EVALUATION OF ECOLOGICAL NETWORK EFFECTIVENESS BY MEANS OF GENE FLOW ANALYSIS

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EVALUATION OF ECOLOGICAL NETWORK EFFECTIVENESS BY MEANS OF GENE FLOW ANALYSIS

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1. Introduction

Biological diversity, or biodiversity, is a term we used to describe the variety of ecosystem, species, populations within species, and genetic diversity within species. The biodiversity of our planet is rapidly decreased as a direct and indirect consequence of human actions: a lot of species are already extinct, while many other are reduced population size that put them at risk, in fact lot of them now require benign human intervention to improve their management and ensure their survival (Frankham et al. 2002).
Despite of the importance of biodiversity is recognized by a long time, anthropic pressure still persist: the five main pressures on natural ecosystems are habitat destruction; introduction of alien species; over-exploitation and unsustainable use of natural resources; climate change; and pollution or excessive nutrient load (Wilcove et al. 1998; Primack, 2010; Groom et al. 2006; Kareiva & Marvier, 2011). All these factors are related to human population size, for this reason the importance of these elements will increase in the future as the human population will continue to grow. Human-related factors usually reduce species to population sizes where they are susceptible to stochastic effects: these ones naturally occurring in all populations, but they increase the risk of extinction in small populations. Stochastic factor may have environmental, catastrophic, demographic, or genetic origins, the latter include inbreeding depression (deleterious effects on reproduction and survival of

offspring result of breeding of related individuals), loss of genetic diversity (that involve the ability to evolve in response of environmental change), genetic drift (random processes that override natural selection as the main evolutionary process) and mutational accumulation. All of these factors rather play cumulatively or interactively so, even if the cause of populations decline is removed, problems associated with small populations will persist.

The research is focus on the problem of habitat destruction. First of all, it is important to underline that the term ‘‘habitat fragmentation’’ is often used ambiguously for several landscape scale processes including habitat loss (physical loss), habitat fragmentation per se (the breaking apart of formerly contiguous habitat *sensu* Fahrig 2003), and disruption in structural connectivity (e.g. disruption in the network of hedgerows connecting patches Fischer & Lindenmayer 2007). Two landscapes with the same amount of habitat may have both different levels of habitat subdivision and different levels of structural connectivity (e.g. hedgerows in forested landscapes; Fischer & Lindenmayer 2007; Radford & Bennett 2007).

All of these cases lead to an overall reduction in animal population size and a reduction of migration rate among patches, therefore we will have fragmented populations (populations that are separated into partially isolated fragments). These are also known as meta-populations (*sensu* Levins 1969) that are defined as a network of spatially structured populations consisting of distinct discrete units (i.e. sub-populations) separated by space or barriers, and connected by dispersal movements: the amount of dispersal between sub-populations represents the degree of their ecological connectivity (Hanski & Simberloff, 1997). The main elements for dispersal in the landscape are the distance and the land use between sites, the presence of corridors, and the barrier effect of landscape (Opdam, 1991).

The genetic impacts of population fragmentation depend critically upon gene flow among fragments: with restricted gene flow, fragmentation typically leads to greater inbreeding and loss of genetic diversity within fragments. Each fragments became isolated so they will arise genetic differentiation due to genetic drift and this

will lead the populations to a greater risks of extinction, in long term, than for a single population of the same total size (Frankham et al. 2002). The knowledge of the ecology of fragmented populations is essential in order to prevent their isolation or even restoring the ecological connectivity between them (Saunders et al. 1987; Burgman & Lindenmayer, 1998).

The improvement of tools for protecting biodiversity requires maintaining habitat connectivity to build efficient ecological networks that facilitate the movement of species under pressure from global change. An ecological network is a framework of ecological components, e.g. core areas, corridors and buffer zones, which provides the physical conditions necessary for ecosystems and species populations to survive in a human-dominated landscape. The networks should be based on functional connectivity (the movement of individuals among patches) rather than on structural connectivity (a measure of how spatially connected the elements of a landscape are, without any reference to any particular ecological process) alone. Ecological networks are usually designed by using expert-based approaches or habitat suitability models. Unfortunately, the effectiveness of ecological networks is seldom adequately assessed, because traditional approaches, like radio-tracking or capture-mark-recapture methods, do not take into account reproductive events (White & Garrott, 1990; Barrett & Peles, 1999; Fagan & Calabrese, 2006). Instead, the presence of inter-breeding populations can be assessed by using DNA molecular markers, that are able to detect gene flow in a meta-population.

It is important to underline that ecological networks need to incorporate habitat connectivity for species with different ecological requirements, for this reason we used focal species that required habitat reconstruction because they are limited by a shortage of critical resources, an inability to move between suitable habitat patches, or insufficient habitat to meet their resource needs (Lambeck, 1997).

Focal species have the following characteristics, they are:

- resource-limited species, when the number of individuals that a region can support is determined by the carrying capacity at the time of lowest resource availability;
- dispersal-limited species, when there are suitable habitat patches to support small populations, but the patches are beyond the distance over which individuals can move or are separated by a matrix that is too hostile to permit movement;
- area-limited species are those for which the patches of appropriate habitat are simply too small to support a breeding pair, or, in the case of colonial species, a functional social group;
- process-limited species, when they depend on ecological processes.

The aim of this project is to study the fragmentation in broad-leaved forests, so the species selected were mainly linked to this ecosystem. Almost all amphibian species are model candidates for studies of fragmentation effects on connectivity (Moore et al. 2011): in fact most amphibian species occur as metapopulations (Smith & Green, 2005); in addition, they are a *taxon* particularly susceptible to isolation, as they generally have low dispersal capabilities (Allentoft & O'Brien, 2010) and are rather philopatric to breeding sites (Blaustein et al. 1994). These characteristics often lead to high genetic differentiation, even at restricted scales (Allentoft & O'Brien, 2010). In particular we choose the Fire Salamander (*Salamandra salamandra, AMPHIBIA, URODELA*) that is strongly linked to broad-leaved forest ecosystems, depends on ecosystem processes (such as those that allow the development of an adequate litter structure); is strongly affected by specific resources (such as hydrology, some chemical and physical water parameters); and it is also limited by a low dispersal capability (Lanza et al. 2007).

However not only amphibian species are affected by habitat fragmentation, also arboreal mammals, such tree squirrels and dormice, are often more threatened than other species due to their low dispersal capability in absence of structural connection between habitat patches (Mortelliti et al. 2009). In European regions, various

single-species studies have been carried out, suggesting similar responses in this guild of mammals (e.g. Bright et al. 1994; Rodriguez & Andrèn, 1999; Koprowski, 2005; Mortelliti, 2013). Among them, the Hazel Dormouse (*Muscardinus avellanarius, RODENTIA, GLIRIDAE*) is an arboreal rodent living in woodland and hedgerows, whose presence is negatively affected by fragmentation (Bright et al. 1994; Mortelliti et al. 2011). For its poor conservation status and negative long-term trend in almost all Europe, the species is included in the Annex IV of the 92/43/ECC Directive (Habitat Directive), concerning species that require a strict protection regime that must be applied across their entire natural range within the EU. For this reason we sampled also the Hazel Dormouse that can be defined as a focal species *sensu* Lambeck (1997) at least for three out four ecological traits (they are limited by resources, dispersal capability, and ecological processes).
We choose two different areas of study, both affected by the habitat fragmentation: Lombardy Region (Northern Italy, where we worked on the Fire Salamander), and Latium Region (central Italy, where we sampled the Dormouse). In both regions we sampled a fragmented forest area and a continuous one, our control.
The use of traditional methods like radio telemetry or capture–mark–recapture (White & Garrott, 1990; Barrett & Peles, 1999; Tracey, 2006) is alone insufficient for understanding ecological connectivity between populations (Moore et al. 2011). In fact, these methods supply information about individuals movement only, besides being particularly time-expensive. Conversely, molecular-markers, i.e. polymorphic proteins or DNA sequences, are widely used for evaluating the effective genetic connectivity between populations, since they can distinguish breeding events, measure migration rates between generations and estimate gene-flow (Avise, 1994; Frankham et al. 2002; Frankham, 2006). Moreover, molecular techniques require a lower sampling effort, as they usually rely on biological samples collected in a single period (Neville et al. 2006).
As molecular marker we chose Microsatellite (or Short Tandem Repeats) that are repeating sequences of few base pairs of DNA. They are typically highly variable, because they are located in non-

coding DNA portions and so they are not subject to natural selection, moreover they are cost-effective.

