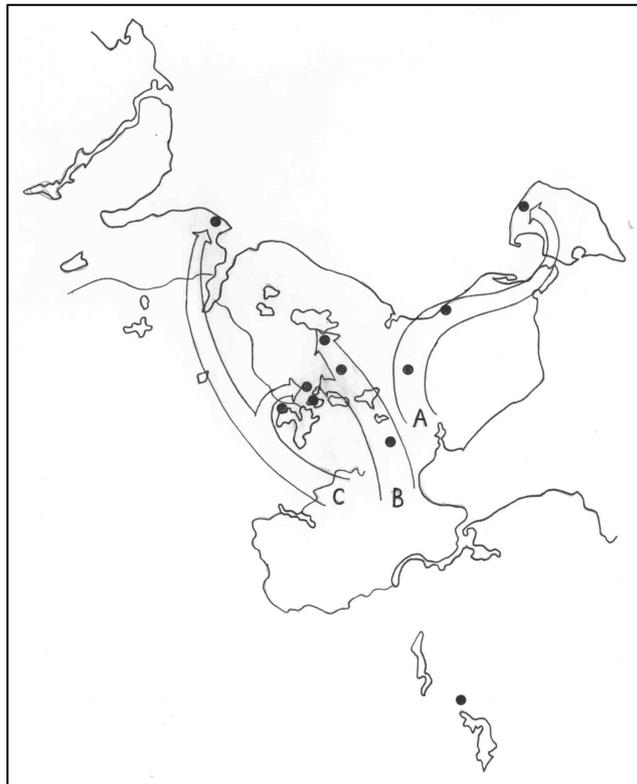


Fig 5: Assumed dispersion of *B. bombina* in Holsteinian lake district, Kåre Fog

In group A, the samples from the Bungsberg area are clearly related to Putlos to the northeast, and very different from those to the south and west, although the geographic distances are approximately the same (10-15 km in all cases). The connection Bungsberg-Putlos may have gone via small isolated nowadays extinct populations like Friederikenhof near the Baltic coast. The connection from Putlos further northwards to Fehmarn is also surprisingly close; the main reason why Fehmarn is differentiated is that it has lost many alleles, probably due to inbreeding.

In group B, Eutin/Röbel, Hohensasel and Gottesgabe-Lammershagen are very similar, although the distance from the first to the last is about 22 km.

In group C, the population at Kühren/Ascheberg is well differentiated from group B. The populations at Schönsee and Rathjensdorf/Lebrade are related to it, but also have some affinity to group B. However, it is remarkable that distance from these localities to Hohensasel (c. 8 km) has been enough to retain some degree of differentiation.

That Dänischer Wohld is very similar to Kühren/Ascheberg is also surprising. Here, the data from mitochondria and those from microsatellites tell rather different stories. The former indicate local diversification, whereas the latter indicate a lack of local diversification; there are no private Microsatellite alleles at Dänischer Wohld.

The division between A, B and C is not reflected in the mitochondrial data, which show a different pattern. However, as the mitochondria in east Holstein are nearly always of only two types, they should be considered much less informative than the microsatellites.

It is strange that the three groups A, B and C have not merged completely due to east-west migrations. A hypothesis that might give a part of the explanation could be that the arrows in the figure reflect past immigration routes. The toads must have immigrated from the southeast, and each line of descent may have spread rapidly in the direction where no ponds were occupied - that is towards northwest - but dispersal in other directions could have been hampered, because neighbouring areas were already occupied by other lines of descent.

2.2.3.3.9. Latvia: Riga Zoo

The assignment test suggests that the genes in the animals kept at Riga Zoo originate exclusively from Daugavpils. If this is true, then the animals may be treated as if they are Daugavpils animals. However, data on MHC (see section 2.3.3.) indicate that there is also a minimal influence of Islice genes.

2.2.3.4. Private alleles

By "private alleles" are understood alleles that occur in the sample we look at, but not in any of the samples that we compare with. The number of private alleles detected depends strongly on sample size and on what other populations are also analysed.

Private alleles may tell about how long the population has been isolated from others, and how valuable it is for the preservation of the total genetic variance. They may also give clues as to the composition of mixed populations.

A special problem is how to treat mirror populations. For example, the mirror population at Røsnæs reflects very well the source population at Nekselø. Nearly all alleles have been transferred in the release process. Thus, there are today no private alleles on Nekselø, because the single allele that was completely private, is now also at Røsnæs. So if one wants to answer the question: "Would any alleles be completely lost if the Røsnæs population disappeared?", then the answer would be no (because they have been preserved in Røsnæs). But if one wants to answer the question: "How much local diversification has happened on Nekselø?" then the answer would be that one allele is found only in this population and its mirror population. A second allele is found only here and in the Swedish populations at Mölle and Frederiksberg. These two Swedish populations have both received offspring from Nekselø, and according to the results from STRUCTURE, it seems that some of the genetic material from Nekselø has actually survived there. It is therefore quite likely that the allele referred to is also a private allele, i.e. it has probably arisen only at Nekselø, from where it has then been transferred by introductions to Røsnæs and Mölle, and from Mölle further to Frederiksberg.

The Potsdam genetics report gives only the number of alleles that are still completely private, i.e. those that have not been transferred elsewhere by any introductions. Here focus will be on signs of local differentiation, and therefore source and mirror populations will be treated as one.

2.2.3.4.1. General overview

Table 1 is an overview over the number of private alleles in the sampled populations, under the condition that source and mirror populations are considered as one.

Population	No. of private alleles	Remarks
DK: Avernakø-Korshavn-Skovsgård	3	
DK: Ærø-Birkholm	1	
DK: Hjortø	1	
DK: Tårup Strand-Klintholm	2	
DK: Nekselø-Røsnæs	1	2, if including one allele also found in Sweden
DK: Agersø-Asnæs	9	
DK: Enø	1	
DK: Knudshoved-Knudsskov	4	
D: Fehmarn	1	
D: Dänischer Wohld	0	
D: Kühren/Ascheberg	0	
D: Schöhsee-Rathejnsdorf/Lebrade	0	
D: Gottesgabe/Lammershagen	2	
D: Hohensasel	0	
D: Eutin/Röbel	0	
D: Bungsberg	1-10	Uncertain because some of the alleles might be due to introduction from Central Europe
D: Putlos	7	
D: Salemer Moor	4	
D: Lakoma	13	
PL: Bialowieza	10	
UA: Lviv	27	
LV: Bauska/Islice	3	
LV: Daugavpils/Riga Zoo	1	Daug. & Riga seen as one pop.
S: Bäckhalladalen	1	
S: Skogshuset	0	

Table 1: No. of private alleles for the whole region considered.

There are some general trends. First we see that the large eastern populations that have been sampled as out-groups have many private alleles. This is very true for Ukraine and less true for Lakoma in eastern Germany and Bialowieza in Poland. This is as expected, when you expect a high genetic diversity in these populations, plus a geographic isolation from the Baltic region.

On the other hand it is very surprising that with respect to private alleles, the small island Agersø in Denmark ranges nearly as high as Bialowieza. Also all other Danish populations have some private alleles, even the two most inbred populations - Hjortø and Enø.

There may be a general trend for local diversification on islands, as also the rather inbred population on Fehmarn has preserved one private allele.

In contrast to this, most samples from mainland Holstein have no private alleles. An obvious explanation for this is that the populations there are so closely connected that any allele that arises by a local mutation will soon have spread to neighbouring populations and therefore not remain private. However, this is not the whole explanation. Even when a sample from Holstein, e.g. Kühren, is only compared with samples from other regions and

not with samples from neighbouring populations, we still observe very few private alleles. And Dänischer Wohld, which is quite isolated, has no private alleles. So it seems that there is indeed little local diversification in Holstein.

