Genetic population structure of the grass snake (*Natrix natrix*) in human-altered landscapes in Switzerland

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TABLE OF CONTENTS

SUMMARY

Both the conversion of natural habitats to farmland and efforts at increasing the yield of existing crops contribute to a decline in biodiversity. As a consequence of land conversion, specialised species are restricted to remnants of original habitat patches, which are frequently isolated. This may lead to a genetic differentiation of the subpopulations. A lack of gene flow may also result in genetically impoverished subpopulations increasing the risk of local extinction. Highly variable genetic markers, like microsatellites, can be used to investigate genetic differences among subpopulations. The grass snake (*Natrix natrix*) primarily feeds on amphibians and is therefore associated with wetlands. As a result of pronounced changes in land use, the area actually occupied by the grass snake in Switzerland is restricted to remnants of pristine habitats and the populations are declining in many regions. A few years ago, microsatellite markers were not available for the grass snake. Therefore, six microsatellite markers were developed for the grass snake (*N. natrix*) and three microsatellite markers of the dice snake (*Natrix tessellata*) were checked for cross-amplification. These microsatellite loci were used to examine the genetic structure of grass snakes sampled in remnants of pristine habitat embedded in an intensively used agricultural landscape and in a former floodplain in the Swiss lowlands, as well as in a rural valley in the Bernese Alps. The three study areas were 30–100 km apart, but were interconnected by the river Aare. At the local scale, no genetic differentiation was found in either of the *N. natrix* populations inhabiting the intensively used agricultural area or the rural alpine valley. However, two subpopulations in the former wetland area were genetically differentiated as indicated by a low but significant F_{ST}-value. This slight genetic differentiation can be explained by isolation by distance. At the regional scale, significant genetic differentiation between *N. natrix* populations inhabiting the three study areas was found. The genetic structure was highly related to isolation by distance with 85% of the among-population genetic variance explained only by the geographical distance between subpopulations. The present findings indicate regular gene flow between *N. natrix* subpopulations. Human activity and habitat alteration do not seem to reduce significantly the movements of grass snakes. These results suggest that conservation actions in landscapes altered by humans should focus on the maintenance of a habitat mosaic with anuran breeding ponds and adequate oviposition sites.

Another aspect of this thesis was to investigate the occurrence and frequency of multiple paternity in the grass snake as a source of genetic diversity. Males can enhance their reproductive success through mating with multiple females. For females, however, one mating is

usually sufficient to inseminate all their ova. Females may benefit from multiple mating by producing genetically more diverse offspring, and by having the opportunity to choose sperm of the genetically most compatible male. The frequency of multiple paternity was assessed in 11 clutches of the grass snake using the microsatellite markers. Two and more fathers were found to sire offspring in 27% of the clutches using a very conservative estimate. However, based on a maximum likelihood, multiple paternity occurred in 91% of the clutches with 2–5 contributing males per clutch. This is the first study demonstrating multiple paternity in a European natricine, with a frequency similar to those found in new world natricines.

To sum up, this thesis demonstrated that the genetic variability in grass snake populations is maintained by regular gene flow between subpopulations, and through multiple mating by females resulting in multiple paternity.

GENERAL INTRODUCTION

Biodiversity, generally defined as diversity of ecosystems, species and genes, is rapidly declining worldwide. The decline is mainly caused by the conversion of natural habitats to farmland and the intensified use of existing crops (Matson et al., 1997; Vitousek et al., 1997). This leads to the destruction of the livelihood of many species and, consequently, to the extinction of the concerned species (Krebs et al., 1999; Pimm and Raven, 2000). Furthermore, human development results in the fragmentation of natural and semi-natural habitats (Foley et al., 2005). Habitat fragmentation leads to the isolation and size reduction of remnant populations, and consequently increases the risk of local extinction (Saccheri et al., 1998). There is growing evidence that relictual populations are subject to significant genetic and demographic changes, which may directly (e.g. via inbreeding depression or allele erosion) or indirectly (e.g. via reduced evolutionary potential) affect individual fitness and population viability (Young and Clarke, 2000; Rusterholz and Baur, 2010). Therefore, habitat fragmentation is known as a threat to genetic diversity, which is a key factor for evolution as it enables organisms to react to a changing environment, e.g. induced by climate change (Kettlewell, 1955).

In animals, dispersing individuals may introduce novel alleles into a population, what enhances genetic diversity. One of the measurements of genetic diversity is the heterozygosity level. In diploid organisms, heterozygosity is expressed as the fraction of individuals in the population with two different alleles at a determined locus in their genome. Nowadays, it is known that the fitness of individuals and the survival of populations are related to the levels of heterozygosity (Madsen et al., 1996; Reed and Frankham, 2003). A high level of heterozygosity can be perpetuated through gene flow. Maintaining gene flow between isolated populations is therefore crucial for the long-term viability of populations. In general, the dispersal rate depends on the population size, the extent of resource competition, the habitat quality, the size and isolation of suitable habitat patches, as well as the species' behaviour (Bennett, 2003).

Landscape genetics is a combination of population genetics, landscape ecology and spatial statistics. This approach is used to investigate how different landscape features influence gene flow (Manel et al., 2003). In landscape genetics, permeability describes the resistance of the landscape matrix to a species' movements, where different land uses can enhance (high permeability) or impede (low permeability) movements (Kindlmann and Burel, 2008). Empirical evidence from a variety of animal taxa indicates that human infrastructure, including highways, roads, and railway lines, reduce landscape permeability (Trombulak and Frissell, 2000; Clark et al., 2010; Holderegger and Di Giulio, 2010; but see Brown et al., 2006).

However, even in the absence of barriers, cumulative effects of different landscape features with low permeability may lead to genetic differentiation of populations. Another aspect is isolation by distance, where close populations are genetically more similar than distant populations. Landscape features such as ridges, rivers and open shrub habitat influence dispersal in water-depending taxa, e.g. amphibians (Funk et al., 2005; Giordano et al., 2007; Spear et al., 2005).

In reptiles, the dispersal pattern is affected by habitat fragmentation (Stow et al., 2001) and genetic differentiation may occur over short distances (Moore et al., 2008). For example, a fine-scale genetic population structure (< 7 km) has been reported in eastern massasauga rattlesnakes (*Sistrurus catenatus catenatus*; Chiucchi and Gibbs, 2010), in adders (*Vipera berus*; Ursenbacher et al., 2009), in eastern fox snakes (*Mintonius* [*Elaphe*] *gloydi,* now *Pantherophis gloydi;* DiLeo et al., 2010) and in smooth snakes (*Coronella austriaca*; Pernetta et al., 2011). In contrast, local populations of the asp viper (*Vipera aspis*) exhibited a low genetic differentiation (measured with RAPD markers) at the regional scale (120 x 45 km; Jäggi et al., 2000). Whereas no genetic differentiation was found in the black rat snake (*Elaphe obsoleta obsoleta,* now *Pantherophis obsoletus*) at a fine-scale (≤ 6 km), a limited differentiation at the local scale (15–50 km), and a high differentiation at a larger scale (465–1900 km; Lougheed et al., 1999) has been detected. These findings indicate that dispersal in snakes is species-specific and highly dependent on the suitability of the habitat patches and the permeability of the surrounding landscape.

Habitat fragmentation can reduce the levels of gene flow between populations, therefore reducing genetic diversity and, consequently, heterozygosity. Another key factor influencing heterozygosity is the mating system. In mating systems without paternal care, males can enhance their reproductive success through mating with multiple females. In these species, mate acquisition is the limiting factor for male reproductive success and strategies to overcome this limitation are numerous (Shine, 2003). For females, however, one copulation might be sufficient to inseminate all ova. Nevertheless, multiple mating by females has been reported in a variety of reptile species, indicating direct and indirect benefits for the females (Zeh and Zeh, 2001). Direct benefits arising from paternal contributions to egg production or parental care are unlikely to play an important role in most reptile species (Uller and Olsson, 2008). However, indirect benefits may arise to multiply mated females from increased genetic quality, higher complementarity, and / or enhanced genetic variation (bet hedging) of their offspring (Madsen et al., 2005; Uller and Olsson, 2008). Therefore, polyandry is a source of genetic diversity and thus of heterozygosity.

FOCUS OF THE THESIS

The aim of this thesis was to examine the permeability of human-altered landscapes for the grass snake (*Natrix natrix*). Landscape structures that impede movements, e.g. through behavioural avoidance or impassability for the grass snake, reduce gene flow and may act as partial, or complete barriers. The identification of structures with barrier effects is crucial for the detection of possible population subdivision. The knowledge of population subdivision is an important information to assess the persistence of populations.

Highly variable genetic loci, like micorsatellites, are the ideal markers to examine the genetic population structure of a species in a given area and to detect genetic discontinuities. A few years ago, microsatellite markers were not available for the grass snake. In **Chapter 1**, the development and amplification of six novel microsatellite markers for the grass snake (*Natrix natrix*), cross-amplification of these microsatellite loci in the dice snake (*Natrix tessellata*) and cross-amplification of dice snake microsatellite markers for the grass snake are described. A set of nine microsatellites, six markers developed for *N. natrix* and three for *N. tessellata*, was used to investigate the genetic population structure of grass snakes in human-altered landscapes (**Chapter 2** and **3**) and the occurrence and frequency of multiple paternity in *N. natrix* (**Chapter 4**) living in different localities in Switzerland.

Although high proportions of the world's surface are used as farmland, our knowledge on the permeability of agricultural fields for snakes is still very limited. In **Chapter 2**, the permeability of an intensively managed agricultural landscape for the grass snake was examined at the local scale. Furthermore, it was investigated whether dispersal among suitable habitat patches occurs frequently enough to prevent genetic differentiation among (sub)populations.

In **Chapter 3**, the dispersal ability of the grass snake was investigated at a larger scale. The genetic population structures of *N. natrix* living in remnants of a former wetland located in the intensively cultivated Swiss lowland and in a rural, scarcely populated valley in the Alps were compared with the genetic population structure of grass snakes living in the intensively used agricultural landscape described in Chapter 2. All three study areas are interconnected by the river Aare over a distance of 100 km.

Gene flow between populations as a result of successful dispersal counteracts the effects of genetic drift and allows the maintenance of genetic diversity. Another mechanism to maintain genetic variation is multiple paternity. In **Chapter 4**, the occurrence and frequency of multiple paternity in natural populations of the grass snake were assessed.

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CHAPTER 1

Novel microsatellite loci in the grass snake (*Natrix natrix*) and cross-amplification in the dice snake (*Natrix tessellata*)

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PERMANENT GENETIC RESOURCES NOTE

Novel microsatellite loci in the grass snake (*Natrix natrix*) and cross-amplification in the dice snake (*Natrix tessellata*)

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Abstract

Six novel polymorphic microsatellite loci are presented for the grass snake (*Natrix natrix*), a species with declining populations in many regions. The number of alleles per locus ranged from two to seven. Four dice snake (*Natrix tessellata*) microsatellites were polymorphic in the grass snake with three to four alleles. At two loci, the expected heterozygosity differed significantly from observed heterozygosity. Cross-amplification of the grass snake markers in the dice snake showed two polymorphic microsatellites with two and four alleles.

Keywords: conservation, dice snake, grass snake, microsatellites, *Natrix natrix*, *Natrix tessellata*

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The grass snake, *Natrix natrix* (Linnaeus 1758), has a wide distribution range (northern Africa, Europe, eastern Asia; Kabisch 1999). In many regions, however, *N. natrix* is threatened as a result of habitat alteration and fragmentation and of the decrease of amphibians, the primary food of the grass snake (Monney & Meyer 2005). Regional conservation strategies developed to preserve the remaining grass snake populations have generated growing interest in genetic research and the need for molecular markers. Here we describe the isolation and characterization of six microsatellite loci in grass snake individuals sampled in an intensively used agricultural area (Grosses Moos, Switzerland) and the results of cross-amplification of these markers in dice snakes (*Natrix tessellata*) collected in the Lavaux (lake Geneva, Switzerland).

Genomic DNA was extracted from shed skin, cut scales or liver tissue using a modified cetyltrimethyl ammonium bromide-based extraction protocol. Dice snake microsatellites (Table 2; Gautschi *et al.* 2000b) were tested for cross-amplification in the grass snake and a new primer pair was designed based on Nt8, which did not amplify (Nt8new, Table 2). We also investigated a subset of microsatellite markers (Ns 2, Ts2 and Ts3) reported to amplify in the grass snake (Hille *et al*. 2002). Selection of these microsatellites was based on their allele sizes with the method of Spreadex® gel electrophoresis and allele resolution (see below). However, none of the microsatellites presented by Hille *et al*. (2002) amplified in five *N. natrix* samples and one *N. tessellata* sample.

In a second step, a set of six novel microsatellites was developed for the grass snake. An enriched library was constructed by ecogenics GmbH (Schlieren, Switzerland) from size-selected genomic DNA ligated into SAULA/SAULB-linker (Armour *et al*. 1994) and enriched by magnetic bead selection with biotin-labelled $(CT)_{13}$, $(GT)_{13}$, $(GTAT)_7$ and $(GATA)_7$ oligonucleotide repeats (Gautschi *et al*. 2000a, b). Of 374 recombinant colonies screened, 108 gave positive signals after hybridization. Plasmids from 46 positive clones were sequenced and primers were designed for 12 microsatellite inserts, all of which were tested for polymorphism. ecogenics GmbH used M13-modified primers to determine the polymorphism of the microsatellite markers (for details see Schuelke 2000 and Armbruster *et al*. 2007). The developed microsatellite loci were tested in *N. natrix* ($n = 20$) and for cross-amplification in *N. tessellata* ($n = 21$).

