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Effect of habitat fragmentation on the genetic structure of slow-worm (*Anguis fragilis*) populations

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ABSTRACT

Fragmentation is the major factor leading to the current, unprecedented biodiversity decline. Fragmentation leads to isolated populations where inbreeding depression and increasing genetic drift can lead to a loss of genetic diversity and an increase in deleterious alleles threatening their fitness and future adaptation to their environment and even to extinction. The relatively new field of landscape genetics represent an effective method to correlate population genetics with habitat features which influence latter structure.

In this one year study the genetic population structure of slow-worms (*Anguis fragilis*) could be assessed in a 16km^2 area in Western Switzerland with a newly developed set of 9 microsatellites. Overcoming the difficulties of the application of an ideal experimental design to the reality of field work 13 populations could be successfully analysed for genetic differentiation. The pairwise genetic differentiation determined among sites using F_{ST} indices appeared to be weak, in addition no distinct population clusters could be assessed in the entire study area.

To identify the factors involved in the fragmentation and which consequently lead to the population genetic structure I took advantage of the relatively new field of landscape genetics with 3 different methods: IBD (isolation by distance), least-cost modelling and a strip-based approach. The first approach, with scale as unique landscape feature, showed a significant IBD effect. Since this effect was not entirely explaining the structure other methods including more landscape variables have been used. Least-cost modelling and strip-based methods were used to assess the effect of 12 landscape variables which were supposed to influence slow-worm dispersal. Generally the models including more variables performed better than IBD showing the importance of the matrix between habitat patches. The compared results of least-cost modelling and the strip-based approach showed some difference, this demonstrated the need to use several approaches. Considering advantages and drawbacks of each method I analysed the effect of each element separately. The negative influence of recent elements resulting from human activity, like the highway or roads, on gene flow could be demonstrated. In addition a lower negative effect of natural elements like rivers could also be detected. On the contrary the results strongly suggest that agricultural areas and forests are potential dispersal corridors.

Finally the results suggest that fragmentation will not endanger slow-worm populations in the future since even if several elements have been showed to have a negative effect on gene flow no inbreeding effect or increased genetic drift has been detected.

INDEX

1. INTRODUCTION

Two thousand and ten has been proclaimed as the "International Year of Biodiversity" by the United Nations. As a matter of fact, the current biodiversity decline is unprecedented, a study about the future of biodiversity showed that the extinction rate is 10 to 1000 times higher than the one before dawn of mankind (Pimm, Russell et al. 1995). The four main general threats to biodiversity are habitat destruction (including habitat fragmentation), introduced species, overexploitation and food chain disruptions (Campbell and Reece 2002). Still, the main causes leading to this loss of biodiversity is the habitat fragmentation (Sala, Chapin et al. 2000).

The fundaments of the theory of habitat fragmentation arose from the "Island biogeographic Theory" in 1967 (MacArthur and Wilson 1967). Ever since fragmentation became an important field in population ecology, conservation ecology and other related fields (Saunders, Hobbs et al. 1991; Debinski and Holt 2000; Driscoll 2004; Arens, van der Sluis et al. 2007). Lenore Fahrig pointed out, citing a recent search of the Cambridge database which revealed over 1600 publications about fragmentation, that there is no consensus about latter definition (Fahrig 2003). In her opinion "the term should be reserved for the breaking apart of habitat, independent of habitat loss". In accordance with this definition there are four effects implied in the fragmentation process: reduction in the amount of available habitat, increase in number of patches, decrease in sizes of habitat patches and increase in isolation of patches (Fahrig 2003). Even if a part of this fragmentation arise from natural barriers like rivers, forests etc. the critical and larger part of habitat fragmentation is due to human alteration by agriculture and urban development including roads and forest clearing (Campbell and Reece 2002).

Habitat fragmentation induces 2 processes, a separation of habitat in "island" patches and for most species a reduction in population size and a reduced migration (gene flow) among these patches (Frankham, Ballou et al. 2010). The effect and the repercussion of theses processes are significantly determined by the connectivity between these patches which is determined 1st by the resistance which the diverse land uses composing the matrix present to animal movement, an 2nd by the configuration of those land uses (Moilanen and Hanski 2001).

The impact of fragmentation on gene flow are species specific and mainly includes the number of population in fragments, distances between fragments and the dispersal ability of the species, migration rates, time since fragmentation, extinction and recolonization rates across fragments (Frankham, Ballou et al. 2010). Thus, the increased habitat fragmentation has a strong impact on gene flow and population structure which degree is influenced by species specific abilities and the degree to which habitat patches are connected. Two major phenomena threatens isolated populations whose gene flow is limited. First, fragmentation increases the likelihood for one individual to mate with a relative, meaning with an individual possessing a similar genotype. This will lead to a loss of heterozygous genotypes in the same

frequency homozygosity increases. Therefore inbreeding implies changes in the mean phenotype within a population which arise from these changes in genotype frequencies and associated fitness effects, a harmful phenomenon referred to as inbreeding depression. The phenotypic changes implies decreased performances, growth, reproduction and even viability and can therefore lead to extinction (Hamilton 2007). Secondly, in small populations random genetic drift reduces the genetic diversity since natural selection isn't acting anymore (Lande 1988). Genetic drift is the stochastic change in allelic frequency in opposition to changes induced by natural selection. In natural population of a sufficient size harmful alleles which are continually introduced by mutation are "purged" by natural selection. In opposition, in small population random genetic drift can overcome natural selection so that deleterious mutations are not kept at low frequencies any more implying a negative phenotypic effect reducing the fitness (Allendorf and Luikart 2007). Since most of these deleterious alleles are recessive their harmful effects are only expressed in homozygotes and therefore the combined effect of inbreeding and increased genetic drift can lead to extinction in small populations. Even if the distribution of a species, as well as it abundance are crucial information for effective conservation issues, these essential genetic issues show how the evaluation of the genetic diversity and gene flow provides an essential knowledge about the degree to which a species is endangered by fragmentation.

Population genetic approaches using multilocus genotypes data are well suited to analyse migration and gene flow. Even if the dispersal "behaviour" a species is not known it has been showed that generally a decreased differentiation calculated with F_{ST} is associated with increased dispersal (Bohonak 1999). Therefore two complementary methods can be used in order to study the effect of fragmentation on gene flow (Manel, Gaggiotti et al. 2005). First, clustering approaches like the one integrated in the software STRUCTURE (Pritchard, Stephens et al. 2000) allow to identify the number of population and assign the origin of each individual. Secondly, to assess the degree of population differentiation Wright's F-statistics (Wright 1951) are the most widely used statistics in population genetics (Manel, Gaggiotti et al. 2005; Holsinger and Weir 2009).

To address population genetic and further ecological questions it is crucial to choose the adapted genetic marker. In the past decade microsatellites have emerged as the most popular choice to answer ecological questions since they also provide information about contemporary effects on gene flow (Selkoe and Toonen 2006). Microsatellites are DNA sequence stretches with tandem repeats of 1-6 nucleotides. They are codominant markers inherited in a mendelian way, randomly distributed across the genome. Only a few microsatellites are under selective pressure, therefore they are neutral markers. In addition they are highly polymorphic in natural populations, mutations occurs at a high rate, 10^{-6} to 10^{-2} per locus and per generation (Schlotterer 2000). For this reason they are well suited to analyse evolutionary changes also on

an ecological scale which is very important for conservation biology which wants to assess recent human induced changes. Analyses of genetic variability can thus be conducted by PCR (polymerase chain reaction) amplification using the stable flanking regions of each microsatellite loci with specific primers. Consequently flanking regions must be highly conserved in contrary to the microsatellite regions. One of the reason of the rapid expansion and power of microsatellites are the recent improvements in new sequencing technologies, genetic analysis and genotyping methods.

Since Frederick Sanger and Walter Gilbert have been awarded with the Nobel Prize in 1980 for there work about sequencing a main goal in this field has been to increase throughput of DNA sequencing. The sequencing technology developed by Sanger to determine the nucleotide sequence of DNA molecules involves *in vitro* synthesizing of complementary strands of the DNA to be sequenced. In this method the molecules to be sequenced are cloned restriction fragments (Campbell and Reece 2002). In the last decade the sequencing revolution took place with the development of next-generation sequencing like the pyrosequencing technique allows fast and cost-effective sequencing for 2 major reasons. One the one hand use of light detection allowed miniaturization, as the reaction volume just has to be high enough to emit detectable levels of light. As a matter of fact in this sequencing method each DNA sequence is bound to a bead, which is further “mixed” to droplets “containing” the “PCR-reaction-mixture” so that PCR amplification occurs within a droplet. The light detection is enabled because of the use of fluorescent labelled tags instead of radioactive labels to detect the terminated ladders. On the other hand this method also enables parallel sequencing enhancing throughput (Mardis 2008; Mardis 2008; Rothberg and Leamon 2008). These improvements allow high throughput sequencing at lower costs and less time-consuming since less material is used, because of the miniaturization and parallelization of the reaction. This new technology provides great improvements for fields lasting from human genetics to ecology (Mardis 2008).

These novel techniques to develop microsatellites markers combined with statistical tools to infer population genetics allow scientists to combine multilocus genotype dataset to analyse the genetic structuration resulting of the configuration of landscape elements. This is the main concern of landscape genetics, a relatively new and interdisciplinary research field combining population genetics, landscape ecology and spatial statistics to correlate latter fields (Manel, Schwartz et al. 2003). The goal is to analyse the effect of landscape variables which can also represent barriers such as land uses, exposition, topology etc. on gene flow. In conservation biology landscape genetics are used in particular for the analysis of human influenced variables such as roads, agricultural areas to explain the anthropic induced contemporary changes including fragmentation on gene flow. Landscape genetics are better to model real world than classical metapopulations studies because not only distances between patches are studied but

also the quality of the matrix in-between (Holderegger and Wagner 2006). Therefore the approach aims to quantify the effects of landscape variables (composition, configuration, matrix quality etc.) on spatial distribution of genes, i.e. populations. This approach involves different fields of research, so that a high level of interdisciplinarity is essential to succeed as it is the rule in landscape genetics (Holderegger and Wagner 2006; Storfer, Murphy et al. 2007). Finally landscape genetics provides an insight in key ecological processes like dispersal influenced by barriers and can be used as a valuable tool for conservation particularly in detecting contemporary, often anthropogenic, landscape effects.
It has been shown that to address landscape genetics questions it is essential to analyse data sets of population genetics with several approaches (Excoffier and Heckel 2006). Further, 3 landscape genetics methods will be introduced: isolation by distance, least-cost modelling and strip-based approach. Since gene flow is directly influenced by the landscape connectivity which "is the degree to which the landscape facilitates or impedes movement of organisms among source patches" (Taylor, Fahrig et al. 1993; Tischendorf and Fahring 2000) it is crucial to proceed to analyses which include the structural connectivity (characteristics of landscape) as well as the functional connectivity (mobility of the organism).
A first approach, IBD (Isolation by Distance (Wright 1943)) only takes into account the distance between patches to describe genetic differentiation, here scale can be seen as the most basic landscape element. Even if an IBD effect is often confirmed it represents in most cases only a part of the explanation of differentiation so that it has been pointed out that in plenty reviewed studies only Euclidean distances are used (Moilanen and Hanski 2001) neglecting the specificity of landscape between patches which influence species dispersion. In order to disentangle the effect of distance and the influence of the matrix between patches further landscape genetics have to be chosen.
The second method, least-cost modelling, is the first alternative to IBD since it includes landscape variables. Least-cost modelling computes EGD (effective geographical distances) between two habitat patches, using friction maps, where each raster-cell is given a special value representing the degree of resistance of the specific landscape type.
The third is a new method; the strip-based approach assesses the effect of landscape elements on gene flow in a linear fashion between sites using defined pairwise strips between these sites. This method has the advantage that no a priori assumptions had to be made (Emaresi, Pellet et al. 2011).

In this study I used new molecular markers and landscape genetic methods to analyse the effect of fragmentation on the genetic population of a poorly known lizard, the slow-worm. Pointing out the fact that ecological knowledge about slow-worms is insufficient Völkl et al. published a book (Völkl and Alfermann 2007) reviewing all known studies about this species and pointing out the future research needs to have a better overview of slow-worm ecology. Since knowledge about this species has often

been acquired during mostly unpublished monitoring experiments or studies performed more than 30 years ago it is difficult to have access to them. Therefore the facts about slow-worms mentioned here are cited according to this book if a different source is not mentioned.

The slow-worm is an elongated legless lizard native from Eurasia and a member of the family *Anguidae* in the *Squamata* order. Slow-worms can be found in a large variety of habitats ranging from natural ones like shrub vegetation, edges of forests to anthropogenic influenced areas like gardens, parks etc. This is probably the reason why slow-worms are the most widespread reptile in Europe. Like all other lizards slow-worms are ectotherm, meaning that they need external heat sources to control their body temperature. Lizards can gain heat in 2 ways, heliotherms gain heat directly by radiation; thigmotherms gain heat by conduction from the substrate. Slow-worms are thought to be thigmothermic occasionally exhibiting a low degree of heliothermy by basking in the sun (Evans and Leszczynski 2009). This is probably one important reason for the importance of microhabitats and microclimate for slow-worms. As a matter of fact even in a favourable habitat type the presence of slow worms will be mainly influenced by several microhabitat factors. The availability of some natural or artificial refugees to gain heat and to hide is the first important condition. Since slow-worms will also need to feed under these refugees it is important that they show humid conditions to attract their prey mostly invertebrates like earthworms and slugs. The structure of soil is also important, first it has to be loose so that slow-worm can burrow into it, and also be suitable to gain heat by conduction.

Slow-worms are active from March to September depending of ambient temperatures which also influence the duration of their winter hibernation period. The mating period occurs in spring, the females are ovoviviparous and they only mate every 2 to 3 years since this breeding strategy requires a large amount of energy; gestation time is about 12 weeks. They give birth to 2 to 23 descendents depending primarily of the size of the female. Like other lizards they are capable of autotomy to escape potential predators.

Only scarce and controversial knowledge about dispersal is known. In the Nederlands (STUMPEL 1985, cited in (Völkl and Alfermann 2007) observed that slow-worms stay mainly in the same location during a capture/ recapture experiment, anyhow he also noticed that one individual moved 80 meters in 7 days, the maximal distance observed was 130m in 2 years. In an other experiment Plattenberg (Plattenberg 1999, cited in (Völkl and Alfermann 2007)) showed that in average slow-worms moved 12m to 16m between the first capture and recapture, the calculated “home ranges” for 2 activity periods were 466 ± 150 m^2. To summarize the authors hypothesized that the adult animals stay mainly in a range of 30-50 meters and that some subadult animals undertake more important migrations and colonize new habitats and are therefore responsible for gene flow. Finally, the need for studies of the dispersal with transmitters and detailed populations genetic analysis in particular studies integrating the effect of contemporary fragmentation are pointed out by the authors (Völkl and Alfermann 2007).