The small populations in Latvia both have at least one private allele.

2.2.3.4.2. Private alleles within Denmark

Table 2 is an overview over the number of alleles that are private for a single locality in Denmark, provided that it is compared only with other Danish localities, neglecting what alleles occur in other countries. The table deals only with populations of pure descent. The occurrence of alleles in populations of mixed origin (Ærø, Birkholm) was disregarded when the table was constructed.

Population	No. of private alleles
DK: Avernakø-Korshavn-Skovsgård	7
DK: Hjortø	2
DK: Tårup Strand-Klintholm	3
DK: Nekselø-Røsnæs	5
DK: Agersø-Asnæs	18
DK: Enø	2
DK: Knudshoved-Knudsskov	12

Table 2: No. of private alleles when only pure populations in Denmark are considered.

Again we see the very large local differentiation in the Agersø population, but this time the local differentiations in Avernakø and Knudshoved are also clear. The small inbred populations (Hjortø and Enø) now both have two private alleles.

2.2.3.4.3. The situation on Ærø

Let us now consider the Danish populations of mixed origin, i.e. Ærø and Birkholm, which were let out of consideration in the preceding paragraph. The results obtained with STRUCTURE, referred to above, were that the main part of the genetic variation here originated from Avernakø, with only small fractions originating from Hjortø. If this were indeed so, we would expect the following: Out of the rare alleles found in Ærø-Birkholm most or all would have been private alleles for the source population on Avernakø until they eventually were brought to Ærø-Birkholm by the introductions. But the pattern that we see is very different from this expectation - see Table 3.

Allele	Found in					Remarks
	Avernakø	Hjortø	Ærø west	Ærø east	Birkholm	
F22: 158			x	x		Probably Hjortø origin
Bv411: 272					x	
Bv411: 274		x	x		x	Certainly Hjortø origin
Bv117: 162				x		
F2: 245					x	

Table 3: The geographical distribution of rare alleles found in Ærø-Birkholm (i.e. private alleles and alleles that were private before introduction to Ærø).

There occur a total of 5 alleles in Ærø-Birkholm that were or are private. Of these alleles, none is also found in Avernakø, but 1 is also found in Hjortø.

The distribution within Ærø-Birkholm is interesting, because animals from Avernakø were mainly released in Ærø west, whereas animals from Hjortø were released both in Ærø west and east, and all the first introductions from Hjortø, performed during 1987-1991, were only released in Ærø east, from where offspring was later introduced to Birkholm. These first introductions include offspring from a mating in 1987 which may have included the last male from Ærø.

Now we see that none of the rare alleles is found only in Ærø west (which might be expected if it was originally an Avernakø allele). Two alleles are found in two locations, either Ærø west + east or Ærø west + Birkholm. For both of these, an origin from Hjortø is most likely, and one of the alleles is very common in Hjortø, but completely absent in Avernakø, so here an origin from Hjortø is practically certain. The remaining three are found only in Ærø east or Birkholm, which suggests an origin from the first introductions to Ærø. It is possible that all these alleles originate from Hjortø, at the time when this population was not yet severely inbred. But that will mean that Hjortø at that time contained a total of 6 private alleles, which is a surprisingly large number for such a small population (cf. Table 2). Therefore, from such very indirect considerations, one may infer that it is fairly likely that a few of the private alleles could have come from the original Ærø population. Thus, the data are compatible with an assumption that the last surviving individual on Ærø has actually contributed with genes to the present population there.

Although such reasoning is very uncertain and speculative, we do have a situation where Ærø-Birkholm contains relatively many alleles that are found nowhere else in Denmark, and one of them nowhere else in Europe. And it seems unlikely that STRUCTURE gives a correct picture of the composition of the mixed population. It would therefore be sensible to consider Ærø as a distinct population, different from others, and to treat it as such. It is not just a simple mirror population of what is now found in Avernakø and Hjortø.

2.2.3.5. Allelic richness

The term 'allelic richness' means the number of different alleles found within a population, calculated as an average per locus. This number depends, of course, on the total number of samples analysed. If many samples are analysed, more rare alleles will be found. It is possible to make statistical computations that compensate for this effect.

Allelic richness is an indicator of genetic variation. It does not say exactly the same as the heterozygosity. One may have two populations with approximately the same heterozygosity where one has many more alleles than the other.

The number of alleles in a population is not, like the number of private alleles, affected by the presence of a mirror population.

2.2.3.5.1. Overview

The average number of alleles per locus, recalculated to a sample size of 13, is as follows:

- Eastern Europe: 7.6
- Schleswig-Holstein: 5.9
- Denmark: Sjælland: 4.9
- Denmark: Fyn: 4.0
- Latvia: 3.8

Obviously, the closer we come to the border of distribution, the fewer alleles we find. The differences between regions are more marked than what is seen for the heterozygosity (see next paragraph). In Eastern Europe, most loci have more than one allele, and most of them even have a very large number of alleles. This extent of genetic variation is not fully expressed in the heterozygosity, because even with fewer alleles per locus, nearly all individuals would still be heterozygous.

2.2.3.5.2. Danish mirror populations

If the establishment of a mirror population were 100 % successful, then all alleles found in the source population would also have been transferred to the mirror population. Let us see how the results actually are (Table 4):

Source population	Mirror population	Source	Mirror
Tårup Strand	Klintholm	2.37	2.49
Avernakø	Korshavn	2.70	2.15
Avernakø	Skovsgård	2.70	2.47
Knudshoved	Knudsskov	3.50	3.43
Agersø	Asnæs	3.98	3.33
Nekselø	Røsnæs	2.68	2.67

Table 4: Allelic richness (alleles/locus, compensated for sample size) in Danish source and mirror populations. Compensations have been made corresponding to a sample size of 6 specimens.

The most successful establishment of a mirror population is that at Røsnæs. From the table it seems as if every single allele has been transferred. That is not quite so, however. Several alleles have been found on Nekselø, but not on Røsnæs, whereas several others have been found on Røsnæs, but not on Nekselø. These differences probably just reflect sampling errors, but it is also possible that a few alleles have actually not been transferred.

Another successful mirror population is that made from Knudshoved. Klintholm, which is a mirror population of Tårup Strand, seems to have been "over successful". The explanation here is that the transfer happened already c. 15 years ago. Since then, the population at Tårup Strand has remained rather small, and due to continued inbreeding, a number of alleles may have been lost, whereas these alleles have survived at Klintholm, where the population size has been larger.

There are two mirror populations of Avernakø. One is Korshavn, which was established already 20 years ago, with a rather limited amount of individuals; and since then, the Korshavn population has remained quite small. It is therefore not surprising that there are so many fewer alleles on Korshavn; rather, it is surprising that the loss of alleles has not been greater. Korshavn is not worthless as mirror population - 8 alleles have been found there which have not been found on Avernakø.

The other mirror population from Avernakø is Skovsgård. This has been stocked recently with a rather large number of animals, and one should therefore expect a rather close identity to the source population. But this is not very much so. It should be added that 7 alleles have been found in Skovsgård which have not been found in Korshavn or Avernakø.

The poorest result is that for Asnæs as a mirror population of Agersø. There is a large unique genetic variation on Agersø, with extraordinarily many private alleles. But out of 18 private alleles, only 3 have been transferred to Asnæs. Evidently, too little material has been transferred.

2.2.3.5.3. Danish mixed populations

Ærø and Birkholm are mixed populations. The allelic richness here, as compared with the source populations Avernakø and Hjortø, is as follows (the figures are adjusted to a sample size of 6 specimens):

- Ærø: 2.83
- Birkholm: 2.53
- Avernakø: 2.70

- Hjortø: 1.78

We see that mixing has caused Ærø to be richest in alleles of all populations in the archipelago south of Fyn.