Polymerase chain reaction (PCR) amplification to determine polymorphism of microsatellite markers was performed in 25 L volume containing 25–50 ng of genomic DNA, 0.3 M of each primer and 1 U of HotStar*Taq* Master Mix (QIAGEN). The thermo-treatment on an Eppendorf Mastercycler® Gradient (Vaudaux-Eppendorf AG) consisted of an initial heat activation at 95 C (15 min), followed by 35 cycles at 95 C (30 s), the locus-specific annealing

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Table 1 Characteristics of six microsatellite markers isolated from *Natrix natrix*

Locus	Primer sequences* (5′ → 3′) F, forward; R, reverse	Repeat motif in sequenced clone	*A*	Size range† (bp)	T_a (C)	*n*	H_O/H_E	Acc. no.
Natnat01	F: GATAAAGGCAACGGCAACTG R: CCAGCAGTTAATGTAAACAGAGG	$(CA)_{17}$	3	176–186	56	20	0.45/0.63	EU517459
Natnat05	F: TCTGCACTGGGGATAGGAAG R: GTCCCTTTTTCAGTGCTGTTG	$(GT)_{16}$	4	182–201	56	20	0.65/0.69	EU517460
Natnat06	F: AATGGCATTCTCTCCAGCTC R: ACCCATATCCGTATCCATATCC	$(GT)_{21}$	5	180–201	56	20	0.50/0.65	EU517461
Natnat08	F: TAAGGATGGTGAAGCCTTGC R: ATCGGTGGTACTGGCAGTTG	$(AT)_2(ATAC)_{13}(AC)_3(ATAC)_4$	2	206–221	60	20	0.00/0.40‡	EU517462
Natnat09	F: TGTAAATAACACTGTACCATTTTGG R: TGACTGGGCAACAGAAAAGC	$(AC)_{22}$	7	126–147	56	20	0.60/0.71	EU517463
Natnat11	F: GGCTGTTTTCCCAGTGAAGC R: GGTCTGGGGAAAAAGAAAGG	$(GA)_{13}$	4	128–141	56	20	0.41/0.44	EU517464

*The forward primers used in PCR were modified with a 18-bp long M13-tail. †Size of the amplicons including the 18 bp M13-tail. *A*, number of alleles; T_a, annealing temperature; *n*, number of individuals tested; H_O, observed heterozygosity; H_E, expected heterozygosity. ‡Indicates significant deviation from Hardy-Weinberg equilibrium (after Bonferroni correction), suggesting the presence of null alleles ($P < 0.001$) or being the result of sample size artefacts, founder effects and/or spatial genetic structure.

Table 2 Cross-amplification of grass snake (*Natrix natrix*) microsatellites in the dice snake (*Natrix tessellata*) and vice versa

			Natrix tessellata		*Natrix natrix*		
Locus	Primer sequences (5′ → 3′) F, forward; R, reverse	Repeat motif in sequenced clone	T_a (C)	Size range (bp) (*A*; *n*)	T_a (C)	Size range (bp) (*A*; *n*)	Acc. no.
Natnat01	F: GATAAAGGCAACGGCAACTG R: CAGCAGTTAATGTAAACAGAGG	$(CA)_{17}$	56	168–174* (2; 21)			EU517459
Natnat11	F: GGCTGTTTTCCCAGTGAAGC R: GGTCTGGGGAAAAAGAAAGG	$(GA)_{13}$	56	129–174* (4; 21)			EU517464
Nt8new‡	F: GTATCGTCCTTCCAGACAAG R: GCAAATCAAATAAATCTCACTGG	$(AC)_{15}$	55	85–93 (3; 21)	55	83–109 (4; 20)	AF269190†
Nt3±	F: GGCAGGCTATTGGAGAAATG R: GGCAAAACTCCAGGTGCTAC	$(AC)_{16}$			63	129–138 (3; 20)	AF269186
Nt5±	F: TGCTTTTCGGATTTGACATTC R: CTGCATTTGAAGCGTGGTAG	$(CA)_2GA(CA)_3GA(CA)_4GA(CA)_{15}$			58	93–103 (4; 20)	AF269187
Nt7±	F: TTTGAAAGGAGAATGAATCGTG R: CGCGAGGAATCAGAATGAAC	$(AC)_{17}$			58	176–186 (3; 20)	AF269189

*Size of the amplicons including the 18 bp M13-tail. ‡New primer designed on the basis of Nt8 (Gautschi *et al*. 2000b). ±See Gautschi *et al*. 2000b. T_a, annealing temperature; *A*, number of alleles; *n*, number of individuals tested. †Accession no. of Nt8 for which a new primer pair has been designed.

temperature (30 s; Tables 1 and 2), and 72 C (30 s). The last cycle was followed by 1 min at the annealing temperature and a 30-min extension at 72 C. Amplified products were loaded on Spreadex® EL-400 or EL-600 gels (Elchrom Scientific AG) and electrophoresis was performed with a SEA 2000 advanced submerged gel electrophoresis equipment (Elchrom Scientific AG). Allelic signals were analysed using GenePop (http://genepop.curtin.edu.au; Raymond & Rousset 1995). No significant linkage was found after using Bonferroni corrections for P values.

All six novel loci were variable in *N. natrix* with two to seven alleles (Table 1) and the dice snake microsatellites (Nt3, Nt5, Nt7 and Nt8new) showed three to four alleles in the grass snake (Table 2). However, loci Nt1, Nt2, Nt6 and Nt10 (Gautschi *et al*. 2000b) did not amplify in the grass snake or could not be optimized. Significant deviations from Hardy–Weinberg equilibrium were observed at loci Natnat08 and Nt5 in *N. natrix*. We used Micro-Checker, version 2.2.3 (van Oosterhout *et al*. 2004) to test for the presence of null alleles, stuttering signals or large allelic

dropout. The presence of null alleles is suggested for loci Natnat08 and Nt5. However, deviation from Hardy–Weinberg equilibrium could also be the result of sample size artefacts, founder effects and/or spatial genetic structure.

Two grass snake microsatellites (Natnat01 and Natnat11) were variable in *N. tessellata* (Table 2); loci Natnat05, Natnat06 and Natnat09 were monomorphic and Natnat08 did not amplify. The primer Nt8new showed three alleles (Table 2). The samples of *N. tessellata* examined were obtained from an introduced population and may therefore exhibit founder effects. They may consist of a reduced number of alleles at some loci. The number of introductions and founding individuals are unknown (S. Ursenbacher, personal communication.).

The developed microsatellite markers are currently being used to investigate the genetic population structure of *N. natrix* in landscapes with different permeability.

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CHAPTER 2

Spatial genetic analysis of the grass snake, *Natrix natrix* (Squamata: Colubridae), in an intensively used agricultural landscape

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Spatial genetic analysis of the grass snake, *Natrix natrix* (Squamata: Colubridae), in an intensively used agricultural landscape

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Both the conversion of natural habitats to farmland and efforts at increasing the yield of existing crops contribute to a decline in biodiversity. As a consequence of land conversion, specialised species are restricted to remnants of original habitat patches, which are frequently isolated. This may lead to a genetic differentiation of the subpopulations. We used seven microsatellite markers to examine the genetic population structure of the grass snake, *Natrix natrix* (Linnaeus, 1758), sampled in remnants of pristine habitat embedded in an intensively used agricultural landscape in north-western Switzerland. The study area, a former wetland, has been drained and gradually converted into an agricultural plain in the last century, reducing the pristine habitat to approximately 1% of the entire area. The grass snake feeds almost entirely on amphibians, and is therefore associated with wetlands. In Central Europe, the species shows severe decline, most probably as a result of wetland drainage and decrease of amphibian populations. We found no genetically distinct grass snake populations in the study area covering 90 km^2. This implies that there is an exchange of individuals between small remnants of original habitat. Thus, gene flow may prevent any genetic differentiation of subpopulations distributed over a relatively large area. Our results show that a specialized snake species can persist in an intensively used agricultural landscape, provided that suitable habitat patches are interconnected. *Biological Journal of the Linnean Society*, 2010, **101**, 51–58.

ADDITIONAL KEYWORDS: fragmentation – intensive agriculture – *Natrix natrix* – population genetic structure.

INTRODUCTION

Habitat loss and fragmentation through conversion of pristine habitats to farmland and intensified land use are major causes of the decline in biodiversity (Foley *et al*., 2005; Ribeiro *et al*., 2009). Habitat fragmentation reduces the area suitable for organisms, and leads to the isolation and decrease in the size of remnant populations of plants and animals, which are exposed to an increased risk of local extinction (Saccheri *et al*., 1998). There is increasing evidence that populations are often subject to significant genetic and demographic changes subsequent to being fragmented, and that these interact via a number of direct (e.g. inbreeding depression and allele erosion) and indirect (e.g. reduced evolutionary potential) linkages to affect individual fitness and population viability (Young & Clarke, 2000; Rusterholz & Baur, 2010). Gene flow is therefore crucial for the long-term viability of populations. In animals, dispersal is thought to be the mean mediator of gene flow, where reproducing dispersers may provide new alleles to a population. The dispersal rate depends on the population size, extent of resource competition, habitat quality, and the size and isolation of the habitat patch (Bennett, 2003). A high-quality habitat patch may support more

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individuals, which show a higher survival rate and higher fecundity than those in a low-quality patch. Furthermore, high-quality habitat patches may attract immigrants and allow successful settlement.

A central topic of landscape genetics is to examine how different landscape features influence gene flow (Manel *et al*., 2003). We examined the genetic population structure of the grass snake, *Natrix natrix* (Linnaeus, 1758), living in remnants of pristine habitat embedded in an intensively used agricultural landscape. Permeability describes the resistance of the landscape matrix to grass snake movements, where different land uses can enhance (high permeability) or impede (low permeability) movement (Kindlmann & Burel, 2008). Landscape structures that impede movement, e.g. through behavioural avoidance or impassability for a certain species, reduce gene flow, and may act as partial, or complete, barriers. The identification of structures with barrier effects is crucial for the detection of possible population subdivision.

Certain structures, such as roads, reduce landscape permeability, as has been demonstrated in a variety of animal taxa (reviewed by Trombulak & Frissell, 2000). However, even in the absence of barriers, cumulative effects of different landscape features with low permeability may lead to genetic differentiation of populations. Different landscape features, including ridges, rivers, and open shrub habitat, influence dispersal in amphibians (Funk *et al*., 2005; Spear *et al*., 2005; Giordano, Ridenhour & Storfer, 2007). In reptiles, the dispersal pattern is affected by habitat fragmentation (Stow *et al*., 2001), and genetic differentiation may occur over short distances (Moore *et al*., 2008). For example, fine-scaled genetic structure has been found in subpopulations of the massasauga rattlesnake (*Sistrurus catenatus catenatus* Rafinesque, 1818) situated less than 2 km apart, as a result of restricted gene flow (Gibbs *et al*., 1997), in subpopulations of adders (*Vipera berus* Linnaeus, 1758) separated by less than 3.5 km (Ursenbacher, Monney & Fumagalli, 2009), and in populations of northern water snakes (*Nerodia sipedon sipedon* Linnaeus, 1758) located less than 2 km apart (Prosser, Gibbs & Weatherhead, 1999). In contrast, only modest genetic differentiation between timber rattlesnake (*Crotalus horridus* Linnaeus, 1758) hibernacula separated by 2–8 km was found (Clark *et al*., 2008), and neighbouring populations of the asp viper (*Vipera aspis* Linnaeus, 1758) exhibited a low genetic differentiation in a study area of 120×45 km (Jäggi, Wirth & Baur, 2000). These findings indicate that dispersal in snakes is species-specific, and is highly dependent on the suitability of the habitat patches and the permeability of the surrounding landscape.

Although high proportions of the world's surface are used as farmland, our knowledge on the permeability of agricultural fields for snakes is still very limited. In a Swedish population, grass snakes crossed arable land to reach suitable habitat patches (Madsen, 1984), but avoided grazed fields in England (Reading & Jofré, 2009), whereas monocultures of cereals and root crop constituted a component of the habitat of female grass snakes in north-western Switzerland (Wisler, Hofer & Arlettaz, 2008). These contrasting findings raise the question whether dispersal in the grass snake is limited in an intensively used agricultural landscape. The aim of this project was to examine the permeability of an agricultural landscape for the grass snake, and to investigate whether dispersal among suitable habitat patches occurs frequently enough to prevent genetic differentiation among (sub)populations.

MATERIAL AND METHODS

Study organism

The grass snake has a wide distribution range (northern Africa and Eurasia; Kabisch, 1999). Despite its wide potential distribution in Switzerland, the area actually occupied by the grass snake is relatively restricted (Monney & Meyer, 2005). Two subspecies can be found in Switzerland: *Natrix natrix natrix* occurs in the north-eastern part of the country, and *Natrix natrix helvetica* occurs in the remaining regions. Both subspecies are declining, and are therefore registered on the red list of Switzerland: *N. n. natrix* as 'endangered' and *N. n. helvetica* as 'vulnerable' (Monney & Meyer, 2005). This study focuses on *N. n. helvetica*, for which a decline in occurrence of over 30% has been recorded during the past century (Monney & Meyer, 2005). The decline is paralleled with pronounced changes in land use and a decrease of amphibian populations, the primary food of *N. natrix* (Reading & Davies, 1996; Gregory & Isaac, 2004). Indeed, the grass snake is associated with habitats that support high densities of amphibians, in particular wetlands. Based on telemetry data, the mean home range of adult females varied from 15.1 to 102.5 ha (mean 39.7 ha) in the same study area (Wisler *et al*., 2008). Some females moved a distance of 500 m to reach oviposition sites.