Since “umbrella species” are often carefully studied and monitored common species, like slow-worms, seem to have been left behind by the scientific community even if some observations about a decline are available (Monney and Meyer 2005). .According to the guidelines of the IUCN (International Union for Conservation of Nature), the slow worm status is considered as “least concern” since 1994 by the Swiss red list of endangered species established by the “BAFU” (Agency of the Environment, Transport, Energy and Communications, previously “BUWAL”) and the “KARCH” (coordination centre for conservation of Swiss amphibians and reptiles). On one hand the authors claim that this species obviously colonizes more than 2000km^2 and that it has, compared to other reptiles, little ecological requirements. For these reasons they considered that the slow-worm is more adaptable to anthropogenic influenced habitat. One the other hand they point out the fact that they have incomplete data about the repartition of the species, and that this species seems to decline in the midland and in the lower part of valleys according to cantonal inventories (Monney and Meyer 2005). Even so the slow-worm is included in the “annexe III”, protected species of the “Bern Convention on the Conservation of European Wildlife and Natural Habitats” ratified in 1979 (Übereinkommen vom 19. September 1979 über die Erhaltung der europäischen wildlebenden Pflanzen und Tiere und ihrer natürlichen Lebensräume (Swiss_federal_Authorities).

As mentioned before the four main threats to biodiversity are habitat destruction (including habitat fragmentation), introduced species, overexploitation and food chain disruptions (Campbell and Reece 2002) the most important being fragmentation (Sala, Chapin et al. 2000). Regarding slow-worms it seems obvious that neither overexploitation does represent a threat since they are of no economic interest, nor introduced species since no invasive species are competing for the same habitat or feeding on slow-worms. Food chain disruptions shouldn’t also not represent an issue for slow-worms since their prey mainly earthworms and slugs are widespread. Therefore the only aspect to be analyse concerning the decrease of slow-worms is habitat destruction, in particular habitat fragmentation since it has be identified as the main general threat to biodiversity (Sala, Chapin et al. 2000). In this study I use fragmentation in the strict sense of a possible isolation of patches by presumable barriers to the dispersion of slow-worms since habitat loss is not possible to assess due to the lack of knowledge concerning slow-worm ecology. The unknown effect of habitat fragmentation, the lack of data and the absence of habitat and dispersal information about slow-worms suggested that it was necessary to carry out a scientific study especially including genetics to get a better insight in the slow-worm ecology.

The first step in this study was to develop a suitable set of microsatellites at moderate cost in a reasonable time period taking advantage of the new sequencing technologies since no molecular marker were available for this species yet.

Further, I performed population identification and genetic differentiation analyses to assess the potential gene flow between habitat patches in the region of interest and the effect fragmentation had yet.

It has been shown that in order to study fragmentation, “in situ” experiments are necessary because the complex environment is difficult to represent in models. In comparison to laboratory experiments observational studies and field experiments gain generality but don’t allow to isolate the effects of the different mechanisms (McGarigal and Cushman 2002). To counteract this generalization and disentangle the different elements leading to genetic differentiation I performed 3 landscape genetic analyses (IBD, least-cost path modelling, strip-based approach) to identify the elements leading to fragmentation. I selected and analysed a set of variables to detect which one impedes gene flow and in which extend each variable representing a barrier influence the gene flow between the populations. Finally, for each method I analysed and compared the results in respect to the advantages and drawbacks of each.

2. MATERIAL AND METHODS

Study area

Sampling has been conducted in the Canton Vaud in the region of “La Côte” region (see Figure 1) from March to September 2010. This region was chosen because of the landscape heterogeneity, the presence of an altitudinal gradient ranging from the lake (372m) to the Jura Mountain (highest sample sites at about 1000m).

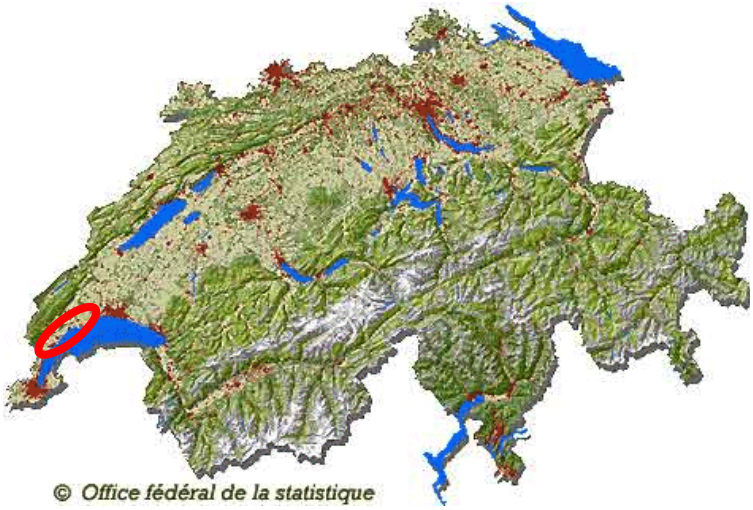


Figure 1 : Swiss map showing the location of the study site in red (Swiss Federal Statistical Office).

The total sampling area measures about 16 km^2 (see Figure 2). The sample region is dominated by agriculture (yellow) throughout the sample site. Another important feature is the dense forest (green) in the middle of the site, and other patches. Other types of forests are only present in very small patches throughout the site (light green). In the South the railway and railroads are crossing the site side by side; more northwards also in parallel one can observe an important vineyard belt (orange). The grey areas represent parts of villages which could be partly suitable habitats for slow-worms like garden parks etc. Shrub and pastures are only present in a very low amount. Throughout the sites two major linear elements can be observed: rivers (blue) and roads (antique pink, highway not included). In the category “others” are included all elements which were only present in marginal amounts like orchards, the airport etc.

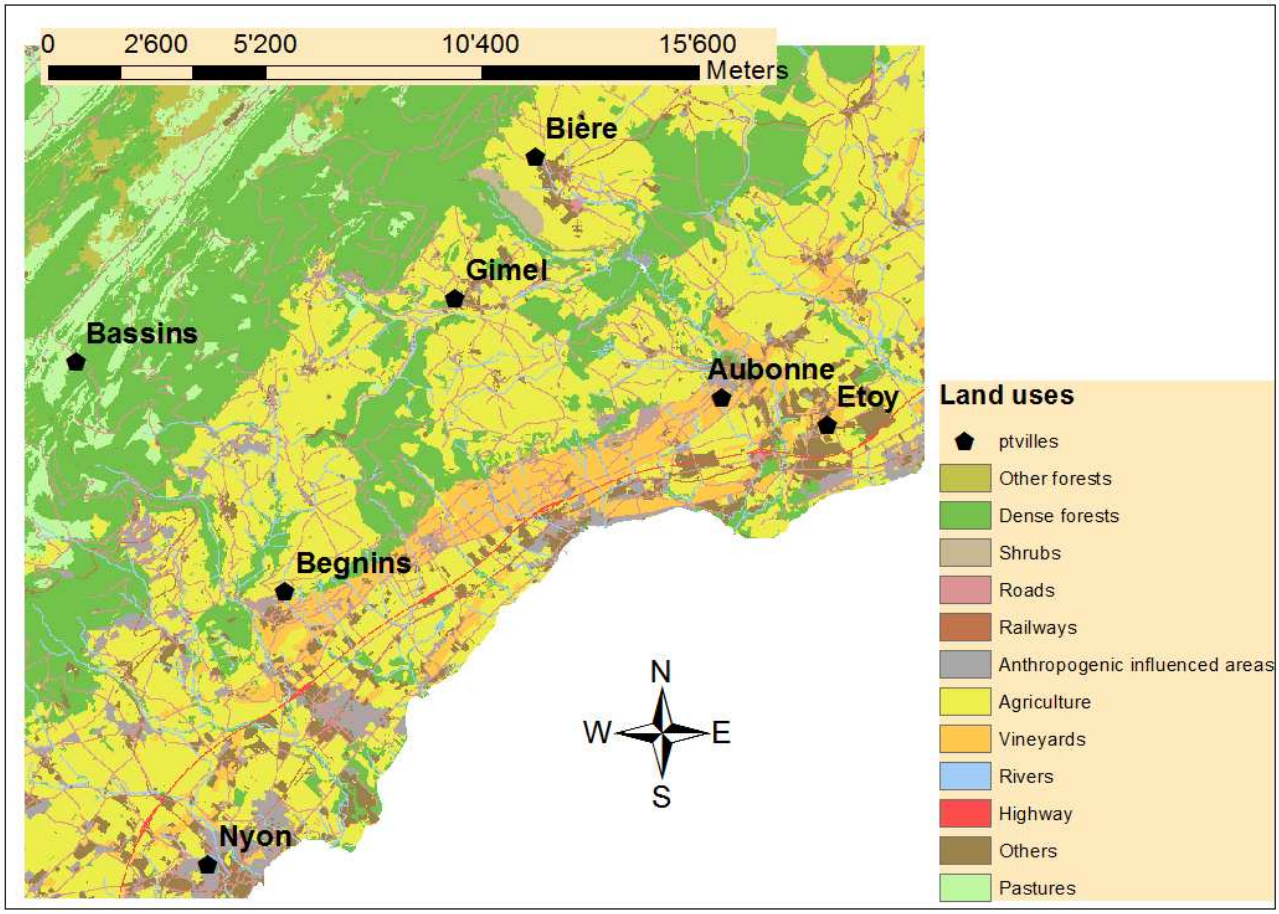


Figure 2 : Raster map of the land uses of the study area clustered in 12 categories of the original map (Lehmann, Maggini et al. 2000).

Experimental design and sampling methods

In order to test the effect of the different land uses on gene flow between sites, the sampling design was conducted to provide sites on each side and in-between the potential fragmentation elements. Several authors recommend a continuous, evenly spaced sampling for landscape studies focused on the detection of spatial boundaries (Guillot, Estoup et al. 2005; Manel, Berthoud et al. 2007). It was also important to have a regular distribution of pairwise distances between sites to test for isolation by distance avoiding bias during the comparison with genetic distances. Moreover sites had to include all different landscape elements in order to test their fragmentation effect separately. According to these prerequisites I had to selected sites favourable for the slow-worms, such as hedges, edges of forest, isolated patches of trees and natural gardens.

Since slow-worms have a semi-fossorial lifestyle and are thigmotherms I used some black undulated tar plates to trap the lizards (see Appendix 1 and Appendix 2). Those plates represent refugees and a heat source for the animals and improve the capturability of the species (Völkl and Alfermann 2007). In

addition in humid conditions they also represent and even help to create a suitable habitat for their prey so that slow-worms would settle down over a longer time period under these plates.
First, a set of 331 were installed in 33 sites from the end of March for the first ones up to May 2010 depending on the sites. The plates have been installed with the goal to find and sample at least more than 20 different animals in each site. In August, 100 plates of the sites without any slow-worm detected were removed and placed in 5 new sites or added in sites where less than 20 animals have been sampled.

Development of new microsatellites for *Anguis fragilis*

Identification of new microsatellites for *Anguis fragilis*

To perform further population genetics analysis between 8 and 15 suitable microsatellites for *Anguis fragilis* were needed since no published microsatellites are available yet.
First, DNA has been extracted of a slow-worm from a previously sampled slow-worm, using the Qiagen DNeasy kit (QIAGEN) following the supplied protocol. This DNA was then subjected to a random 454 shotgun sequencing (1/16 run, on the “Roche FLX Genome Sequencer” with Titanium chemistry by Microsynth AG). The reads obtained were screened for potential microsatellites using MSATCOMMANDER v0.8.2 (Faircloth 2008) and the final selection was made by eye based on the length and the homogeneity of their repetitions with SPOTLIGHT (Mac OS X 10.6) to select about 150 sequences. In the next step I designed primers for further amplification by PCR when flanking regions of the selected sequences enabled it. Once primers have been obtained the optimization has been performed by amplifying the potential microsatellites performing PCR on a “Mastercycler Gradient” (Eppendorf) with variable conditions. PCR amplifications were then tested on agarose gel (1%) electrophoresis. Further, polymorphisms between samples were assessed using a multicapillary electrophoresis system (“QIAxcel System”, QIAGEN). For this analysis I used a QIAxcel DNA High Resolution cartridge combined with the QX Alignement Marker 15bp/500bp and the QX DNA Size Marker 25-450bp which enables a separation of the fragments with a resolution of 3-5 bp.

Testing the newly identified microsatellites

The samples obtained during the field work in this study have been extracted with the Qiagen DNeasy kit (QIAGEN) following the supplied protocol. Three main steps have been changed: incubation has been performed overnight at 56°C, a supplementary centrifugation step after removing the buccal swab and elution has been done twice each time with 100 µl of the provided AE buffer to obtain a higher amount of DNA.

Once the potential microsatellites have been identified and the 118 samples been extracted it was possible to test the microsatellites for null alleles, large allelic dropout and stutter errors. For this analysis I used the software Micro-checker (Van Oosterhout, Hutchinson et al. 2004), which performs analysis for each "population" and locus. First the software checks for accuracy of the dataset, indicating possible typing errors when reading the sequences. Then I selected a 95% confidence Interval for the Monte Carlo simulations of homozygotes, this value is then compared to the observed value of homozygotes. Afterwards the software compares the observed allele frequency to the estimated allele frequency with four different algorithms. In this case it was especially important to check for allelic dropout because of the relatively low DNA concentration after the buccal swabs extractions. This low level of DNA could prevent the amplification; respectively impede the lecture of an allele after sequencing implying a deficit of heterozygotes. Observed (Ho) and expected heterozygosity were calculated for each microsatellite using FSTAT 2.9.3.2 (Goudet 1995).

Population genetics of the slow-worm

Laboratory analysis

Genetic material was collected from saliva and blood using buccal swabs. This approach is the less invasive method to obtain sufficient amounts of DNA for further analysis, as for instance microsatellite markers (Miller 2006; Beebee 2008). Individual analysis had to be conducted to avoid multiple analysis of a single individual. Since all simple marking methods (e.g. with nail polish) used for reptiles failed due to their smooth skin I used individual photographic identification (see Appendix 4), which allowed a long-term identification during the study period.

Once all samples have been collected during the field work they have been extracted with the Qiagen DNeasy kit (QIAGEN) following the supplied protocol. Three main steps have been changed: incubation has been performed overnight at 56°C, a supplementary centrifugation step after removing the buccal swab and elution has been done twice each time with 100 µl of the provided AE buffer to obtain a higher amount of DNA. Further all individuals have been amplified for each locus with the previously determined PCR conditions. Fluorescent labelled primers for the microsatellites to be tested have been ordered and after a last optimization all amplified loci for all animals have been analysed on the AB3130xl sequencer (Applied Biosystems) in 2 multiplex PCR. Scoring of alleles has been performed by identifying visually the microsatellite peaks on Peak Scanner™ Software v1.0 (Applied Biosystems).

