The adder (*Vipera berus*)

Problematic isolation in Dutch populations?

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Contents

Forword

Worldwide animal populations are decreasing due to habitat destruction and fragmentation. Population sizes are declining, and the effects of this decline on the genetic health of have been studied in some species.

In the Netherlands many of the nature areas have strongly decreased in size within the last decades, and connectivity of populations is sometimes very low. To test the effect of this fragmentation on animal species, genetic studies are needed. The habitat of the adder (heathland and bogs) is one of the vegetation types which are severely decreased and fragmented. Studies in Sweden showed the adder to be a strong example of the importance of genetic diversity for species survival, and proved that the adder is a vulnerable species in this aspect, making it an excellent indicator species for questioning the fragmentation of habitats.

Despite the fact that the reptile populations are well monitored, thus far no genetic research was directed towards the populations of the adder in the Netherlands. With this pilot study I tried to fulfil a gap in the empirically based knowledge on the genetic status of the adder populations. Perhaps this study will provide enough reason to sustain repeatedly genetic monitoring of more reptile species in the Netherlands.

1. Introduction

1.1 Species description

The adder, *Vipera berus* (L. 1758), is a heavily build snake, with a relative short tail and a clear distinction between head and body. The adder belongs to the family Viperidae and is the only poisonous snake species in the Netherlands. Adults have a total length of 45 until 75 cm, depending on the area in which they live. The most characteristic parts are the red eyes with a vertical pupil, a black zigzag pattern on the back and keeled ventral and lateral scales. The body coloration is strongly variable, depending on origin. Males are predominantly grey(ish brown) and strongly patterned, while females are more reddish brown. The ventral scales are large, with a grey coloration (Lenders *et al*. 2002). Sometimes melanistic adders are found, more often in Germany and Austria, but also in the Netherlands they occur. Forsman (1993) found no effect of melanism on growth or survival of individuals. Often scale anomalies like small scales in between, or divided scales can be found. Scale anomalies were suggested to indicate genetic inbreeding, but evidence is weak, since scale anomalies are commonly found in many snake species and heritability is low (Forsman *et al.* 1994).

1.2 Ecology

Adders primarily forage on mice, voles, reptiles, amphibians and nest birds. Preferred prey is strongly depending on region, due to prey species availability (Lenders *et al.* 2002). Young adders forage predominantly on young frogs (*Rana temporaria* and *R. arvalis*) and lizards (*Zootaca vivipara*) (Dorenbosch & Van Hoof 2000). Like a large number of predators, optimal body size (Forsman 1991) and reproduction success depend largely on fluxes in prey availability. Following a year with high prey abundance, adders will give birth to a higher number of hatchlings (Lenders *et al.* 2002). Adders are only able to grow rapidly when prey are abundant, if not most energy will be stored as reserve for unsuitable periods (Forsman & Lindell 1991).

In spring (February-March) the males start to wake up from their hibernation, and spent a lot of time warming up, and go through spermatogenese (riping of sperm cells). Females become active a few weeks later. After the first moult of the males, usually in April, the mating season starts. Males look for females, and covered distances vary strongly from 100 m (Thomas 2004) to more than 1 km per day (Madsen *et al.* 1996). This is the period in which the most observations are done, since migrating adders are better visible, and less precautious. The mating season takes place more or less during the month May. Both males and females mate multiple times (Höggren & Tegelström 1996), which increases the reproduction success (Jennions 1997). Pregnant females don't forage and hardly migrate. Adders are ovoviviparous, the eggs hatch in the body of the females, and babies hatch fully developed. Since the females live on their reserves, they participate in reproduction once every two years, and increase their reserves the other year. Females start mating at the age of 4, but no relation was found between age and clutch size directly, although there was a relation found between body size and clutch size(Madsen 1988, Madsen & Shine 1992).

About migration of non-breeding females and males in summer almost nothing is known. Dispersal between summer and winter habitat is usually limited to 200 m (Thomas 2004), and sometimes females breed in the same place as they hibernate (Andersson 2003). Due to this limited ability to disperse it's important that areas are large enough to support a viable population size, or are well connected to other suitable habitats without physical barriers in between. Density of adders in a suitable habitat is usually about one adult per hectare, although in core populations densities of about 7 animals per hectare are reached (Thomas 2004, Wollesen & Schwartze 2004). Within a population the sex ratio is more or less equal (1:1) (Thomas 2004).