Study area

The study area called 'Grosses Moos' is an intensively used agricultural landscape of approximately 90 km^2 in the western part of the Swiss lowlands, located between the lakes of Murten, Neuenburg, and Biel (46°59′N, 7°08′E; Fig. 1). As a result of deforestation in the Middle Ages, the meandering river Aare frequently inundated the area, which led to an extensive wetland (Nast, 2006).

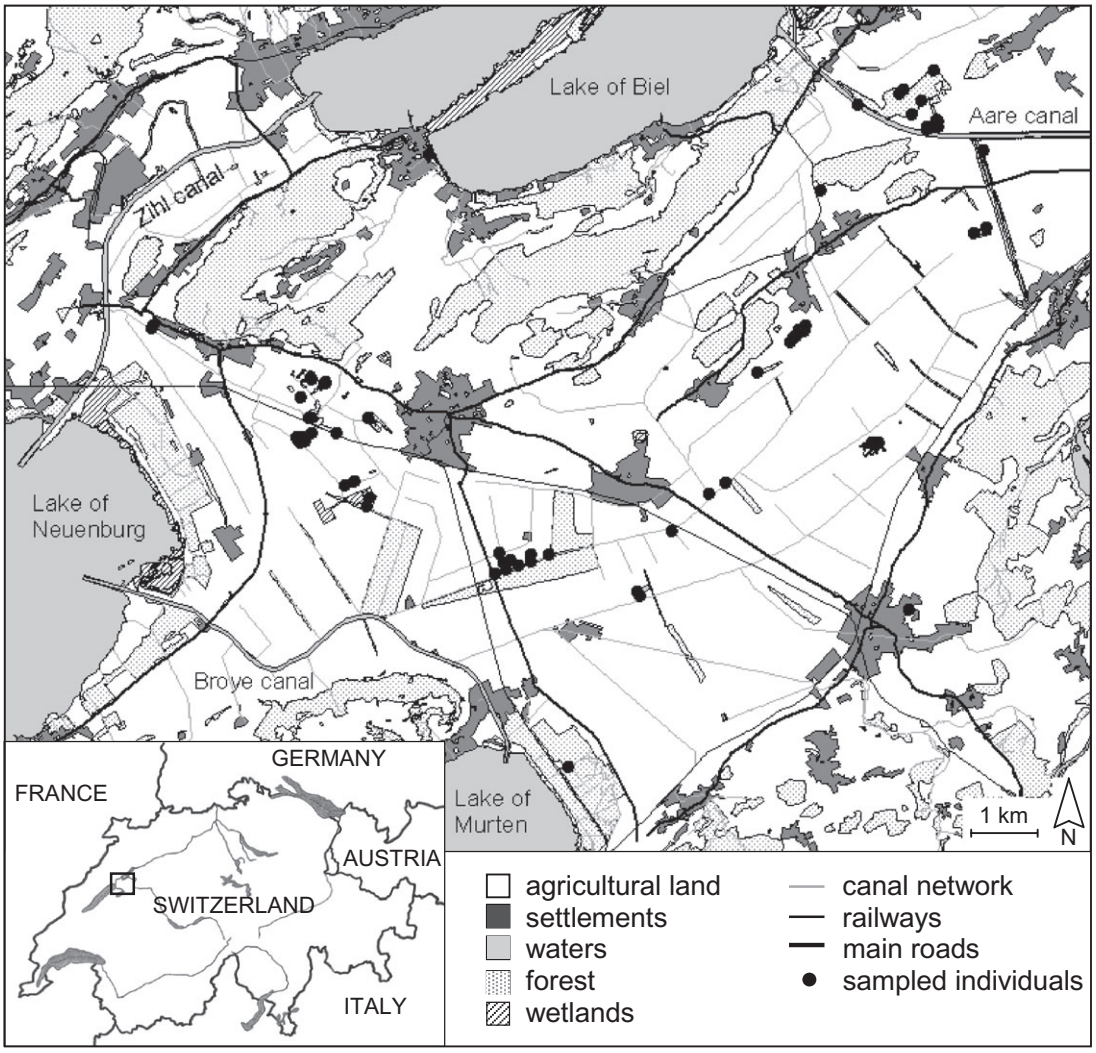

Figure 1. Location in Switzerland and overview of the study area.

Between 1868 and 1891, an extensive drainage system was built converting the wetland into arable land. The main river system (Aare), with numerous branches, slow-flowing meanders, and sand and gravel flats, was transformed into a fast-flowing canal that was redirected to lake Biel (Fig. 1). At the same time two smaller rivers (Broye and Zihl) were channelled. The increased discharge resulted in a lowered water table of lake Biel by 2.5 m. As a consequence of the agricultural use and the drainage of the peat soil, the terrain sunk approximately 1 m (in certain places up to 4 m). Thus, the difference between water and soil level was reduced, enhancing the possibility of flooding. Indeed, several floods caused severe damage in the 20th century. As a consequence, the canals of the rivers were widened and deepened in the 1960s, and a weir was built to regulate the water level of the lakes (Nast, 2006). Further alterations of the landscape include the construction of the railways between 1876 and 1917 (Fig. 1), and the enlargement of the road system.

Pristine habitats are now only represented in small remnants with a scattered distribution, surrounded by intensively used farmland and settlements, interspersed with drainage canals. Wetland habitats cover approximately 1% of the study area. The agricultural fields are cultivated with vegetables, cereals, root crop, and pastures in crop rotation. The mean size of single crop fields is approximately 1 ha, an exception being two farms owned by public authorities with fields of up to 20 ha. A dense network of unpaved roads intersecting the study area is regularly used by farmers and by the local people for leisure activities.

Sampling

Samples of *N. n. helvetica* were collected between April and September in 2006 and 2007. The inaccessibility of the crops prevented a random sampling of the study area. In a preliminary study (U. Hofer, unpubl. data), set-asides, riparian zones, embankments, wetland remnants, and forest edges were sampled within half the cells of the 1-km^2 cells (31 cells) in the core area of our study area. Sampled cells were arranged like the black squares of a chequerboard. Sample locations with proofs of *N. natrix* were further investigated in our study. In the remaining parts of the study area, we searched for *N. natrix* in the same habitat types. The occurrence of these habitats in combination with (incidental) findings of road kills and shed skins determined the distribution of the sampled sites across the study area. The most distant sample sites within the triangle-shaped study area are separated by approximately 13.6 km, Euclidian distance.

Snakes were hand-captured by walking along the edges of the habitats and by controlling artificial shelters laid out to attract snakes (Fitch, 1992). Tissue of snakes was obtained by clipping ventral scales. Snakes were sexed, aged (subadult/adult), based on their size, and a picture of the ventral side of the head and the fore body was taken, showing unique markings for identification after recapture (Carlström & Edelstam, 1946). Shed skins and dead snakes were also collected. Snake tissue was preserved in 80% ethanol and stored at 5 °C until genetic analysis. The coordinates of snake locations were taken with GPS (Garmin GPS 12 Personal Navigator™).

Genomic DNA was extracted using a modified cetyltrimethyl ammonium bromide-based extraction protocol, and genotyped using a set of nine microsatellites (Natnat01, Natnat05, Natnat06, Natnat08, Natnat09, Natnat11, μNt3, μNt7, and μNt8new) developed for the grass snake or the dice snake (*Natrix tessellata*), respectively (Gautschi, Widmer & Koella, 2000; Meister *et al*., 2009). Polymerase chain reaction amplification and electrophoresis of amplified products were conducted as described by Meister *et al*. (2009).

Data analysis

The occurrence of null alleles, stuttering signals, or large allelic dropout was examined using MICRO-CHECKER 2.2.3 (Van Oosterhout *et al*., 2004), and the frequencies of putative null alleles were calculated with GENEPOP using the expectation-maximization algorithm (Dempster, Laird & Rubin, 1977). We calculated the Hardy–Weinberg equilibrium and linkage disequilibrium probabilities for each marker using GENEPOP (http://genepop.curtin.edu.au; Raymond & Rousset, 1995), and applied Bonferroni corrections for *P* values in multiple tests. Population subdivision was evaluated using a Bayesian clustering approach (STRUCTURE; Pritchard, Stephens & Donnelly, 2000). The program was run with a burn-in period of 10 000 repetitions and 100 000 iterations (Markov chain Monte Carlo). The predicted number of populations (*K*) ranged from one to ten, with ten independent runs for each *K*. However, this approach does not incorporate the geographic location of the samples. Therefore, we analysed the data using GENELAND 3.0.2 and R 2.7.2 (Guillot, Mortier & Estoup, 2005) with 100 000 iterations and a thinning of 100. The number of possible populations was set to 1–10, with 100 independent runs. In addition, a spatial autocorrelation analysis was performed using SPAGeDi 1.2 (Hardy & Vekemans, 2002). We tested for correlations between geographic distances (Euclidian distances and nearest distance along water bodies) and pairwise relationship coefficients. Distance intervals were chosen in order to have similar numbers of pairwise comparisons in each interval. Ninety-five percent confidence intervals were calculated with 1000 permutations. Input files were prepared using CONVERT 1.31 (Glaubitz, 2004).

RESULTS

Population genetic diversity

The number of alleles ranged from three (both in μNt3 and μNt7) to 12 (Natnat09) in the 91 individuals analysed (Table 1). Significant deviations from the Hardy–Weinberg equilibrium were observed at the loci Natnat01 and Natnat08. MICRO-CHECKER indicated the occurrence of null alleles for these two loci. The frequency of null alleles estimated with

Table 1. Genetic diversity at seven microsatellite markers in a population of the snake *Natrix natrix*

Locus	*A*	H_O	H_E	F_{IS}	Null allele frequency
Natnat05	4	0.50	0.59	+0.154	0.11
Natnat06	6	0.66	0.66	–0.002	0.02
Natnat09	12	0.56	0.64	+0.123	0.06
Natnat11	5	0.39	0.42	+0.065	0.01
μNt3	3	0.23	0.27	+0.163	0.05
μNt7	3	0.35	0.42	+0.165	0.16
μNt8new	9	0.64	0.65	+0.011	0.05
Mean	6	0.47	0.52	+0.086	

A, number of alleles; F_{IS}, heterozygote deficit within population; H_E, expected heterozygosity; H_O, observed heterozygosity; null allele frequency, estimated with GENEPOP using the expectation-maximization algorithm.

GENEPOP was 0.36 for Natnat01 and 0.36 for Natnat08, and ranged from 0.01 (Natnat11) to 0.16 (μNt7) for the remaining loci (Table 1). Consequently, the loci Natnat01 and Natnat08 were excluded from further analyses, and observed (H_O) and expected (H_E) heterozygosity, as well as heterozygote deficit (F_{IS}), were calculated for the remaining seven loci (Table 1). No linkage disequilibrium was detected.

Observed and expected heterozygosity varied between 0.23 (μNt3) and 0.66 (Natnat06), and between 0.27 (μNt3) and 0.66 (Natnat06), respectively (Table 1). Mean values calculated over all seven loci were 0.47 (H_O) and 0.52 (H_E) (Table 1). Heterozygote deficit (F_{IS}) ranged from –0.002 (Natnat06) to +0.165 (μNt7), with a mean of +0.086 (Table 1).

Genetic structure

STRUCTURE revealed that all individuals of *N. natrix* sampled in an area of 90 km^2 belong to a single population, as estimates of the logarithm of the probability of the data [ln Pr($X|K$)] were maximal for $K = 1$ (Fig. 2). The program GENELAND showed a range of predicted populations (K) from 1 to 6 in 100 independent runs. The majority of runs (60) revealed $K = 1$, whereas the remaining runs showed $K = 2$ (23), $K = 3$ (11), $K = 4$ (3), $K = 5$ (2), and $K = 6$ (1). Both approaches showed that the grass snakes in the study area belong to a single population.

The spatial autocorrelation between Euclidian distance and relatedness (measured as Moran's index) was significant for the distance group 0–1000 m (mean distance = 438.4 m, $P < 0.05$, see Fig. 3), but not for larger distance groups. This indicates that some individuals are related to each other within a distance of 1000 m. Distance along connecting water bodies did not explain relatedness better than Euclidian distance.

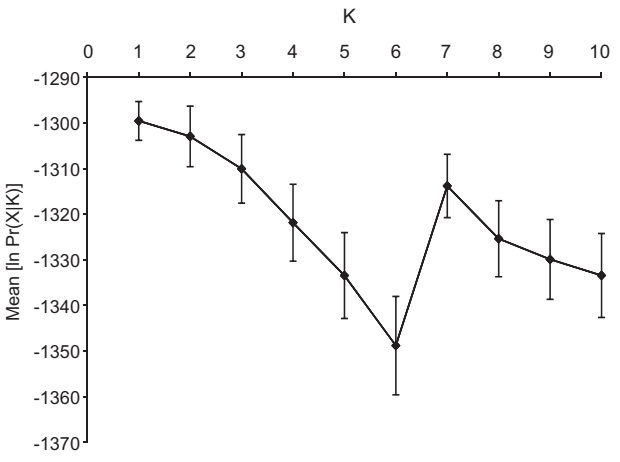

Figure 2. Mean estimates of the logarithm of the probability of the data, mean [ln Pr($X|K$)], and standard deviation for ten independent runs for each K revealed by STRUCTURE, with K ranging from 1 to 10.