Genetic diversity and population differentiation

All following analysis have been performed with the previously extracted and scored individuals. One condition to use microsatellite is the random distribution of the markers across genome, if 2 or more loci are linked the influence of this zone of the genome is to important and can bias further genetic analysis. Therefore I first assessed the absence of linkage disequilibrium between pairs of loci have been tested using FSTAT 2.9.3.2 (Goudet 1995). Conformity to Hardy-Weinberg has been assessed with a test randomising alleles within samples based on F_{IS}, since microsatellites are neutral markers they should not be under selection; this can be confirmed if loci are in Hardy-Weinberg equilibrium. Furthermore F_{IS} values (Weir and Cockerham 1984) has been calculated to test for inbreeding depression. Finally the analysis of the degree of population differentiation have been performed with F_{ST} values (Weir and Cockerham 1984). Since gene diversity is highly dependent of the sample size they are not discussed here. Therefore I used the allelic richness which is corrected for sample size and gives better results when there are important differences in sample size (Leberg 2002).

To asses the presence of different populations with the multilocus genotype data I needed to infer clusters without any a priori structure assumption. This structure has been analysed with STRUCTURE 2.3.3 (Pritchard, Stephens et al. 2000). Using a Bayesian clustering approach this software clusters individuals assumed to be in Hardy Weinberg equilibrium based on the similarity of their alleles. The software computes the estimated log probability for each K clusters, where K is fixed between a given range (here from 1 to 10). Here the model with admixture has been used with 100’000 iterations and a burn-in period of 20’000 iterations. In our case the patterns of dispersal among populations are probably not homogenous as they could be influenced by landscape and space in a hierarchical system. In this case the interpretation of STRUCTURE likelihood values results is doubtful. Therefore in this study, I used the approach of Evanno et al. (Evanno, Regnaut et al. 2005) which infer a more realistic number of clusters based on the results of STRUCTURE.

Landscape genetics

Identification of the variables to be analysed

Correlating genetics to landscape features requires a choice of maps which take into account the structural connectivity but also imply the functional connectivity by taking into account the perception of the studied organisms. The commonly available source of land use information in Switzerland is a photo-interpretation map which assigns one of 74 categories to cells of a 100m grid (Swiss Federal Office of Statistics). In this type of maps a land use is assigned to each bottom-left corner of the 100m cell which made it impossible to assign more detailed land-uses to each cell for example by dividing it. Thinking at the perceptual range of slow-worms this type of maps does not suite to analysis of species with such a little perception of its environment. Therefore I used a raster map with cell size of $25m^2$ to take into account the perceived environment of the species. This downscaled land use map has been obtained by combining the highly precise Swiss national map (1:25000 scale) with a land use classification from photo-interpretation which possessed a high level of thematic details (Lehmann, Maggini et al. 2000). The result is a precise and detailed map with a resolution of 25m integrating 61 categories which can be used for studies at a regional scale.

For further modelization steps it is crucial to have the same projection for the different inputs, here all maps, grids and shapefiles has been projected with the LV1903 Swiss coordinate system, map units have always been set to meters.

As mentioned before the chosen map contained over 60 different land use types, therefore it was important to extract and cluster elements of interest for the analysed species. It was also important to use variables which were present in a sufficient amount of pixels to be correctly analysed with the different statistical methods used in this study.

Isolation by distance

Isolation by distance (IBD, Wright 1943) can be seen as the null hypothesis in landscape genetics, the scale being here the first landscape variable tested which influences population differentiation. It has been shown that a corrected F_{ST} (Rousset 1997), $F_{ST}/(1-F_{ST})$ compared to the logarithm of the distance gave more realistic results therefore this corrected F_{ST} has been used here. To test the correlation between these two elements a mantel test (10’000 permutations) has been performed between the logarithm of Euclidian distances and the corrected F_{ST} values for the 13 sites for which F_{ST} has been calculated. The mantel test has been performed with the software R (Team 2011) using the package ncf (Ottar N. Bjornstad, ncf: spatial nonparametric covariance functions, R package version 1.1-3, 2009). The results have then been

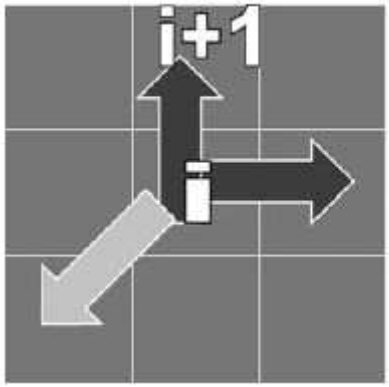
plotted and analysed with a linear regression. The regression coefficient (R^2) has been calculated to show the percent of explained variance of the genetic structure by Euclidian distances.

Least-cost Modelling

Least-cost modelling, originated from the graph theory, gained in attention in the last decade because of the availability of softwares to calculate it (Adriaensen, Chardon et al. 2003). It is a widely used method in ecology (Broquet, Ray et al. 2006; Epps, Wehausen et al. 2007; Schwartz, Copeland et al. 2009); in this study it was particularly interesting since my goal was to recognize potential barriers to gene flow without having a detailed knowledge of the variables implicated. For this method friction maps are created, where each raster-cell is given a special value representing the degree of resistance of the specific landscape type. In choosing a raster with a pixel size in respect to the perception of the species of interest and allocating costs with respect to the species ecology this method allows to account for the structural connectivity as well as for the functional connectivity.

For this method several scenarios had to be tested to recognize first which variables had a negative or positive effect on dispersal and which is the strength of this impact. On the one hand this approach aims to test the effect of the chosen variables on gene flow, respectively the landscape units which are "fragmenting the populations". And, on the other hand it allows to test if it is possible to analyse least-cost path deduced from available literature about habitat, since a suitable habitat and a possible dispersal path are probably not the same.

To modelize least-cost paths in a pairwise fashion I used the extension PATHMATRIX (Ray 2005) in ARCVIEW 3.X (Environmental Science Research Institute, Redlands, USA) to compute matrices of EGD (effective geographical distances) among the 13 sample sites where F_{ST} has been calculated previously using a least-cost path algorithm. EGDs have then been computed according to a friction map where each cell (representing a landscape unit) has been given a cost which reflects the difficulty to traverse it. The software computes then a path which minimizes the sum of frictions of all cells along this path. The method is based on a simple eight-neighbour-cell algorithm (see Figure 3, ESRI, 1996). From each cell N_i to N_{i+1} the cumulative cost is calculated as sum of the cost to reach Ni and the cost to move through N_i and N_{i+1}.



$$N_{i+1} = N_i + (r_i + r_{i+1})/2$$

or

$$N_{I+1} = N_i + 2^{**}0.5 * (r_i + r_{i+1})/2$$

N_i = accumulated cost in cell i
r_i = resistance value in cell I
i: source cell
i+1: target cell

Figure 3 : The algorithm underlying least-cost modelling (Adriaensen, Chardon et al. 2003).

To make least-cost modelling more effective it is important to test multiple least-cost models based on different landscape characteristics (Epps, Wehausen et al. 2007). This is particularly important in the case of slow-worms where only very few and contradictory knowledge about dispersal and habitat is available. Therefore I tested the following scenarios:

Scenario 1: Test of the following hypothesis: "Dense forests, highway and rivers are the main barriers to gene flow, respectively the landscape units which are fragmenting the populations."

For this purpose I "extracted" 3 elements of my clustered land use raster: Dense forest, highway and rivers and allocated them several different costs to test the sensitivity of this model.

Scenario 2: Test of following hypothesis: Primary and secondary habitat according to Völkl and Alfermann (Völkl and Alfermann 2007) are capable to describe dispersal.

In this scenario I "translated" each of the 61 categories of the raster to primary and secondary habitats according to Völkl and Alfermann (Völkl and Alfermann 2007) in costs to produce a friction map.
For this analysis I clustered the results in categories (for details see Appendix 8):

1. Primary habitat which is always suitable for slow-worms
2. Primary habitat which can be partly suitable for slow-worms or suitable depending on the intensity of use.
3. Secondary habitat which is always suitable for slow-worms.
4. Secondary habitat which can be partly suitable for slow-worms or suitable depending of the intensity of use.
5. Other land-use which should not be a potential habitat.

According to these weights I computed several scenarios weighting the 5 categories in different ways either with ascending weights for category 1 to 4 or equal costs for 1 to 4, category 5 always having the highest value as it shouldn't represent a potential habitat (see details in Appendix 8).

Scenario 3: Disentangling the effect of the 12 different variables one by one and creating a scenario which includes all 12 selected variables with respect to previous analysis.

In this scenario I first analysed each of the 12 variables one by one with different costs. I allocated costs of 2,4,8,10,15,20,30,40,60 and 80 to each variable, all other land uses each with a cost of 1. To test the results I also tested the contrary in giving the cost 1 to the variable and a higher cost (2,4,10,20,30,60) to all other land uses. I then compared the correlation coefficients which showed a significant (5%) p-value, and chose for each variable the "best" cost according to the correlation coefficient. In the case 2 values had the same correlation coefficient I choose the one with the lowest p-value.
Afterwards I combined in different ways these values with respect to their effect assessed before.
Scenarios 3A, 3B, 3C and 3D has been used to test again effects of several land uses for which the result wasn't very clear before, the other scenarios were computed to test the sensitivity of the model.

- Scenario 3a: Allocating to each category the best cost assessed before
- Scenario 3b: Removing the effect of "dense forest"
- Scenario3c: Removing the effect of agricultural areas
- Scenario 3e: Increase of the effect of "dense forest"
- Scenarios 3D, 3F, 3G, 3H, 3I, 3J, 3K changing the costs of fragmenting elements in respect to their ratio.

To select the best model and test the sensitivity of each version of the scenarios I compared the genetical distances (corrected Fst) to the logarithm of EGDs with the mantel test (Mantel 1967) using the software MANTELN (Nicolas Ray, 2003) with 10'000 permutations. The mantel test uses permutations to determine the linear relationship (r) of both matrices (Corrected Fst and logatrithm of EGDs).
Even if mantel tests are controversial due to a potential underestimation of type I error (false positive results) they still are commonly used when correlating distance matrices with genetic matrices (Epps, Wehausen et al. 2007; Wang, Yang et al. 2008). According to Rousset (Rousset 1997) it is more effective to compare log-transformed geographic distances to genetic distances therefore for all analyses I used the corrected version of F_{ST} (F_{ST} /(1- F_{ST})) to correlate it with the logarithm of EGDs.
Once the EGDs have been computed and analysed it was important to disentangle the part of the model explained by landscape elements alone without the effect of distance described by IBD. For this purpose I

calculated correlation by partialling out the distance with a partial mantel test included in the R package ncf (Ottar N. Bjornstad, ncf: spatial nonparametric covariance functions, R package version 1.1-3, 2009).

Strip-based approach

In the strip-based approach the goal was to recognize the main landscape elements influencing gene flow overcoming issues of unknown dispersal knowledge since all variables are analysed without a priori assumptions. In this method landscape element are analysed in straight-line strips of varying width among each pair of the analysed populations.

For this analysis I used the previously showed raster in 12 categories. In the first step I transformed this raster in a set of boolean maps with Arcgis 9.3 (ESRI, 2008). In this step it was important to verify that linear elements where conserved. To extract land use densities in each strip I used FRICTIONNATOR (http://www2.unil.ch/biomapper/frictionnator/frictionnator.html) which requires the IDRISI raster format. To obtain this format I transformed the data with the tool “AV 2 IDRISI” (www.terracs.com/en/products/software/av-2-idrisi.html) which can be added as an extension in ARCview (ESRI, 2003). The second input includes genetic data (F_{ST}) and the geographic coordinates of each site. The output of this software is a table with the genetical distances, the Euclidian distances and for each one the sum of pixels of every tested strip type and land-use for each chosen kind of stripes. Since I was interested in the density of each land use, I calculated the percentage of pixels of each land-use in each strip (pixel/pixel). To select the best strip width and analyse in more details the effect of the different land uses on dispersal I performed linear regressions. All statistical analysis has been performed with R 2.10.0 (R development Core Team 2010). The F_{ST} values have previously been transformed and successfully tested for normality with the Shapiro-Wilk test. In the first step the relation of F_{ST} against distance alone has been analysed. Afterwards each land-use has been added to this null model one by one:

« Lm (Fst~ ”Euclidian distances”) »

« Lm (Fst~ ”Euclidian distances” + ”Landuse 1”) »

« Lm (Fst~ ”Euclidian distances” + ”Landuse 2”) »

…

Two strip types can be chosen; here following strips have been tested:

- fixed width: strips of 75m, 125m, 275m and 525
- width: length ratio: 1:1, 1:3, 1:5 and 1:7

To choose the best fitting strip width I calculated the mean Aikaike’s information criterion (AIC) to select the best model. Since pairwise F_{ST} are not independent significance is biased, therefore the information-theoretic approach with AIC is better. The smallest AIC value indicated the model which was the closest to the “true model”. Afterwards the best model has been analysed in more details with weighted AICs

(wAIC), which assessed the relative likelihood of one regression compared to all others in this model. The squared correlation coefficient (R^2) which explained the proportion of explained variation for each variable has also been calculated to assess the importance of the impact of each variable. Finally the sign of the regression coefficient estimating whether a variable impede (+) or facilitate (-) gene flow has been printed.

3. RESULTS

Experimental design and sampling methods

Sites are evenly spaced so far as it was possible in an outdoor experiment which large anthropized areas and they were successfully placed in respect of the different major landscape elements (see Figure 4). On latter map the sites where plates have been installed are shown with black asterisks and pink dots (depending of the successful capture of animals). The histogram of Figure 5 shows the experimental design which should allow an evenly spaced sampling of animals. Since animals have been found in 13 sites described by pink dots the effective sampling results did not exactly correspond to the experimental design. Nevertheless most landscape elements are included but there is a lack of sites between the highway, the vineyard belt and railroads. However the distribution of distances which could effectively be used showed a distribution which allowed further analysis even if a lack of distances over 12000m could be observed (see Figure 6).