1.3 Habitat

Since adders are ectotherm the habitat choice is largely influenced by the possibilities to maintain the preferred body temperature (about 30 ºC). The preferred habitat of adders consists typically of transition zones between wet and dry vegetation, often between forest and open habitats. The occupied habitats vary slightly throughout the total distribution. In the Netherlands adders can be found in heathlands, bogs, and fen areas, in which *Molinia caerulea* is often a dominating plant species. While in other countries (sparse birch-pine) forests and montane areas also form part of the preferred vegetation types, borders and edges of different vegetations remain important (Grillitsch & Cabela 2004). At higher latitude or altitude adders can predominantly be found on south facing (warmer) slopes, but transition zones between different types of vegetation form the most important habitat throughout the distribution. (Wollesen & Schwartze 2004, Thomas 2004)

The adder needs in principle 4 key habitats: hibernation (winter) site, spring/autumn warming up site, mating/reproduction site and summer site. When the habitat is fragmented, distances between these sites could be more than a kilometre. Hibernation habitat and mating site are probably the most important (critical) of these sites, and can be determined as key habitats. In Germany almost half of the hibernation sites were found in the border of the forest, the rest within *Molinia* bogs between 20 and 60 m from the forest edge (Thomas 2004). Mostly old nests of mice or niches underneath tree roots are used as hibernacula (hibernation sites). Usually 1-4 adders use the same hibernacula, sometimes more (Lenders *et al.* 2002, Thomas 2004). For summer sites, climatic conditions are of less importance, so these could change easily (Völkl & Kornacker 2004). In most cases adders have just a distinct hibernation and summer habitat. But in the study of Wollesen and Schwartze (2004) in Germany the adders were found throughout the year in the same area, which therefore served as both hibernation and summer habitat. As reproduction sites bog areas were used, which were open enough to warm up. Looking at the size of the areas that are needed for individual adders, males were found to develop a home range of up to 5.2 ha, while reproductive females just used 0.76 ha (Neumeyer 1987), although Thomas (2004) only found home ranges of resp 0.4 and 0.7 ha for males and females. After the reproduction period the sizes were equal between sexes. Characteristic for sites of pregnant females is the more southerly oriented exposition, which these females need for sustaining a slightly higher body temperature (Neumeyer 1987). An interesting observation was done by Herczeg *et al.* (2007), who found a lower preferred body temperature in juvenile *V. berus*, compared to adults. This is probably due to the higher predation risk for juveniles, which therefore are forced to maintain a lower body temperature.

1.4 Distribution

The adder (*Vipera berus*) is probably the most widespread snake species in the world; it occurs throughout a huge range on the Eurasian continent (Carlsson *et al*. 2003). It can be found from Great Britain in the west until the island Sachalin (Siberia) in the east, from within the arctic circle in the north until northern Italy and Greece in the south (Dorenbosch & Van Hoof 2000). But within this enormous range the species is often distributed in small populations inhabiting discrete regions (Madsen *et al*.1996).
Within the Netherlands two large core populations exist (Fig.1), the largest in the north, at the border between Friesland and Drenthe (Fochteloërveen, Bargerveen, and Appelscha), the second one at the Veluwe (mainly National Park the Hoge Veluwe and Kootwijk). Two small populations occur isolated in Limburg (De Meinweg) and in the east of Overijssel (Haaksbergerveen) (Van den Berg & Van Kuijk 2002). Formerly, adders were much more common, and occurred in a larger range of areas within the Netherlands. Nowadays its distribution is limited to the mentioned core populations. The last ten years the populations fluctuated strongly, but seem to be relatively stable, with a slight increase in the last years 2003-2006 (Fig 1). Population trends differ per population; in 2006 most populations increased while only the population at De Meinweg decreased.