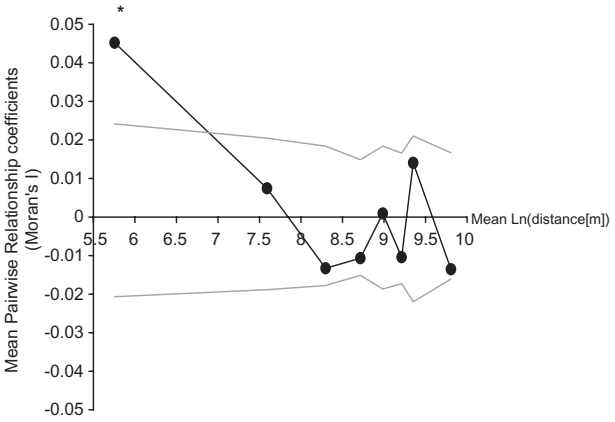

Figure 3. Spatial autocorrelogram of a *Natrix natrix* population based on Euclidian distances. Grey lines indicate 95% confidence intervals determined by SPAGeDi. *Significant value $P < 0.05$.

DISCUSSION

The present study did not reveal any genetically distinct grass snake population in the study area covering 90 km^2. This implies that there is a considerable exchange of individuals between small remnants of pristine habitat used by the grass snakes, thereby preventing any genetic differentiation. The lack of genetic structure is obviously not related to a limited variability in the microsatellite markers, because up to 12 different alleles were observed (mean allele number = 6; Table 1). Furthermore, the large size of the study area (90 km^2) related to the home range of the females (mean, 39.7 ha: see Wisler *et al*., 2008) cannot explain the absence of identifiable genetic clusters. The genetic homogeneity is a surprising result, and can be explained by the high connectivity of the matrix surrounding the habitat patches. Interspersed with drainage canals, our study area is characterized by monocultures of cereals and root crops, with pesticide and fertilizer input, repeated disturbance by agricultural machinery, and severe structural modifications within a single season. Our results suggest that the permeability of any habitat types within the study area is not limiting *N. natrix* dispersal. In fact, all female grass snakes used monocultures in the course of the tracking period (Wisler *et al*., 2008). It is suggested that because of a combination of suitable basking sites, favourable foraging opportunities, and low pressure from avian predators, monocultures may provide at least temporary advantages over more natural

habitats, with a seasonal shift in functional relevance induced by the time of oviposition. In contrast, agricultural land use negatively affected reptile biodiversity in Spain, including *N. natrix* (Ribeiro *et al*., 2009). Compared with the Spanish study, our study area may be more heterogeneous and consist of smaller arable fields, therefore enabling snakes to disperse through the landscape.

Female grass snakes had substantially larger home ranges in our study area (40 ha; Wisler *et al*., 2008) than gravid females in a Swedish population (25 ha 'total home range'; Madsen, 1984), or snakes in England (0.18–9.41 ha; Reading & Jofré, 2009). Whereas arable land was the dominant habitat type (accounting for at least 50%) in the Swiss and the Swedish study, the English study area consisted of deciduous woodland and pastoral fields. Snakes in our study area may be confronted with a lower density of prey and oviposition sites. Less productive habitats are thought to lead to larger home ranges, as snakes have to travel greater distances to cover the same area of suitable habitat (Stickel & Cope, 1947). In the study of Wisler *et al*. (2008), the radio-tracked individuals inhabited the embankment of a drainage canal, which could lead to long-distance movements.

The present study suggests that the connectivity of the matrix has to be considered when the genetic structure of snake populations in remnant habitat patches is examined. Furthermore, distance alone is an insufficient predictor for levels of gene flow between suitable habitat patches. For example, urban development interrupted gene flow between black rat snake (*Elaphe obsoleta* Say, 1823) hibernacula separated by only 1.6 km (Prior, Gibbs & Weatherhead, 1997), and movements of timber rattlesnakes were influenced by the location of basking sites (Bushar, Reinert & Gelbert, 1998; Clark *et al*., 2008). No genetic population structure was detected in small-eyed snakes (*Rhinoplocephalus nigrescens* Günther, 1862) within a distance of 16 km (Keogh, Webb & Shine, 2007), and significant levels of gene flow were found between timber rattlesnake hibernacula separated by 2–8 km (Clark *et al*., 2008). Both studies were conducted in undisturbed natural habitat, and the study sites were therefore highly connected. In contrast, two garter snake species (*Thamnophis elegans* Baird and Girard, 1853 and *Thamnophis sirtalis* Linnaeus, 1758) exhibited low but significant population differentiation and asymmetric gene flow between water bodies varying in degree of isolation (Manier & Arnold, 2005). Black rat snakes showed significant differentiation between sites separated by 15–50 km (Prior *et al*., 1997; Lougheed *et al*., 1999). On the other hand, populations of both massasauga rattlesnake and northern water snake exhibit microgeographic genetic structure, even in connected habitats (Gibbs *et al*., 1997; Prosser *et al*., 1999). This may be the result of limited dispersal, e.g. northern water snakes have a mean home range of 1–4 ha, indicating a very sedentary lifestyle (Roe, Kingsbury & Herbert, 2004; Pattishall & Cundall, 2008). This outlines the effect of the behaviour of a species on genetic structure. Even in a connected habitat, a species may exhibit genetic differentiation as a result of a sedentary lifestyle and restriction to a certain habitat type. In contrast to water snakes, however, the grass snake is much more agile and less restricted to water.

Our study also showed that grass snakes sampled within a distance of 1000 m are more closely related than individuals separated by larger distances. Grass snakes have been shown to use the same home range during successive years (Madsen, 1984; but see Reading & Jofré, 2009), and females have been reported to use the same (communal) oviposition sites (Wisler *et al*., 2008), even during successive years (Kabisch, 1999). Furthermore, the distance of 1000 m is similar to the maximal distance travelled by a female in the same study area (Wisler *et al*., 2008). Therefore, it is likely to find siblings and half-siblings close to each other. The movement activity of females is most extensive during the oviposition period from late June to July. During that period, females move approximately 114 m a day (Madsen, 1984), or 17 m (June) to 35 m (July) per hour, during their long-distance movements to reach the oviposition sites (up to 500 m; Wisler *et al*., 2008). After that period, females are more sedentary in August, moving 3 m per hour (Wisler *et al*., 2008). In contrast, males cover the largest distances during the mating season, with daily movements of 55 m, whereas males are more sedentary after that period (daily movements of 13 m; Madsen, 1984). Based on telemetry and capture–mark–recapture data the distance of 1000 m seems to be the upper limit of directly observable movement of adult snakes. The present study demonstrates, however, that there is sufficient gene flow between patches to avoid local differentiation, even when the snakes do not move longer distances.

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CHAPTER 3

Grass snake population differentiation over different geographic scales

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GRASS SNAKE POPULATION DIFFERENTIATION OVER DIFFERENT GEOGRAPHIC SCALES

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ABSTRACT: The loss and fragmentation of pristine habitat restrict specialized species to remnants of original habitat patches in a less suitable landscape. This may lead to a genetic differentiation of the subpopulations and to a decline in biodiversity. We used seven microsatellite markers to examine the genetic population structure of the Grass Snake, *Natrix natrix*, sampled in remnants of pristine habitat in a former wetland in the Swiss lowlands and in a rural valley in the Alps. On a regional level, the population structures of *N. natrix* in these two areas were compared with that of Grass Snakes living in an intensively used agricultural area. The three study areas were 30–100 km apart, but were interconnected by the river Aare. At the local scale, no genetic differentiation was found in either of the *N. natrix* populations inhabiting the rural alpine valley or the intensively used agricultural area. However, two subpopulations in the former wetland area were genetically differentiated with a low but significant measure of genetic differentiation between subpopulations, F_{ST}. This slight genetic differentiation can be explained by isolation by distance. At the regional scale, we found significant genetic differentiation between *N. natrix* populations inhabiting areas separated by 30–100 km. The genetic structure was highly related to isolation by distance with 85% of the among-populations genetic variance explained by the geographical distance between subpopulations. Euclidean distance explained genetic differentiation of Grass Snake populations better than the distance following watercourses. Our findings indicate regular gene flow between *N. natrix* subpopulations and show that this species also moves across intensively used terrestrial habitat. The genetic structure of Grass Snakes is mainly affected by geographic distance, while human activity and habitat alteration do not seem to reduce the snakes' movements. Our results suggest that conservation actions in landscapes altered by humans should focus on the maintenance of a habitat mosaic with anuran breeding ponds and adequate oviposition sites.

Key words: Grass Snake; Isolation by distance; Microsatellite DNA; *Natrix natrix*; Population structure

HUMAN activities result in the loss and fragmentation of pristine habitat (Foley et al., 2005). Habitat fragmentation reduces the suitable area for organisms, leads to the isolation and size reduction of remnant populations, and increases the risk of local extinction (Saccheri et al., 1998). There is growing evidence that relictual populations are subject to significant genetic and demographic changes, which may directly (e.g., via inbreeding depression or allele erosion) or indirectly (e.g., via reduced evolutionary potential) affect individual fitness and population viability (Young and Clarke, 2000; Rusterholz and Baur, 2010). Maintaining gene flow is therefore crucial for the long-term viability of populations. In animals, dispersing individuals may introduce novel alleles into a population. The dispersal rate depends on the population size, extent of resource competition, habitat quality, and size and isolation of suitable habitat patches, as well as the species' behavior (Bennett, 2003).

A central topic of landscape genetics is examination of how different landscape features influence gene flow (Manel et al., 2003). Empirical evidence from a variety of animal taxa indicates that human infrastructure, including highways, roads, and railway lines, reduces landscape permeability (Trombulak and Frissell, 2000; Clark et al., 2010; Holderegger and Di Giulio, 2010; but see Brown et al., 2006). However, even in the absence of barriers, cumulative effects of different landscape features with low permeability may lead to genetic differentiation of populations. Landscape features such as ridges, rivers, and open shrub habitat influence dispersal in amphibians (Funk et al., 2005; Spear et al., 2005; Giordano et al., 2007). Dispersal in reptiles is affected by habitat discontinuities (Stow et al., 2001). As a consequence, the genetic population structure of reptile species is influenced by the size and configuration of suitable habitat patches and the permeability

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of the surrounding landscape (DiLeo et al., 2010). For example, a fine-scale genetic population structure (<7 km) has been reported in Eastern Massasauga Rattlesnakes (*Sistrurus catenatus catenatus*; Chiucchi and Gibbs, 2010), in Adders (*Vipera berus*; Ursenbacher et al., 2009), and in Eastern Foxsnakes (*Pantherophis gloydi*, formerly *Elaphe gloydi;* DiLeo et al., 2010). In the Eastern Ratsnake (*Pantherophis alleghaniensis*, formerly the Black Ratsnake, *Elaphe obsoleta obsoleta*), no genetic differentiation was found at a fine scale (≤6 km), a limited differentiation was found at the local scale (15–50 km), and a high differentiation was present at a larger scale (465–1900 km; Lougheed et al., 1999). However, local populations of the Asp Viper (*Vipera aspis*) exhibited a low genetic differentiation (measured with random amplification of polymorphic DNA markers) at the regional scale (120 × 45 km; Jäggi et al., 2000).

These findings show that dispersal in snakes seems to be species-specific and partly depending on characteristics of the landscape in which they live. Although our knowledge of the conservation genetics of reptiles has increased during the last decade (King, 2009), it is still lacking behind of other taxonomic groups (Jehle, 2010). Here we examine the genetic population structure of the Grass Snake, *Natrix natrix*, at two spatial scales in agricultural areas in Switzerland. A previous study revealed a lack of genetic population structure in *N. natrix* in an intensively used agricultural area at a local scale of <14 km (Meister et al., 2010). This first result raises the question of the dispersal ability of the species at a larger scale. We compared the genetic population structure of Grass Snakes living in remnants of a former wetland located in the intensively cultivated Swiss lowlands and in a rural, scarcely populated valley in the Alps with that of the intensively used agricultural area reported in Meister et al. (2010). All three study areas are along and consequently interconnected by the river Aare and its riparian natural vegetation over a distance of 100 km. Considering this larger regional scale, this study compared the findings of an individual-based Bayesian clustering approach with the results of *F* statistics based on predefined populations. Finally, because the Grass Snake is a wetland-associated species, we tested whether watercourse distance better explains isolation by distance than Euclidean (straight-line) distance.

Materials and Methods

Study Organism

The Grass Snake (*Natrix natrix*) has a wide distribution (northern Africa and Eurasia; Kabisch, 1999). Despite its wide potential distribution in Switzerland, the area actually occupied by the Grass Snake is relatively restricted (Monney and Meyer, 2005). Two subspecies exist in Switzerland: *Natrix natrix natrix* occurs in the northeastern part of the country and *Natrix natrix helvetica* occurs in the remaining regions. Both subspecies are declining, and are therefore registered on the red list of Switzerland: *N. n. natrix* as "endangered" and *N. n. helvetica* as "vulnerable" (Monney and Meyer, 2005). This study focuses on *N. n. helvetica* for which a decline in abundance of over 30% has been recorded during the past century (Monney and Meyer, 2005). The decline is paralleled by pronounced changes in land use and a decrease in amphibian populations, the primary food of *N. natrix* (Reading and Davies, 1996; Gregory and Isaac, 2004). Indeed, the Grass Snake is associated with habitats that support high densities of amphibians, in particular wetlands. Based on telemetry data, the home range size of adult females varied from 15.1 to 102.5 ha (mean 39.7 ha) in an intensively used agricultural area in Switzerland (Wisler et al., 2008) where some females moved a distance of 500 m to reach oviposition sites.