2.2.3.5.4. Swedish mixed populations

Table 5 illustrates allelic richness in the Swedish populations:

Most important source population(s)	Mirror population	Source(s)	Mirror
Avernakø	Bäckhalladalen	2.70	2.49
Nekselø	Skogshuset	2.68	2.67
Tårup Strand, Avernakø	Frederiksberg	2.37, 2.70	3.78
Knudshoved, Agersø, Nekselø	Mölle	3.50, 3.98	4.22

Table 5: Allelic richness (alleles/locus) in Swedish mixed populations compared with some of the Danish source populations. Compensations have been made corresponding to a sample size of 6 specimens.

In general, we should expect higher allelic richness in mixed populations than in source populations. But this is not so here. The only population with an increased allelic richness is Frederiksberg. This may clearly be due to the presumed additional transfer of individuals from Mölle to this locality. The release into Bäckhalladalen has genetically not been very successful. On the other hand, the transfer to Skogshuset has been surprisingly successful, in consideration of the results from STRUCTURE that the only population that has effectively contributed to this mirror population is Nekselø. Therefore, both Skogshuset and Røsnæs may be considered as good mirror populations of Nekselø.

The highest allelic richness is seen at Mölle, although it is not as high as might be expected from the introduction of Central European genes.

2.2.3.6. Heterozygosity

Heterozygosity is an important measure of genetic variation. What is measured is mostly not the observed heterozygosity, but rather the expected heterozygosity, called H_e , which is calculated from the observed allele frequencies under the assumption of genetic equilibrium.

2.2.3.6.1. Overview

The average heterozygosity H_e is as follows in various regions:

- Eastern Europe: 0.76
- Schleswig-Holstein: 0.65
- Denmark: Sjælland: 0.48
- Denmark: Fyn: 0.40
- Latvia: 0.55 (natural populations)

As expected, H_e is highest in the large populations in eastern Europe, and lower in small populations near the border of distribution. It is lowest in Denmark, especially in Fyn county.

Within Fyn, it is lowest in Hjortø - 0.21, which is an extraordinarily low value, in agreement with the severe inbreeding that has affected this population. Among the other populations of Fyn county, it ranges from 0.29 as the lowest, in Tårup Strand, to 0.43 as the highest, in Avernakø.

Within Sjælland, H_e ranges from 0.34 in Nekselø to 0.48 in Enø and 0.56 in Knudshoved

and Agersø. It is surprising that the small and isolated population Enø lies relatively high, whereas the population at Nekselø, which seems to have a relatively high fitness, ranks as the lowest. But there are reasons to suspect that the analysed sample from Nekselø was not representative of the whole Nekselø population. All analysed animals are from a single large population at the south end of Nekselø, a population which was founded by very few immigrants around 1990. We should expect that a larger genetic variation would be present in the north end of Nekselø, where Bombina has been continuously distributed among a series of ponds for a long time.

Within Holstein, the lowest value of H_e is 0.45 in Schöhsee/Behler See. There are also relatively low values in the small and isolated populations Fehmarn and Dänischer Wohld (0.51 in both cases). The highest value in Holstein proper is 0.62 in Putlos. Salemer Moor in Lauenburg lays even higher (0.66). There is an even higher H_e value at Bungsberg, but this must be due to the effect of introduction of foreign genes.

The highest of all recorded values is at Bialowieza (0.70), which lies marginally higher than Lviv in Ukraine.

2.2.3.6.2. Danish mirror populations

Table 6 compares the source and mirror populations in Denmark:

Source population	Mirror population	Source	Mirror
Tårup Strand	Klintholm	0.29	0.32
Avernakø	Korshavn	0.43	0.36
Avernakø	Skovsgård	0.43	0.39
Knudshoved	Knudsskov	0.56	0.54
Agersø	Asnæs	0.56	0.49
Nekselø	Røsnæs	0.34	0.37

Table 6: Expected heterozygosity, H_e , in Danish source and mirror populations.

The conclusions are more or less the same as with allelic richness. We see once more that Asnæs is an incomplete mirror of Agersø. Successful mirror populations are Klintholm, Skovsgård, Knudsskov and Røsnæs.

2.2.3.6.3. Danish mixed populations

Ærø and Birkholm are mixed populations. The H_e values here, as compared with the source populations Avernakø and Hjortø, are as follows:

- Ærø: 0.42
- Birkholm: 0.36
- Avernakø: 0.43
- Hjortø: 0.21

We see that mixing has caused Ærø to have a fairly high heterozygosity. Considering that Birkholm is a small isolated population, founded by few introduced animals, the heterozygosity is also fairly high here.

2.2.3.6.4. Swedish mixed populations

Table 7 illustrates H_e in the Swedish populations:

Most important source population(s)	Mirror population	Source(s)	Mirror

Avernakø	Bäckhalladalen	0.43	0.43
Nekselø	Skogshuset	0.34	0.47
Tårup Strand, Avernakø	Frederiksberg	0.29, 0.43	0.61
Knudshoved, Agersø, Nekselø	Mölle	0.56, 0.56	0.63

Table 7: Expected heterozygosity, H_e , in Swedish mixed populations compared with some of the Danish source populations.

Bäckhalladalen seems to be a successful mirror of Avernakø. Skogshuset has considerably larger variation than Nekselø; this cannot be explained by any marginal contributions from Knudshoved or Agersø to this population, because alleles that are especially characteristic of any of these two source populations are absent or rare in Skogshuset. A more likely explanation is that the sample from Nekselø was not representative. The source material for the introduction to Skogshuset was from the north part of Nekselø, which has constantly had a widely distributed Bombina population, whereas the present sample from Nekselø is from a population at the south end of the island, which has been founded by rather few animals.

The high heterozygosities at Frederiksberg and Mölle are most likely due to the introduction of foreign genes.

2.3. The MHC-Complex:

2.3.1. General remarks

The genes of the MHC complex (major histocompatibility complex) are believed to have a mutation rate intermediate between that of the two other types of DNA analysed here, wherefore they allow analysis of diversification on an intermediate time scale.

However, the rate of accumulation of mutations in the MHC complex does not depend in a simple way on how often mutations arise. This is because they may therefore be used to study genetic differentiation on a time scale shorter than that analysed by data on mitochondria, and longer than that analysed by data on microsatellites. The MHC genes, unlike neutral markers, are subject to selective pressures and are supposed to be adaptive. It is hoped that by analysing MHC it is possible to see if inbred populations may suffer a loss of fitness.

The genes analysed in this study were the MHC class II genes of Exon 2. Exon 2 encodes the variable peptide binding site ($\beta 1$ domain) and is responsible for the binding of foreign proteins, mainly derived from extra cellular pathogens like bacteria or helminths. That is why a high variability is believed to be adaptive, because the more divergent alleles an individual has, the more pathogens can be recognised. This might be a fitness advantage and many alleles can be maintained through balancing selection.

Analysis of MHC is technically more difficult than the other methods, and although the first results with the method were obtained 20 years ago, fewer studies have been carried out than with mitochondria and microsatellites.

There were two sources of information on the MHC genes of the sampled toads. First the general report from the institute in Potsdam, and second a diploma work made by Ina Pokorny at the same institute. In the two investigations, the genes recorded were not all identical. In a letter, Ina Pokorny explains the differences as follows:

“First, for my analysis I used a new primer mostly because the old ones were too unspecific. We decided to amplify all populations from the report again, to be able to compare all data sets using the same conditions. Furthermore, I used a proof-reading polymerase, which is able to detect amplification errors and corrects them, resulting in more reliable alleles. “

2.3.2. Geographical differentiation

The general report shows an overall trend that the alleles shift gradually along the route Latvia - Poland - East Germany - Holstein - Denmark. The overall pattern is that geographical distance and genetic distance are well correlated here.