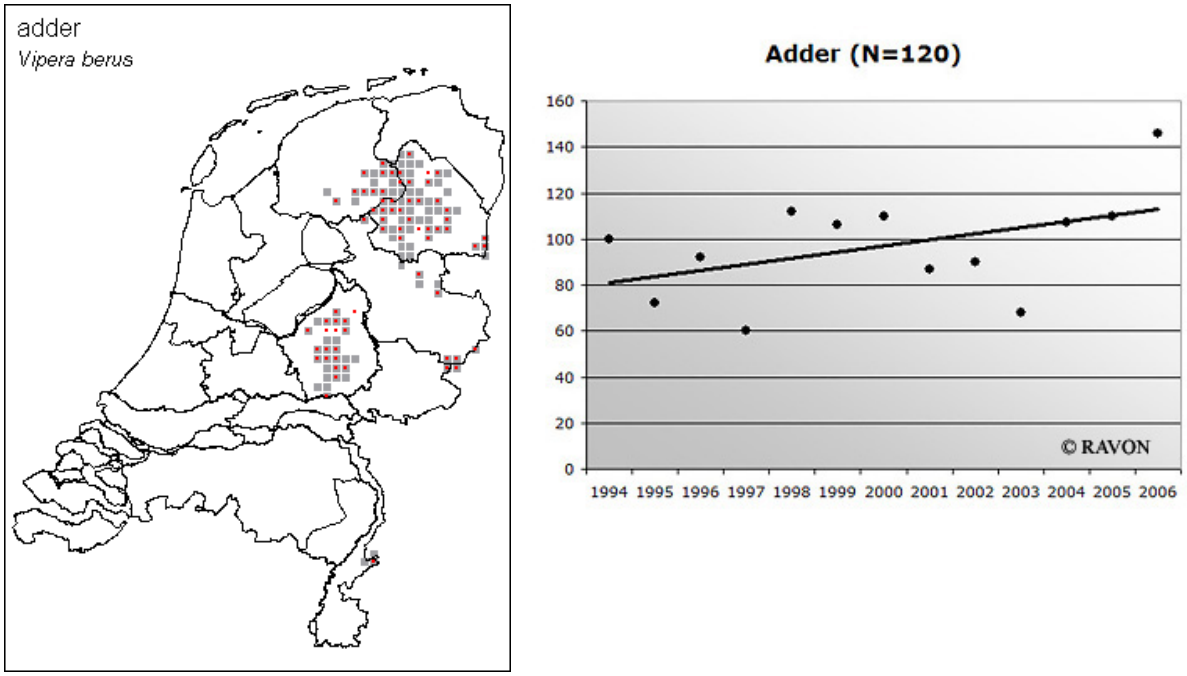

Figure 1. Distribution of the adder in the Netherlands. Grey: 1997-2005, red: 2006. In the graph right the trend of the adder populations in the Netherlands, indexed with 1994 as basis. Source: RAVON

1.5 Genetic background

Human pressure, resulting in particularly habitat destruction and fragmentation, causes many species, including the adder, to decline worldwide (Caughley 1994, Ursenbacher *et al*. 2009). Extinction risks are particularly high for reptile species, of which many are endangered (Corbett 1989). Snake species are often persecuted as potentially dangerous to human, while habitat fragmentation increases the human pressure on these animals (Ursenbacher *et al.* 2009). A large number of snakes are killed on roads by traffic, but the amount is variable, depending on season, species, population, sex and age. Mate searching males and newborn juveniles are at higher risks due to higher dispersing activity (Bonnet 1999). These factors, predominantly diminishing habitats, lead to a decrease in population sizes, negatively affecting the genetic diversity of species (Frankham 1995, 2005). Small populations are vulnerable for extinction due to coincidental combinations of negative environmental or demographic changes. Population size is negatively correlated with stronger forms of inbreeding and genetic drift. To prevent stochastic processes negatively influencing reproduction fitness, populations should be larger than the minimal viable population size (MVP). Genetic drift is an important process determining genetic diversity. In passing on genes tot the next generation, some genetic variation might be lost. Next to the influence of genetic drift on a population, also selection, migration, inbreeding and bottlenecks could have an effect on genetic diversity. Low genetic diversity could lead to a lower fitness of individuals in a population. Growth, life expectancy and reproductive success could indicate a low genetic diversity. This decline in genetic diversity has been shown for many threatened species that consist of small (isolated) populations (Frankham 1996). The low adaptive potential causes a higher risk of local extinction, especially when changes in environmental conditions occur (Frankham *et al*. 2002). However, low genetic diversity is not per definition bad, deleterious alleles could be purged (lost by selection), and the genetic base of the population is optimized for the current environment. But changes in the environment could have dramatic effects on the population dynamics, since the populations are not able to adjust due to the low genetic variability (Blitterswijk *et al.* 2005). Bringing in not adapted individuals of other populations to enrich the genetic diversity, could lead to an outbreeding depression, a loss of local adaptation and a decreasing fitness (Frankham *et al.* 2002).

Whether these issues of low genetic diversity applies to the populations of the adder, *V. berus,* in the Netherlands will follow from this study.

1.6 Goals of the study

In this study, we aimed to fulfill the following goals:

- Gain insight in the state of genetic diversity of the Dutch populations
- Understand the dispersion and genetic differentiation between populations
- Give recommendations for ensuring sustainability of different metapopulations within the Netherlands (possibilities for dispersion/translocations)

To met the above mentioned goals, I formulated the following research questions:

- What is the genetic diversity in the populations of the adder in the Netherlands?
- What is the diversity in the subpopulations of the Fochteloërveen (northern) metapopulation?
- To what extent are the populations differentiated?
- Is there further management action needed to sustain the genetic viability of the populations?