REFERENCES

Allentoft ME, O'Brien J (2010) Global amphibian declines, loss of genetic diversity and fitness: a review. *Diversity*, **2**, 47 71.

Avise JC (1994) *Molecular Markers, Natural History and Evolution*. Chapman & Hall, New York.

Barrett GW, Peles JD (1999) *Landscape Ecology of Small Mammals*. Springer-Verlag Inc., New York.

Blaustein AR, Wake DB, Sousa WP (1994) Amphibian declines: judging stability, persistence, and susceptibility of populations to local and global extinctions. *Conservation Biology*, **8**, 60 71.

Bright PW, Mitchell P, Morris PA (1994) Dormouse distribution: survey techniques insular ecology and selection of sites for conservation. *Journal of Applied Ecology*, **31**, 329 339.

Burgman MA, Lindenmayer DB (1998) *Conservation biology for the Australian environment.* Chipping Norton, NSW: Surrey Beatty.

Fagan WF, Calabrese JM (2006) Quantifying connectivity: balancing metric performance with data requirements In K.R. Crooks, M. Sanjayan (red.), *Connectivity Conservation*, pp. 297-317. Cambridge University Press, Cambridge.

Fahrig L (2003) Effects of habitat fragmentation on biodiversity. *Annu Rev Ecol Evol Syst*, **34**, 487 515.

Fischer J, Lindenmayer DB (2007) Landscape modification and habitat fragmentation: a synthesis. *Glob Ecol Biogeogr*, **16**, 265 280.

Frankham R (2006) Genetics and landscape connectivity. In K.R. Crooks, M. Sanjayan (red.), *Connectivity Conservation*, pp. 72-96. Cambridge University Press, Cambridge.

Frankham R, Ballou JD, Briscoe DA (2002) *Introduction to Conservation Genetics*. Cambridge University Press, Cambridge.

Groom MJ, Meffe GK, Carroll RC (2006) *Principles of Conservation Biology, Third Edition.* Sinauer Associates, Sunderland (USA).

Hanski I, Simberloff D (1997) The metapopulation approach, its history, conceptual domain, and application to conservation. pp. 5–26. In: I. A. Hanski & M. E. Gilpin (eds.), Metapopulation Biology. Academic Press, San Diego, Californina.

Kareiva P, Marvier M (2011) *Conservation Science: Balancing the Needs of People and Nature.* Roberts and Company Publishers, Colorado (USA).

Koprowski JL (2005) The response of tree squirrels to fragmentation a review and synthesis. *Anim Conserv*, **8**, 369 376.

Lambeck RJ (1997) Focal Species: A Multi-Species Umbrella for Nature Conservation. *Conservation Biology*, **11**, 849 856.

Lanza B, Andreone F, Bologna MA, Corti C, Razzetti E (2007) *Fauna d'Italia. AMPHIBIA*. Ed. Calderini, Bologna.

Levins R (1969) Some demographic and genetic consequences of environmental heterogeneity for biological control, *Bulletin of the Entomological Society of America*, **15**, 237 240.

Moore JA, Tallmon DA, Nielsen J, Pyare S (2011) Effects of the landscape on boreal toad gene flow: does the pattern–process relationship hold true across distinct landscapes at the Northern range margin? *Molecular Ecology*, **20**, 4858 4869.

Mortelliti A (2013) Targeting habitat management in fragmented landscapes: a case study with forest vertebrates. *Biodiversity And Conservation*, **22,** 187 207.

Mortelliti A, Santulli Sanzo G,·Boitani L (2009) Species' surrogacy for conservation planning: caveats from comparing the response of three arboreal rodents to habitat loss and fragmentation. *Biodiversity Conservation*, **18**, 1131 1145.

Mortelliti A, Amori G, Capizzi D, Cervone C, Fagiani S, Pollini B, Boitani L (2011) Independent effects of habitat loss, habitat fragmentation and structural connectivity on the distribution of two arboreal rodents. *Journal of Applied Ecology*, **48**, 153 162.

Neville H, Dunham J, Peacock M (2006). Assessing connectivity in salmonid fishes with DNA microsatellite markers. Pages 318-342 in K. Crooks and M. A. Sanjayan, editors. *Connectivity Conservation*. Cambridge University Press, Cambridge.

Opdam P (1991) Metapopulation theory and habitat fragmentation: a review of holarctic breeding bird studies. *Landscape Ecology*, **5**, 93 106.

Primack RB (2010) *Essentials of Conservation Biology, Fifth Edition*. Sinauer Associates, Sunderland (USA).

Radford JQ, Bennett AF (2007) The relative importance of landscape properties for woodland birds in agricultural environments. *J Appl Ecol*, **44**, 737 747.

Rodriguez A, Andrèn H (1999) A comparison of Eurasian red squirrel distribution in different fragmented landscapes. *Journal Of Applied Ecology*, **36**, 649 662.

Saunders DA, Arnold GW, Burbridge AA, Hopkins AJ (1987) *Nature conservation: the role of remnants of native vegetation.* Chipping Norton, NSW: Surrey Beatty.

Smith AM, Green DM (2005) Dispersal and the metapopulation parading in amphibian ecology and conservation: are all amphibian populations metapopulations? *Ecography*, **28**, 110 128.

Tracey JA (2006) Individual-based modeling as a tool for conserving connectivity. In K.R. Crooks, M. Sanjayan (red.), *Connectivity Conservation*, pp. 343-368. Cambridge University Press, Cambridge.

White GC, Garrott RA (1990) *Analysis of Wildlife Radiotracking Data*. Academic Press, London.

Wilcove DS, Rothstein D, Dubow J, Philips A, Losos E (1998) Quantifying threats to imperiled species in the United States -- assessing the relative importance of habitat destruction, alien species, pollution, overexploitation and disease. *Bioscience*, **48**, 607 615.

2. Fire Salamander population structure in fragmented landscape in Northern Italy

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ABSTRACT

Human activities often cause environmental changes such as habitat loss and fragmentation that reduce animal populations' size and banish them to residual habitat patches. Small and isolated populations has a higher probability of extinction, because of less effective response to environmental variability due to demographic and genetic problems. In order to counteract isolation, meta-populations should be conserved maintaining or restoring the functional connectivity between fragmented populations. Amphibians are good model candidates for studying habitat fragmentation since most of them generally have low dispersal capabilities, are rather philopatric to breeding sites, and thus highly threatened by isolation, derived from habitat loss and fragmentation. The characterization of the population structure of Fire Salamander (*Salamandra salamandra, AMPHIBIA*, *URODELA*) in Lombardy (Northern Italy) was the goal of this study. We compared genetic structure of populations living in a fragmented and a continuous forest area, respectively the foothill lowland and Prealps of the Region. Thirty-one sampling populations were identified, according to a habitat suitability model, as those suitable habitat patches where at least three sample was collected. Biological sample of 471 salamander larvae collected at breeding sites were genotyped at 16 specie-specific autosomal microsatellite *loci* (STR). Genetic population structure was assessed by using STRUCTURE 2.3.4. We found two main populations of origin, one in the Prealpine belt, which maintain connections with the populations of the Eastern foothill lowland, and another inhabiting the Western foothill lowland. While the first main population was characterised by a Western-Eastern gradient, the second one resulted structured in four populations of origin. These areas are surrounded by large conurbation and a wide watercourse that separates them from the Prealpine belt and the Eastern foothill lowland. The genetic approach allowed us outlining the critical isolation of all the populations living in the Western foothill area, which appeared to be further fragmented and partially isolated among themselves.