2.3.3. Latvia: the Riga zoo population

In Pokorny's report, the population at Islice has a few more alleles than the populations at Daugavpils and in Riga Zoo. It gives no clear clue as to the origin of the zoo population. The general report, however, gives important information about the artificial population in Riga Zoo. The allele frequencies in the zoo population agree fairly well with those found in the Daugavpils population, which supports the results obtained with microsatellites. But in contrast to the microsatellites, the MHC also indicates a small influence from the Islice population in that two rare alleles that otherwise have been found only at Islice, are also found in the zoo. Though the frequencies of these alleles is very small, however, so the contribution from Islice is minimal detectable in the general report (but not in Pokorny's report).

2.3.4. The question of common and uncommon alleles

In the general report, 22 different alleles are recorded. These fall into two groups, viz. those that are found in many (at least 13) individuals, and those that have been found in few (maximally 3) individuals.

Many alleles that deviate from very common alleles by only 1 or 2 mutations could not be verified in Pokorny's analysis, and thus may be due to methodological errors in the first report (some of these errors may be due to the taq-polymerase, which from time to time inserts wrong nucleotides). For this reason, and because the number of investigated toads is too small to draw conclusions from the appearance of rare alleles, the discussion of the data from the general report will concentrate on the common alleles.

2.3.5. Diversity of common alleles in the general report

On the basis of the preceding considerations, we will only count consider the alleles called 1, 10 12, 16, 19 and 21 in the general report alleles contributing to fitness. The following table gives the number of such alleles found in each population:

Population	No. of alleles	No. of equally frequent alleles
LV: Daugavpils	3	2.67
LV: Riga Zoo	3	2.85
LV: Islice	4	3.32
PL: Bialowieza	4	3.33
D: Lakoma	5	3.79
D: Salemer Moor	4	2.69
D: Dänischer Wohld	2	1.84
DK: Knudshoved	4	2.48
DK: Enø	4	2.65
DK: Tårup Strand	4	1.82
DK: Hjortø	3	1.66
S: Skogshuset	4	2.19

Table 8. Results on MHC alleles according to the general report. The number of common MHC alleles found in each population, and the number of equally frequent alleles calculated from the Shannon-Weaver formula.

What is important is presumably not just the number of relevant alleles, but also their frequency. For instance, at Islice in Latvia, we have 4 relevant alleles. They all occur in fairly high proportions, varying from 10 % to 40 % of the total. For comparison, Skogshuset in Sweden also has 4 alleles, but these occur in a very skewed ratio, with one allele being found in 75 % of all clones, whereas the others are found in only 16, 6 and 3 % of the clones, respectively. So, effectively, this works as if there were fewer than 4 alleles. A way to express this situation is to calculate the genetic diversity with the Shannon-Weaver index, H , and then to calculate $\exp(H)$, which gives the computed “number of equally frequent alleles”.

The number of equally frequent alleles is seen to be lowest in Hjortø, which is also the most inbred population.

However, too much emphasis should not be put on these figures. In a letter, Pokorny explains: “In the [general] report, the number of clones which carry an allele was displayed. . . . this is misleading, because the amplification of alleles depends on the primers. It is possible that the amplification of alleles is biased because [a] primer [may] fit better on one allele than on another. “

2.3.6. Diversity of alleles in Pokorny’s report

Pokorny’s report records a larger number of alleles (viz. 32) than the general report. Of these, only 9 are common to both studies. Pokorny counts each allele once for every time it is found in one animal. With five animals analysed per population, each allele may be counted maximally five times per population, in contrast to the general report, where the number of clones was counted.

Pokorny’s report presents various measures of diversity of MHC alleles. One is called gene diversity. Here, all alleles count equally, irrespectively of whether they are very similar (e.g. when they differ by only one mutation) or very different. Another measure is called nucleotide diversity. Here, the diversity is calculated on the basis on the nucleotide bases, which means that when an allele differs by many mutations from another allele, it contributes much to the diversity. The nucleotide diversity seems to be the most informative, partly because an animal with two very different MHC alleles is fit for a more diverse response to pathogens than one with two very similar MHC alleles that may be identical in their function. And partly because the results show that the nucleotide diversity diverges more between inbred and outbred populations than gene diversity does.

In addition, Pokorny reports the average number of alleles recorded per population and per individual.

The following table lists the number of alleles and the nucleotide diversity of those populations that were investigated by Pokorny:

Population	No. of alleles found in population	No. of alleles per individual	Nucleotide diversity
LV: Daugavpils	4	3	0.073
LV: Riga Zoo	4	3	0.070
LV: Islice	6	3.2	0.074
PL: Bialowieza	11	3.6	0.079
D: Lakoma	7	3	0.075
DK: Knudshoved	10	4.6	0.068
DK. Knudsskov	8	3.4	0.072
DK: Enø	9	4.2	0.074
DK: Agersø	9	4.2	0.064
DK: Asnæs	11	4.2	0.066
DK: Neksælø	6	2.75	0.073

DK: Røsnæs	6	2.6	0.063
DK: Tårup Strand	6	3.6	0.074
DK: Klintholm	8	4	0.060
DK: Hjortø	5	2.6	0.045
DK: Ærø Øst	7	3.4	0.069
DK: Ærø Vest	9	3.6	0.058
DK: Avernakø	6	3.2	0.057
S: Skogshuset	4	2.4	0.060
S: Frederiksberg	8	3.8	0.084

Table 9. Results on MHC alleles according to Pokorny's report. The total number of alleles found in each population (in five individuals), the number of alleles per individual and the nucleotide diversity.

Concerning the number of alleles per individual, the lowest figures are seen for Hjortø, which once more corroborates the inbreeding in this population. The highest figures are seen in Knudshoved, which strangely ranges much higher than Bialowieza and Lakoma. The highest nucleotide diversity is seen in Frederiksberg. This is due to the presence there of two alleles that differ by many mutations from the other alleles. This once more indicates the intrusion into this population of genes of foreign origin (probably from central Europe). Apart from that, the two highest nucleotide diversities are found in Bialowieza and Lakoma.

2.3.7. Comparison of original populations and mirror populations

In Table 9, we may compare the original and mirror populations in the pairs Knudshoved – Knudsskov, Agersø – Asnæs, Nekselø – Røsnæs and Tårup Strand – Klintholm. There is no general trend that the mirror populations have lower numbers of alleles or lower diversity.

2.3.8. Mixed populations

In Table 9, the high nucleotide diversity for the population at Frederiksberg must be due to the admixture of foreign genes, as explained above.

The population at Skogshuset, on the other hand, has a low diversity and a low number of alleles. In this respect, it reminds of Nekselø and Røsnæs, and this agrees with the results from microsatellites, namely that it originates mainly from Nekselø. Certain details indicate, however, that the population at Skogshuset also contains some alleles that originate from Knudshoved or Agersø. It is not a 100 % pure Nekselø replicate.

Of special importance are the results for the mixed population at Ærø. It has higher numbers of alleles and a higher diversity than both constituent populations Avernakø and Hjortø. A detailed inspection shows quite a number of alleles on Ærø that have neither been found on Hjortø nor on Avernakø. This result is discussed by Pokorny in her report, and she writes that it may possibly be due to the small sample size – i.e. the private Ærø alleles might turn up in the other populations if more animals were analysed. But she also writes that “it is also possible that these alleles originate from the Ærø male that was used in the rearing in 1986, and have been retained by balancing selection”. To this may be added a third possibility, namely that they originate from the Hjortø population before this became genetically impoverished by inbreeding in the late 1990s.