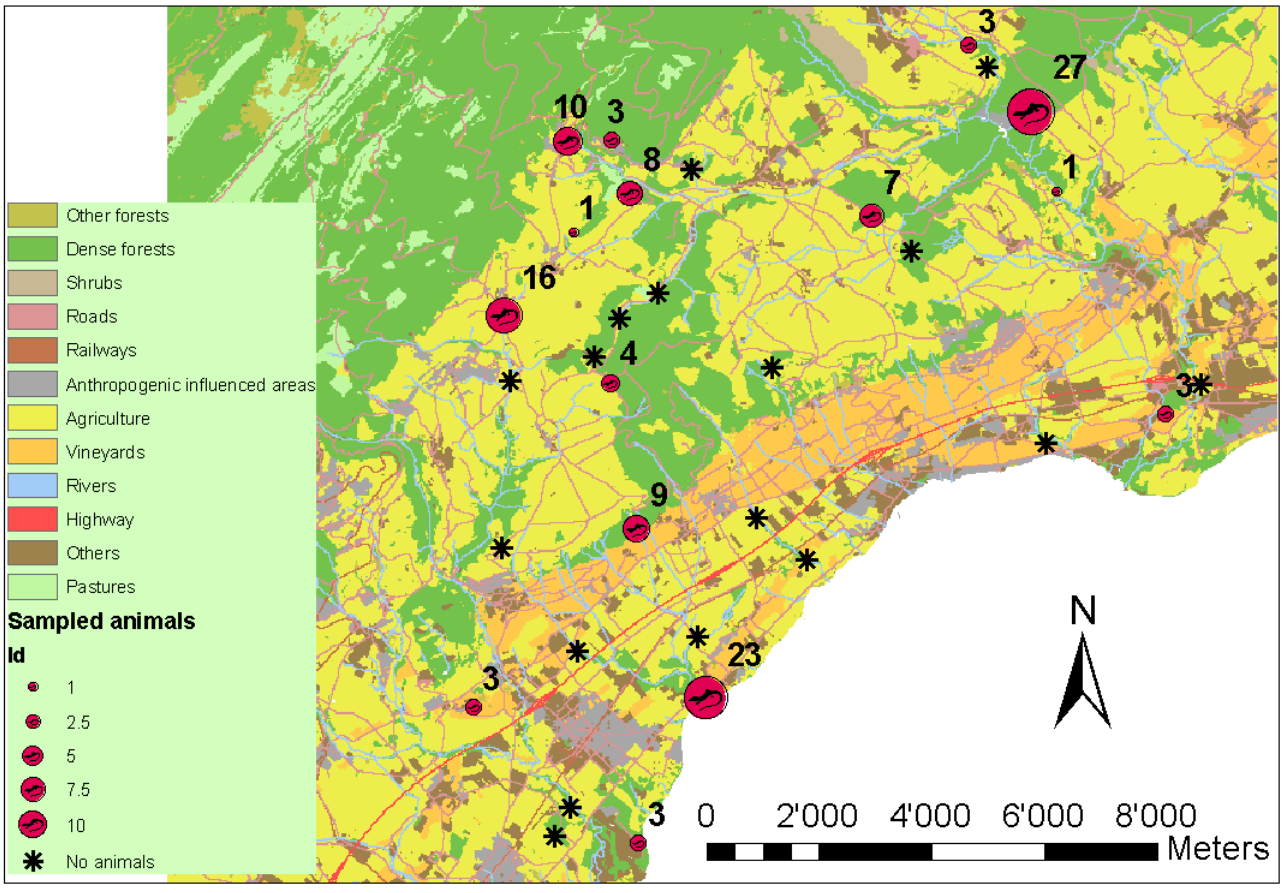


Figure 4 : Land use map of the study sites were plates have been installed in respect to presence or absence of slow-worms. The pink dots represent sites with presence of slow-worms; the amount of sampled slow-worms sampled is printed beside. Black asterisks represent sites were plates have been installed but no animals have been captured.

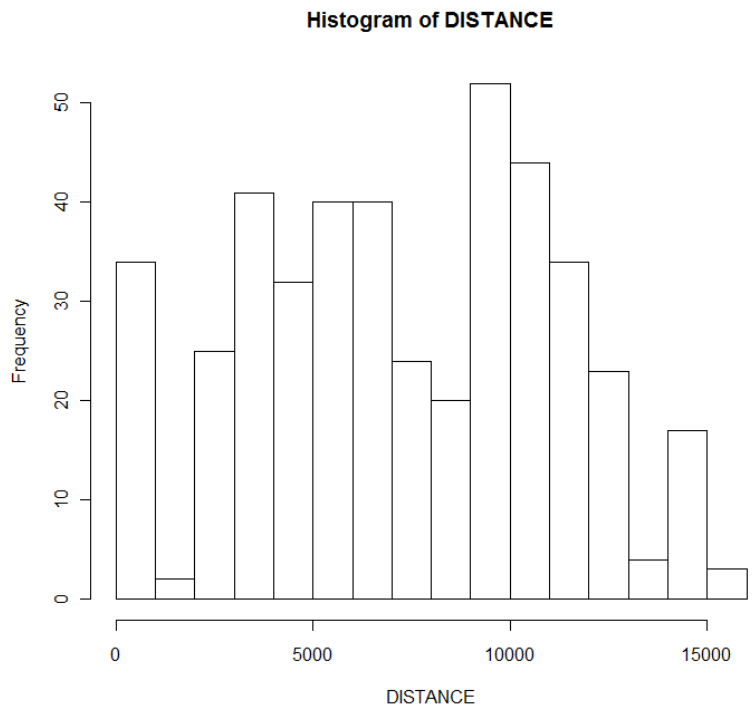


Figure 5 : Histogram of the pairwise distances (in meters) between all plates corresponding to the experimental design.

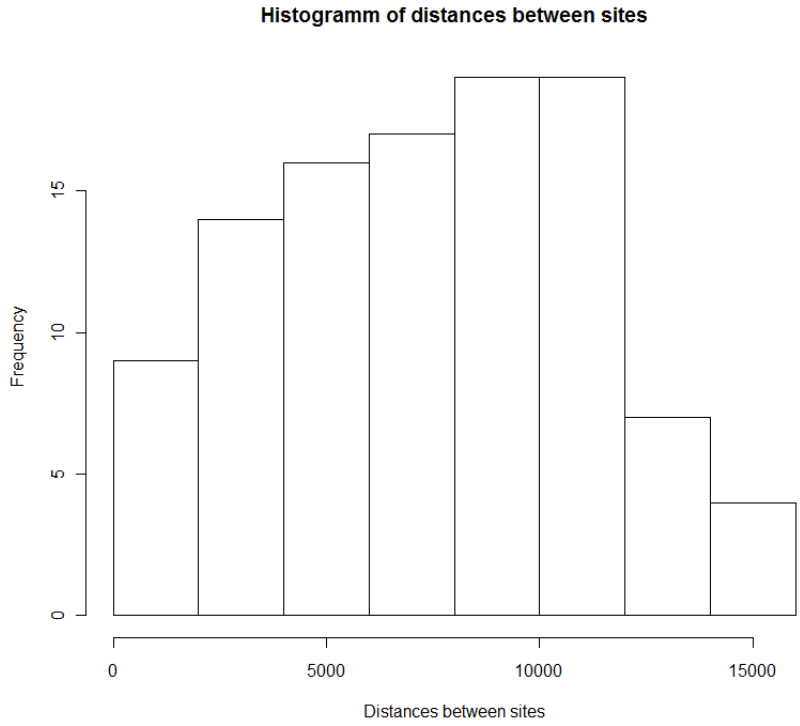


Figure 6 : Histogram of the distances between the 15 sites where animals have been sampled, distances in [m].

Slow-worms have been captured in 15 sites, for 17 sites no slow-worm has neither been seen nor sampled (see Table 1). In the sites with slow-worm presence between 1 and 27 animals have been sampled (see Table 1). A total of 118 animals have been sampled. No animals have been found in the additional sites installed in August. The plates which have been added to other sites in order to get more animals were also not colonized.

The capture success was highly dependent on the location of sites and microclimate around it but not of the amount of plates installed. The linear correlation between sites and number of slow-worms was positive, mainly due to one outlier (site St with 27 plates and 26 slow-worms trapped). Nevertheless, the adjusted R-squared of 0.046 showed the low quality and non significativity (p-value=0.12) of this relation. The Grass snake (*Natrix natrix)* was also commonly found under tar plates in the most successful sites. The photographic identification has been a very valuable method to identify the animals, even juveniles, so that no animals have been sampled twice.

Table 1 : Names, codes and geographic coordinates of sites, number of sampled animals, number of plates installed and presence of *Natrix natrix* under the plates. Coordinates of sites correspond to the center of the area when sites placed in a semi-circular way, or the middle of the segment when plates where installed in a linear way.

Capture sites	Code	Coordinates X	Coordinates Y	Captured individuals *A. fragilis*	Amount of "plates"	Observation of *Natrix natrix*
Allaman	Al	520295	147299	3	11	No
Bois des Ursins	Bu	515059	150818	7	11	No
Coinsins	Cc	508007	142099	3	6	No
La Curtillode, Vinzel	Cu	510880	145269	9	10	No
Forteresse, Gland	Fo	510938	139688	3	8	No
Private garden, Marchissy	Ge	508537	149037	16	4	Yes
Maison Rouge	Ps	510451	147855	4	13	Yes
Rucher, Longirod	Ru	510779	151221	8	15	No
Saint-George	Sg	509679	152126	10	1	No
Bois du Crêpon, Saint-Livre	Sl	517883	152660	27	26	Yes
STEP, Gland	St	512122	142271	23	12	Yes
Côtette, Saint-George	Tt	509773	150503	1	18	No
Tuilerie, Bière	Tu	516797	153835	3	16	No
Côte Viry, Saint-George	Vi	510459	152162	3	3	No
Volaille, Saint-Livre	Vo	518348	151230	1	6	No
Aérodrome, Prangins	Ar	509456	139822	0	13	No
Le Courtillet, Pizy	Co	515782	150189	0	11	No
Corbière, Gimel	Cr	511881	151639	0	11	No
Les Côtes, Essertines-sur-Rolle	Cs	513327	148126	0	6	No
Bois Guyot, Bière	Da	517139	153446	0	6	No
Chemin de fer, Etoy	Fe	520920	147824	0	6	No
Sous la Dolle, Gilly	Fs	513944	144712	0	11	No
Le Fossy, Bursins	Fy	513993	143352	0	11	No
Grange des bois, Prangins	Gb	509731	140329	0	18	No
Grandes Tattes, Burtigny	Gt	510612	148999	0	6	No
Inversins	Iv	510163	148322	0	14	No
Longeraie, Gilly	LgEx	513032	145469	0	9	No
Loirin, Gland	Lo	509855	143088	0	9	No
Moulin de Boutecul, Burtigny	Mc	508667	147901	0	6	No
Château de Perroy, Perroy	Pe	518178	146781	0	11	No
Prémondavaux, Burtigny	Px	518178	146781	0	15	No
Prés de Vaux, Begnins	Vx	513327	148126	0	8	No

Development of new microsatellites for *Anguis fragilis*

Identification of new potential microsatellites for *Anguis fragilis*

Out of the 18’190 reads provided by the 454 sequencing selection with MSATCOMMANDER and by eye provided 150 potential microsatellites sequences. For 33 of these sequences primers could be designed. Optimization with different temperatures (50°C, 52°C, 55°C, 57°C or 58°C, 60°C, 62°C) and $MgCl_2$ concentrations (1.5 and 3) allowed the successful amplification and visualisation by gel electrophoresis of 27 (80%) of these sequences. The polymorphism analysis has been performed with the extracted DNA of 11 animals of the study area. 13 microsatellites (40% of the sequences for which primer have been ordered) showed polymorphism in the region of interest according to comparisons of the peaks of the 11 animals on the QIAxcel electropherogram.

Testing of newly identified microsatellites

Analysis with MICRO-CHECKER has been performed with 118 animals in 13 populations and one single locus has shown occurrence of null alleles: locus Af19 in the population “St”, there the software assessed a homozygote excess (expected 18.542, observed 22). Otherwise no other locus in any population showed a large allelic dropout, stuttering errors or evidence of null alleles.
More details about the handling of the microsatellites in the Appendix 5.

Table 2 : The 10 microsatellites developed for *Anguis fragilis.*

Locus	Primer sequence	repeated motif	Occurrence of null alleles
Af19	CAG TGA TTG TGT GGT GTT TAT CTC TCT AGG AGT CTG AGT TTC GGC	$(CAA)_{13}$	Yes
Af22	CAG ATT GCT GAC TGG GAC C GTG ATC TCT GGG AAG TGC CTC	$(TTAT)_{8}$	No
Af24	GCT AGG TAG CGT TCT CC GGGACAGAGCACTTTGTGTG	$(ATT)_{8}$	No
Af34	CCA CAC TCT ACA TGG ACT GC CAC TCT GGA TTA AGT CAA GG	$(GT)_{11}$	No
Af37	GCA TAC ATC AAG TAA CC TCC CTT GTA AAC TGC CCT G	$(GAT)_{14}$	No
Af38	AGA CAG ATA TTT CCC TTG TCA ACC CCA TTG TCG CAG CCA GGC AC	$(ATT)_{12}$	No
Af44	GCC AGG GAA AAC ATA GAT GC CTG TAA ACT GCC GAG TGA G	$(TCTT)_{7}$	No
Af46	GTT GCC TTC TAT GTC ATG TCT CT GCC AAA CAT CAT TAC AAG C	$(ATT)_{9}$	No
Af47	GGT GGT AGA ATG AAC TG CTG GAT GTT GGT GTA GAT G	$(ACC)_{11}$	No
Af50	GTC TTG TAG CCC TTT TCC GTC TGT GAA CTT AGT GTC CG	$(CA)_{18}$	No

Population genetics of the slow-worm

Laboratory analysis

As expected buccal swabs were an efficient and minimally invasive method to obtain genetic material, after extraction of the 118 individuals with the “Qiagen DNeasy kit” most samples contained DNA concentrations of about 20-40 ng/µl, only few samples had less than 10ng/µl. All samples have been successfully amplified by PCR in two multiplex with fluorescent labelled primers with the previously determined conditions. All individuals could be scored for all loci except one single individual which couldn’t be correctly scored for one locus after 6 replications.

Genetic diversity and population differentiation

Following analyses have been performed with the individuals of 15 populations, a total of 118 sampled and scored animals. The dataset contained one missing loci for one individual, meaning 0.8% missing values for Af34.

One locus, Af46, showed significant linkage therefore all further analysis has been performed without Af46. The averaged significance of genotypic linkage disequilibrium of all remaining 9 markers after removing Af46 was 0.203; non-independency between loci can therefore not be rejected. Hardy-Weinberg equilibrium could be accepted for all microsatellites excepted for the locus Af34 were the p-value of 0.003 allowed to accept a deviance from Hardy-Weinberg expectations. Removing this microsatellite didn't change significantly F_{ST} values, in addition analyses with MICRO-CHECKER didn't show any abnormality, therefore this allele has been used for further analyses.

Number of alleles, expected and observed heterozygosity, F_{IS} and F_{ST} for each locus are provided in Table 3. The number of alleles per locus ranged from 3 to 7 with expected heterozygosity ranging from 0.244 to 0.649. F_{IS} values ranged from -0.224 to 0.157 while F_{ST} values ranged from 0.022 to 0.133.

Table 3 : Number of alleles for each locus, Nei's estimations of heterozygosity and F_{IS} and F_{ST} values for each of the 9 loci.