Study Areas

The study area Gadmental (10 km^2; 46°43′N, 8°19′E; datum = WGS84) is a rural, scarcely populated valley located in the Bernese Alps (620–1300 m above sea level [asl]; Fig. 1a). Traditional agriculture with pasture farming over many centuries led to a small-scaled, highly structured landscape with numerous habitats suitable to reptiles (dry stone walls, clearance cairns, woodpiles, clipping piles, hedges, clearings). Since the 1960s, however, remote pastures and meadows have been abandoned resulting in an increase of

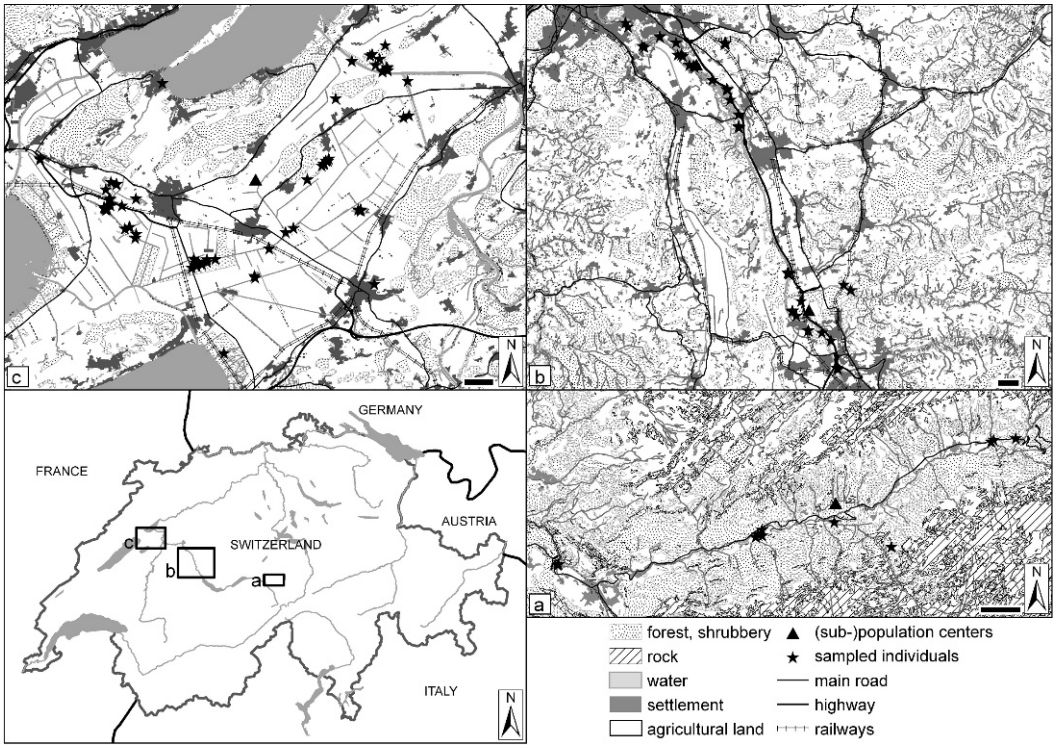

FIG. 1.—Location in Switzerland and overview of the study areas: (a) Gadmental, (b) Aaretal, (c) Grosses Moos. The black bars in a, b, and c indicate 1 km.

bushes and forest cover. At the same time, the use of agricultural areas at the bottom of the valley has intensified (Meyer, 2004).

The study area Aaretal (50 km^2) is located between the cities of Thun (560 m asl; 46°45′N 7°37′E) and Bern (520 m asl) in the Swiss lowlands (Fig. 1b). In past centuries, the widely branching, slowly flowing river Aare frequently inundated the area, which led to severe crop failures. Between 1824 and 1859 and between 1871 and 1892 the river was transformed into a fast-flowing canal fixed with dams (Zurschmiede, 2007). Nowadays, only small portions of the original floodplain remain. Settlements with intensively used farmland interspersed with scattered forest and a dense network of roads and a highway (constructed in 1971) dominate the study area. A large riparian forest belonging to a nature reserve borders the river Aare.

The study area Grosses Moos is an intensively used agricultural landscape of approximately 90 km^2 in the western part of the Swiss lowlands, located between the lakes Murten, Neuenburg, and Biel (46°59′N 7°08′E; Fig. 1c). As a result of deforestation in the Middle Ages, the meandering river Aare frequently inundated the area, which led to an extensive wetland (Nast, 2006). Between 1868 and 1891 an extensive drainage system was built converting the wetland into arable land. The main river system (Aare) with numerous branches, slow-flowing meanders, and sand and gravel flats was transformed into a fast-flowing canal that was redirected to Lake Biel (Fig. 1c). Pristine habitats are nowadays only represented in small remnants with a scattered distribution, surrounded by intensively used farmland and settlements interspersed with drainage canals. Wetland habitats cover approximately 1% of the study area. In the present study, we included data from this area (Meister et al., 2010) to compare genetic distances in *N. natrix* over a geographic distance of 100 km.

Sampling

Samples of *N. n. helvetica* were collected between April and September in 2006–2008.

Snakes were hand-captured by walking along edges of the habitats and by sampling cover boards (corrugated sheets measuring 50 cm × 50 cm or 50 cm × 100 cm) laid out to attract snakes (Fitch, 1992). In the study area Gadmental, 18 cover boards were established in a forest, along a river, and along a pond. Three additional cover boards were placed around an artificial oviposition site. In the study area Aaretal, the forest along the river Aare was sampled using 30 transects each consisting of 10 cover boards. Starting points of transects were chosen at random and cover boards were placed 10 m apart from each other. In addition, the banks of the two tributaries, Kiese and Rotache, were sampled using three transects along each tributary, with each transect consisting of 3–5 cover boards. Furthermore, the nature reserve Rüfenachtmoos (ca. 3 ha), consisting of an artificial pond, moats, a marsh area, and both humid and dry, nutrient-poor grasslands, was sampled using 20 cover boards. For the study area Grosses Moos, we used samples and data analyzed in Meister et al. (2010). The inaccessibility of the crops prevented a random sampling of the study area. In a preliminary study, set-asides, riparian zones, embankments, wetland remnants, and forest edges were sampled in 31 cells measuring 1 km^2 (U. Hofer, personal observation). Sampled cells were arranged corresponding to the black squares of a checkerboard. Sample locations with proof of *N. natrix* were further investigated. In the remaining parts of the study area, we searched for *N. natrix* in the same habitat types. The most distant sample sites within the triangle-shaped study area are separated by approximately 13.6 km following Euclidean distance.

Samples were obtained by clipping ventral scales. Snakes were sexed, aged (subadult/adult) based on their size, and photographed; pictures of the ventral side of the head and fore body (this part shows unique markings in this species; Carlström and Edelstam, 1946) were taken for individual identification. Shed skins and dead snakes were also collected. Snake tissue was preserved in 80% ethanol and stored at 5°C until genetic analysis. Exact coordinates of each snake captured were determined using a global positioning system device (Garmin GPS 12 Personal Navigator™).

Genomic DNA was extracted using a modified cetyltrimethyl ammonium bromide–based extraction protocol (Doyle and Doyle, 1987), and genotyped using a set of seven microsatellites (Natnat05, Natnat06, Natnat09, Natnat11, µNt3, µNt7 and µNt8new; Meister et al., 2009). Polymerase chain reaction amplification was performed in 25 µL reaction volume containing 25–50 ng of genomic DNA, 0.3 µM of each primer, and 1 U of peqGOLD Taq-DNA-Polymerase (Axon Lab). The thermo-treatment on an Eppendorf Mastercycler® Gradient (Vaudaux-Eppendorf AG) consisted of 2 min at 95°C, followed by 35 cycles at 95°C (30 s), the locus-specific annealing temperature (30 s; see Meister et al., 2009), and 72°C (30 s). The last cycle was followed by 1 min at the annealing temperature and a 30-min extension at 72°C. Amplified products were loaded on Spreadex® EL-400 or EL-600 gels (Elchrom Scientific AG) and electrophoresis was performed with an SEA 2000 advanced submerged-gel electrophoresis equipment (Elchrom Scientific AG). The analysis was performed with the same method as described in Meister et al. (2010), except for the use of another variety of Taq-DNA-polymerase. Some samples from the previous work were reanalyzed to check for result similarity.

Data Analysis

We evaluated population subdivision for all snakes in all three study areas and for each study area separately by using a Bayesian clustering approach (STRUCTURE, version 2.3.3; Pritchard et al., 2000). The program was run with a burn-in period of 50,000 repetitions and 100,000 iterations (Markov chain Monte Carlo). We tested a number of clusters (K) between 1 and 8, with 10 independent runs for each K.

We tested for departures from Hardy-Weinberg equilibrium (HWE) expectations for each marker in each subpopulation using GENEPOP (http://genepop.curtin.edu.au; Raymond and Rousset, 1995), and applied Bonferroni corrections for *P* values in multiple tests. Linkage disequilibrium probabilities for each marker were calculated using FSTAT 2.9.3.2 (Goudet, 1995). The occurrence of null

alleles, stuttering signals or large allelic dropout was examined using MICRO-CHECKER (version 2.2.3, Van Oosterhout et al., 2004). We calculated observed and expected heterozygosity (H_O, H_E) and allelic richness (A_R, rarefied to 15 individuals) per locus in every subpopulation using FSTAT and differences of these genetic diversity measurements between subpopulations were tested with analyses of variance (ANOVAs) using PASW statistics 18.0 core system (SPSS Inc., Chicago, Illinois, USA.)

Genetic differentiation between subpopulations (F_{ST}) was calculated with FSTAT and *P* values for pair-wise tests of differentiation were obtained after 6000 permutations. The nominal level for multiple tests was set to 0.001. The geographic distance between subpopulation centers was measured as Euclidean distance and as watercourse distance, measured with the path function implemented in Google Earth 5 (earth.google.com). Isolation by distance was calculated using a Mantel test (Mantel, 1967), implemented in the program FSTAT. We compared pair-wise ($F_{ST}/[1 - F_{ST}]$) values with pair-wise Euclidean distances and watercourse distances (ln transformed).

On the individual level, pair-wise spatial and genetic distances between individuals (genetic distance *a*; Rousset, 2000) were calculated with SPAGeDi (version 1.2; Hardy and Vekemans, 2002). We tested for isolation by distance using a Mantel test. Additionally, spatial autocorrelation analysis was performed using SPAGeDi. We tested for correlations between geographic distances (Euclidean distances) and pair-wise relationship coefficients. Distance intervals were chosen in order to have similar numbers of pair-wise comparisons in each interval. Ninety-five percent confidence intervals were calculated with 10,000 permutations. Input files were prepared using CONVERT (version 1.31; Glaubitz, 2004).

Results

We sampled a total of 172 individuals (19 individuals in Gadmen, 62 in Aaretal [19 in Thun, 43 in Bern], and 91 in Grosses Moos; Table 1). Considering data of all snakes sampled, the STRUCTURE analysis showed the occurrence of three clusters, as the highest likelihood was observed for K = 3 (Fig. 2). The three clusters corresponded to the three geographic areas (Gadmental, Aaretal, Grosses Moos) examined. Subsequent STRUCTURE analyses with each area did not reveal any further substructure. This suggests that the snakes inhabiting a study area belong to the same population.

In the Gadmental and Grosses Moos populations, no significant deviations from the HWE were found at any locus. In the Aaretal population, however, significant deviations from HWE were found after Bonferroni corrections for the loci Natnat06, Natnat09, and Nt8new. MICRO-CHECKER suggested the presence of null alleles at the locus Natnat06, but not at the other two loci. Deviations from HWE could also be the result of heterogeneity in genetic structure. Indeed, in the Aaretal area, snakes were sampled in two different subareas separated by 7.8 km and no individuals were found in between (Figs. 1b and 3). When data from the two subareas were analyzed separately, a significant deviation from HWE was only found for locus Natnat09, which was not a result of null alleles. This suggests that two subpopulations of *N. natrix* may occur in the Aaretal area: the Thun subpopulation and the Bern subpopulation (Fig. 3).

Table 1.—Genetic diversity in two populations and two subpopulations of Grass Snakes (*Natrix natrix*) and the sample size for each population and subpopulation. H_O, observed heterozygosity; H_E, expected heterozygosity; A_R, allelic richness; estimated with FSTAT 2.9.3.2 (Goudet, 1995) and *N*, sample size. Allelic richness is calculated for 15 individuals in each population and subpopulation. Standard errors are given for all diversity measures.