2.1 INTRODUCTION

Anthropic pressure plays an important role in shaping landscapes through habitat destruction and degradation and often determines a fragmentation process that affects the spatial distribution of remaining populations by confining them to residual habitat fragments.

The effects produced by the overall habitat loss are often complex to understand because many impacting factors do not act separately, but rather they play cumulatively or interactively, affecting the dynamics of populations (Gilpin & Soulé, 1986; Lindenmayer, 1995; Young et al. 1996). Indeed, the habitat loss and degradation produce a reduction of population size; small populations are typically more vulnerable to intrinsic demographic and genetic threatening factors. Small populations are characterized by a higher variance of birth and death rates that leads to a higher probability of extinction. Moreover, they have a less effective demographic response to environmental stochasticity. Small populations suffer from a higher genetic drift and inbreeding, leading to the loss of heterozygosity and genetic variability. The mating of closely related individuals leads to the inbreeding depression that has negative effects on demography (e.g. juvenile fitness and mortality rate among offspring; see Ralls et al. 1988; Lacy, 1993; Lacy & Lindenmayer, 1995), reducing population growth rates and thus the population size. In addition, the decrease of genetic diversity makes populations less adaptable to environmental variability (Frankham et al. 2006). The smaller the population is, the more important are the effects of intrinsic threatening factors (Gilpin & Soulé, 1986).

Fragmentation processes generate metapopulations that are defined as a network of spatially discrete populations (i.e. sub-populations) linked by dispersal (Hanski & Simberloff, 1997). The amount of dispersal between sub-populations represents the degree of their ecological connectivity. Several species live in metapopulations, whose long-term persistence could be threatened by anthropogenic habitat fragmentation. This process could lead to isolation, that is the disruption of dispersal movements and, consequently, the halting of the gene flow between sub-populations, further emphasizing the

negative effects produced by the habitat loss. The knowledge of the ecology of fragmented populations is essential in order to prevent their isolation or even restoring the ecological connectivity between them (Saunders et al. 1987; Burgman & Lindenmayer, 1998).

Amphibians are model candidates for studies of fragmentation effects on connectivity (Moore et al. 2011): most amphibian species occur as metapopulations (Smith & Green, 2005); in addition, they are a *taxon* particularly susceptible to isolation, as they generally have low dispersal capabilities (Allentoft & O'Brien, 2010) and are rather philopatric to breeding sites (Blaustein et al. 1994). These characteristics often lead to high genetic differentiation, even at restricted scales (Allentoft & O'Brien, 2010).

The goal of this study was to characterize the population structure of Fire Salamander *Salamandra salamandra* (*AMPHIBIA*, *URODELA*) in a fragmented and a continuous forest area, respectively the foothill lowland and Prealps of Lombardy (Northern Italy). The species is strongly linked to broad-leaved forest ecosystems, depends on ecosystem processes, such as those that allow the development of an adequate litter structure; is strongly affected by specific resources, such as hydrology, some chemical and physical water parameters; and it is also limited by a low dispersal capability (Lanza et al. 2007).

The use of traditional methods like radio telemetry or capture–mark–recapture (White & Garrott, 1990; Barrett & Peles, 1999; Tracey, 2006) is alone insufficient for understanding ecological connectivity between populations (Moore et al. 2011). In fact, these methods supply information about individuals movement only, besides being particularly time-expensive. Conversely, molecular-markers, i.e. polymorphic proteins or DNA sequences, are widely used for evaluating the effective genetic connectivity between populations, since they can distinguish breeding events, measure migration rates between generations and estimate gene-flow (Avise 1994; Frankham et al. 2002; Frankham 2006). Moreover, molecular techniques require a lower sampling effort, as they usually rely on biological samples collected in a single period (Neville et al. 2006).

In this research, we chose microsatellites as molecular markers. They pertaining to a non-coding DNA part of genome, with no known function. This "neutral" region of DNA is thus particularly useful because it could change over time without bias induced by selection pressures. We anyway stress that microsatellites has sometimes been suspected to be non-completely neutral, meaning that at least some of the variation observed within and among populations may be attributed to selection (Kauer et al. 2003). Microsatellites markers generally have high mutation rates resulting in high standing allelic diversity (Selkoe & Toonen, 2006). For this reason, when used for evaluating the ecological connection between populations, they should be identified in sequences with mutation rates that are low relative to the migration rates of individuals (Beebee & Rowe, 2004).

2.2 METHODS

2.2.1 Study area and sampling design

The study area is located in the Pre-alpine belt and in the foothill lowland of Lombardy (Northern Italy). These areas were originally covered by extensive broad-leaved forests, that has been progressively removed and fragmented, especially during last century, particularly in the foothill lowland, where forests has been replaced by a conspicuous urban sprawl (figure 1). We consider the Prealps as a continuous forest area, while the foothill lowland as a fragmented one.

In the study area 168 sampling sites were identified according to Fire Salamander ecology and range distribution in Prealpine and foothill areas of Lombardy: 71 sampling sites were located in the Prealps, while 97 in the foothill lowland. Sampling sites correspond to breeding ponds and slow-flowing streams located in forest areas, where tissues were collected by cutting the tip (about 3-4 mm) of the salamander larvae tail. Tissue samples were stored in 95% ethanol, in the field, and subsequently kept in laboratory at −20 °C. One to 4 biological samples were collected in each sampling site, all year

around, from 2010 to 2013, for a total of 471 samples. Salamander larvae were captured and handled with permit of the Lombardy regional administration (P. T1.2009.0016990 decreed on 2009/09/16 by D.G. Ambiente, Parchi e aree protette for 2010-2012 and administrative order 964 decreed on 2013/02/11 by D.G. Agricoltura for 2013-2014). Possible bias deriving from full-sibling individuals can occur when population genetic structure is inferred by sampling larvae in breeding sites (Goldberg & Waits, 2010). For this reason we sampled no more than 4 individuals per site, and sites were then grouped in sampling population (see "*Fire Salamander population structure"* in Methods section). These populations represent the unit used for the genetic population structure analysis.

2.2.2 DNA extraction and analyses of microsatellite markers

DNA was extracted with the Quick-g DNA™ MiniPrep kit (Zymo Research, USA), eluted in 180 µL of TE buffer (10 mM TrisHCl, pH 8; 0.1 mM EDTA) and stored at -20°C until subsequent handlings.

All samples were genotyped by Polymerase Chain Reaction (PCR) for 20 species-specific microsatellite markers (Table S1; Steinfartz et al. 2004; Hendrix et al. 2010); *locus* designation, primer sequences, label, repeat motif, annealing temperature are summarized in the supplementary material.

PCR amplifications were carried out in 10-µL mix reactions with: 1 µL genomic DNA solutions from tissue extractions, 1 µL of 10x PCR buffer with 2.5 mM Mg^{2+}, 2 µL of Bovine Serum Albumin (2%), 0.4 µM of dNTPs, and 0.2 or 0.3 µL of primer mix 10 µM (forward and reverse) plus 0.05 units of Taq polymerase (5 PRIME Inc., Gaithersburg, USA) and purified water. PCR conditions were optimized for each primer pair, amplifications were performed in a 9700 ABI thermal cycler using the following protocol: (94°C x 2'), a number of cycles between 30 and 40 at (94°C x 30'') (annealing temperature x 30'') (72°C x 30''), and a final extension at 72°C for 10'; some primer pair (SalE8, SalE12, SalE14, SST-A6-II, and SST-B11) were amplified by using a touchdown PCR.