Locus	Number of alleles	H_0	H_S	F_{IS}	F_{ST}
Af19	4	0.375	0.373	0.034	0.103
Af22	5	0.660	0.552	-0.224	0.133
Af24	3	0.375	0.395	-0.005	0.044
Af34	7	0.512	0.649	0.157	0.064
Af37	3	0.244	0.222	-0.103	0.087
Af38	5	0.352	0.352	-0.033	0.022
Af44	4	0.252	0.265	0.014	0.046
Af47	4	0.476	0.452	-0.047	0.079
Af50	5	0.642	0.601	-0.081	0.051

Further, analysis has been performed on the populations, Table 4 provides the mean pairwise F_{IS} and F_{ST} and the allelic richness. These values could only be calculated for the 13 populations with more than 3

sampled animals. Averaged F_{IS} between populations was -0.035, this means that no inbreeding effects have been observed. Two populations Cc and Cu showed low levels of inbreeding (0.2 respectively 0.123). The average allelic richness was low A = 2.10. The total F_{ST} between all populations was also very low (F_{ST} = 0.077) indicating a low degree of genetic differentiation. Differences in sample sizes could imply that some population differentiations were biased, therefore I compared all mean pairwise F_{ST} (Figure 7), it is visible that no population was significantly different of the others. This can assure that there is no outlier and no bias due to the sample size.

Table 4 : Average F_{IS}, F_{ST} and allelic richness (A) values for each sample site.

SITE	Fis	Fst	A
Al	-0.300	0.140	1.80
Bu	-0.175	0.099	2.00
Cc	0.200	0.082	1.90
Cu	0.123	0.043	2.17
Fo	0	0.104	1.90
Ge	-0,086	0.073	2.11
Ps	0.053	0.033	2.46
Ru	0.027	0.038	2.24
Sg	-0.047	0.079	2.11
Sl	-0.011	0.060	2.10
St	-0.001	0.083	2.22
Tt	NA	NA	2.11
Tu	0	0.080	NA
Vi	-0.021	0.092	2.11
Vo	NA	NA	NA

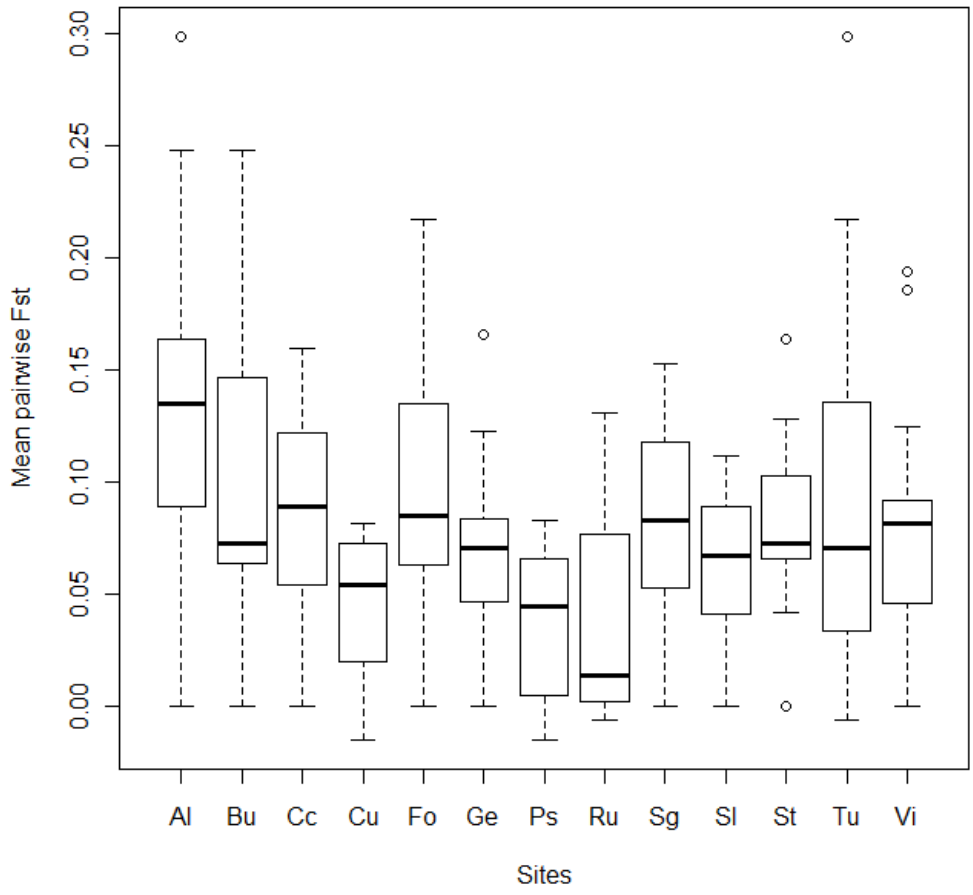


Figure 7 : Mean pairwise F_{ST} values between all 13 sites.

The inference of the number of clusters in the study area with STRUCTURE did not indicate that the study areas show different populations. Inference corrected with the Evanno method (Evanno, Regnaut et al. 2005) showed also the highest likelihood for 1 cluster, therefore all 118 individuals belong to one population.

Landscape genetics

Identification of the variables to be analysed

The first step in the landscape genetic approach was to select and adapt the map for further analysis. The result of the variable analysis and of the possibilities given by the available map led to 12 broad categories which should play a role in slow-worm dispersal based on field work observation and literature (mainly (Völkl and Alfermann 2007)). For this purpose I first reclassified the map in the 12 new categories (see Figure 8), more details of the clustering of each provided in the Appendix 7):

1. **Otherforest**: All type of forests including edges and small forest patches which are not dense and which should in contrary to dense forests represent a suitable habitat for slow-worms and not a barrier, this category contained 13753 pixels and could therefore be analysed with all methods

2. **Dense forest**: Contained only the dense forests present in large patches with a total of 166640 pixels this variable could be correctly analysed. For this variable an edge effect could have been interesting to analyse, since in the map clearings and broad footpaths were not included, buffering the outside edges would have biased the analyses since slow-worms are not able to dissociate clearings or paths from the outside edges. In addition the buffer size of suitable edges would have been difficult to assess and the amount of pixel values probably to low for accurate analysis.

3. **Shrub and bush vegetation**: Contained all types of shorter height vegetation which are thought to be one of the best habitats for slow-worms. Because of this reason these land uses have been clustered together, due to the low amount of this variable (1927 pixels) it could not be analysed with all methods

4. **Roads**: Cluster of all types of roads (38940 pixels) without the highway. Since road killing is an issue for reptiles and amphibians and roads could be an obstacle to dispersal it was important to test the effect of this variable. Since the effect of roads depend highly from their width but also from the profile of its borders it was not possible to assess these effects separately and the general effect of roads have been tested.

5. **Railways**: Included all elements of the railroad network (1903 pixels). It is known that railways represent a suitable habitat for slow-worms, but it is not shown if the width and difficulty to pass them could represent a barrier therefore it was interesting to analyse their effect.

6. **Anthropogenic influenced area**: Included potential anthropized habitats like gardens, parks etc., 15579 pixels. It can be taken for granted that single natural garden and parks are good habitats for slow-worms while intensively used and treated areas are probably not. With this variable I tested the overall effect of anthropogenic influenced area.

7. **Agriculture**: Represent the largest area (191470 pixels) and include all land uses used for agriculture and surroundings. Here there is probably also a difference depending of the intensitivity of use of these areas. Since it is not possible to make a difference between the different agriculture types the overall effect has been tested.

8. **Vineyards**: All types of vineyards (25733 pixels). Same remark as for agriculture, no difference could be made between exploitation types and the overall effect has been tested.

9. **Rivers**: Since slow-worms are not known to swim, crossing of rivers is probably a difficulty. Therefore all rivers of the regions have been clustered in one broad category (14938 pixels).

10. **Highway**: The highway (1616 pixels), crossing the study site and built in the sixtieth represent probably a total barrier to dispersal for slow-worms since no wildlife bridges are crossing it. Therefore this variable had to be tested separately of the other roads.

11. **Other land uses**: Represent land uses which are not interesting for this study or available in a marginal amount (25413 pixels), for example the airport, orchards etc.

12. **Pastures**: Meadows and pastures could represent a suitable habitat and are present in a large amount (30605 pixels). The fact that they are mainly not directly present between sites made the analysis difficult in particular with the strip-based approach.

Former 12 elements are the variables which has been analysed by landscape genetics with the goal to compare there effects to the IBD null model.

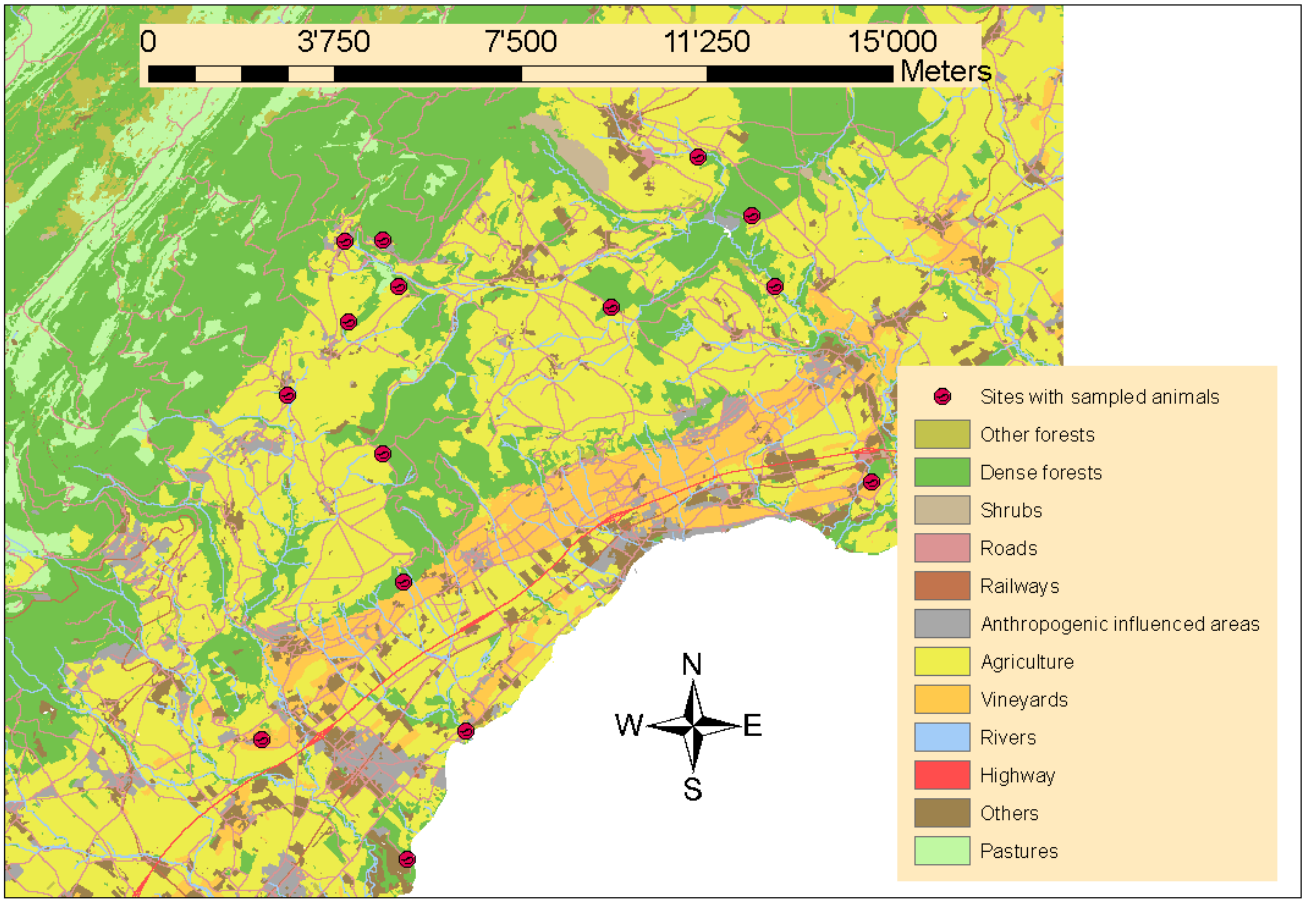


Figure 8 : An overview of the sample region with the raster grid in 12 categories of interest and the sites with sampled animals in pink.

Isolation by distance

The correlation of corrected F_{ST} and the logarithm of distance calculated with mantel tests was positive and significant (r = 0.25, p-value= 0.022) suggesting that the genetic differentiation increases with the geographical distance. As expected from IBD the proportion of explained variance by the model was very low (R^2 = 0.049) but significant (p-value = 0.028).

Least-cost Modelling

For all analysis I used the along least-cost distances in meters to perform the tests as they generally performed better than least-cost distances.

Scenario 1: Test of the following hypothesis: "Dense forests, highway and rivers are the main barriers to gene flow, respectively the landscape units which are fragmenting the populations."

As shown in Figure 9 allocating a cost of 4 to these 3 elements gave the best correlation (correlation = 0.262, p-value = 0.015). Clearly, allocating higher costs to these 3 landscape elements compared to all others had lower positive correlation when compared with the corrected F_{ST}, the highest allocated cost of 50 did not even show significant p-values.

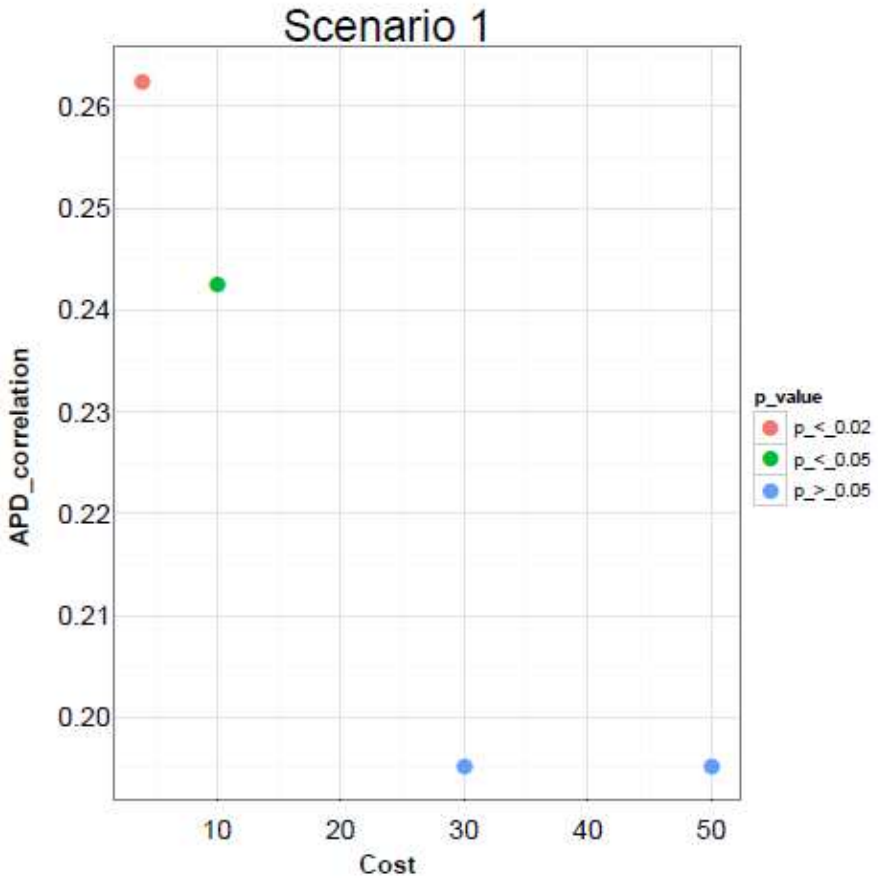


Figure 9 : Along least-cost path correlations of scenario1 and corresponding p-values depending on the allowed costs to dense forests, highway and rivers on the x axis; in blue the points without significant p-value.