2.3.9. Comparison between small and large populations

There is no clear trend that small populations have smaller diversity of MHC alleles than larger populations.

For example we may compare Enø and Knudshoved. They are closely related genetically,

as demonstrated by the microsatellite analysis. They also have much the same MHC alleles. But both in Table 8 and Table 9, Enø has a slightly higher diversity than Knudshoved. This is a very unexpected result considering that the Enø population has been through a narrow bottleneck (7 or 9 animals), whereas the Knudshoved population has not been below 180 animals as far as we know (but in the distant past, it may have been founded by very few animals). Enø should therefore be much more affected by inbreeding, but evidently this is not so. A possible explanation is that animals that survived the bottleneck there and contributed offspring to the future population were the last survivors in three different, widely separated ponds. So alleles that might have died out in one pond, might have survived in another, and vice versa. In addition, this substructure might cause a sampling effect, i.e., the MHC variability in Knudshoved across all ponds might be higher than reported here. In conclusion, one should not consider the Enø population as hopelessly inbred. And indeed, when rearing offspring from Enø, there is little sign of inbreeding depression.

When we go up one step further in population size and compare Knudshoved which is isolated and contains less than 1,000 animals with Lakoma and Bialowieza which are not isolated and contain thousands of animals, we see in Table 9 that the number of alleles per individual in the larger populations is not as high as in Knudshoved. And if we compare Lakoma and Bialowieza with small, isolated populations like Islice, Daugavpils, Enø and Tårup Strand, we see that they have practically the same nucleotide diversity as the larger populations.

Thus, it is as if nearly all populations have approximately the same diversity of MHC alleles. This result is different than the results obtained with neutral genetic markers, and it may be due to the adaptive nature of the MHC alleles. There may be some kind of balancing selection that maintains a high MHC diversity whenever possible. This is the same conclusion as from several other studies on inbred populations of vertebrates from the latest years: a decline in neutral marker variability and a maintenance of MHC variability through some kind of balancing selection.

Out of the populations shown in Table 9, only one is clearly impoverished, viz. Hjortø. This is also the only population with concrete explicit signs of inbreeding depression. These signs are concerned with the mortality of eggs and tadpoles, and the number of malformed offspring. This is not directly related to the immune system, but if inbreeding has negative effects in some features, it may also have so in others.

In Table 8, it seems also that Dänischer Wohld is impoverished. It may be mentioned in this connection that during the rearing of offspring from Dänischer Wohld, relatively many eggs or tadpoles have died from mold infections.

But apart from these two exceptions, it seems that all other populations, including Enø, Islice and Daugavpils which are or have been very small, have a full complement of MHC genes. If there is any correlation between MHC and fitness, then these small populations should have good prospects, and it should not be considered hopeless to restore large and healthy populations there.

3. Conclusions for population management in the LIFE-project

3.1. Results of a workshop

The primary purpose of the genetic analyses was to assist and offering helping tools before drawing the right conclusions and right decisions on how to manage specific populations. The results were discussed on a workshop with the involved field herpetologists and Dr. Susanne Hauswald, a member of the team Prof. R. Tiedemann (University of Potsdam) in September 2006. On this basis a catalogue of conclusions has been made. It is presented in the following table.

SURVEY OF CONCLUSIONS FROM GENETIC ANALYSES

Population	Mirror or reception area	Observed in genetic report	Observed in the field	Suggestions
Nekselø	Røsnæs	Transfer of genetic diversity to the mirror population has been rather successful, but genetic material from north Nekselø is under-represented and transfer of the AAC mito. haplotype has failed.	Both populations are expanding.	Additional release of offspring from north Nekselø could be carried out.
Agersø	Asnæs	Agersø has remarkable number of private alleles. Very incomplete transfer of genetic diversity to Asnæs.	Agersø suffers from intensive agriculture; population is now growing.	Transfer more from Agersø to Asnæs
Knudshoved Odde	Knudsskov	Transfer of genetic diversity to the mirror population has been successful.	Only small breeding success at Knudsskov	Maybe later supplementary release at Knudsskov.
Enø	Svinø	According to data on MHC, the genetic diversity on Enø is probably not so low as to make the population unhealthy.	Establishing the Svinø population started only in 2005.	Go on with the Svinø project.
Tårup Strand	Klintholm	Mit haplotypes: Tårup 2, Klintholm 3. Microsats: Very low H_e , lowest at Tårup. MHC at Tårup: Only one common allele.	The population at Klintholm thrives fairly well, but is still not large enough.	Make further habitat improvements near Klintholm.
Klintholm	Tårup Strand	The genetic diversity is slightly higher at Klintholm than at Tårup Strand, i.e. some alleles have probably been lost recently at Tårup.	Population size at Tårup restricted due to lack of habitat.	Improve the habitat near Tårup, and boost population size there. Transfer genes from Klintholm to Tårup.

Population	Mirror or reception area	Observed in genetic report	Observed in the field	Suggestions
Avernakø	Korshavn	Korshavn is genetically impoverished relative to Avernakø.	Korshavn develops well	Transfer more material, but not so much that the existing population is swamped with Avernakø material.
Avernakø	Skovsgård	The genetic diversity at Skovsgård is nearly as high as on Avernakø.	Skovsgård develops positively, but slowly	No
Avernakø	Ærø	Genetic diversity on Ærø as high as on Avernakø; Ærø is dominated by Avernakø genes more than Hjortø genes	Population on Ærø does not grow well, but recent habitat improvements may help.	Do not transfer genes; the proportion of Avernakø alleles is already very high.
Birkholm	Hjortø	Hjortø has lost many genes. The Birkholm population has a high proportion of Hjortø genes (maybe c. 50 %), many of which have disappeared from Hjortø now. Analysis of mitochondria is still lacking. It might show the proportion of animals with Hjortø origin.	The Hjortø population is barely viable. This may be due to local problems, but also to lack of genetic variation.	Remove threats on Hjortø, if possible. If analyses of mitochondria demonstrate a high proportion of Hjortø genes in the oldest animals on Birkholm, then one might transfer offspring from these to Hjortø.
Birkholm	Ærø	On Birkholm a relatively high proportion of Hjortø genes, maybe about 50 %. Many alleles in Birkholm are not found in Ærø. Two of these are private (found nowhere else in Denmark)	Population on Ærø does not grow well, but recent habitat improvements may help.	Rear offspring from Birkholm, especially from the oldest animals, and release on Ærø to increase the genetic variation there and the proportion of supposed Hjortø alleles.

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Population	Mirror or reception area	Observed in genetic report	Observed in the field	Suggestions
Ærø	Birkholm	There are a number of private alleles on Ærø. Many of these may originate from Hjortø, and some maybe even from the last animal in the original Ærø population. These private alleles are mostly present in Ærø east. But overall, the proportion of the genetic variation on Ærø that originates from Hjortø is low. Analyses of mitochondria are still missing for Ærø east; they might further clarify this.	The Birkholm population is small, but has good prospects.	Carry out some transfer from Ærø east to Birkholm, but wait a few years to do this. Meanwhile, do some rearing from old animals on Ærø and release back in the same area. Do not transfer from Avernakø to Birkholm.
Copenhagen Zoo	Hjortø	The zoo population is genetically much impoverished.	The Hjortø population is barely viable	Do not transfer more material now.
Copenhagen Zoo	Birkholm	The Birkholm population contained maybe c. 50 % Hjortø genes and 50 % Avernakø genes at the time of sampling. Since then, more animals have been released from Avernakø.	The Birkholm population is small, but has good prospects.	Release more animals of zoo origin on Birkholm to increase the proportion of Hjortø genes there.
Copenhagen Zoo	Strynø Kalv		Living conditions are probably better on Strynø Kalv than on Hjortø	Release animals of pure Hjortø origin on Strynø Kalv to see if they thrive there. If yes, then something is wrong with the environment on Hjortø
Dänischer Wohld	Stodthagen	Dänischer Wohld: Mito-DNA: 3 haplotypes, including one unique type. H _e from Microsats: slightly lowered. MHC: not promising	Trend at Dänischer Wohld? Stodthagen: quite promising.	Complete the ongoing transfer, as fully as possible.