PCR products were analysed in an Applied Biosystems 3130XL DNA sequencer (Life Technology) and allele sizes were estimated using the software GENEMAPPER 4.0 (Life Technology). Positive (known genotypes) and negative (no DNA) controls were used to check for laboratory contaminations, which never occurred. A 10% randomly selected subset of the other samples were PCR-replicated two times to check for allelic drop-out and false alleles. Each *locus* was checked for null alleles (alleles that are present in a sample, yet are not amplified) using MICRO-CHECKER (Van Oosterhout et al. 2004). Four microsatellite *loci* (Sal29, SST-F10, SST-G6 and SST-G9) were excluded from the analysis because we were not able to obtain PCR products that can be clearly interpreted.
The estimation of population genetic parameters, such as number of genotyped individuals (N), number of different alleles (Na), number of effective alleles (Ne), the allelic range (AR), observed (Ho) and expected heterozigosity (He), fixation index (F), was performed for all individuals and for each *locus* using GenAlEx v. 6.501 (Peakall & Smouse 2006, 2012). We calculated the Probability of Identity (PI, the probability of two independent samples having the same identical genotype) and the Probability of Identity among full sibs dyads (PIsibs) at a *locus*, for increasing *locus* combination, in order to check if the number of *loci* was suitable to identify univocally the individuals.

2.2.3 Fire Salamander population structure

The analysis of genetic population structure, when data have a weak signal, can be improved by the knowledge of the sampling population of each individual. Nevertheless, when sampling sites are chosen according to a cluster design (sampling units are groups of close sites), it is difficult to assign sampled individuals to a unique sampling population, since even close sites could be actually belong to different populations separated by barriers (i.e. roads) or not suitable areas. In order to assign each individual to a single sampling population, we grouped all sampling sites included in the same continuous habitat patch identified by a habitat suitability model.

Before to realize a habitat suitability model, the scale at which environmental variables affect species biology should be assessed. This is usually done using dispersal data, available from literature, and for the Fire Salamander it was seldom obtained using mark-recapture methods (Denoël 1996; Schulte et al. 2007). In our study we evaluated the effectiveness of genetic data in identifying the spatial scale of individual dispersal. Indeed, the spatial autocorrelation of genotypes can represent the distance at which gene flow may take place and the shortest distance class showing significant differences in Moran's Index (Moran 1950) may be used to deduce the spatial scale at which individual dispersal occur. We calculated the spatial autocorrelation of individual genotypes (based on 16 polymorphic *loci*) using GenAlEx 6.5 (Peakall & Smouse 2006, 2012).
As salamander data are presence-only, the MaxEnt software (Maximum Entropy modelling; Phillips et al. 2005) was used. This software evaluates how a predefined set of environmental variables may affect the probability of presence of a studied species, comparing variable values in presence sites with those of a set of 10,000 points randomly extracted in the study area. Since the Fire Salamander requires two different habitats during its life cycle, we first developed a breeding habitat model (aquatic phase), able to identify suitable reproductive sites for the whole study area. Secondly, we built an adult/dispersal model (terrestrial phase) around suitable reproductive sites. The two steps approach was required because suitable dispersal areas were identified only around suitable breeding sites. The model could not be done in one step as the modelled areas of the two phases differed in extent: the first model was developed only for all potential breeding sites, while the second one for a larger area around only suitable breeding sites. This difference also affected the area within which pseudo-absences (random points) was extracted.
The breeding habitat model was developed for all streams in forest areas, considering as environmental variables the stream order (Strahler method) and, in a 60 m buffer centred on the stream, the fractional cover of four main second level land uses classes (all but

forests, from the digital land use map of Lombardy, DUSAF 2.1, ERSAF 2010, Figure 1). We also considered mean elevation, slope and aspect, calculated in the buffer (Table 2a). We assumed that the 60 m buffer is the extent at which environmental variables may affect the breeding site suitability. The output of the breeding suitability model was the presence probability of salamander larvae in every streams in the forests of the study area.
The adult/dispersal model was built for all areas within a buffer, corresponding to the dispersal spatial scale, from all streams. The environmental variables were the mean breeding site suitability (i.e. the presence probability of salamander larvae estimated by the first model), the land use fractional cover, the road density, the mean elevation, slope and aspect, in the buffer (Table 2b). The output of the adult/dispersal model is the presence probability of the salamander for its whole life cycle in the study area.
We then identified as suitable patches of habitat all those areas with a probability of presence of salamander, derived from adult/dispersal model, higher than 25%.
Finally, we assigned at the same sampling population each individual pertaining to the same patch of suitable habitat.
Genetic population structure was performed analysing the biparental multilocus genotypes using a Bayesian clustering procedure implemented in STRUCTURE 2.3.4 (Pritchard et al. 2000; Falush et al. 2003), which was designed to identify the populations of origin (K) of the sampled individuals. This analysis gave the assignment probability of each individual (Q) to pertain to each of the identified populations of origin. Populations were constructed by minimizing the departures from Hardy–Weinberg equilibrium (HWE) and linkage equilibrium (LE), which could result from recent admixtures, migration or hybridisation.
We ran STRUCTURE using "no-admixture" model because it is more powerful than "admixture" one at detecting subtle population structure (Pritchard et al. 2010). Moreover, in our study area, it was unlikely that individuals from different sampling populations share recent common ancestors (Falush et al. 2003), because the distances between sampling populations were higher than dispersal distance

for several orders of magnitude. We used LOCPRIOR information (sampling population) in order to help clustering procedure. In the first step, we ran STRUCTURE using the "independent allele frequencies" model, in order to identify populations highly divergent from the others (Pritchard et al. 2010) and reduce the likelihood of overestimating K (Hale et al. 2013). After removing divergent populations, it was then possible to use the correlated model (Pritchard et al. 2010), that allows to increase the power to detect distinct populations even when they are closely related by gene flow (Falush et al. 2003; Rosenberg et al. 2005). In the second step, we re-ran STRUCTURE using the "correlated allele frequencies" for each population of origin identified in the first step, assuming that allele frequencies in different populations are likely to be similar due to shared ancestry and migration. This second analysis had the aim to detect the weaker sub-structure of the main populations of origin identified in the first step.

All simulations were run with a *burnin* period of 10,000 and 100,000 MCMC (Hastings, 1970; Green, 1995), replicated 20 times. The optimal K values were selected by means of STRUCTURE HARVESTER (Earl & vonHoldt 2012) following the Evanno method (Evanno et al. 2005), based on the second order rate of change in the log probability of data between successive K values. All the analyses were performed setting K from 1 to 10, using 471 samples genotyped at 16 microsatellites.

In order to evaluate the loss of genetic diversity, we then calculated genetic parameters for all populations of origin identified in structure analyses.

2.3 RESULTS

2.3.1 Genetic variability

The Probability of Identity (PI) for increasing *locus* combinations (16 *loci*) resulted in value as low as $7.7*10^{-12}$, while the Probability of Identity among full sibs (PIsibs) was $2.0*10^{-5}$, meaning that only 2 salamanders in 100,000 siblings are expected to share by chance an

identical genotype. The panel of microsatellites thus supported reliable individual genotype identification. All *loci* were polymorphic, with the number of different alleles (Na) ranging from 5 to 23, and the effective number of alleles (Ne) varying between 1.03 and 5.20. All these parameters, with allelic range (AR), observed (Ho) and expected (He) heterozigosity and fixation index (F), for each *locus*, are shown in table 1.