2. Methodology

For studying genetic diversity in adder populations DNA samples are needed. Most studies use blood samples of caught individuals (e.g. Carlsson *et al.* 2004, Ursenbacher *et al*. 2009). Since this could be a stressful method and the adder is already a vulnerable species in the Netherlands, a minimal disturbance was preferred. We used two forms of non-invasive sampling, the collection of shed skins found in the field, and road-killed snakes. Both methods deliver useful DNA (Jones *et al.* 2008), although DNA from old skins might be degraded and long fragments are more difficult to analyse (Fetzner 1999). Adders usually shed skins two or three times a year for respectively males and females (Neumeyer 1987), and although shed skins could be difficult to find in the field, these skins could be very valuable for DNA sampling. Even parts of adders found in raptor faeces could be used. With these two methods, samples were collected in various sites in the Netherlands. Besides these Dutch populations, the large but isolated population at Groot Schietveld (Antwerpen) in Belgium and reference samples from Sweden were used in the analyses.

Small pieces of skins or tissue (about 25 mg) were used to extract DNA using the DNeasy Tissue Kit of Qiagen. For the analyses micro satellite markers were applied. As primers I used five primers developed for *Vipera berus* in the study of Carlsson (2004) in Sweden (Vb-3, Vb-11, Vb-37, Vb-64, Vb-71) and two primers developed for *Vipera berus* by Ursenbacher (2009) in Switzerland (Vb-A8 and Vb-D'10) (Table 1).

Table 1. Description of the used primers, with basepair sequence, repeated motif, annealing temperature (T_a) and allele length.

Locus	Primer sequence (5'-3')	Nucleotide repeat	T_a (ºC)	Allele size (bp)
Vb-3	CAAGAAATGGAGATGAGC GAAACCTATGAGCCAGTA	$(AC)_{16}$	52	164-176
Vb-11	GCAGCAGTCAGGACCGTTA CCCCTTTCCTCTCCTTCTT	$(TC)_{32}$	60	133-163
Vb-37	CTAAAGATGTCTTAGGGTCACT ATCCAGCCAGAACTGAT	$(CT)_7TT(CT)_{20}$	49	305-395
Vb-64	AGGCTCTGCTAAATGACC GATCCCCTGAATTGATTA	$(TG)_5TT(TG)_{21}$	56	241-263
Vb-71	TTGGCAAGAATCGAGGAGCTG TGTGCCGACTTTTTGTGCTGA	$(AC)_9TC(AC)_5$	62	119-123
Vb-A8	ATTTCACCATGCCTCCAGAA GGTACACTCATTGTGATGAAC	CA	55	194-224
Vb-D'10	GTCCTCCTTATCATCTATCC CCTGGGTGCTCTCTCAG	AAG	58	369-407

Parts of DNA were amplified with Polymerase Chain Reaction (PCR). DNA was 5 times diluted before use, and amplified in a mix of total 10 µl on a MJ Research DNA-engine. All loci were prepared with 1µl 10x PCR buffer (200 mM Tris-HCl, pH = 8.4, 500 mM KCl), 1.5 mM MgCl2, 0.16 µl W-1%, 0.16 µl BSA (20 mg/ml), 200 µM dNTP's-cocktail, 0.13 µM primer and 0.3 U Taq-DNA polymerase (Invitrogen). The PCR conditions consisted of an initial denaturation of 5 minutes at 94 ºC followed by

30 cycles of 30 seconds at 95 ºC, 30 seconds at annealing temperature (Ta) depending on locus (table 4) and 45 seconds at 72 ºC, followed by a final elongation of 10 minutes at 72 ºC. For the primers Vb-A8 and Vb-D'10 the PCR conditions were slightly different. For these, the PCR conditions consisted of 35 cycles of 30 seconds at 95 ºC, 30 seconds at annealing temperature (Ta) depending on locus (table 4) and 45 seconds at 72 ºC, followed by a final elongation of 5 minutes at 72 ºC. The 5' -end of each Forward-primer was synthetized with a IRD 800 nm label. 3 µl of the amplicon was after denaturation in 20 µl loading buffer (99.6 % Formamide, 10 mM EDTA and 0.1 % Broomphenol blue) for 5 minutes at 95 ºC, analyzed on a 25 cm long sequencing gel (6.5 % polyacrylamide, 7 M Urea and 1xTBE) with a LICOR 4200 DNA analyzer. During electrophoreses positive controls were used to be able to score the fragment lengths of the samples.