Study area	Population or subpopulation	H_O	H_E	A_R	N
Gadmental	Gadmen	0.48 ± 0.10	0.48 ± 0.08	3.64 ± 0.51	19
Aaretal	Thun	0.57 ± 0.07	0.55 ± 0.04	3.56 ± 0.43	19
	Bern	0.57 ± 0.05	0.54 ± 0.03	3.67 ± 0.41	43
Grosses Moos	Grosses Moos	0.47 ± 0.06	0.52 ± 0.06	4.31 ± 0.61	91

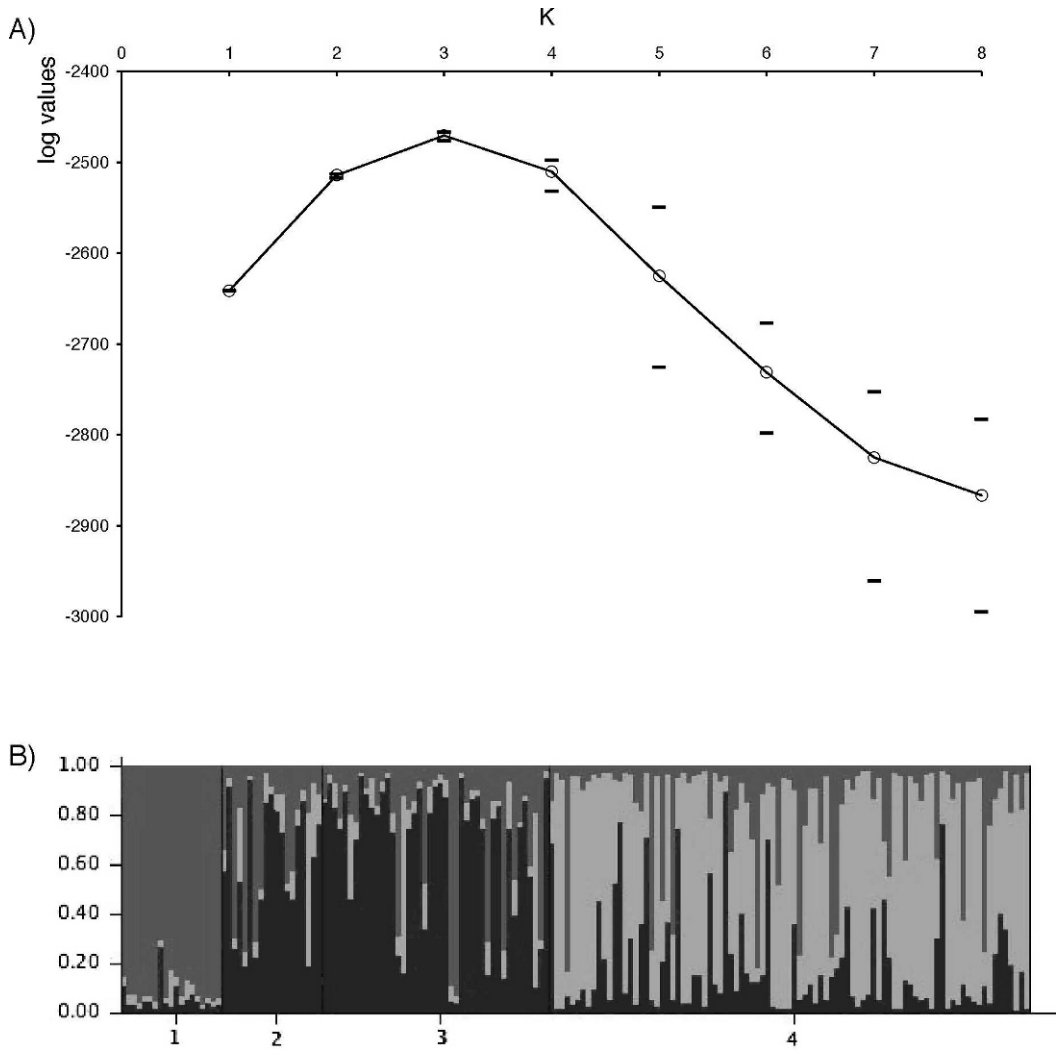

FIG. 2.—(A) Mean, maximum, and minimum likelihood values of 10 simulations conducted with STRUCTURE (Pritchard et al., 2000) for all individuals of Grass Snakes (*Natrix natrix*), and (B) individual assignment to three clusters corresponding to the four study areas (1: Gadmen; 2: Thun; 3: Bern; 4: Grosses Moos) for the simulation with the highest likelihood value (K = 3).

F_{ST} values were calculated for the two populations, Gadmental and Grosses Moos, and for the two subpopulations, Thun and Bern, based on data from either all seven microsatellites or from only six microsatellites (Natnat09 excluded). The results of both analyses were similar and therefore only the results based on seven microsatellites were presented. A low but significant F_{ST} (0.012; Table 2) was found between the two subpopulations Thun and Bern, confirming our previous finding. Consequently, all further analyses were conducted with four units, the two populations Gadmental and Grosses Moos and the two subpopulations Thun (Aaretal) and Bern (Aaretal; Fig. 3). The most distant study area, Gadmental, which is located in the Alps, showed the highest F_{ST} values (Fig. 3; Table 2). The genetic differentiations between Gadmental and the two subpopulations in the Aaretal or the population in the Grosses Moos are similar (F_{ST} between 0.147 and 0.158). The F_{ST} values between the two subpopulations in the Aaretal and the Grosses Moos are approximately half of the F_{ST}

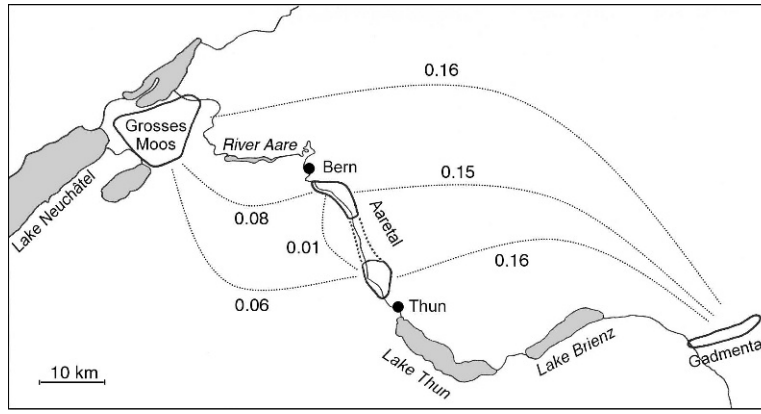

FIG. 3.—Schematic overview of the Grass Snake (*Natrix natrix*) populations of Gadmental, Aaretal (with the two subpopulations Thun and Bern), and Grosses Moos with the corresponding F_{ST} values. The study areas are interconnected by the river Aare.

values (0.062 and 0.078; Table 2) of the remaining population comparisons.

No linkage disequilibrium was found in any of the populations and subpopulations examined. Observed heterozygosity (H_O) ranged from 0.47 to 0.57, expected heterozygosity (H_E) from 0.48 to 0.55, and allelic richness (A_R) from 3.56 to 4.31 (Table 1). No significant difference was found between the populations and subpopulations (ANOVA: H_O: $F_{3,24} = 0.55$, $P = 0.65$; H_E: $F_{3,24} = 0.30$, $P = 0.82$; A_R: $F_{3,24} = 0.48$, $P = 0.70$).

Significant isolation by distance was recorded between populations and subpopulations (Fig. 4). Euclidean distance ($r^2 = 0.85$, $P < 0.02$; Fig. 4) explained genetic differentiation slightly better than watercourse distance ($r^2 = 0.62$, $P < 0.03$). At the individual level, a highly significant isolation by distance was found between the pair-wise genetic distance *a* (Rousset, 2000) and the natural logarithm of the Euclidean distance ($r^2 = 0.05$, $P < 0.001$). Spatial autocorrelation revealed a strong relationship between geographical distance and relatedness measured as Moran's Indices (Fig. 5). Individuals separated by less than 16 km are more related to each other than expected by random sampling (Fig. 5). This distance encompasses the maximum size of the study areas: snakes were sampled within a distance of 12.5 km in the study area Gadmental, within 5.6 km in the subpopulation Thun/Aaretal, within 7.5 km in the subpopulation Bern/Aaretal, and within 13.6 km in the study area Grosses Moos.

DISCUSSION

The present study revealed significant genetic differentiation between *N. natrix* populations separated by 30–100 km in a landscape altered by humans. The genetic structure was highly related to isolation by distance with 85% of the among-population genetic variance explained only by the geographical distance between population units. These results are consistent with the genetic population structure in other natricine snakes. For instance, individuals of two species of garter snakes (*Thamnophis elegans* and *Thamnophis sirtalis*) exhibited low but significant population differentiation over a distance of 50 km (Manier and Arnold, 2005) and populations of Saltmarsh Watersnakes (*Nerodia clarkii compressicauda*) were fragmented into isolated neighborhoods measuring 50–80 km (Jansen et al., 2008). On the other hand, DiLeo et al. (2010) found no genetic structure in Eastern Gartersnakes (*Thamnophis sirtalis sirtalis*) over a distance of 100 km. Isolation by distance, sometimes measured as a cost-based distance including potential basking sites between hibernacula, has also been recorded in other snake species (Bushar et al., 1998; Manier and Arnold, 2005; Ridenhour et al., 2007; Clark et al., 2008; Jansen et al., 2008). The pattern of isolation by distance observed at the population level was

TABLE 2.—Pair-wise F_{ST} estimates (upper diagonal) based on seven microsatellite loci in two populations and two subpopulations of the Grass Snake (*Natrix natrix*), and Euclidean distances (in km, lower diagonal) between population and subpopulation centers.

Study area	Population or subpopulation	Gadmen	Thun	Bern	Grosses Moos
Gadmental	Gadmen		0.156**	0.146**	0.158**
Aaretal	Thun	56.2		0.012*	0.061**
	Bern	64.7	13.7		0.078**
Grosses Moos	Grosses Moos	94.2	40.8	29.6	

* $P < 0.05$, ** $P < 0.01$. Significance was determined by 6000 permutations.

confirmed by the analyses conducted at the individual level. The spatial autocorrelation indicated that snakes within 16 km are more related to each other than expected by random sampling. In an agricultural landscape covering less than this distance (<14 km), snakes captured less than 1 km apart were more related to each other than expected, this observation being explained by the use of the same home ranges and oviposition sites (Meister et al., 2010).

At the local scale, the significant deviations from HWE found in the two Aaretal subpopulations indicate genetic heterogeneity between them. This was confirmed by the small but significant F_{ST} between the two subpopulations. In contrast, the STRUCTURE analysis did not reveal any genetically structured population in the areas examined. This result is in accordance with the finding of Latch et al. (2006), who demonstrated that the threshold for detecting differentiation between clusters using the algorithms implemented in STRUCTURE is close to $F_{ST} = 0.03$. In our study, the F_{ST} between the Thun and Bern subpopulations was 0.012. Therefore, differentiation between clusters in our study was not detectable using STRUCTURE.

Distance effects can also explain the slight genetic differentiation between the Thun and Bern subpopulations in the Aaretal area. Based on the isolation by distance line shown in Fig. 4, a geographic distance of 13.7 km (the distance between the two subpopulation centers) results in a F_{ST} of 0.004 (observed F_{ST}: 0.012). However, this F_{ST} value also indicates limited but regular gene flow between the subpopulations, which may counteract the effects of genetic drift. Our lack of captures between the subpopulations may have resulted from the placement of cover boards in suboptimal habitat (because the placement was determined by the randomly chosen starting points of the transects). Furthermore, the low detectability of Grass Snakes (Kéry, 2002) may have prevented random observations when walking along edges of the habitat.

In our study, Euclidean distance explained genetic differentiation of Grass Snake popu-

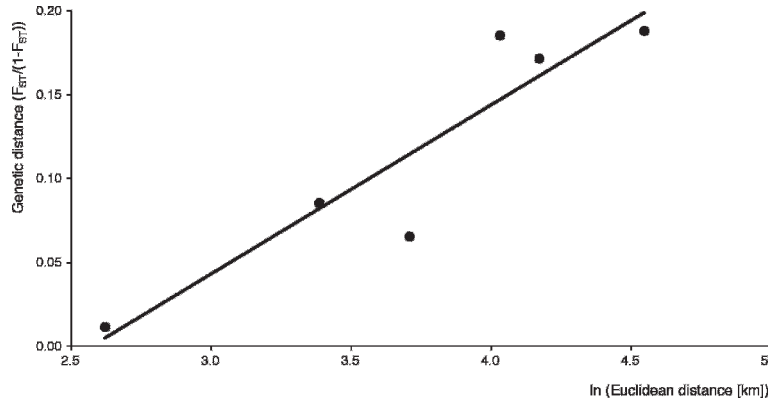

FIG. 4.—Isolation by distance in two populations and two subpopulations of Grass Snakes (*Natrix natrix*; $r^2 = 0.85$, $P < 0.02$). Comparisons are pair-wise between all populations and subpopulations.

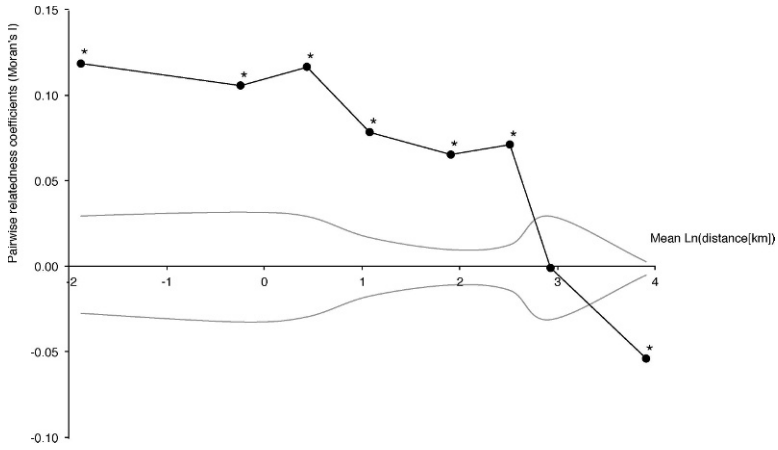

FIG. 5.—Spatial autocorrelogram of 172 samples of Grass Snakes (*Natrix natrix*) in Switzerland. Distances between individuals correspond to Euclidean distances. Grey lines indicate 95% confidence intervals determined by SPAGeDi. * indicates a significant value, $P < 0.001$. Note that the distance is ln-transformed.

lations slightly better than the distance following watercourses. Even if this species is mainly associated with ponds, rivers, and other aquatic habitats (Kabisch, 1999), our findings indicate that Grass Snakes do not obligatorily disperse along watercourses, but also cross terrestrial habitats as demonstrated by telemetry (Wisler et al., 2008; Reading and Jofré, 2009). Grass Snakes should therefore be regarded as generalists whose movements are not restricted to a certain habitat type.