Population	Mirror or reception area	Observed in genetic report	Observed in the field	Suggestions
Dänischer Wohld	Gelting	Dänischer Wohld: Microsats: slightly impoverished. MHC: probably the most impoverished of all populations.	Many ponds are ready at Gelting.	Material transferred to Gelting could be a mixture of Dän. W. and some other locality to increase the genetic diversity of microsats and MHC, recommended: strong Danish populations from Fyn
Holstein	Holstein	The populations are genetically rather similar (as to micro-satellites), although there is some fine structure (division into types A, B and C). There are differences in mitochondrial haplotypes. Genetic diversity is lowest in Schöhsee.		Probably transfer from elsewhere to Schöhsee.
Putlos	Fehmarn	Fehmarn is related to Putlos. The genetic diversity is reduced on Fehmarn, but still higher than in any population in the Fyn region.	Few or no individuals have survived in the wild on Fehmarn; about 13 adult individuals are kept in captivity.	Captive animals should be brought to breed, and offspring released on north Fehmarn and maybe also near Wallnau. No transfer of genes from Putlos should be made now, at least not in all sites on Fehmarn, in order not to swamp the Fehmarn genes, but supplementary transfer from Putlos may be carried out at a later step.
Mölle	Mölle	Nearly 90 % of the genetic material is probably of central European origin.		Do not breed from this origin. Let the population thrive on it's own.
Sjælland	Skogshuset	Allelic richness is rather poor at Skogshuset. The AAD mit. haplotype does not exist anywhere in Denmark (anymore). All other genes originate from the Sjælland region, and nearly exclusively from Neksælø.	The population is expanding.	Additional genetic material from Agersø, Enø and Knudshoved could be released.

Population	Mirror or reception area	Observed in genetic report	Observed in the field	Suggestions
Fyn	Frederiksberg	There are signs of introduction of genes from Central Europe, probably by release of animals from Mölle. The rest of the genetic material is mainly from Tårup Strand. Due to the mixed nature of the population, allelic richness and H_e are fairly high.	The population is expanding very much.	No ponds should be made between this population and that at Skogshuset, because we do not want too much spread of genes of Central European origin. If more genetic material from Denmark is released in order to increase the "Danish" genetic element, then this could be from Avernakø or Ærø.
	Bäckhalladalen	Heterozygosity and especially allelic richness is poor at Bäckhalladalen. At the sampling spot, the genetic material is mostly from Avernakø. Genes from Sjælland may exist at another nearby site where animals of Sjælland origin were released.	The population has remained rather small, and it is doubtful if it has colonised the ponds that were recently made. If not, it may be feared that the population dies out again.	If it is considered important that a population survives here, then release of additional genetic material at the new ponds may be necessary. The offspring released should be from somewhere else than Avernakø or Ærø.
Riga Zoo	Bauska, Islice	Genetically, the populations at Islice and Riga Zoo are very different.	The population at Islice is small and threatened.	Genetic material from Riga Zoo should not, or not now, be released at Islice.
Riga Zoo	Daugavpils, Ilgas	The genetic material at Riga Zoo nearly exclusively descends from Daugavpils, but according to the data on MHC, there may also remain a small fraction of genes from Islice.	The population at Daugavpils is small and threatened.	Genetic material from Riga Zoo could be used for supportive breeding to strengthen the population at Daugavpils.

Table 10: Survey of results and their consequences for each population

3.2. Comments

3.2.1. Denmark/Sweden

An important result is that two of the four investigated populations in Sweden contain genetic material of "foreign" origin - probably from Central Europe. This is most clearly evident from the occurrence of the DEG haplotype of mitochondria, but is also supported by the presence of certain characteristic microsatellite alleles, and from the occurrence of animals with a phenotype that resembles *Bombina variegata*. The proportion of such "foreign" genetic material is high at Frederiksberg and very high at Mölle.

The locality at Frederiksberg/Svartskylle is connected to other *Bombina* localities via stretches of landscape that are favourable to the spreading of these animals: hilly terrain grazed with cattle, various types of extensive common, scattered with large and small lakes. Going two kilometres to the north via this favourable landscape we reach a region (Ållskog, Svinahøjden) where *Bombina* has proliferated very much since around 2000, and colonised many ponds. From here, there is a stretch of only about 1.5 km of not unfavourable landscape to the locality at Skogshuset. It is obvious that some specimens of *Bombina* will be able to cross these distances, and indeed there is an indication that one of the sampled specimens at Frederiksberg had come from one of the other localities. If migration is possible in one direction, it will also be possible in the opposite direction. It is therefore only a question of time before the alleles at Frederiksberg diffuse into the neighbouring populations. The population at Frederiksberg seems to thrive particularly well, and to have had a tremendous breeding success in 2006. This may lead to the hypothesis that animals with mixed genetic background have a higher overall fitness than animals of purely Danish origin. If this hypothesis holds true, then the frequency of genes of "foreign" origin will steadily increase in the future in the whole region.

There are only two areas in Scania where the (re)introduction of *Bombina* has been so successful that large, proliferative populations have arisen. These are Mölle and Skogshuset-Frederiksberg. We now know that on a longer term none of these will genetically be a mixture of purely Danish origin.

One might imagine that some time in the future, something could go generally wrong for *Bombina* in Denmark. Increasing sea levels and increased frequency of storm surges could wipe out or weaken many populations on small islands and/or close to the sea. Climatic change with warmer and wetter winters and longer periods of drought in summer could be unfavourable to *Bombina* and cause a further weakening of the populations. If all populations are isolated, it could be that none of them were able to adapt genetically to the changed climate. In the end, this could conceivably lead to the extermination of *Bombina* in Denmark.

Up to now we have believed that if things went as wrong as described here, then it would be possible to use the mixed *Bombina* population in Sweden as a mirror population for the whole Danish population. The adaptations that were not possible in the isolated Danish populations could be possible in Sweden, due to the mixed composition there. Therefore it could be sensible to bring animals back from Sweden to Denmark.

We now know that this possibility is lost. The mixing of Danish populations in Sweden has not been efficient. Most of the analysed populations contain Danish alleles that originate from only one or little more than one population. On the other hand, those populations that thrive are "polluted" with alleles that are not of Danish origin. So a functioning mirror population for all Danish *Bombina* populations does not exist.

If such a mirror population should be made, then it would have to be made once more, this time in Denmark.

3.2.2. Germany, Schleswig-Holstein

In Schleswig-Holstein four management units of *B. bombina* populations were identified by the genetic investigations (Tiedemann et al 2006): Dänischer Wohld, Holsteinian lake district, Island of Fehmarn and Schaalsee area.

3.2.3. The situation in Dänischer Wohld – Schleswig-Holstein

The genetic results are that the population at Dänischer Wohld is most probably of purely indigenous origin. The original population is threatened, but transfer of offspring to the newly established locality at Stodthagen not far from there seems to work well.

There is an interest in reintroducing Bombina to Gelting in Schleswig, where the original population died out rather recently, and where many new ponds have been created since then. The introduction of animals from Dänischer Wohld has already started.