The primers Vb-37 and VB-A8 were left out after electrophoreses due to the large number of not-scoring samples. Samples which missed alleles at more than 2 loci were removed from the analyses. Also populations for which less than 5 samples were available after scoring were left out of the analyses. After the mentioned deleted loci and samples, the data set consisted of 154 samples from 7 populations, tested for 5 loci. Sampling was relatively biased towards the populations in the north of the Netherlands. 61 Samples were analyzed from Fochteloërveen, 25 from Appelscha (Drents Friese Wold) and 19 from the Duurswouderheide. The Veluwe samples consisted of Hoge Veluwe (1), Planken Wambuis (2), Hoog Soeren (1), De Bieze (1), Kootwijkseveld (1) and the Toverberg in Ermelo (1). These samples were grouped in further analyses (and referred to as Veluwe samples) to be able to include these in the analyses. Of the small population in Wolfheze 5 samples were available, and these samples were analyzed separately (analyses don't work for populations smaller than 5 samples). Besides these Dutch populations, also 30 samples of the large but isolated population at Groot Schietveld (Antwerpen) in Belgium. Also reference samples from Sweden (7) were used in the analyses.

Genetic variation is characterized by a mean number of alleles (N_a), the number of effective alleles (N_e) and the percentage of heterozygosity (H_e (expected) and H_o (observed)) in the different populations. The number of alleles per microsatellite is a measure for the number of varieties of a gen or locus. The comparison of N_e and N_a indicates the number of alleles independent of sample size, and therefore more useful for comparisons between populations. The heterozygosity represents the genetic diversity within a population. Analyses were done by use of the program GenAlEx 6.2 (Peakall & Smouse 2006), followed by the program FSTAT 2.9.3 (Goudet 1995). With GenAlEx the multivariate Principal Coordinate Analysis (PCA) was done, which provided an image representing the genetic distance between the individual samples, showing the clustering of differentiated populations.

Differentiation of the various adder populations was tested with the program FSTAT. F_{st} is a relation between the variation within a population and the variation among populations, and indicates the degree of differentiation of populations. The F values are situated between 0 and 1, 0 means complete genetically overlap, so no differentiation, while a F of 1 indicates complete differentiation. Values lower than 0.05 mean no differentiation, while values larger than 0.15 points towards severe isolation and differentiation.

3. Results

3.1 Genetic diversity

The result of the diversity analyses are presented in table 2, including sample size (n), number of alleles(N_a), effective number of alleles (N_e), observed (H_0) and expected (H_e) heterozygosity and variation within populations (F). The adders of the reference population (Sweden) had only 2.4 effective alleles per locus (N_e), while the large Dutch populations in Drenthe had more than 3 effective alleles (Fochteloërveen largest with 3.8 effective alleles), so did the population of Groot Schietveld (Belgium). The grouped samples from the Veluwe reached the same genetic variability as the population in Sweden, while the Wolfheze population showed a lower genetic diversity. Due to the unequal sample size the results might be slightly balanced, but the parameter for effective alleles should take that in account, and function independent of sample size (Table 2). Heterozygosity was low overall, and het observed heterozygosity was for all populations lower than the expected. The large adder population at Groot Schietveld showed the lowest heterozygosity (H_o of 0.22), while Fochteloërveen represented the largest heterozygosity (H_o of 0.38). For some loci the allele lengths varied strongly between populations (Vb-11, Vb-D'10), while others were relatively constant (Vb-71) (Fig. 2).

Table 2. Genetic diversity of the different adder populations, based on 5 loci. n, number of samples; N_a, number of alleles; N_e, number of effective alleles; H_o, observed heterozygosity; H_e, expected heterozygosity; F, variation within population.

Population	n	N_a	N_e	H_o	H_e	F
Appelscha	25	6.8	3.6	0.28	0.63	0.558
Duurswouderheide	19	5.6	3.2	0.31	0.56	0.369
Fochteloërveen	61	8.6	3.8	0.38	0.59	0.333
Groot Schietveld (B)	30	5.8	3.0	0.22	0.43	0.336
Veluwe	7	3.2	2.4	0.30	0.51	0.476
Sweden	7	3.4	2.4	0.30	0.54	0.368
Wolfheze	5	2.0	1.7	0.28	0.35	0.149

3.2 Differentiation

The results of the differentiation analyses of the adder populations can be found in table 3. Remarkable is the strongest differentiation of the population in Belgium (Groot Schietveld), while looking at the geographic distance the population in Sweden was expected as ultimate outgroup (which turned out to be not true for every population). The Belgian population seemed to be further differentiated from the Veluwe than from the population in the north of the Netherlands, which is from a geographic perspective not explicable, since the Veluwe is situated in between. This

might be caused by the sampling of the Veluwe (samples from different populations grouped as a single large population and therefore as group more divers and less differentiated than the single subpopulations). The populations in the north of the Netherlands (Appelscha, Duurswouderheide and Fochteloërveen) showed no differentiation among each other, suggesting that genetic exchange still happens between them, or very recently was aborted. Wolfheze differentiated lowest from the Veluwe, while clear differentiation showed up towards the north of the Netherlands and the foreign populations, even more than the Swedish population compared with the other populations.