Locus	**N**	**Na**	**Ne**	**AR**	**Ho**	**He**	**F**
SalE2	471	11	2.34	216-262	0.569	0.572	0.006
Sal3	471	11	2.49	181-327	0.563	0.598	0.058
SalE5	471	5	1.03	180-190	0.030	0.029	-0.012
SalE6	471	6	2.34	277-297	0.531	0.573	0.073
SalE7	471	12	2.73	184-232	0.637	0.633	-0.006
SalE8	469	10	4.34	143-181	0.736	0.770	0.044
SalE11	471	6	2.24	238-258	0.529	0.553	0.044
SalE12	464	16	4.43	223-307	0.636	0.774	0.179
SalE14	471	6	2.17	237-257	0.522	0.539	0.031
Sal23	448	9	2.47	282-320	0.558	0.595	0.062
SST-A6-I	471	6	1.76	207-231	0.431	0.432	0.003
SST-A6-II	471	8	2.32	193-221	0.561	0.568	0.014
SST-B11	470	23	5.20	149-263	0.772	0.808	0.044
SST-C2	470	13	3.06	194-246	0.615	0.673	0.087
SST-C3	471	5	1.69	207-227	0.414	0.409	-0.012
SST-E11	471	13	4.27	233-311	0.694	0.766	0.093
Mean	468.88	10.00	2.80	-	0.55	0.58	0.04
SE	1.46	1.20	0.29	-	0.04	0.05	0.01

Table 1. Population genetic parameters: number of genotyped individuals (N), number of different alleles (Na), number of effective alleles (Ne), allelic range (AR), observed (Ho) and expected heterozigosity (He), and fixation index (F).

2.3.2 Fire Salamander population structure

The spatial autocorrelation analysis showed that individual genotypes were significantly autocorrelated from 50 m to 1500 m, although there was a significant difference in Moran's I between the 50 m and 500 m distant classes (figure 2). For this reason, we assumed that 500 m could be used as a measure of individual dispersal distance. This result is in accordance with literature data, where the maximum distance covered by adults during dispersal is equal or lower than 500 m (Denoël 1996; Schulte et al. 2007). This value was used to define a 1000 m buffer centred on streams (500 m for each side) within which environmental variables were evaluated, in order to build the adult/dispersal habitat suitability model.

The breeding habitat suitability model, developed with MaxEnt, showed a good predictive ability (AUC = 0.828). Moreover, the threshold to balance sensitivity and specificity of the model equalled 0.450, which allowed a correct classification of about 75% of the samples with a *Training Omission Rate* of 0.246. The results of this model, according to the permutation importance, that is a robust measure of variable contribution (Phillips & Dudik, 2008), showed how the most important environmental variables affecting the breeding site suitability were elevation (41.9%), slope (23.8%) and the fractional cover of urban areas (11.8%) (Table 2a). The breeding suitability decreased when elevation increased, and the larvae presence probability approaches zero at 1500 m. The optimal slope seemed to be between about 5° and 20°, while the presence probability was close to zero for slope higher than 40°. Urban areas showed an almost linear negative effect on the presence of salamander larvae (Figure S2a).

The adult/dispersal model showed a good predictive ability as well (AUC = 0.879) and the threshold value given by the software to balance sensitivity and specificity was 0.368, which implies that a correct classification of about 80% of the samples was possible with a *Training Omission Rate* of 0.201. The most important variables affecting salamander presence were the fractional cover of broadleaved (22.0% of permutation importance) and mixed forests

(13.5%) and the breeding site suitability (10.3%). All these variables positively affected the probability of presence of salamanders (Figure S2b).

We identified as continuous suitable habitat patches for the whole study area, all those areas with a salamander presence probability higher than 25%. Our sampling sites were located in 31 of these patches (Figure 3). All sites pertaining to the same patch were assigned to the same sampling population (i.e. LOCPRIOR in STRUCTURE analyses), with the number of individual assigned to a unique patch ranging from 3 to 61.

The first step of population structure analysis, performed on all samples (31 sampling populations, 471 individuals), evidenced two distinct clusters (K=2), Ln Pr (X|K) = -18066.0) (Figure S3a). The first cluster included all the Prealpine area and the Eastern foothill lowland (PEF), with 26 sampling populations (339 individuals), while the second one corresponded to the Western foothill lowland (WF) with 5 sampling populations (132 individuals) (Figure 4).

In the second step we re-ran population structure analysis separately for each of the two clusters. The analysis of the PEF sub-sample revealed two populations of origin (K=2), corresponding to a Western-Eastern gradient, Ln Pr (X|K) = -12961.0) (Figure S3b). Conversely, the analysis of the WF sub-sample showed 4 distinct populations of origin (K=4), Ln Pr (X|K) = -4654.3) (Figure S3c).

PEF population had a significantly larger number of sampled individuals than WF and a higher number of private alleles (52 in PEF and 11 in WF): in particular, the SalE5 *locus* became monomorphic in the WF sub-sample. The mean number of different alleles (Na) was higher in PEF (9.31 ± 1.20 SE) than in WF (6.75 ± 0.79 SE), while mean number of effective alleles (Ne) were comparable between the two populations (2.80 ± 0.30 SE in PEF and 2.73 ± 0.29 SE in WF).

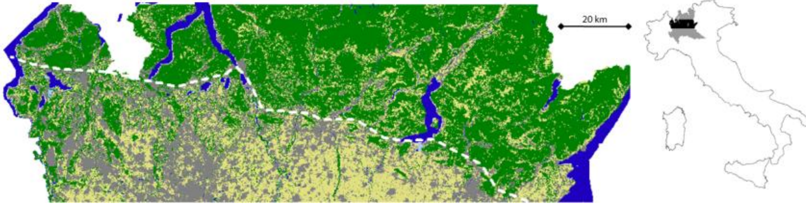

Figure 1. On the right, study area (black), within Lombardy (grey) in Northern Italy. On the left, colours represent main land use classes (DUSAF 2.1): forests in green, farmland and other open habitats (grasslands and shrubs) in yellow, urban areas in grey, lakes and rivers in blue, wetlands in azure. White dashed line separates the Prealps in the North from the foothill lowland in the South.

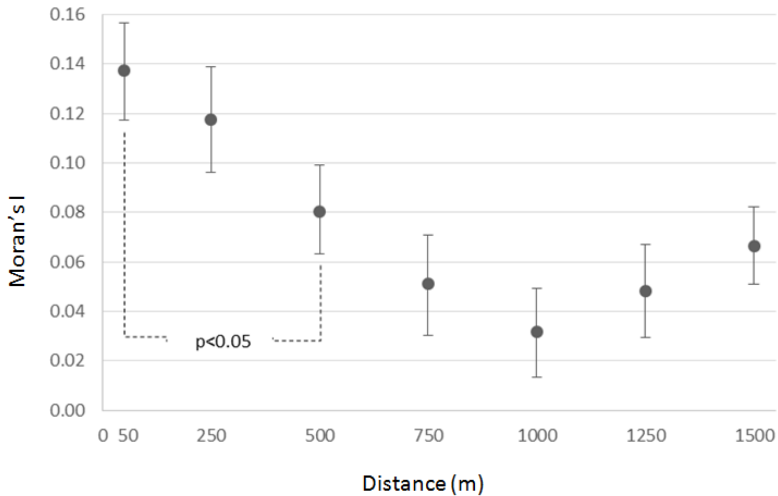

Figure 2. Spatial autocorrelation of individual genotypes among 7 distance classes. Dashed line shows the first distance class significantly different from the first class.

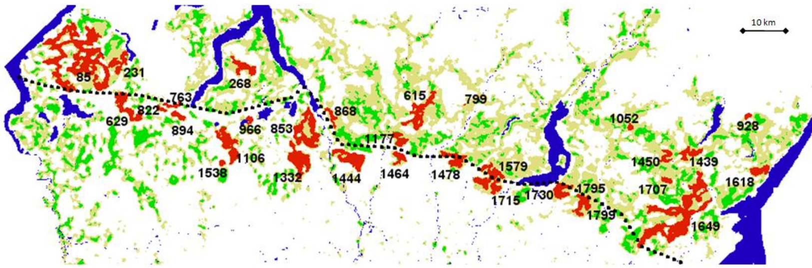

Figure 3. Fire Salamander presence probability estimated by the adult/dispersal habitat suitability model: light brown, $0.05 \leq p < 0.25$; green, $p \geq 0.25$. Red areas are the 31 continuous patches (sampling populations) where biological samples were collected. The black dashed line separates the Prealps (North) from the foothill lowland (South) of Lombardy. Main lakes and rivers in blue.