Scenario 2: Test of following hypothesis: Primary and secondary habitat according to Völkl and Alfermann (Völkl and Alfermann 2007) are capable to describe dispersal.

The friction maps with a weight of 60 for the « non-habitat » land-uses (see Appendix 8) showed to perform poorly, some were even non-significant (2c, 2f, 2i). Weighting the habitats in ascending order performed better (models 2c-2i), with the best model being "2g", with a "non-habitat" cost of 20

(correlation: 0.256, p-value: 0.0203), there the difference of cost values allocated to habitats and non-habitats are quite small.

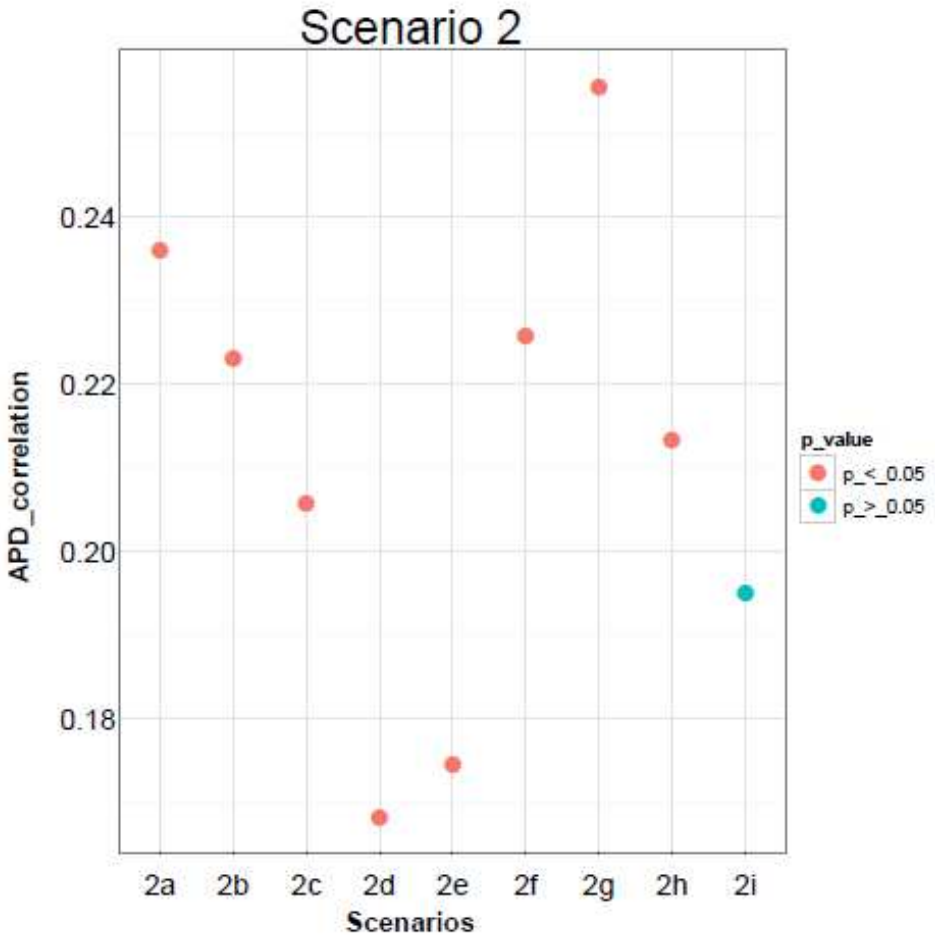


Figure 10 : Results of the APD correlations and their respective p-value when comparing potential primary and secondary habitats to "non-habitat" land-use.

Scenario 3: Disentangling the effect of the 12 different variables one by one and creating a scenario which includes all 12 selected variables with respect to previous analysis.

For each variable I tested different costs and selected the highest correlation for each value (details not shown), when values were equal I chose the value with the lowest p-value (see results in Table 5). In this analysis 6 elements showed a positive effect. Dense forests had only a small correlation, roads, vineyards and railways showed the highest correlation. Rivers and the highway showed an intermediate effect.

Table 5 : Results of the APD correlations and their respective p-values for all elements which showed a fragmentating effect when analysing all different land uses one by one. Beside each land-use the cost with the best correlation is mentioned.

Land use	APD correlation	p-values
Dense forest (Cost of 2)	0.251	0.021
Roads (Cost of 90)	0.253	0.020
Railways (Cost of 40)	0.258	0.020
Vineyards (Cost of 80)	0.260	0.016
Rivers (Cost of 18)	0.260	0.020
Highway (Cost of 15)	0.258	0.016

In the next step I compared the combined friction maps of the best costs assessed previously (see Table 5). It is visible in Figure 11 that the scenario 3A (friction map combined with the exact costs assessed before) performed well (APD_correlation = 0.266, p-value = 0.020). Some costs needed to be checked again when combined, as there allocation (Table 5) was not totally clear before (3B, 3C, 3E), these models showed worse correlation and p-values (see Figure 11) confirming the ranking of the values assessed previously. The test of sensitivity showed clearly that 3J performed the best (APD_correlation = 0.284, p-value = 0.013), in this scenario I changed the values from scenario 3A in respect to their ratio (see Appendix 10).

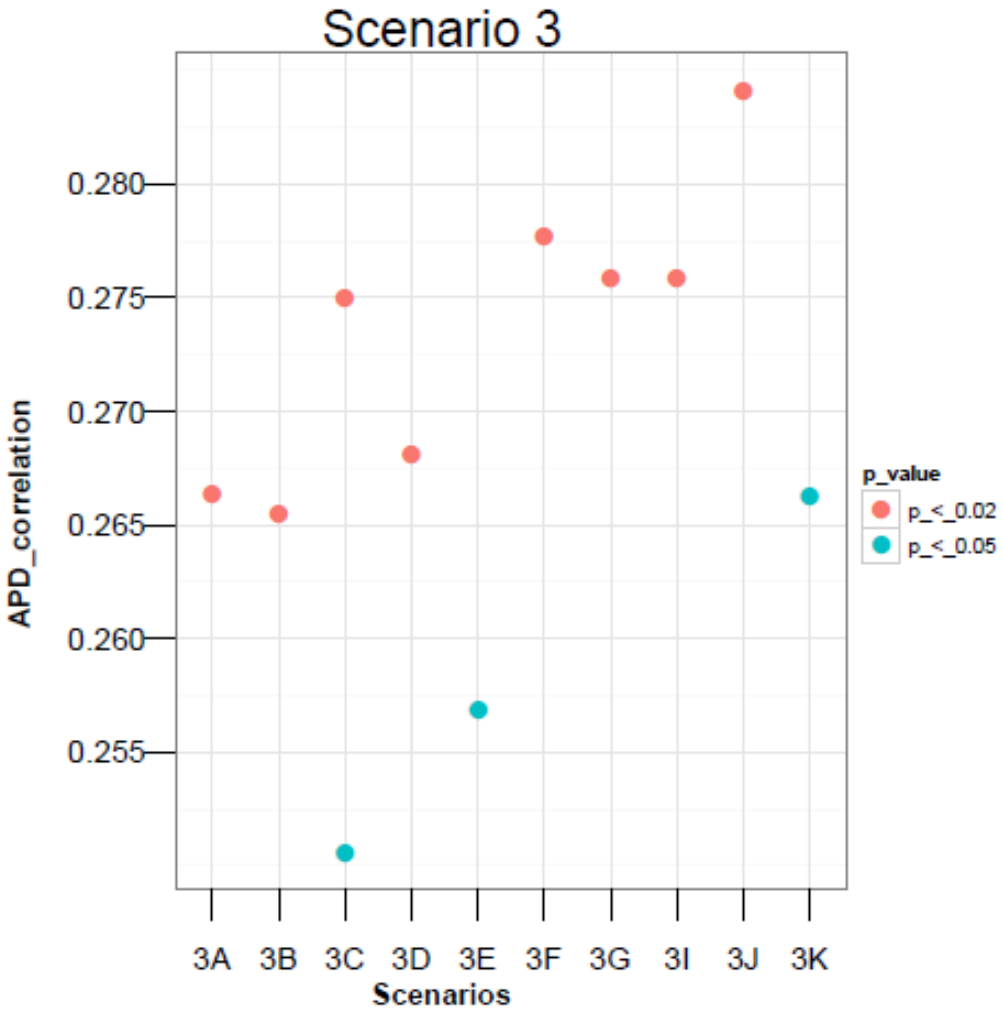


Figure 11 : APD correlations and corresponding p-value of the different scenarios of the third model

The best scenario (3J) showed the highest correlation for a cost of 75 for roads, railways and vineyards (see Table 6). Rivers and the highway showed the best correlation for an intermediate cost of 40. The other elements showed the best results when allocating low costs of 1 or 2 for dense forests.

Table 6 : Land uses and allocating costs of scenario 3J, which showed the best correlation comparing all scenarios

Land use	Costs
Other forest	1
Dense forest	2
Shrub and bush vegetation	1
Roads	75
Railways	75
Anthropogenic influenced areas	1
Agriculture	1
Vineyards	75
Rivers	40
Highway	40
Other land uses	1
Pastures	1

Comparing the 3 scenarios the last scenario (3), with previous analysis of each variable one by one performed best (APD_correlation = 0.284). Scenario 2, based on the habitat knowledge, showed the worst results. The intermediate scenario was the first, which only considered roads, dense forests and rivers as fragmenting land uses.

To disentangle the effect of distance alone and addition of EGDs I performed a partial mantel test on the best model, scenario 3J (see Table 7). The genetic distances are stronger correlated with along least-cost distances (r =0.284) than with Euclidian distances alone (r = 0.248). Along least-cost path distances are strongly correlated with Euclidian distances (not shown here), partialling out the Euclidian distances the genetic distances are still highly correlated with landscape elements (r = 0.218) even if the p-value is only marginally significant.

Table 7 : Correlations of mantel test and partial mantel test removing the effect of Euclidian distances (GeoDist) of the best scenario 3J with 10'000 permutations. In the first column the correlation of genetic distances and along least-cost distances. In the second column the correlation between Euclidian distances and genetic distances. In the third column the correlation of the genetic distance with APD-correlation when partialling out the Euclidian distances.

Analysed variables	APD/ GenDist	GeoDist/ GenDist	GenDist/APD| GeoDist
Correlation	0.284	0.248	0.218
p-value	0.016	0.0270	0.057

After identification of highway being an important element leading to fragmentation I made the same tests with scenario 3J analysing the 10 populations on the northern part of the highway (see results in Table 8). The results showed that the model performed not as good as before since no p-value was significant it was impossible to verify the model in this way. It is possible that too few values biased this analysis.

Table 8 : Analysis with model 3J, when removing the populations in the southern part of the highway.

Analysed variables	APD/ GenDist	GeoDist/ GenDist	GenDist/APD| GeoDist
Correlation	0.218	0.211	0.066
p-value	0.106	0.115	0.325

Strip-based approach

In the first step the different strip type were tested. Models with fixed strips performed slightly better than ratio fixed strips regarding the mean AICs (see Table 9). The best strip model was the one with a fixed width strip of 525m, this model had the lowest mean AIC value of 32.05. In all models the rounded averaged proportion of explained variance was of 10%.

I also tested a model with a 75m strip as many variables showed a very high amount of null values and the results were highly biased I discarded this model for further analyses.

Table 9 : The 9 different strips models analyzed: 3 with a fixed strip width and 5 with different width to lengths ratios. For each model mean AIC has been calculated as well as the averaged proportion of explained variance (Mean R^2).

Class	Width	Mean AIC	Mean R^2 (%)
Fixed	125 m, 5 pixels	34.11	10
Fixed	275 m, 11 pixels	33.86	10
Fixed	525 m, 21 pixels	32.05	10
Ratio	1:1	33.88	10
Ratio	1:3	33.97	10
Ratio	1:5	34.20	10
Ratio	1:7	33.97	10
Ratio	1:9	33.96	10

Regarding all models the best AIC for a single land-use was also in the strip model of 525m, there railways showed an AIC of 28.32. The highest explained part of variance was also in the same model; there the percentage of explained variance reached 16.31 % for the railways.

In the 525m strip model (see Table 10) 7 variables had a higher effect on F_{ST} than distance: railway, highway, other land-use, anthropogenic influenced areas, agriculture, dense forests, other forests and roads. Among these variables 5 had a negative influence on gene flow. Due to their low weighted AIC rivers and vineyards only have a marginal influence in this model.

The best model (see Table 10) with 525m strips showed that railways had the highest impact on gene flow (16.31%), as it is partly correlated with railways it is relevant to analyse both variables together, both account for 28.94% of variance in the model. Both variables had a positive correlation with F_{ST}, meaning a negative effect on gene flow. Other land-use and anthropogenic influenced areas account also to ~ 23% of explained variance, also influencing negatively gene flow. Only two variables had a positive effect on gene flow: agriculture and dense forests, accounting for ~20% of variance.

Table 10 : Analyses of the strip 21, 525m, model strip. Si : Sign of the regression coefficient.

Variable	S_i	AIC	wAIC	R^2 (%)
Railway	+	28.32	0.313	16.31
Highway	+	31.67	0.059	12.63
Other land-use	+	31.7	0.058	12.60
Anthropogenic influenced areas	+	33.61	0.022	10.43
Agriculture	-	33.87	0.019	10.13
Dense forests	-	34.47	0.014	9.45
Other forests	+	35.08	0.011	8.73
Roads	+	35.21	0.010	8.59
Distance	+	32.05	0.048	7.77
Rivers	+	36.42	0.005	7.15
Vineyards	+	36.67	0.005	6.85

Summary of the landscape genetics results

When comparing the 3 landscape genetic models it appeared that the IBD model performed poorly compared with models integrating more landscape elements than only the scale. In the least-cost modelling this has been showed by partialling out the Euclidian distances and for the strip-based approach the distance was poorly explaining the model according to R^2 and wAIC.

To compare both models integrating land uses I compared the degree to which each variable impeding gene flow influenced the models. For the least-cost path approach I calculated percentual costs of elements impeding gene flow. For the strip-based approach I used the pecentual R^2 of all elements impeding gene flow. Since both methods are based on different statistical approaches this method is not strictly correct, but as I am interest in the degree to which each variable act as barrier it was an acceptable method to compare them. As a matter of fact, in both methods the distance is partly included in the results; the EGDs took in account distances (strong correlation) and the regressions of the strip-based method the distance is added in each regression.