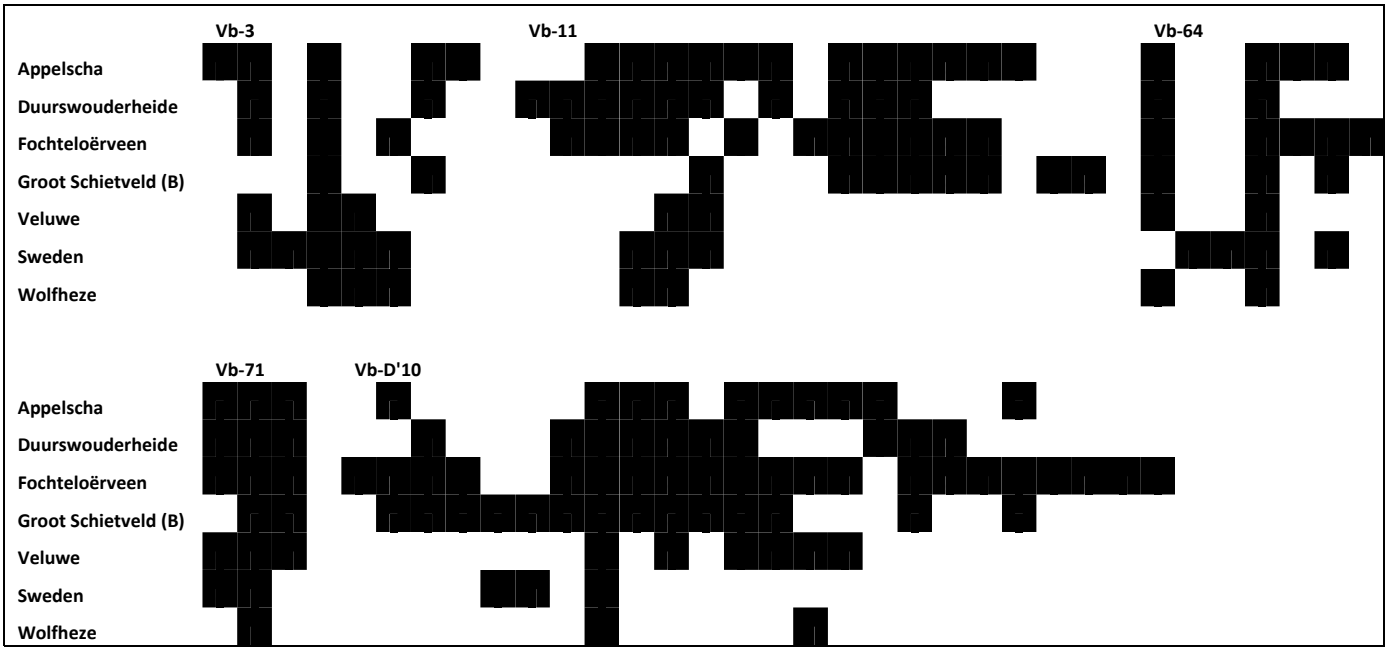

Figure 2. Presence (black) and absence (white) of alleles of 5 loci, for each population.

Table 3. Differentiation among adder populations (F_{st} values). Appel=Appelscha, Duur=Duurswouderheide, Focht=Fochteloërveen, Groot S=Groot Schietveld. F_{st} values larger than 0.15 (severe differentiation) are printed in bold.

	Appel.	Duur.	Focht.	Groot S.	Veluwe	Sweden	Wolfheze
Appel.	0.0000						
Duur.	0.0320	0.0000					
Focht.	0.0345	0.0550	0.0000				
Groot S.	**0.2251**	**0.2712**	**0.2599**	0.0000			
Veluwe	0.0956	**0.1672**	0.0750	**0.3934**	0.0000		
Sweden	0.1048	**0.2376**	**0.1629**	**0.3489**	0.1086	0.0000	
Wolfheze	**0.1813**	**0.2896**	**0.2128**	**0.3948**	0.0832	**0.1844**	0.0000

With a Principal Coordinate Analysis (PCA) a representation is pictured in two dimensions (Fig 3). Due to the multiple dimensions of the analyses, the first two axes explain most of the variation, but not all. The clearest outgroup is formed by the population at Groot Schietveld, which also showed up in the F_{st} values in table 3. Also the Wolfheze samples are grouped, as are the populations of Sweden and the Veluwe. The populations in the north of the Netherlands show no differentiation, which was also found in the F_{st} values. The axes explain 47.8 percent of the total variation.