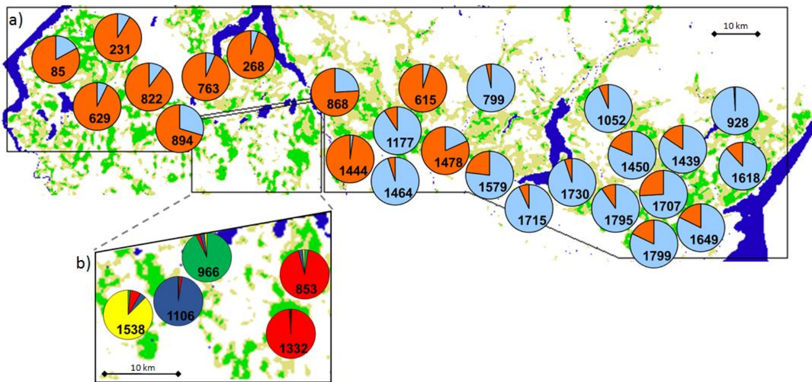

Figure 4. Fire Salamander population structure in Lombardy (Northern Italy). Pie graphs show the assignment probability (Q) to clusters identified by STRUCTURE 2.3.4. Two main clusters were identified in the first step: (a) Prealpine and Eastern foothill lowland (PEF) and (b) the Western foothill lowland (WF). a) PEF was further divided in two clusters along a gradient from West (orange) to East (azure). b) WF was divided in three clusters composed by a single population (yellow, blue and green) and one cluster composed by two populations (red). Underlying map shows the probability of presence of Fire Salamander: light brown, $0.05 \leq p < 0.25$; green, $p \geq 0.25$ with main lakes and rivers in blue.

Variable	*Contribution (%)*	*Permutation importance*	*Variable range (presence sites)*	*Variable range (random points)*
Elevation	32.4	41.9	246 - 1502	68 - 2010
Slope	29.2	23.8	0 - 42.12	0 - 77.31
Urban areas	10.1	11.8	0 - 0.4	0 - 1
Farmlands	10.4	7.4	0 - 0.8	0 - 1
Stream order	5.6	4.7	1 - 4	1 - 8
Grasslands	4.3	4.7	0 - 1	0 - 1
Shrubs	4.7	2.6	0 - 0	0 - 1
Aspect (E-W)	1.5	1.8	-0.80 - 0.67	-1 - 1
Aspect (N-S)	1.9	1.3	-0.84 - 0.91	-1 - 1

Table 2a. Reproductive habitat suitability model: analysis of variable contribution and ranges of variable values.

Variable	*Contribution (%)*	*Permutation importance*	*Variable range (presence sites)*	*Variable range (random points)*
Broadleaved forests	13.9	22	0 - 1	0 - 1
Mixed forests	5.0	13.5	0 - 0.94	0 - 1
Breeding site suitability	39.9	10.3	0.01 - 0.34	0 - 0.39
Elevation	4.5	9.8	242 - 1562	75 - 2049
Coniferous forests	7.6	7.9	0 - 0.2	0 - 1
Road density	6.4	6.5	0 - 0.28	0 - 0.57
Shrubs	4.1	6.3	0 - 0.14	0 - 0.87
Grasslands	1.8	6	0 - 0.56	0 - 0.93
Urban areas	5.2	5.7	0 - 0.35	0 - 0.98
Slope	3.9	5	1.71 - 38.72	0 - 48.49
Farmlands	6.7	4.9	0 - 0.58	0 - 0.97
Aspect (N-S)	0.7	2.0	-0.42 - 0.05	-0.84 - 0.4
Aspect (E-W)	0.3	0.2	-0.04 - 0.26	-0.31 - 0.54

Table 2b. Adult/dispersal habitat suitability model: analysis of variable contribution and ranges of variable values.

Population		N	Na	Ne	Ho	He	F
PEF	Mean	337.06	9.31	2.80	0.551	0.577	0.034
	SE	1.45	1.20	0.30	0.043	0.047	0.015
WF	Mean	131.81	6.75	2.73	0.545	0.571	0.042
	SE	0.10	0.79	0.29	0.044	0.047	0.020

Table 3. Mean population genetic parameters for the two cluster (PEF and WF) identified in the first step of population structure analysis: number of genotyped individuals (N), number of different alleles (Na), number of effective alleles (Ne), allelic range (AR), observed (Ho) and expected heterozigosity (He), and fixation index (F).

2.4 DISCUSSION

This research allowed us to analyse the population structure of the Fire Salamander in Lombardy, comparing sub-populations living in continuous forests with those living in fragmented ones.
The first step of the analysis concerned the estimation of the individual dispersal distance, in order to develop a habitat suitability model at an appropriate spatial scale. The measure of 500 m, resulted from the spatial autocorrelation of genotypes, was confirmed by literature data (Denoël 1996; Schulte et al. 2007) and can represent a proof of the power of microsatellites data for dispersal estimation respect to traditional methods (radio tracking, capture-mark-recapture).
According to the Fire Salamander ecology (Griffiths, 1995; Lanza et al. 2007), the habitat suitability model showed that elevation and slope of breeding sites and the presence of forests and urban areas around them, play an important role in determining the presence probability of the species.
Population structure analysis underlined a strong differentiation of Fire Salamander sub-populations in the Prealpine/foothill lowland of Lombardy (Northern Italy). We first identified two main clusters,

PEF and WF: the first one can be further divided in two populations of origin connected along an Western-Eastern gradient (Figure 4a). All the sampling populations of the fragmented Eastern foothill lowland were instead assigned to the PEF cluster with all the Prealpine sampling populations. The same happened for the sampling populations of the extreme Western foothill lowland. Although these sub-populations appeared inhabiting areas where salamander habitat is fragmented, genetic data showed that they still maintain an ecological connection with those inhabiting the Prealpine areas.

Conversely, in the Western-central foothill lowland, the sampling populations were grouped in a clearly different cluster, WF (Q higher than 0.605 for all sampling populations). Wide urban areas in its Northern and Western boundaries and a large watercourse (Adda river) in the East, separate this cluster from PEF. WF was further subdivided in four clusters in the second step of population structure analysis. These four clusters almost reproduce the spatial configuration of the five sampling populations of WF. Although they are geographically very close, three out of five sampling populations appeared strongly isolated, as they were assigned at three different clusters with a probability of assignment higher than 0.982 for all individuals. The other two sampling populations were grouped in a unique cluster ($Q>0.986$ for all individuals) (Figure 4b).

PEF and WF clusters were composed by a different number of individuals ($N_{PEF} = 339$; $N_{WF} =132$) and PEF showed both a higher number of private alleles and a higher mean number of different alleles (Na) than WF, so that the SalE5 *locus* became monomorphic in WF. Nevertheless, the number of effective alleles (Ne) and the observed heterozigosity (Ho) were very similar, suggesting that currently there wasn't a loss of genetic diversity (Table 3). These results may be explained by the hypothesis of a short-term fragmentation in the WF cluster: in this case, we cannot currently detect a loss of alleles, but in the long-term, lacking gene flow, it may arise, mainly in small and/or isolated populations, due to genetic drift, inbreeding or local extinctions. Conversely, it is likely that the PEF cluster will maintain a high number of alleles in the long-term,

because it is represented by many large populations, connected along a Western-Eastern gradient.

REFERENCES

Allentoft ME, O'Brien J (2010) Global amphibian declines, loss of genetic diversity and fitness: a review. *Diversity*, **2**, 47 71.

Avise JC (1994) *Molecular Markers, Natural History and Evolution*. Chapman & Hall, New York.

Barrett GW, Peles JD (1999) *Landscape Ecology of Small Mammals*. Springer-Verlag Inc., New York.

Beebee T, Rowe G (2004) *An introduction to molecular ecology*. Oxford University Press, New York.