Analysis of the MHC complex suggests that the population at Dänischer Wohld is more impoverished in this part of the genome than others. We do not know if this has any consequences for fitness - the animals at Dänischer Wohld do not appear to be unhealthy. But in theory, they might be more vulnerable than others if they are exposed to diseases or parasites that are new to them. Therefore it seems wise to make a mixed population at Gelting. The other populations in Schleswig-Holstein have to a great extent the same alleles as that at Dänischer Wohld, as far as microsatellites are concerned. So one strategy would be to mix with a population, which is genetically somewhat further away.

One possibility could be Ærø in Denmark. A rationale behind this could be that Gelting is geographically midway between Dänischer Wohld and Ærø, and it could then become so even genetically. Ærø has a larger MHC diversity than the neighbouring populations (Avernakø and Hjortø), and thus should be better as a source of introduction. Unfortunately, the MHC complex of Ærø and Dänischer Wohld has not been analysed by comparable methods, but when we look at other populations from Fyn county that have been analysed by the same methods as Dänischer Wohld (Hjortø, Tårup Strand), then these both contain the alleles 16 and 21 that are lacking in Dänischer Wohld. This might speak in favour of a release from Ærø or some other place in Fyn County.

3.2.4. The situation in Holstein

All analysed samples from Holstein are fairly similar regarding microsatellite alleles. This leads to the not very surprising conclusion that they may until fairly recently have been parts of one coherent population. The populations are not completely alike - there is some indication of a fine structure, with more gene flow in the direction southeast - northwest than in other directions.

Concerning mitochondria, the situation is different. The frequencies of the two main haplotypes are very different in different populations.

Especially noteworthy is the finding in a single specimen at Ascheberg/Kühren with the mitochondrial haplotype AAF which is found nowhere else. It could be a recently arisen mutation. It might be interesting, because of this, to use Ascheberg/Kühren as a source for introduction to other places in Holstein, e.g. a supplementary introduction to an already existing, but genetically impoverished population. Such a population exists at Schöhsee/Behler See not very far from Ascheberg.

In a small area an illegal release of *B. bombina* was identified by the same Mitochondrial-DNA Type (DEG) as in Sweden. For the future management it is necessary to develop a strategy to avoid the spreading of these genes within the Holstein population.

3.2.5. Fehmarn

Genetically, the population of Fehmarn is obviously related to the nearest population on the continent, viz. Putlos. If the Fehmarn population is considered so weakened by

inbreeding that supportive introductions should be made, then Putlos would be the obvious source. An advantage of Putlos is also that out of the natural populations in Holstein, it has the highest allele richness, the highest number of private alleles, and the highest heterozygosity.

On the other hand, the situation for the original Fehmarn population, although it is in a difficult phase right now, is not hopeless from a genetic viewpoint. The heterozygosity (concerning microsatellites) is 0.51, which is at a level with other apparently healthy populations in Holstein, like Rathjensdorf, and approaches the level of the largest Danish populations. So it would seem premature at present to initiate introductions to Fehmarn of genetic material from elsewhere.

3.2.6. Schaalseegebiet

Mitochondrial-DNA data indicate a position between the Holstein population and the population from the central distribution range of *B. bombina*, e. g. Poland and Brandenburg. This is in line with the historic data. The populations in the Schaalsee-region seem to show up in the 1930^s and 1940^s for the first time. So at that time there might have been an outspread of a *B. bombina* population from the river Elbe valley or from Mecklenburg-Vorpommern north-west wards.

The heterozygosity (concerning microsatellites) is as high as in big populations from Ukraine and Poland. Similar are the data for the MHC-Complex. All data are indicating a meta-population structure within the Schaalsee region until recent time.

3.2.7. Latvia

Both Latvian populations are small and threatened. This is reflected e.g. in a slightly reduced heterozygosity, but when it comes to the MHC complex, the situation seems surprisingly good. There is therefore no reason in advance to expect a low fitness.

Although the captive population at Riga Zoo was thought to be a population of mixed origin (Islice/Daugavpils), the genetic analysis indicates that those individuals who were actually the ancestors of this population, were practically only from Daugavpils, maybe with just a very small genetic contribution from Islice. But it seems fair to conclude that the population is so close to being a pure Daugavpils population, that it can be used for supportive introductions to Daugavpils.

4. Conclusions for future management concepts on landscape level

4.1 Population size

Population size is important for the genetic composition of populations. This has long been known, and it has been confirmed also in the present study. Even in time spans as short as 20 years, genetic drift in small isolated populations may create considerable genetic distance between populations that were formerly identical, and cause "erosion" of the genetic variation. This is likely to have consequences for the fitness of the animals, although little is known about this. At the genetic level, there are also negative consequences: conservation of biodiversity includes conservation of genes, and these are lost at an increasing rate when populations become smaller. Furthermore, loss of genes means erosion of genetic patterns, whereby it becomes more difficult to study the pedigree and past history of populations.

How large must a population be to avoid such phenomena? There is no precise answer to this, but a useful rule of thumb is that the effective population size, N_e , must be at least 500 to 1,000 animals. We do not know the size of N_e for a given census population size, N , in *Bombina bombina*. It would be useful to carry out precise investigations of this, but these have not been made up to now.

As long as precise information is lacking one may start with an assumption that N_e/N is no larger than 1/4. This means that one should aim at census population sizes of 2,000 - 4,000 adult animals.

What does it require to satisfy this criterion? One of the important parameters is the number and size of ponds. There are cases when *Bombina* occurs in high densities with less than 5 m² pond surface per individual, but we also know that in such cases the animals are often undernourished and do not breed satisfactorily. In good ponds, 10 m² pond surface per individual may suffice, but to be on the safe side, one should reckon 20 m² pond surface per individual. So one of the criteria to be fulfilled is that the combined pond area should be around 20,000 - 80,000 m² in each population. If for instance half of the ponds are 400 m² and half are 800 m², then this means between 33 and 133 ponds per population. Few populations today fulfil this criterion.

If each isolated population has one mirror population, and if from time to time genetic material is exchanged artificially between the original and the mirror population, then these may be considered as one population in genetic terms. It would then suffice to have between 16 and 67 ponds per population. But even this may be difficult to realize. In many cases, the most promising option is to connect neighbouring populations by functioning dispersal corridors in the landscape.

From the above considerations it is evident that there is a need for further habitat improvements in nearly all localities. But, of course, this need is most urgent in the smallest and most isolated populations.

4.1.1. Conclusions for Denmark

Much has already been done in Denmark to enlarge and improve areas with living conditions for *Bombina*. There still remains something to be done. From a genetic point of view, the following localities are of special interest:

An unusually high genetic variation was found in the population on Agersø. It is therefore unfortunate that the population size there is still only about 200 adults, and the number of breeding ponds is only 2-3. More should be done to improve the population size there. It is also unfortunate that the transfer of genetic material to Asnæs has been very incomplete, and that the number of suitable ponds there is moderate. More rearing and release at Asnæs should be carried out, and the efforts to improve the habitats should continue.

The mixed population on Ærø contains considerable genetic variation that is not represented elsewhere. It is therefore unfortunate that the population size there is still less than 100 adults, and not increasing. The efforts to improve the habitats there should continue.

Remains of the former Hjortø population still exist, but the individuals are strongly inbred. It is uncertain if it will be possible to make a population thrive on Hjortø. There are arguments for the idea of releasing Hjortø animals on Strynø Kalv and creating suitable habitats there. Maybe this gives us the best chance to re-establish a naturally functioning population with genes exclusively of Hjortø origin.

Continued efforts to improve habitats are also relevant at the other Danish populations. The establishment of a mirror of the Enø population at Svinø has only just started within the frames of the present LIFE project and should continue as planned.