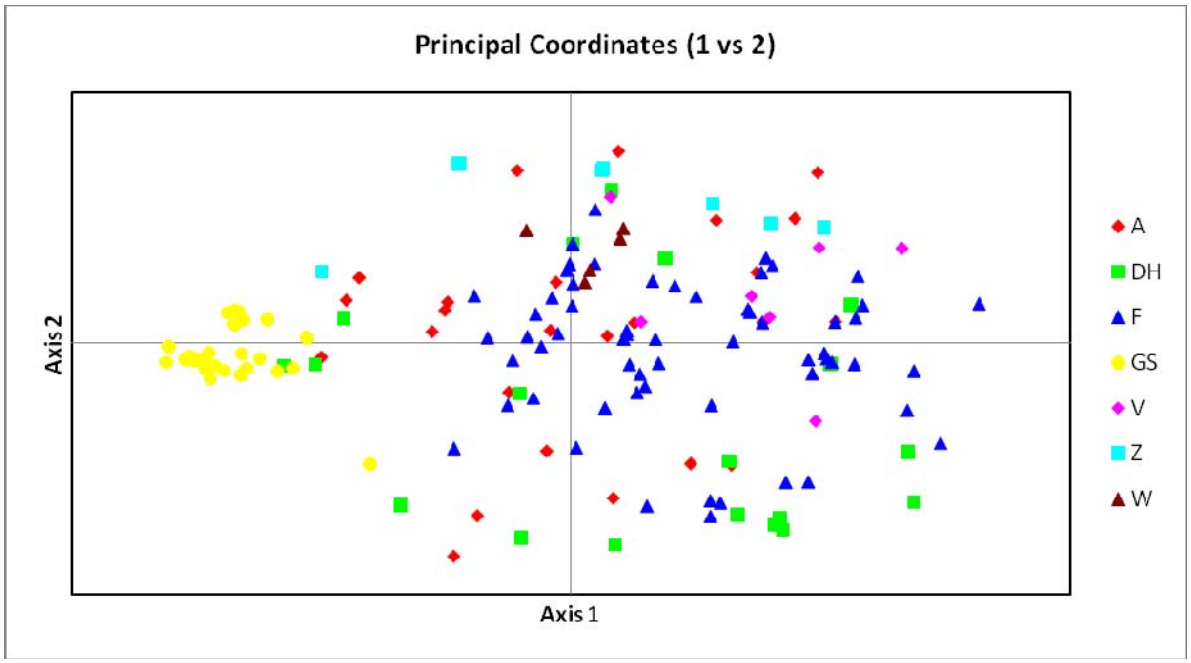

Figure 3. Scores of individuals samples on the first and second ordination axes. These axes explain 47.8% of total variation. A=Appelscha, DH=Duurswouderheide, F=Fochteloërveen, GS=Groot Schietveld, V=Veluwe, Z=Sweden, W=Wolfheze.

4. Discussion

This study showed that the adder populations in the north of the Netherlands, as Fochteloërveen, Appelscha en Duurswouderheide, are genetic diverse and showed no or almost no differentiation among each other. The connectivity between these populations is apparently large enough to provide sufficient gene flow or the population sizes are much larger than the minimal viable population size (MVP) and therefore genetic erosion does not occur. Apparently the intermitting distances are small enough for dispersing males to overcome and encounter females from other nearby populations. If isolation plays a role in these populations, it has to be from very recent origin, otherwise it would probably be found in the diversity and differentiation analyses.

The population at the Veluwe is a different case. All populations at the Veluwe were isolated from each other sooner or later, leading to a differentiation of these populations from all other populations. The isolated populations genetically eroded due to a lack of gene flow with other areas and random processes within the populations (local adaptation). This has led to a higher genetic diversity within the Veluwe when the populations are grouped, but lower diversity within the subpopulations. The Wolfheze population is a strong example for this. This population showed to be strongly differentiated (least from the other Veluwe populations due to the latest isolation), and genetic diversity within the population was low; not as low as the inbred populations studied by Madsen *et al.* (1996), but probably strongly heading towards that status. This strong differentiation results in inbred populations, which in turn causes health and reproduction problems at the longer run, and in the end local extinction. To prevent this from happening, a genetic flow should be created between the subpopulations of the Veluwe, either by connection of habitats or artificially translocation of individuals between populations.