Blaustein AR, Wake DB, Sousa WP (1994) Amphibian declines: judging stability, persistence, and susceptibility of populations to local and global extinctions. *Conservation Biology*, **8**, 60 71.

Burgman MA, Lindenmayer DB (1998) *Conservation biology for the Australian environment.* Chipping Norton, NSW: Surrey Beatty.

Denoël M (1996) Phénologie et domaine vital de la salamandre terrestre *Salamandra salamandra* terrestris (*Amphibia, Caudata*) dans un bois du Pays de Herve (Belgique). *Chaiers d'Ethol*, **16**, 291 306.

Earl DA, vonHoldt BM (2012) STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output and implementing the Evanno method. *Conservation Genetics Resources*, **4**, 359 361.

ERSAF (2010) Uso del suolo in Regione Lombardia: i dati DUSAF. Ente Regionale per i Servizi all'Agricoltura e alle Foreste.

Evanno G, Regnaut S, Goudet J (2005) Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. *Molecular Ecology*, **14**, 2611 2620.

Falush D, Stephens M, Pritchard JK (2003) Inference of population structure using multilocus genotype data: linked *loci* and correlated allele frequencies. *Genetics*, **164**, 1567 1587.

Frankham R (2006) Genetics and landscape connectivity. In K.R. Crooks, M. Sanjayan (red.), *Connectivity Conservation*, pp. 72-96. Cambridge University Press, Cambridge.
Frankham R, Ballou JD, Briscoe DA (2002) *Introduction to Conservation Genetics*. Cambridge University Press, Cambridge.
Giplin ME, Soulé ME (1986) Minimum viable populations: processes of species extinctions. In: *Conservation biology: the science of scarcity and diversity*: pp. 19-34. Soulé, M.E. (ed.). Sunderland, MA: Sinauer Associates.
Goldberg CS, Waits LP (2010) Quantification and reduction of bias from sampling larvae to infer population and landscape genetic structure. *Molecular Ecology Resources*, **10**, 304 313.
Green PJ (1995) Reversible jump Markov chain Monte Carlo computation and Bayesian model determination. *Biometrika*, **82**, 711 732.
Griffiths R (1996) *Newts and salamanders of Europe*. T & AD Poyser Natural History, London.
Hale JM, Heard GW, Smith KL, Parris KM, Austin JJ, Kearney M, Melville J (2013) Structure and fragmentation of growling grass frog metapopulations. *Conservation Genetics*, **14**, 313 322.
Hanski I, Simberloff D (1997) The metapopulation approach, its history, conceptual domain, and application to conservation. pp. 5–26. In: I. A. Hanski & M. E. Gilpin (eds.), *Metapopulation Biology*. Academic Press, San Diego, California.
Hastings WK (1970) Monte Carlo sampling methods using Markov chains and their applications. *Biometrikam*, **57**, 97 109.
Hendrix R, Hauswaldt S, Veith M, Steinfartz S. (2010) Strong correlation between cross-amplification success and genetic distance across all members of ‘True Salamanders’ (*Amphibia: Salamandridae*) revealed by *Salamandra salamandra*-specific microsatellite *loci*. *Molecular Ecology Resources*, **10**, 1038 1047.
Kauer M, Dieringer D, Schlotterer C (2003) Nonneutral admixture of immigrant genotypes in african *Drosophila melanogaster* populations from Zimbabwe. *Molecular Biology and Evolution*, **20**, 1329 1337.

Lacy RC (1993) Impacts of inbreeding in natural and captive populations of vertebrates: implications for conservation. *Prespectives in Biology and Medicine*, **36**, 480 496.

Lacy RC, Lindenmayer DB (1995) A simulation study of the impacts of population subdivision on the mountain brushtail possum *Trichosurus caninus ogilby* (*Phalangeridae, Marsupialia*) in South-Eastern Australia. 2. Loss of genetic variation within and between subpopulatins. *Biological Conservation*, **73**, 131 142.

Lanza B, Andreone F, Bologna MA, Corti C, Razzetti E (2007) *Fauna d'Italia. AMPHIBIA*. Ed. Calderini, Bologna.

Lindenmayer DB (1995) Disturbance, forest wildlife conservation and a conservative basis for forest management in the mountain ash forests of Victoria. *Forest Ecology and Management*, **74**, 223 231.

McRae B (2006) Isolation by resistance. *Evolution*, **60**, 1551 1561.

Moore JA, Tallmon DA, Nielsen J, Pyare S (2011) Effects of the landscape on boreal toad gene flow: does the pattern–process relationship hold true across distinct landscapes at the Northern range margin? *Molecular Ecology*, **20**, 4858 4869.

Moran PAP (1950) Notes on Continuous Stochastic Phenomena. *Biometrika*, **37**, 17 23.

Neville H, Dunham J, Peacock M (2006) Assessing connectivity in salmonid fishes with DNA microsatellite markers. Pages 318-342 in K. Crooks and M. A. Sanjayan, editors. *Connectivity Conservation*. Cambridge University Press, Cambridge.

Peakall R, Smouse PE (2006) GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. *Molecular Ecology Notes*, **6**, 288 295.

Peakall R, Smouse PE (2012) GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research-an update. *Bioinformatics*, **28**, 2537 2539.

Phillips SJ, Anderson RP, Schapire RE (2005) Maximum entropy modeling of species geographic distributions. *Ecological Modelling*, **190**, 231 259.

Phillips SJ, Dudik M (2008) Modeling of species distribution with MaxEnt: new extensions and a comprehensive evaluation. *Ecography*, **31**, 161 175.

Pritchard JK, Stephens M, Donnelly PJ (2000) Inference of population Structure using multilocus genotype data. *Genetics*, **155**, 945 959.

Pritchard JK, Wen X, Falush D (2010) Documentation for STRUCTURE software. [Documentation file]. Available with the program at http://pritch.bsd.uchicago.edu/structure.html.

Ralls K, Ballou JD, Templeton AR (1988). Estimates of lethal equivalents and the cost of inbreeding in mammals. *Conservation Biology*, **2**, 185 193.

Rosenberg NA, Mahajan S, Ramachandran S, Zhao C, Pritchard JK, Feldman MW (2005) Clines, clusters, and the effect of study design on the inference of human population structure. *PLoS Genetics*, **1**, 660 671.

Saunders DA, Arnold GW, Burbridge AA, Hopkins AJ (1987) *Nature conservation: the role of remnants of native vegetation.* Chipping Norton, NSW: Surrey Beatty.

Schulte U, Kuesters D, Steinfartz S (2007) A PIT tag based analysis of annual movement patterns of adult Fire Salamanders (*Salamandra salamandra*) in a middle European habitat. *Amphibia-Reptilia*, **28**, 531 536.

Selkoe KA, Toonen RJ (2006) Microsatellites for ecologists: a practical guide to using and evaluating microsatellite markers. *Ecology Letters*, **9**, 615 629.

Smith AM, Green DM (2005) Dispersal and the metapopulation parading in amphibian ecology and conservation: are all amphibian populations metapopulations? *Ecography*, **28**, 110 128.

Steinfartz S, Küsters D, Tautz D (2004) Isolation and characterization of polymorphic tetranucleotide microsatellite *loci* in the Fire Salamander *Salamandra salamandra* (*Amphibia: Caudata*). *Molecular Ecology Notes*, **4**, 626 628.

Tracey JA (2006) Individual-based modeling as a tool for conserving connectivity. In K.R. Crooks, M. Sanjayan (red.), *Connectivity Conservation*, pp. 343-368. Cambridge University Press, Cambridge.

Van Oosterhout C, Hutchinson WF, Wills DPM, Shipley P (2004) Microchecker: software for identifying and correcting genotyping errors in microsatellite data. *Molecular Ecology Notes*, **4**, 535 538.

White GC, Garrott RA (1990) *Analysis of Wildlife Radiotracking Data*. Academic Press, London.