In

Table 11 the percentual effect of each element impeding gene flow is assessed comparing both methods. In both methods railway and highway showed the highest impact. Three elements (“Other land use”, “Anthropogenic influenced areas” and “Other forests”) showed a negative effect using the strip-based approach but no negative effect in the least-cost path modelling. In particular “Other land use” and

“Anthropogenic influenced areas” showed a high negative effect in the strip-based approach while no effect has been detected in the least-cost path modelling. “Agriculture” and “dense forests” showed a similar low negative or positive effect on gene flow. Roads and vineyards showed a high effect in the least-cost path modelling but only a marginal effect in the strip-based approach. Rivers had a relatively low effect in both analyses.

Table 11 : Comparison of the elements impeding gene flow in the least-cost path and the strip-based approach

Variable	Least-cost modelling	Strip-based approach
Railway	24%	20%
Highway	13%	15%
Other land use	No	15%
Anthropogenic influenced areas	No	13%
Agriculture	No	No
Dense forests	< 1 %	No
Other forests	No	10%
Roads	24 %	10%
Rivers	13 %	9%
Vineyards	24 %	8%

4. DISCUSSION

Experimental design and sampling of slow-worms

The goal of the experimental design was to have an evenly spaced sampling which includes all landscape elements. A first limitation arose from the fact that it was also essential to target suitable habitats for slow-worms like, for instance, forest edges which were not available everywhere in this highly anthropized area. In addition authorizations from each parcel owner had to be obtained, often several per site, this represented the second challenge when passing from the ideal experimental design to practical field work. Regarding the sites where plates have been installed the prerequisites of experimental design have been entirely respected. Since animals have been found in 15 of the 33 sites the effective sampling did not correspond to ideal experimental design. Even so the spacing of sites with sampled animals allowed an unambiguous analysis of isolation by distance. In addition all landscape elements have been covered and could be analysed separately for their participation to fragmentation with the exception of vineyards, the highway and the railway since no animals have been sampled in-between. Concerning the new plates installed in August they were not colonized probably because this period represent the end of the activity period of slow-worms, this shows the importance of installing plates for such experiments at the beginning of their activity period.

The capture method with tar plates has been effective even if animals have been expected to colonize more capture sites since suitable habitat has been targeted. It was also expected to find much more animals per sites once slow-worms were present. Some reptiles chose their artificial refugees on very specific thermal properties whereas some other species are rather unselective (Thierry, Lettink et al. 2009) it appears also clearly, that other factors are influencing species when choosing a refuge like the physical properties or the food availability below it. Considering thermal criteria the choice of the black tar plates seemed to be good since they absorb a high amount of the heat which slow-worms can gain by conduction. This effect is even amplified by the undulated form of the plates which allows slow-worm to fit between the undulations increasing the contact zone to gain heat. The major factor to find slow-worms in a site was certainly their presence in the surrounding area, but even so they “chose” the plates to colonize and colonized the plates in different ways depending probably on microhabitat conditions. As a matter of fact in some sites slow-worms were clustered under one or a few tar plates during all field work period whereas in other sites they were distributed under the plates moving regularly from one plate to the others in a one week time span. Since in these situations insolation conditions were the same (same expositions, comparable umbrage) microhabitats conditions seemed to be the decisive factor for colonizing plates. In particular plates in humid conditions were preferred, this can partly be explained by higher food availability; also soil composition seemed to play a role since different soils absorb heat

differently and slow-worms need loose soil configuration in which they can burrow. Finally tar plates seems also to be effective since all life stages and both sexes have been observed under the plates confirming that no bias are introduced due to a lack of young or old sampled animals. Besides the preferences due to the habitat properties others factors could also play a role. It has been shown that slow-worms are able to discriminate conspecific scents (Gonzalo, Cabido et al. 2004) this could explain the absence of slow-worms in some sites where alternative, older refugees were present. This could be observed in 2 sites where slow-worms colonized older artificial refugees and did not colonize the new tar plates placed around the older refugees, even when placing the slow-worms under the new plates after sampling they returned to the older plates, often colonized by other individuals, the next day. If slow-worms showed the same "behaviour" with natural refugees which could not be detected this could explain the absence of slow-worm in several sites.

Population genetics of the slow-worm

Next-generation sequencing allowed developing a set of 9 suitable microsatellites for a species for which no markers were available yet in about 3 month. For 8 microsatellites I found no occurrence of null alleles (one microsatellite, Af19, showed possible occurrence for null alleles in one single population), no significant linkage disequilibrium could be showed between all markers which generally respected Hardy-Weinberg expectations and where therefore suitable for further genetic population analyses.

The sampling size allowed calculating F-statistics for 13 of the 15 sites where animals have been sampled. F-statistics are generally sensitive to the sampling size therefore the results had to be tested for possible bias in populations with a lower amount of animals. As a matter of fact differences in the F-statistics results could arise because the sampled populations are only a subset of all populations that could be sampled or because the sampled populations are only one possible outcome of an underlying stochastic evolutionary process (Holsinger and Weir 2009). To be able to control for this effect I compared the mean F_{ST} of all sites, since no outliers were found all 13 remaining populations have been used for further analysis since no difference could be attributed to the low sample size.

According to the F_{ST} values overall differentiation was low ($F_{ST} = 0.077$) in this 16 km^2 sample region in Western Switzerland since it has been shown that an F_{ST} of above ~ 0.15 can be considered as an indication of significant differentiation (Frankham, Ballou et al. 2010). In this study no mean F_{ST} value exceeded 0.15. In addition, results also showed no inbreeding depression in the sample sites. These results are confirmed by the individual assignment method which was unable to gather some individuals together, not even animals belonging to the same study site. These results suggested a strong gene flow between sites. Even if the exact dispersal "behaviour" of slow-worms is not known it has been showed for

different species that generally a decreased differentiation calculated with F_{ST} is associated with increased dispersal (Bohonak 1999). These facts suggests that the dispersal capacities of slow-worms are underestimated and support the hypothesis that some individuals are migrating to allow gene flow (Völkl and Alfermann 2007). In addition the absence of inbreeding tends to show an under-detection of slow-worms since the small number of animals sampled would probably lead to consanguineous mating and lead to an inbreeding depression which could not be showed here. The low degree of differentiation measured with F_{ST} and the absence of inbreeding measured with F_{IS} are both measures related to the variance in allele frequency since they reflect a possible reduction in heterozygosity when compared to Hardy-Weinberg expectations (Holsinger and Weir 2009). Most deleterious alleles are recessive, their harmful effect being only expressed in homozygotes. Since no reduced heterozygosity could be demonstrated here it can be conclude that slow-worm populations are not endangered by fragmentation and the effects of subsequent genetic drift and inbreeding depression in small populations.

Landscape genetics

The first sensitive step in the landscape genetics was to select a set of variables adapted to the species and the scale of the sample area. In addition due to the scarce knowledge about habitat and dispersal of slow-worms the set of variables had to be chosen in respect to their possible effect on slow-worm dispersal and resulting gene flow. The literature analysis and field work observation used to select the 12 variables showed to be efficient since all major elements showed to have an impact on gene flow. Nevertheless it was essential to cluster the suitable habitats in broader but similar categories, for example separating dense forests which are thought to be a barrier and clustering all other types of favourable forests types. With this set of variable the broad effect of each land use have been tested, further analysis should also include more specific maps to study also finer effects, for example edge effects which have not been studied here, since the scale of the map did not allow it.

The ultimate goal of the landscape genetics methods was to analyse these variables comparing them to the results of the population analysis results. Since it has been suggested to analyse data sets of population genetics with several approaches (Excoffier and Heckel 2006) here 3 different approaches will be discussed: isolation by distance, least-cost modelling and the strip-based approach.
The first method, which can be seen as the simplest approach, in which scale is the only landscape variable is the isolation by distance (IBD) model, which can also be seen as the null model for further methods. A significant IBD effect has been detected; regarding to the size and the dispersal abilities of lizards it seems logical that at the scale of the sample region higher distances implied a higher genetic

differentiation since gene flow is related to dispersal. Nevertheless the rather low effect of IBD justified landscape genetics since scale was just partly explaining gene flow.
The least-cost modelling showed the importance of previous analysis of each variable. The models using habitat knowledge to explain gene flow performed poorly. This can be explained by a poor knowledge of habitat preferences and the fact that temporary dispersal habitat can be different of the definitive habitat. The best model was therefore the model in which each variable has been assessed separately excluding as far as possible the bias due to the scarce knowledge.

Further the main result of this study was the detection of the elements which represent barriers to gene flow analyzing the best models of each method. Both landscape genetics methods showed the highly negative effect of the highway and railroads which are crossing the sample site, these elements, in less extend also vineyards, were partly correlated and disentangle their effect statistically was impossible. Since railroads are known to be a potential habitat for slow-worms and are not very broad in this region this is probably not the main barrier here. It is more likely that the negative effect of gene flow is caused by the highway built in the sixties since in this region there are no real possibilities to traverse it indirectly for example with a wildlife crossing, and the mortality by crossing this broad element is probably very high. Another hypothesis could be that there is a combined effect of the vineyard belt, the railroad and the highway which are creating a broad barrier. This could be supported by the fact that plenty of sites have been placed between these elements without any presence of slow-worms. However if this hypothesis should be confirmed suitable habitat possibilities exist on both sides and populations will probably be more and more differentiated on each side in the future but not endangered since large amount of suitable habitat exist on both sides.
In a less extend roads and rivers represented also a barrier even if in this case they impede gene flow rather than really stopping it. This is supported by the fact that both elements especially rivers are not recent elements, if their effect had been higher, a higher differentiation would probably be assessed nowadays. Here again the broad effect of these elements could be assessed. Clearly slow-worms can not cross a large river, but it could be possible that they cross the bridges or shallow areas. Roads represent probably a barrier in particular when they are large and the border difficult to cross for slow-worms. As a matter of fact even if a slow-worm cross a road it will probably not be able to “climb” on sidewalks and remaining on the road increase the mortality risk.
Dense forests in contrary had a positive effect on gene flow; they seemed to be used partly as dispersal corridors. It is not shown if only the edges are used for dispersal, but these forests are crossed by several trails and clearings which obviously allow dispersal but are not detectable with the used map. Agriculture seemed also to represent a suitable corridor, even if during field work it did not seem to be a suitable long term habitat. Both of these elements used as corridors would probably not be the primary dispersal

corridor for slow-worms when other more favourable elements were present. Nevertheless, since the study area is strongly influenced by the human activity these facts strongly suggest that slow-worms use the most suitable habitats represented here by agricultural areas and forests to disperse. In the end these areas, probably mainly the edges, allowed slow-worms to find natural or artificial refugees to hide, gain heat and feed and showed the ability of slow-worms to adapt to anthropized areas.

"Other land use", "anthropogenic influenced areas" and "other forests" showed a high effect in the strip-based approach but no negative effect in the least-cost path modelling method. These 3 variables are distributed in patches, there the density plays an important role and it is probable that least-cost path did not detect them due to the distribution of sampled populations, but would eventually detect them if the sites were placed in a different way. For these reasons, they will not be discussed in relation with the methods here.

Since the different methods showed some different results for different landscape elements the importance of using several approaches for landscape genetics and to analyse each result taking in account strength and drawbacks of each method has been demonstrated here.

The advantage of the least-cost path method was to be able to detect linear elements much better than the strip-based method which detected linear elements only when the effect on gene flow is very high like for the railways and the highway. This could be observed for the variables roads and rivers, these 2 elements have been far less detected by the strip-based approach since for these elements the shape is more important than the density regarding dispersal of an organism. As a matter of fact if a barrier is present in a low amount of pixels it will highly impede gene flow if this element cuts a dispersal corridor and this can not be assessed in a linear relation.

Since least-cost path computes the most probable path based on a cost grid it could happen that it did not detect several patchy elements when 2 sites were not directly enclosing it. Least-cost modelling would probably perform better and detect more elements with sites covering more landscape elements.

Concerning the strip-based approach the amount of sites did not seem to be a limitation. Here the limitation occurs for strongly zero-inflated elements which could not be analysed and for linear elements which were under-detected due to the linearity of the analysis.

Regarding previous assumptions an important drawback of least-cost modelling is that it is based on expert knowledge; here I overcame the scarce knowledge about the species by analysing not only paths based on expert knowledge but also each variable one by one. In addition the calculated route is the most probable one, but effective dispersal route is unknown. In contrary the strip-based approach does not need expert knowledge on dispersal, but important assumptions have also to be made when using straight strips as dispersal corridors. Even if these different straight width strips are tested statistically to select the best fitting one they are only correct when we act on the assumptions that we are analysing gene flow in infinite populations which would allow having a straight gene flow by chance. This could explain that the

best model was the one with the fixed strip width of 525m, showing that the animals need broad corridors to disperse.

In addition since the strips are analysed in a pairwise fashion we had to assume that “migration” happened in all directions equally. Therefore the larger the strip size the more probable that individuals dispersed in this area and that the model analysed fit to the analysed data, this probably explains that the best strip size was the widest fix strip size of 525m. The results showed that the width of “strips” computed by least-cost path was often much larger than the 525m and that the path did not always start in the direction of the next site. The least-cost path modelling is therefore better reflecting the dispersal “decision” of individuals than directly gene flow, but it is probable that the highest fraction of slow-worms are using the most probable route between 2 points this way least-cost paths are also representing gene flow.

Finally, since the strip-based approach include directly distances in the regressions, no further analysis are necessary to disentangle the effect of distance versus all other variables. In contrary in the least-cost path modelling further analysis with partial mantel test with subsequent possible bias are necessary for the same analysis.

5. CONCLUSION AND OUTLOOK

Even if studies about fragmentation gained in interest, reptiles are still an understudied group regarding this aspect (McGarigal and Cushman 2002). With this study a deeper insight in the effect of fragmentation on population genetics of the slow-worm could be obtained to enlarge the knowledge about fragmentation in this group.

Knowledge about slow-worms is particularly scarce; the development of genetic markers widened the knowledge from observational studies to the genetic structure of populations and will hopefully also allow further studies in this direction. The low differentiation flow assessed here, suggests an important gene flow between sites and tend to support the hypothesis (Völkl and Alfermann 2007) that some individuals migrate to allow this gene flow. Future studies should be performed in particular at different scales to understand the overall evolutionary mechanisms leading to this structure, but also in similar areas to avoid local interpretation.

The efficient approach with landscape genetics using several approaches could also be demonstrated, since it allowed identifying successfully different landscape elements leading to the genetic structure. These approaches were also powerful overcoming the lack of prior knowledge about habitat preferences of this species by careful analysis of each variable one by one.