4.1.2. Conclusions for Germany

The genetic analyses show that the populations in the Holstein lake district east of Kiel are all genetically rather similar, and they are obviously relicts of a large coherent population which has become fragmented only recently. There are good reasons to try to strengthen the now isolated populations by connecting them via dispersal corridors in the landscape.

The population at Putlos is isolated from the others by such a distance that connection via dispersal corridors will not be easy. To preserve the relatively high genetic variation there,

it is important to secure that the population will in the future stay at least around 2,000 animals. This may require habitat improvements.

A special situation exists for the populations at Bungsberg, because out of the two local populations there, one, at Neutestorf, is indigenous, whereas the other, at Neuhamrsdorf, 4 km from the first, contains many alleles that are believed to be of foreign origin, probably from Central Europe. At Neutestorf, the living space for *Bombina* has recently been extended by the creation of a series of new ponds. Further pond projects should not extend in the direction of Neuhamrsdorf, and not be carried out near Neuhamrsdorf. If this were done, the population with foreign genes would multiply and spread out into the neighbourhood, with a risk that migrants reach the area at Neutestorf and spread the foreign genes also to this population. The 4 km barrier between the two localities should remain as inhospitable to *Bombina* as possible.

The populations on Fehmarn and at Dänischer Wohld have already been helped by the creation of many new ponds, but more could be made.

Today in the Schaalsee region the meta-population structure of *B. bombina* seems to be fragmented into sub-populations which are not connected. In future a meta-population structure has to be re-established or secured by a management on landscape level.

4.1.3. Conclusions for Sweden

The original idea was that the Swedish populations should only contain genes from the nearest possible populations, i.e. the Danish ones. This idea has obviously been corrupted, as the genetic analysis has shown that two of the Swedish populations contain a large proportion of "foreign" genes, presumably from Central Europe. There is no wish to promote the spreading of these genes further. This leads to the conclusion that only little or nothing should be done to improve the habitats around the two "infested" localities, Mölle and Frederiksberg.

If habitat improvements are omitted at Frederiksberg, this will delay the spreading of foreign genes, but it will not prevent it. On a long term, the foreign genes will spread to all localities in the core area in south-central Scania. That is, from the point of view of preserving Danish genetic material in Sweden, focus should shift away from Mölle and south-central Scania, and shift to the remaining localities.

One remaining locality, where no introgression of foreign genes has been detected, is that at Bäckhalladalen. At present, the situation here is not promising. If one wants a functioning reserve population for Danish genes here, then a new effort should be made to make this population function. This could include further habitat improvements.

Another remaining locality is at Gislöv near Glimminge in southeast Scania. This population has not been analysed genetically. A proposal could be to investigate the possibilities for large-scale habitat improvements here, and stocking with more genetic material from Denmark.

4.1.4. Conclusions for Latvia

The analyses have shown that the two populations in Latvia are genetically rather different, which is not surprising, considering the geographic distance between them (c. 175 km). This means that they should be treated as two separate entities, and that each entity must be brought to a population size where it is out of danger. This will require considerable habitat improvements at both localities.

5. General conclusions and recommendations

5.1. General conclusions

Up to now, it has been unusual that practical conservation work with habitat improvements etc. have been coupled to genetic investigations. The present project, which is a co-operation between field herpetologists and a DNA laboratory, is therefore to some degree a pilot project. It is therefore worthwhile to draw some general conclusions.

First of all, the cooperation has worked well. The working group at the DNA laboratory has been interested in understanding the situation and the geographic distribution of the sampled populations, and the field herpetologists have been interested in the results of the genetic analyses.

An important point has been that the most important results did not always come from the first genetic analyses. Rather, the first analyses left many important points unanswered, and the feedback from the field herpetologists was that information from more analyses was needed. An important point for the obtained success has been the flexibility in the DNA laboratory, with their willingness to engage in necessary additional analyses, in line with increasing awareness among the field herpetologists as to what information would be crucial.

An example of this regards one of the most important questions: which populations are indigenous, and which go back, wholly or partially, to illegal introduction of animals of foreign origin. The first analyses of mitochondrial DNA included most of the relevant populations, but detected only one case of "pollution" with foreign genes. It was only with the latest analyses of additional populations that a total of three cases of such "pollution" was detected.

Another conclusion is that the results of the genetic analyses have indeed, in some cases, led to a change of strategy. Again, this is most obvious as regards the detected cases of illegal introductions. In these cases, the new information has led to shifted priorities not only regarding rearing and release of animals, but also regarding where to place new ponds and how to prioritise the efforts between different localities. On the other hand, there are several populations where introduction of foreign genes might have been suspected in advance - not as a result of actual knowledge, but rather as a result of loose speculation. However, in these cases, the genetic results were reassuring, and it was concluded that the populations were certainly or very probably indigenous, and could be treated as such.

Another example of a benefit from genetic analyses was the conclusion that the captive population kept at Riga Zoo, which was believed to be a mixed population with contributions from both Latvian populations, consisted nearly 100 % of genetic material from only one of the two populations.

5.2. MHC analyses

The analysis of MHC alleles is a relatively new technique, and the analysis was in the beginning associated with technical problems and a need for basic investigations, before the method was sufficiently developed to work also for *Bombina bombina*. Up to now there exists less experience with the interpretation of MHC data than with data on neutral DNA markers, wherefore the conclusions drawn here may be somewhat tentative.

The interesting thing about MHC is that the genes are subject to selective pressure and are related to the fitness of the animals. During recent years, studies on other vertebrates (fish, birds, mammals) have shown that small isolated populations may retain a high variability of MHC, probably because the variability is preserved by balanced selection. The conclusions drawn here with regards to *Bombina bombina* are in line with this. Nearly all *bombina* populations, regardless of size and degree of isolation, had nearly the same variability of MHC genes (number of alleles, nucleotide diversity).. There were only few exceptions from this. A Swedish population with admixture of foreign genes had an

elevated MHC diversity. And a few small isolated populations had markedly reduced MHC variability. This was definitely true for Hjortø and maybe also for Dänischer Wohld.

This suggests, for *B. bombina* and for other vertebrates, that even rather small and isolated populations are not weakened in their ability to withstand infections and pathogens. We cannot say with certainty yet if this conclusion is correct. If it is, then small populations may have preserved a higher fitness than has been thought up to now, at least with respect to pathogens. This implies that the preservation of small isolated populations as pure populations without admixtures may often be worthwhile.

It would be useful in future studies to get more experience with the interpretation of data on MHC, and do more to establish a link between data on MHC and actual fitness of the organisms.

5.3. Further recommendations

A number of further investigations in this subject would be useful:

- 1) It is a problem that we do not know the ratio between the effective population size, N_e , and the census population size, N , in *Bombina bombina*, or in other European species of amphibians. Knowledge of N_e is important for setting criteria on recommended minimal population sizes. It would therefore be useful to initiate a study of this. This requires cooperation between a DNA laboratory and field biologists and it requires careful long-time planning because such a study must extend over an interval of a considerable number of years.
- 2) The situation in south-central Scania in Sweden, where one population has been affected by introduction of foreign genes, whereas another nearby population is not so affected up to now, invites to a study of the spreading of genes. Samples could be taken at intervals of a few years to follow the spreading of genes in the landscape.
- 3) It is of considerable interest to know if there are marked differences in fitness and/or competitive ability between animals from different isolated populations. It would therefore be interesting to follow one or several mixed populations, if possible from the beginning of their existence, to see if there is a systematic trend that certain alleles increase in frequency at the expense of others. Along the same line, one could investigate *Bombina* populations that have been mixed with foreign (Central European?) genes and see if the proportion of these genes increases systematically over the years.

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