No geographic connection was found between the Veluwe and the populations in and around Fochteloërveen, these metapopulations differentiated from each other more than within both metapopulations. In between these two core populations some small populations exist such as Staphorst, but connectivity between these “stepping stones” is too small to be useful for exchange of genetic info. To draw strong conclusions from the genetic status of the population at the Veluwe and the different subpopulations, and confirm if other subpopulations have the same severe inbreeding as Wolfheze showed, a more thorough study is needed. In the data set for this study the Veluwe consists of a group of single samples from several subpopulations (excluding Wolfheze), therefore the image of the Veluwe might not be complete enough.

The high differentiation of the adders from Groot Schietveld in Belgium was remarkable. Looking at the distance to the nearest population some differentiation was expected. But it showed an even higher genetic distance to the Dutch populations than the Swedish samples did. Looking at the relation with the populations in the Netherlands, the closest relatives are found in the north, in and around Fochteloërveen. The intermediate populations of the Veluwe showed slightly higher differentiation from the Belgian samples, but that might be the result of the low number of samples from the Veluwe, which is therefore less reliable for strong

conclusions. Besides that, the large differentiation between Groot Schietveld and the Veluwe (and Wolfheze as single population within the Veluwe) is strengthened by the differentiation of the Veluwe populations as well, which is not the case for the populations in Drenthe. The population at the Groot Schietveld showed a low genetic diversity (heterozygosity), which indicates a relatively strong isolation. This effect is remarkable, since the population is relatively large and therefore expected to be less vulnerable for genetic drift.

In the population in Smygehuk in southern Sweden, which was studied by Madsen *et al*. (1996) the genetic diversity (effective number of alleles and heterozygosity) was much lower than that of the populations of our study. The study we executed was not complete enough to be sure that all populations in the Netherlands are genetically diverse enough to survive without further management actions. The small populations at the Veluwe (but with Wolfheze as single example) and the larger populations at the Meinweg in Limburg and Haaksbergerveen in Overijssel were not included in this study due to lack of available DNA. These populations might reveal a low genetic diversity and strong differentiation due to long isolation as was shown for Wolfheze, but to test this further large scale research is needed. The other populations analysed by Madsen *et al*. (1996), showed comparable diversity as the samples in this study. The study of Ursenbacher *et al*. (2009) revealed much higher heterozygosity in the Swiss and French populations, but the indicators for differentiation among the populations were in the same order of magnitude as in this study.

Overall can be concluded, that this study worked as a valuable pilot for further research. To evaluate all adder populations in the Netherlands, much more samples are needed (preferably 30 per location, according to Blitterswijk *et al.* (2005)), and more microsatellite loci (minimal 10, following Blitterswijk *et al.* (2005)) should be applied to strengthen the analyses. This study however, provided some strong indications. The adder populations in the north of the Netherlands have a viable perspective to survive. This study showed no shortcomings in genetic diversity or connectivity. The subpopulations at the Veluwe required more research to ensure the genetic healthiness. The Wolfheze population serves as an example for an isolated populations which is heading towards severe inbreeding. If the other Veluwe populations turn out to follow the same trend: lowering genetic diversity and increasing differentiation, management actions should be taken to improve first of all population sizes (to numbers larger than the MVP. Secondly corridors between populations should be improved, targeting natural gene flows. If this is not possible at the shorter term and monitoring results indicate a need for urgent action, translocation of one or a few individuals from one population to the other might provide an alternative for exchange of DNA. The same applies for the population of Groot Schietveld in Belgium, which seems to be large enough to survive, but repeated monitoring might show a decline in genetic diversity. Attention must be paid to the strong differentiation of this population. It deviates genetically from all relatively nearby populations, so there is a risk for outbreeding depression, which is a loss of adaptation to the local conditions.

5. Recommendations

From this study a number of recommendations come forward, both towards research and management perspective.

Monitoring:

- More sampled populations needed, preferably all Dutch populations for a complete overview
- More samples per population needed to strengthen the analyses
- More loci needed, to test the samples for
- Population sizes should be taken into account, combination with genetic data is important
- Based on this combination, a relation should be found between genetic variation and population size, including level of isolation

Management:

- Aim for increase in habitat size to allow population growth over MVP
- Defragmenting habitats remains critically important
- Use genetic diversity to monitor viability of small isolated populations to be on time for action

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