Grass Snakes are relatively large-bodied snakes with a length of up to 120 cm (maximal total length 205 cm; Kabisch, 1999). For females, the moving activity is most extensive during the oviposition period, in which individuals move up to 114 m in a day (Madsen, 1984) or 500 m in total to reach the oviposition sites (Wisler et al., 2008). After that period, females are more sedentary (Wisler et al., 2008). In contrast, males cover the largest distances (up to 55 m per day) during the mating season and are more sedentary afterwards (daily movements of 13 m; Madsen, 1984). Females had slightly larger home ranges in agricultural areas in Switzerland (39.7 ha; Wisler et al., 2008) than in Sweden (25 ha "total home range"; Madsen, 1984), or in England (0.18–9.41 ha; Reading and Jofré, 2009). This discrepancy could perhaps influence the genetic structure and result in more structured (higher F_{ST} values) populations. In general, Grass Snakes might be more mobile than other species of watersnakes (Natricinae) of similar size. Populations of the Northern Watersnake (*Nerodia sipedon sipedon*), for instance, exhibit microgeographic genetic structure, even in connected habitats (Prosser et al., 1999). The interspecific difference in genetic structure could be a result of differences in dispersal and/or feeding behavior. Northern Watersnakes are strongly bound to watercourses resulting in a mean home range of 1–4 ha (Roe et al., 2004; Pattishall and Cundall, 2008).

The very small number of recaptured individuals in our study highlights the fact that *N. natrix* is mobile or occurs in large numbers, which further explains the low genetic differentiation of the populations. The Dice Snake (*Natrix tessellata*), which is the more-aquatic sister species of the Grass Snake (Guicking et al., 2006), exhibited strong genetic differentiation, even between nearby localities, as a result of small population sizes, enhanced genetic drift, and successive bottlenecks during postglacial range expansion (Guicking et al., 2004).

Genetic diversity, measured as observed and expected heterozygosity and allelic richness, was similar in all three study areas. This finding contrasts with the reduced genetic diversity observed in the Adder (*Vipera berus*) in the Swiss Jura Mountains and Alps compared to French populations (Ursenbacher et al., 2009), where the reduction results

from the postglacial recolonization pattern. The Grass Snakes in our study areas belong to the western subspecies (*Natrix natrix helvetica*), which derived from glacial refuges from Iberia and Italy (Thorpe, 1984). We found a similar extent of genetic diversity in the lowland (Grosses Moos and Aaretal population) and in the Alps (Gadmental), even if the Alps were later colonized. This confirms our finding that a considerable amount of gene flow between Grass Snake populations prevents peripheral populations from loosing genetic diversity.

Our study showed that the genetic structure of Grass Snakes is mainly affected by distance. Interestingly, human activity and habitat alteration do not seem to confine this species and reduce its movements. However, this does not imply that there is no need for natural habitat, because the Grass Snake prefers habitat boundaries for basking sites and requires specific oviposition sites (Madsen, 1984; Wisler et al., 2008; Reading and Jofré, 2009). The conservation of this species should focus on the maintenance of a habitat mosaic with anuran breeding ponds and adequate oviposition sites. However, further fragmentation of Grass Snake habitat should be avoided.

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CHAPTER 4

Frequency of multiple paternity in the grass snake (*Natrix natrix*)

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Frequency of multiple paternity in the grass snake (*Natrix natrix*)

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Abstract. Males can enhance their reproductive success through mating with multiple females. For females, however, one mating is usually sufficient to inseminate all of their ova. Females may benefit from multiple mating by producing genetically more diverse offspring, and by having the opportunity to choose sperm of the genetically most compatible male. We used five microsatellite loci to investigate the occurrence and frequency of multiple paternity in 11 clutches of the grass snake (*Natrix natrix*) in Switzerland. Using a very conservative estimate (program GERUD), two or more fathers were found in 27% of the clutches. However, based on the maximum likelihood estimate (program COLONY), multiple paternity occurred in 91% of the clutches with 2-5 contributing males per female. This is the first investigation demonstrating multiple paternity in a European natricine, with a frequency similar to those found in new world natricines.

Keywords: COLONY, GERUD, microsatellite DNA, Natricinae, paternity analysis.

In mating systems without paternal care, males can enhance their reproductive success through mating with multiple females. In these species, mate acquisition is the limiting factor for male reproduction and strategies to overcome this limitation are numerous (Shine, 2003). For females, however, one copulation might be sufficient to inseminate all ova. Nevertheless, multiple mating by females has been reported in a variety of reptile species, indicating direct and indirect benefits for the females (Zeh and Zeh, 2001). Direct benefits arising from paternal contributions to egg production or parental care are unlikely to play an important role in most reptile species (Uller and Olsson, 2008). However, indirect benefits may arise to multiply mated females from increased genetic quality, higher complementarity, and/or enhanced genetic variation (bet hedging) of their offspring (Madsen et al., 2005; Uller and Olsson, 2008).

In squamates, multiple paternity is common, and occurs at high levels in natural populations (Uller and Olsson, 2008). Oviductal sperm storage has been reported in females for numerous reptilian taxa with varying durations (Birkhead and Møller, 1993; Sever and Hamlett, 2002).

To our knowledge, the occurrence and extent of multiple paternity have not yet been examined in the grass snake (*Natrix natrix*). In this study, we used five microsatellite loci to investigate the occurrence and frequency of multiple paternity in three natural populations of *N. natrix* in Switzerland.

The grass snake has a wide distribution (northern Africa and Eurasia; Kabisch, 1999). Despite its wide potential distribution in Switzerland, the area actually occupied is relatively restricted and a decline in abundance of over 30% has been recorded during the past century (Monney and Meyer, 2005). The species is therefore registered on the red list of Switzerland as "vulnerable" (Monney and Meyer, 2005). The decline is paralleled by pronounced changes in land use and a decrease in amphibian population sizes, the primary food of *N. natrix* (Reading and Davies, 1996; Gregory and Isaac, 2004). Mating occurs in spring (April-May) shortly after emergence from hibernation. Occasionally, grass snakes mate a second time in autumn (September-October; Kabisch, 1999). Many snake species that live in temperate regions and copulate in fall store sperm in tubules until ovulation in spring (Sever and Hamlett, 2002; Uller, Stuart-Fox and Olsson, 2010). Female grass snakes can store sperm up to 180 days (Birkhead and Møller, 1993). During copulation, male grass snakes release a white viscous fluid (Olsson and Madsen, 1998). One possible function of such fluids is the formation of a copulatory plug in the female cloaca that prevents further copulations or sperm transfer (Olsson and Madsen, 1998; Uller, Stuart-Fox and Olsson, 2010). Finally, oviposition takes place between July and August in Switzerland, and progeny hatch 7-9 weeks later (Kabisch, 1999).

In this study eleven pregnant females of *N. n. helvetica* were collected in June and July in the years 2006-2008

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Table 1. Number of fathers contributing to the clutches of *Natrix natrix* collected in three natural populations and the number of offspring sired by different fathers (indicated by different letters). The same letter in different mother-offspring arrays does not refer to the same father.

Mother[§]	Number of offspring analysed	Minimum number of fathers*	Total number of fathers[‡]	Total number of offspring sired by different fathers[‡]
GM 51	8	1	2	6a, 2b
GM 126	35	3	5	14a, 11b, 5c, 3d, 2e[†]
GM 240	12	1	1	12a
GM 244	13	2	2	11a, 2b[†]
A 117	16	1	3	8a, 6b, 2c
A 247	11	1	3	7a, 2b, 2c
G 72	7	1	3	4a, 2b, 1c
G 74	8	2	4	3a, 2b, 2c, 1d
G 76	6	1	2	5a, 1b
G 140	8	1	3	5a, 2b, 1c
G 255	17	1	4	8a, 5b, 3c, 1d
Mean	*12.8*	*1.4*	*2.9*	–

[§] GM: Grosses Moos, A: Aaretal, G: Gadmen.
[*] Parentage reconstruction using GERUD.
[‡] Likelihood assessment based on genetic diversity using COLONY.
[†] Different fathers sired different numbers of offspring.

in three different areas in Switzerland: an intensively used agricultural area called Grosses Moos, in the Aaretal, an agricultural area with remnants of wetland along the river Aare between the cities of Thun and Bern, and in Gadmental, a valley in the Alps. The three areas are situated 30-100 km apart from each other (for a detailed description see Meister, Ursenbacher and Baur, 2012). Snakes were hand-captured by walking along edges of the habitats and by controlling coverboards laid out to attract snakes (Fitch, 1992). Pregnant females were kept at the zoo "Dählhölzli" in Bern until oviposition and then released at their catching places. Clutches were incubated and neonates were kept in boxes until first ecdysis after which they were released at the catching place of their mother. DNA was obtained from cut scales and shed skins of juvenile snakes. Unhatched eggs were checked for undeveloped embryos, which were used for DNA extraction. If no embryo could be detected, the eggs were regarded as unfertilised. Tissue of female snakes was obtained by clipping ventral scales. Scale tissue was preserved in 80% EtOH and stored at 5°C until genetic analysis.

Genomic DNA was extracted using a modified cetyltrimethyl ammonium bromide-based extraction protocol (CTAB; Doyle and Doyle, 1987) and genotyped using a set of five microsatellite loci (Natnat05, Natnat06, Natnat09, Natnat11 and µNt8new; Meister et al., 2009). The genetic analysis was performed as described in Meister, Ursenbacher and Baur (2012). Microsatellite characteristics were assessed using GenAlEx, version 6.1 (Peakall and Smouse, 2006). Probabilities for departures from Hardy-Weinberg equilibrium expectations, linkage disequilibrium, and occurrence of null alleles, stuttering signals or large allelic dropout were previously examined for these populations in Meister, Ursenbacher and Baur (2012). The DNA of eight juveniles in four clutches did not amplify any loci. These individuals were excluded from the analyses. We genotyped a total of 141 offspring from 11 mothers (table 1). The proportions of offspring included in the paternity analyses averaged 80.2% (range: 50.0%-100.0%) of the total number of eggs produced (including eggs that were later regarded as unfertilised). Considering exclusively fertilised eggs, the proportions of offspring included in the paternity analyses averaged 93.2% (range: 72.7%-100.0%). Sibship analysis and parentage reconstruction of the mother snakes and their progeny were performed with GERUD, version 2.0 (Jones, 2005) and COLONY, version 2.0 (Wang, 2004). GERUD uses multiple-locus data for reconstruction of the contributing paternal genotype(s) from mother-offspring arrays. The software does not distinguish between potential fathers of the same genotype. Consequently, the number of paternal genotypes estimated is equal to a minimum number of involved fathers. The maximum likelihood software COLONY was used to assess the total number of fathers contributing their gametes to a progeny array. For the paternity assignment, the genotypes of the individuals sampled in the populations were used (Grosses Moos: 91 individuals, Aaretal: 62 individuals, Gadmental: 19 individuals; Meister et al., 2010; Meister, Ursenbacher and Baur, 2012). COLONY provides the most probable configuration of paternity including assignments of every offspring to one of the estimated paternal genotypes. We calculated Spearman rank correlation to test whether the number of fathers contributing to a clutch is correlated with the number of offspring analysed. Differences in levels of paternity between populations were investigated with a one-way ANOVA. These statistical analyses were conducted using PASW®

Table 2. Summary statistics for five microsatellite loci used in *Natrix natrix* populations.

Locus	N	N_C	Exclusion probability*
Natnat05	3	3	0.33-0.33
Natnat06	4	7	0.33-0.54
Natnat09	4	5	0.33-0.68
Natnat11	3	3	0.22-0.42
µNt8new	5	7	0.36-0.42

N: Number of alleles per locus for mother snakes; N_C: Number of alleles in clutches.
* Range for all three populations. Combined exclusion probability for all five loci ranged 0.86-0.98.

statistics 18.0 core system (SPSS Inc., 2009). We examined whether multiple fathers sire equal numbers of eggs in a given mother using contingency tests for the clutches exhibiting multiple paternity and with more than 10 offspring analysed (we considered that the statistical power in this particular test is too low in clutches with less than 10 offspring).

The polymorphism level of the five microsatellite loci ranged from three to five alleles in the mother snakes and from three to seven alleles in their progeny (table 2). Paternity exclusion probability for each microsatellite ranged from 0.22 to 0.68 (table 2) and from 0.86 to 0.98 for all microsatellite loci combined.

Occurrences of multiple paternity were detected in three of the eleven clutches using GERUD (table 1). This conservative analysis reveals the minimum number of contributing fathers, which ranged from 2 to 3 (table 1). Using COLONY, multiple paternity was found in ten of the eleven clutches. This analysis estimates the total number of fathers, which ranged from 2 to 5 per clutches (table 1). The number of fathers per clutch was not correlated with the number of offspring analysed (Spearman rank correlation, GERUD: $r_s = 0.38$, $N = 11$, $P = 0.25$; COLONY: $r_s = 0.39$, $N = 11$, $P = 0.24$), and the three populations considered did not differ in the level of multiple paternity (GERUD: $F_{2,8} = 1.12$, $P = 0.37$; COLONY: $F_{2,8} = 0.38$, $P = 0.70$).

Patterns of sperm utilisation were examined in the five clutches consisting of more than 10 offspring and showing multiple paternity (COLONY). In two of them, the distribution of paternity was non-random (contingency-test, in all cases, $P < 0.05$; table 1). In the remaining three clutches, no significant difference in the number of offspring sired by each father was found ($P > 0.05$).