Since gene flow can be linked with dispersal the landscape genetic approaches identified the major dispersal corridors and barriers for slow-worms which could be useful in future conservation approaches for this species. In particular it could be showed that slow-worms could adapt to human activities in using for example agricultural areas as corridors. Nevertheless to get a complete insight in slow-worm behaviour, habitat preferences should be assessed in a scientific approach and exact dispersal capacities should be tested, e.g. with telemetry.

Finally as it has been pointed out in 2009 by Balkenhol et al. (Balkenhol, Waits et al. 2009) there is a real need to improve statistical methods for landscape genetics, in particular developing non-linear multivariate methods because the methods commonly used today in particular the mantel test produce a high amount of type I errors. This was also an issue here, since the mantel test is used to compare the genetic data to least-cost paths. In a new study Legendre et al. (Legendre and Fortin 2010) compared the mantel test to other methods and advised to use multiple regressions when investigating environmental and spatial response variables. This fact has been taken into account in the analysis of the strip-based approach where genetical data have been analysed with multiple linear regressions. However, to be able to assess in which situations each method perform best, and to disentangle the differences between both methods a simulation study would be necessary in the future

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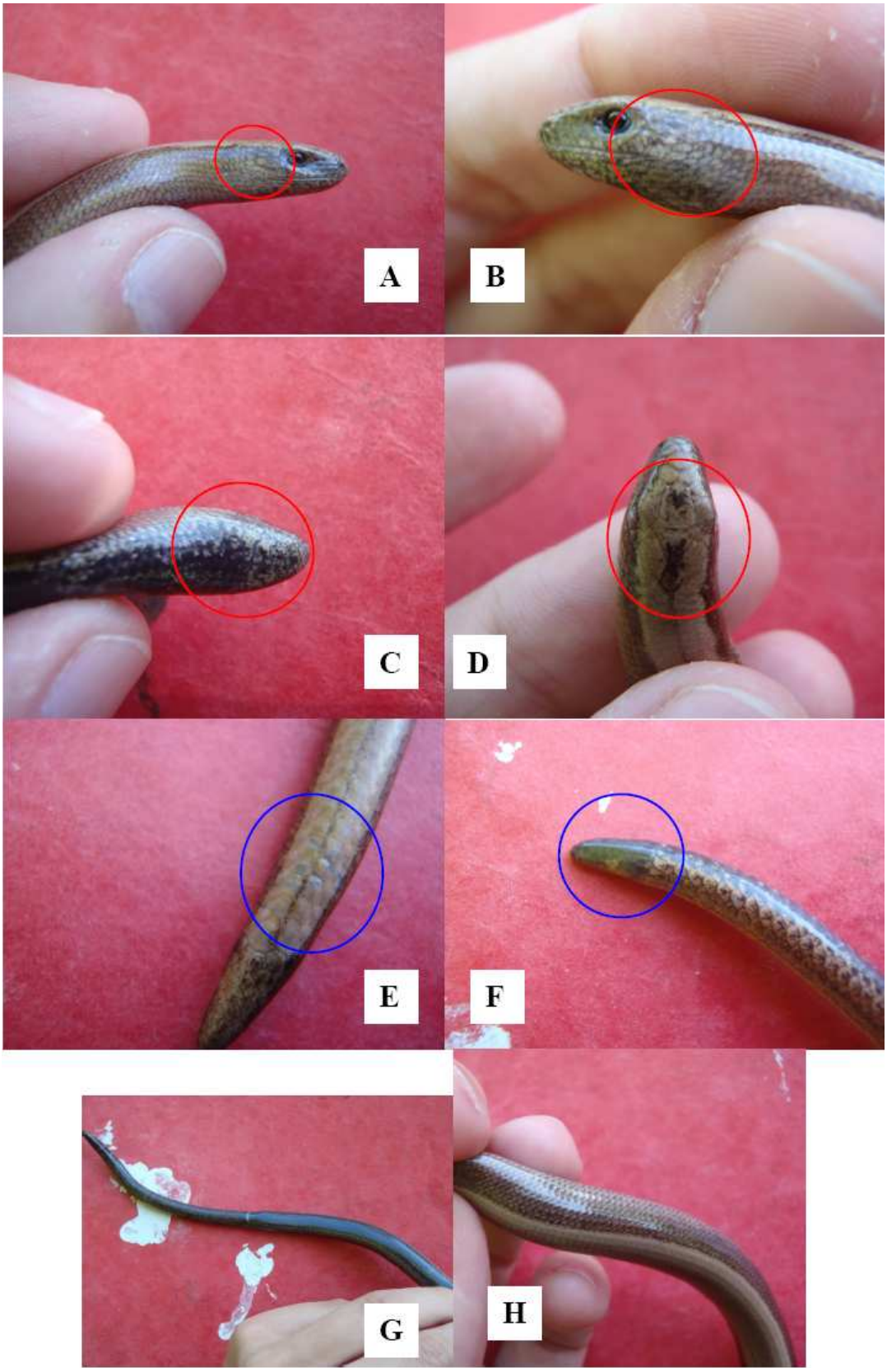
8. APPENDIX

Appendix 1 : Tar plates used to « trap » slow-worms.

Appendix 2 : Sheets which has been fixed on each plate to inform passers-by not to remove the plates.

Appendix 3 : Buccal swabing of a slow-worm.



Appendix 4 : Photographic identification of individual 122 of the site "Sl". To identify an individual I used the ornamentation patterns on the head (A-D), patterns at the head side allowed to identify also juveniles at an individual level properly. Picture E and G shows all particularities of the animals here scars (E) and probably traces of previous autotomy (F). For each animal I took a picture of underneath and the difference between the dorsal and ventral coloration.

Appendix 5 : Details about the handling of the microsatellites. Number of PCR cycles, annealing temperature, $MgCl_2$ concentration, theoretical length of the microsatellites, colour of the fluorescent label, multiplex reactions and the amount of each diluted primer in the reaction.

Locus	Number of PCR cycles	Annealing temperature [°C]	$MgCl_2$ [mM]	Length [bp]	Flurorescent label	Multiplex reaction	Amount of each primer in 10 µl PCR-mix
Af19	40	55°C	3 mM	144	Black	2	0.5 µl
Af22	40	55°C	3 mM	247	Black	2	0.5 µl
Af24	40	50°C	1.5 mM	130	Black	1	0.5 µl
Af34	40	55°C	3 mM	226	Green	2	0.3 µl
Af37	40	55°C	3 mM	146	Red	2	0.3 µl
Af38	40	50°C	1.5 mM	197	Red	1	0.5 µl
Af44	33	60°C	3 mM	154	Green	2	0.3 µl
Af46	40	52°C	3 mM	331	Black	1	0.5 µl
Af47	40	52°C	3 mM	183	Green	1	0.3 µl
Af50	40	52°C	1.5 mM	154	Red	1	0.4 µl

Appendix 6 : Pairwise F_{ST} values calculated between the 13 sites where more than 2 individuals have been analysed.

	Al	Bu	Cc	Cu	Fo	Ge	Ps	Ru	Sg	Sl	St	Tu	Vi
Al	0.000	0.2669	0.1667	0.0433	0.1304	0.1771	0.0772	0.1524	0.1617	0.0991	0.1745	0.2771	0.1
Bu		0.000	0.1198	0.0648	0.2058	0.1111	0.0601	-0.0129	0.1442	0.052	0.0673	-0.0023	0.2041
Cc			0.000	0.0705	0.1549	0.0678	0.0513	0.0068	0.1204	0.0731	0.1351	0.0625	0.0333
Cu				0.000	0.0573	0.0868	-0.0301	0.0004	0.0718	0.0312	0.078	-0.0087	0.099
Fo					0.000	0.0979	0.0244	0.1169	0.046	0.0812	0.1083	0.2258	0.1
Ge						0.000	0.0279	0.0496	0.0365	0.0717	0.0694	0.0912	0.0578
Ps							0.000	-0.0062	0.0177	0.0674	0.0764	0.0338	0.0302
Ru								0.000	0.0876	0.0074	0.043	-0.0419	0.0951
Sg									0.000	0.0988	0.0899	0.1077	0.0443
Sl										0.000	0.0468	0.0454	0.1032
St											0.000	0.0555	0.1377
Tu												0.000	0.1894
Vi													0.000

Appendix 7 : Clustered categories of the 61 original land uses in 12 categories.

	Original Pixel Value	New category
Forest fresh cuts	9	Other forest
Other forest	10	Other forest
Normal dense forest	11	Dense forest
Open forest (on unproductive area)	12	Other forest
Open forest (on agricultural areas)	13	Other forest
Forest stripes, edges	14	Other forest
Brush forest	15	Shrub and bush vegetation
Scrub Vegetation	16	Shrub and bush vegetation
Clusters of trees (on agricultural areas)	18	Other forest
Other woods	19	Other forest
Motorways	31	Highway
Roads and paths	33	Roads
Parking areas	34	Roads
Railway station grounds	35	Railways
Railway lines	36	Railways
Airports	37	Other land uses
Airfields, green airports environs	38	Other land uses
Industrial ground	41	Other land uses
Land around 25	45	Anthropogenic influenced area
Land around 26	46	Anthropogenic influenced area
Land around 27	47	Anthropogenic influenced area
Land around 28	48	Agriculture
Land around 29	49	Other land uses
Sport ground	51	Other land uses
Garden allotments	52	Anthropogenic influenced area
Camping, caravan sites	53	Anthropogenic influenced area
Golf courses	54	Other land uses
Cemeteries	56	Other land uses
Public parks	59	Anthropogenic influenced area
Other energy building	61	Other land uses

Other supply or waste treatment plants	62	Other land uses
Waste water treatment plants	63	Other land uses
Discharges	64	Other land uses
Quarries, mines, dumps	65	Other land uses
Green railway environs	67	Railways
Green roads environs	68	Other land uses
Regular vineyards	71	Vineyards
“Pergola” vineyards	72	Vineyards
Extensive vines	73	Vineyards
Intensive orchards	75	Other land uses
Rows of fruit trees	76	Other land uses
Horticulture	78	Other land uses
Favourable arable land and meadows	81	Agriculture
Other arable land and meadows	82	Agriculture
Farm pastures	83	Agriculture
Brush meadows and farm pastures	84	Agriculture
Mountain meadows	85	Pastures
Brush alpine pastures	86	Pastures
Remote and steep alpine meadows and pastures	87	Pastures
Favourable alpine pastures	88	Pastures
Rocky alpine pastures	89	Pastures
Glacier	90	Other land uses
Lake	91	0
River	92	Rivers
Wetlands	95	Other land uses
Water shore vegetation	96	Rivers
Unproductive grass and shrubs	97	Shrub and bush vegetation
Bare rocks	99	Other land uses
	NoData	0

Appendix 8 : Habitat categories of scenario2.

	Original Pixel Value	Scenario 2
Forest fresh cuts	9	Primary habitat type 1
Other forest	10	Primary habitat type 1
Normal dense forest	11	Not potential habitat
Open forest (on unproductive area)	12	Primary habitat type 1
Open forest (on agricultural areas)	13	Primary habitat type 1
Forest stripes, edges	14	Primary habitat type 2
Brush forest	15	Primary habitat type 1
Scrub Vegetation	16	Primary habitat type 1
Clusters of trees (on agricultural areas)	18	Primary habitat type 2
Other woods	19	Primary habitat type 1
Motorways	31	Not potential habitat
Roads and paths	33	Not potential habitat
Parking areas	34	Not potential habitat
Railway station grounds	35	Secondary habitat type 2
Railway lines	36	Secondary habitat type 1
Airports	37	Not potential habitat
Airfields, green airports environs	38	Secondary habitat type 2
Industrial ground	41	Not potential habitat
Land around 25	45	Secondary habitat type 2
Land around 26	46	Secondary habitat type 2
Land around 27	47	Secondary habitat type 2
Land around 28	48	Secondary habitat type 2
Land around 29	49	Secondary habitat type 2
Sport ground	51	Secondary habitat type 2
Garden allotments	52	Secondary habitat type 1
Camping, caravan sites	53	Secondary habitat type 2
Golf courses	54	Secondary habitat type 2
Cemeteries	56	Secondary habitat type 1
Public parks	59	Secondary habitat type 1
Other energy building	61	Not potential habitat
Other supply or waste treatment plants	62	Not potential habitat

Waste water treatment plants	63	Not potential habitat
Discharges	64	Not potential habitat
Quarries, mines, dumps	65	Secondary habitat type 2
Green railway environs	67	Secondary habitat type 1
Green roads environs	68	Secondary habitat type 1
Regular vineyards	71	Secondary habitat type 1
“Pergola” vineyards	72	Secondary habitat type 1
Extensive vines	73	Secondary habitat type 1
Intensive orchards	75	Not potential habitat
Rows of fruit trees	76	Not potential habitat
Horticulture	78	Not potential habitat
Favourable arable land and meadows	81	Not potential habitat
Other arable land and meadows	82	Not potential habitat
Farm pastures	83	Not potential habitat
Brush meadows and farm pastures	84	Primary habitat type 1
Mountain meadows	85	Primary habitat type 1
Brush alpine pastures	86	Primary habitat type 1
Remote and steep alpine meadows and pastures	87	Primary habitat type 1
Favourable alpine pastures	88	Primary habitat type 1
Rocky alpine pastures	89	Primary habitat type 1
Glacier	90	Not potential habitat
Lake	91	0
River	92	Not potential habitat
Wetlands	95	Primary habitat type 2
Water shore vegetation	96	Primary habitat type 1
Unproductive grass and shrubs	97	Primary habitat type 1
Bare rocks	99	Not potential habitat

Appendix 9 : Table of the different friction maps tested for scenario 2

Scenarios	Cost of category 1	Cost of category 2	Cost of category 3	Cost of category 4	Cost of category 5
2a	1	1	1	1	20
2b	1	1	1	1	40
2c	1	1	1	1	60
2d	1	2	3	4	20
2e	1	2	3	4	40
2f	1	2	3	4	40
2g	3	4	10	11	20
2h	3	4	10	11	40
2i	3	4	10	11	60

Appendix 10 : Different friction maps for scenario 3.

	A	B	C	D	E	F	G	H	I	J	K
Land use											
Other forest	1	1	1	1	1	1	1	1	1	1	1
Dense forest	2	1	2	2	5	2	2	2	2	2	2
Shrub and bush vegetation	1	1	1	1	1	1	1	1	1	1	1
Roads	90	90	90	50	75	20	75	90	30	75	75
Railways	40	40	40	50	30	20	75	90	30	75	75
Anthropogenic influenced areas	1	1	1	1	1	1	1	1	1	1	1
Agriculture	2	2	1	1	1	1	1	1	1	1	1
Vineyards	80	80	80	50	75	20	75	90	30	75	75
Rivers	18	18	18	10	15	5	20	30	10	40	20
Highway	15	15	15	10	15	5	20	30	10	40	20
Other land uses	1	1	1	1	1	1	1	1	1	1	1
Pastures	1	1	1	1	1	1	1	1	1	1	1