Multiple paternity was found in at least 27% of the grass snake clutches (using GERUD), but most probably occurred in more than 90% of the clutches (using COLONY). Most studies on multiple paternity in natricines considered new world species: multiply sired litters have been reported in *Thamnophis sirtalis*, with 37.5%-75.0% of the litters exhibiting multiple paternity (Schwartz, McCracken and Burghardt, 1989; McCracken, Burghardt and Houts, 1999; Garner et al., 2002), and in *Nerodia sipedon*, in which 58.0%-85.7% of the litters were sired by more than one father (Barry, Weatherhead and Philipp, 1992; Prosser et al., 2002; Kissner, Weatherhead and Gibbs, 2005). In most natricine species investigated so far multiple paternity has been documented: *T. elegans*, *T. butleri*, *T. radix*, *T. sauritus*, *N. rhombifer*, *Regina septemvittata*, *Storeria occipitomaculata*, *S. dekayi* (Wusterbarth et al., 2010). However, this study is the first to demonstrate the occurrence and level of multiple paternity in a European natricine.

Interestingly, Madsen and Shine (1993) observed in a mating experiment with captive grass snakes that males abandoned the female after successful copulation and did not court a previously mated female. If a female mates only with one male per mating season (as observed by Madsen and Shine, 1993), the presence of multiply sired clutches, as found in this study, could be explained by mating in the previous mating season(s) or year(s), combined with long-term sperm storage. Given that grass snakes have been reported to store sperm for a maximum of six months (Rollinat, 1946; Birkhead and Møller, 1993), the presence of two sires could be explained by one autumn mating in the previous year and one spring mating in the current year, according to the obser-

vation of a single copulation per mating season (Madsen and Shine, 1993). Our study, however, demonstrated that more than two males sired eggs in the same clutch. Consequently, our findings demonstrate that males copulate with previously mated females in the wild. Otherwise the paternity by more than two males in a clutch could not be explained. Sexual unattractiveness of mated females due to a copulatory plug formed by gelatinous material has been reported in *Thamnophis* species (Ross and Crews, 1977; but see Shine, Olsson and Mason, 2000). In *T. sirtalis*, multiply mated females with two or more plugs have been found in the field (Shine, Olsson and Mason, 2000). Nevertheless, they produced multiple-sired clutches (see above). Obviously, mating plugs do not completely prevent further sperm transmission and female natricines seem to have developed strategies to overcome forced chastity, as found in Viperidae (Ursenbacher, Erny and Fumagalli, 2009).

In the present study, different fathers sired different numbers of offspring in two out of five clutches. This could be a result of different numbers of sperm delivered by different males, the ability of the female to choose among sperm from different males (cryptic female choice), or of "topping off", in which the female accepts a large amount of sperm from the first male, while sperm from further males are only kept in the sperm storage tubules if space allows. Once the tubules are filled, sperm from consecutive mates admix (Jones, Adams and Arnold, 2002). Our data do not allow to distinguish between these explanations.

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GENERAL DISCUSSION

The rapid growth of the human population enhances the pressure on natural habitats, as more surface is required for agriculture to supply sufficient food (Tilman et al., 2001). Human altered landscapes are expected to have a pronounced effect on species from higher trophic levels, like the grass snake (Holt et al., 1999). Therefore, the knowledge of how landscape features and anthropogenic barriers influence patterns of genetic structure and gene flow is crucial for the evaluation of the long-term viability and persistence of existing populations. In addition, the genetic aspect is more and more taken into account by decision makers in conservation biology. The aim of this thesis was to investigate the genetic population structure of the grass snake in three different areas in Switzerland. A further aspect was to examine the occurrence and frequency of multiple paternity in wild grass snake populations as a source of genetic diversity.

The development of grass snake microsatellite markers used for genetic analyses and their cross-amplification in the dice snake (*N. tessellata*), as well as the cross-amplification of dice snake microsatellite loci in the grass snake are described in **Chapter 1**. The developed microsatellite markers were variable allowing to investigate the genetic population structure of grass snakes in different landscapes and to assess the frequency of multiple paternity in these populations.

In the **Chapters 2** and **3**, the genetic structure of grass snake populations in human-altered landscapes was examined at the local the regional scale. No genetically distinct grass snake population was found in an intensively used agricultural landscape in the Swiss lowlands covering 90 km^2 and in a rural valley in the Bernese Alps covering 10 km^2. These results imply a considerable exchange of individuals between small remnants of pristine habitat used by the grass snakes, thereby preventing any genetic differentiation. The genetic homogeneity of populations indicates that the permeability of any habitat types within the study areas is not limiting the dispersal of *N. natrix*. In fact, all female grass snakes monitored in the landscape with intensive agriculture in the Swiss lowlands (the same area as in **Chapter 2**) used monocultures in the course of a telemetry study (Wisler et al., 2008). In contrast, agricultural land use negatively affected reptile biodiversity in Spain, including *N. natrix* (Ribeiro et al., 2009). Compared with the Spanish landscape investigated, the study areas reported here may be more heterogeneous and consist of smaller arable fields, interrupted by more favourable areas, therefore enabling snakes to disperse through the landscape. On the other hand, grass snakes in a former floodplain in the Swiss lowlands covering

50 km^2 exhibited weak but significant genetic differentiation. The slight genetic differentiation between the two subpopulations (the centres are separated by 13.7 km) can also be explained by distance effects. However, the low F_{ST}-value between the two subpopulations also indicates limited but regular gene flow, even if no snake was captured between the two subpopulations. In the present study, the starting points of the sampling transects were chosen randomly. Consequently, it is possible that the coverboards were placed in suboptimal habitat. Furthermore, the low detectability of grass snakes (Kéry, 2002) may have prevented random observations when walking along edges of the habitat.

The connectivity of the matrix has an important impact on the genetic structure of snake populations in remnant habitat patches. Furthermore, distance alone is mostly an insufficient predictor for levels of gene flow between suitable habitat patches. For example, urban development interrupted gene flow between black rat snake (*Elaphe obsoleta*, now *Pantherophis obsoletus*) hibernacula separated by only 1.6 km (Prior et al., 1997), and movements of timber rattlesnakes (*Crotalus horridus*) were influenced by the location of basking sites (Bushar et al., 1998; Clark et al., 2008). In undisturbed natural habitat with highly connected study sites, no genetic population structure was detected in small-eyed snakes (*Rhinoplocephalus nigrescens*) within a distance of 16 km (Keogh et al., 2007) and significant levels of gene flow were found between timber rattlesnake hibernacula separated by 2–8 km (Clark et al., 2008). On the other hand, populations of both massasauga rattlesnake (*Sistrurus catenatus catenatus*) and northern water snake (*Nerodia sipedon sipedon*) exhibit micro-geographic genetic structure, even in connected habitats (Gibbs et al., 1997; Prosser et al., 1999). This may be the result of limited dispersal, e.g. northern water snakes have a mean home range of 1–4 ha, indicating a very sedentary lifestyle (Roe et al., 2004; Pattishall and Cundall, 2008). This outlines the effect of a species' behaviour on the genetic structure. Even in a connected habitat, a species may exhibit genetic differentiation as a result of its sedentary lifestyle and restriction to certain habitat types. In general, grass snakes might be more mobile than other water snake species (Natricinae). For female grass snakes, the moving activity is most extensive during the oviposition period, in which individuals move up to 114 m within a day (Madsen, 1984) or 500 m in total to reach the oviposition sites (Wisler et al., 2008). After that period, females are more sedentary (Wisler et al., 2008). In contrast, males cover the largest distances (up to 55 m per day) during the mating season and are more sedentary afterwards (daily movements of 13 m; Madsen, 1984). Females had slightly larger home ranges in agricultural areas in Switzerland (39.7 ha; Wisler et al., 2008) than in Sweden (25 ha "total home range"; Madsen, 1984), or in England (0.18–9.41 ha; Reading and Jofré, 2009). This discrepancy could perhaps influence the genetic structure and result in less structured populations (smaller F_{ST}-values) in Switzerland.

At the regional scale, significant genetic differentiation between the above mentioned grass snake populations was found. The genetic structure was highly related to isolation by distance with 85% of the among-populations genetic variance explained only by the geographical distance between population units. These results are consistent with the genetic population structure in other natricine snakes. For instance, two garter snakes (*Thamnophis elegans* and *Thamnophis sirtalis*) exhibited low but significant population differentiation over a distance of 50 km (Manier and Arnold, 2005) and populations of salt marsh snakes (*Nerodia clarkii compressicauda*) were fragmented into isolated neighbourhoods measuring 50–80 km (Jansen et al., 2008). On the other hand, DiLeo et al. (2010) found no genetic structure in eastern garter snakes (*Thamnophis sirtalis sirtalis*) over a distance of 100 km. Isolation by distance, sometimes measured as a cost-based distance including potential basking sites between hibernacula, has also been recorded in other snake species (Bushar et al., 1998; Manier and Arnold, 2005; Ridenhour et al., 2007; Clark et al., 2008; Jansen et al., 2008; reviewed by King, 2009).

The present thesis showed that the genetic structure of grass snakes is mainly affected by distance. Interestingly, human activity and habitat alteration do not seem to confine this species and reduce its movements. However, this does not imply that there is no need for natural habitat, because the grass snake prefers habitat boundaries for basking sites and requires specific oviposition sites (Madsen, 1984; Wisler et al., 2008; Reading and Jofré, 2009). The conservation of this species should focus on the maintenance of a habitat mosaic with anuran breeding ponds and adequate oviposition sites. Further fragmentation of grass snake habitat should be avoided. The genetic results reported here have probably not yet been impacted by recent fragmentation, e.g. through roads. In large populations, it takes a long time until genetic differentiation between two demes can be detected.

The high dispersal ability of this species and, consequently, gene flow between grass snake populations counteracts any effects of drift. A further mechanism maintaining genetic diversity is mating with different partners resulting in multiple paternity. In **Chapter 4**, the occurrence and frequency of multiply sired clutches in the grass snake were investigated. Multiple paternity was found in at least 27% of the grass snake clutches (using a conservative estimate), but most probably occur in more than 90% of the clutches (using a maximum likelihood estimate). Most studies on multiple paternity in natricines considered new world species: multiply sired litters have been reported in *Thamnophis sirtalis*, with 37.5–75.0% of the litters exhibiting multiple paternity (Schwartz et al., 1989; McCracken et al., 1999; Garner et al., 2002), and in *Nerodia sipedon*, where 58.0–85.7% of the litters were sired by more than one father (Barry et al., 1992; Prosser et al., 2002; Kissner et al., 2005). In most natricine species investigated so far multiple paternity has been documented: *T. elegans, T. butleri, T. radix, T. sauritus, N. rhombifer, Regina septemvittata, Storeria occipitomaculata, S. dekayi*

(Wusterbarth et al., 2010). However, this study is the first to demonstrate the occurrence and level of multiple paternity in a European natricine.

Interestingly, Madsen and Shine (1993) observed in a mating experiment with captive grass snakes that males abandoned the female after successful copulation and did not court a previously mated female. This suggests that multiply sired clutches might be the result of mating in the previous year(s) and long-term sperm storage, although grass snakes have been reported to store sperm for only six months (Birkhead and Møller, 1993). The present findings demonstrate that males copulate with previously mated females in the wild. Otherwise the paternity by more than two males in a clutch could not be explained. Sexual unattractiveness of mated females due to a copulatory plug formed by gelatinous material has been reported in *Thamnophis* species (Ross and Crews, 1977; but see Shine, Olsson and Mason, 2000). In *T. sirtalis*, multiply mated females with two or more plugs have been found in the field (Shine, Olsson and Mason, 2000). Nevertheless, they produced clutches with multiple paternity (see above). Obviously, mating plugs do not completely prevent further sperm transmission and female natricines seem to have developed strategies to overcome forced chastity, as found in Viperidae (Ursenbacher et al., 2009).

OUTLOOK

This thesis revealed that the genetic structure of grass snake populations in Switzerland is mainly affected by distance. However, the habitat matrix in Switzerland is very diverse with rather small arable fields, frequently intercepted with hedgerows, fallow land, or forest stands. Grass snakes strongly prefer edge habitats over monocultures (Wisler et al., 2008). Therefore, the habitat heterogeneity occurring in the study areas may have facilitated the dispersal of grass snakes within these landscapes by enhancing the permeability. In contrast, arable fields in other European countries are much larger containing fewer heterogeneous landscape elements. Consequently, further studies are needed to investigate the genetic population structure and, hence, the dispersal ability of grass snakes in landscapes with even more pronounced human impact. Furthermore, a species may exhibit different genetic structures, depending on the characteristics of the studied habitats (Stow et al., 2001).

Data on sex-specific dispersal in snakes are scarce. Here, the low number of sampled males did not allow an assessment of a sex-specific dispersal pattern in the grass snake. Male-biased dispersal has been reported in some species (Rivera et al., 2006; Keogh et al., 2007; Dubey et al., 2008), while Lane and Shine (2011) report inter-population variation in the sex-specific dispersal pattern of two sea snake species, depending on the availability of sex-specific resources. During the mating period, male grass snakes move long distances in

search for mates (Madsen, 1984). This moving activity combined with the high frequency of multiply sired clutches in this species suggests a male-biased contribution to gene flow. On the other hand, females move long distances to reach oviposition sites (Madsen, 1984; Wisler et al., 2008), especially when adequate oviposition sites are scarce. The scarcity of oviposition sites may enhance long distance movements by females. This suggests that dispersal in the grass snake could also be female-biased. However, females may return to their previous home-ranges after oviposition, as certain individuals have been found in the same home-ranges over successive years (Madsen, 1984). In contrast, Reading and Jofré (2009) did not report such phylopatry. These contrasting results indicate the importance to study the movements of male and female grass snakes over successive years in order to gain insight to the dispersal pattern of this species. In addition, sex-biased dispersal should be assessed over a broad geographic range in different habitats to elucidate possible variation in the sex-specific moving activity due to varying resource availability, as reported in sea snake species (Lane and Shine, 2011). Otherwise, it is not possible to draw any conclusions about a general pattern.

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