Young AG, Boyle T, Brown AHD (1996) The population genetic consequences of habitat fragmentation for plants. *Trend in Ecology and Evolution*, **2**, 413 418.

SUPPLEMENTARY MATERIALS

Locus	Primer sequence (5’ → 3’)	Label	Repeat motif	Ta (°C)	Reference
SalE2	F: CACGACAAAATACAGAGAGTGGATA R: ATATTTGAAATTGCCCATTTGGTA	HEX	(GATA)$_6$(GACA)$_5$(GATA)$_{12}$	55 °C	Steinfartz et al. 2004
SalE5	F: CCACATGATGCCTACGTATGTTGTG R: CTCCTGTTTACGCTTCACCTGCTCC	FAM	(GT)$_{14}$	55 °C	Steinfartz et al. 2004
SalE6	F: GGACTCATGGTCACCCAGAGGTTCT R: ATGGATTGTGTCGAAATAAGGTATC	FAM	(GATA)$_2$GATG(GATA)$_{15}$	55 °C	Steinfartz et al. 2004
SalE7	F: TTTCAGCACCAAGATACCTCTTTTG R: CTCCCTCCATATCAAGGTCACAGAC	HEX	(GATA)$_6$(GACA)$_{11}$(GATA) (GACA)(GACA)(GATA)$_{12}$	55 °C	Steinfartz et al. 2004
SalE8	F: GCAAAGTCCATGCTTTCCCTTTCTC R: GACATACCAAAGACTCCAGAATGGG	FAM	(TATC)$_{16}$	(TD) 55	Steinfartz et al. 2004
SalE11	F: CACAGTTCATTATTTCCACTACTGA R: AGGACCTCAAGACCTGGCTCTTCAA	FAM	(CTAT)$_{15}$CCAT(CTAT)$_5$	55 °C	Steinfartz et al. 2004
SalE12	F: CTCAGGAACAGTGTGCCCCAAATAC R: CTCATAATTTAGTCTACCCTCCCAC	TET	(CTAT)$_{15}$	(TD) 55	Steinfartz et al. 2004
SalE14	F: GCTGCCCTCTCTGCCTACTGACCAT R: GCCAAGACATGGAACACCCTCCCGC	TET	(CTAT)$_{16}$	(TD) 55	Steinfartz et al. 2004
Sal3	F: CTCAGACAAGAAATCCTGCTTCTTC R: ATAAATCTGTCCTGTTCCTAATCAG	FAM	(GAGT)$_{15}$	55 °C	Steinfartz et al. 2004
Sal23	F: TCACTGTTTATCTTTGTTCTTTTAT R: AATTATTTGTTTGAGTCGATTTTCT	HEX	(GACA)$_8$(GATA)$_4$	55 °C	Steinfartz et al. 2004

Table S1. *Salamandra salamandra* microsatellite *loci* used for the genetic analysis

Locus	**Primer sequence (5’ → 3’)**	**Label**	**Repeat motif**	**Ta (°C)**	**Reference**
Sal29	F: CTCTTTGACTGAACCAGAACCCC	TET	(GATA)$_{14}$	60 °C	Steinfartz et al. 2004
	R: GCCTGTCGGCTCTGTGTAACC				
SST-A6-I	F: TTCAGTGCTCTTGCAGGTTG	HEX	(ATCT)$_{9}$ATCA(ATCT)$_{10}$	55 °C	Hendrix et al. 2010
	R: AGTCTGCAAGGATAGAAAGATCG				
SST-A6-II	F: ATTCTCTCTGACAAGGATTGTGG	FAM	(TATC)$_{11}$	(TD) 60 °C	Hendrix et al. 2010
	R: GGTAGACAGACATCAAGGCAGAC				
SST-B11	F: TCAAACGGTGCCAAAGTTATTAG		(TATC)$_{14}$	(TD) 60 °C	Hendrix et al. 2010
	R: TTAATTGGCAGTTTTCTTTCCAG	HEX			
SST-C2	F: CTTTGGGTCAGCCCTCTTC		(TATC)$_{16}$	55 °C	Hendrix et al. 2010
	R: CAGAGCAACATTGGATGTATCAG	FAM			
SST-C3	F: CCGTTTGAGTCACTTCTTTCTTG		(TAGA)$_{7}$TAGG(TAGA)$_{3}$	55 °C	Hendrix et al. 2010
	R: TTGCTTTACCAACCAGTTATTGTC	HEX			
SST-E11	F: AGACAAAAATGGGGACTAACCAC		(TAGA)$_{10}$AAGATAGG(TAGA)$_{5}$	55 °C	Hendrix et al. 2010
	R: TGTCTACCTGTTTGTTTATCTACTGG	HEX			
SST-F10	F: GGCCAACGTCAGAGGTTTC		(TAGA)$_{13}$	60 °C	Hendrix et al. 2010
	R: TCATATTCCTCTTATGTTCCTACTCC	HEX			
SST-G6	F: GAGGCCCATTTTCTTTACTTACC	FAM	(TATC)$_{12}$	60 °C	Hendrix et al. 2010
	R: GTAAAGAGGGCCGCTTAGTTG				
SST-G9	F: CCTCGTCAGGGGTTGTAGG	FAM	(ATCT)$_{13}$	65 °C	Hendrix et al. 2010
	R: CTTTCCAGGAAGAAACTGAGATG				

Table S1. *Salamandra salamandra* microsatellite *loci* used for the genetic analysis.

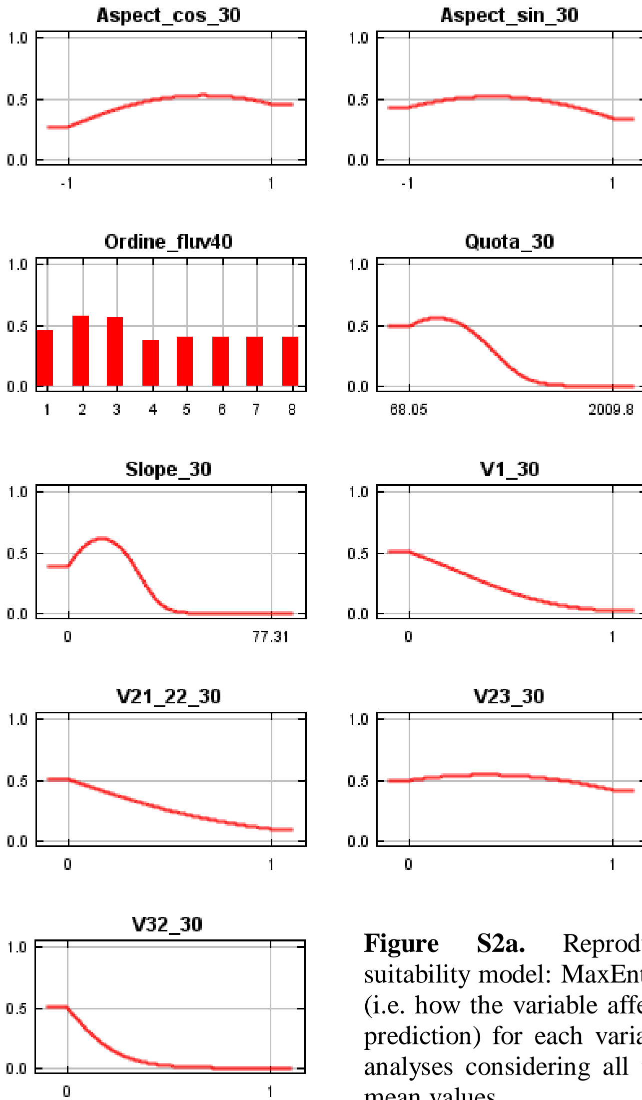

Figure S2a. Reproductive habitat suitability model: MaxEnt response curve (i.e. how the variable affects the MaxEnt prediction) for each variable used in the analyses considering all the other at the mean values.

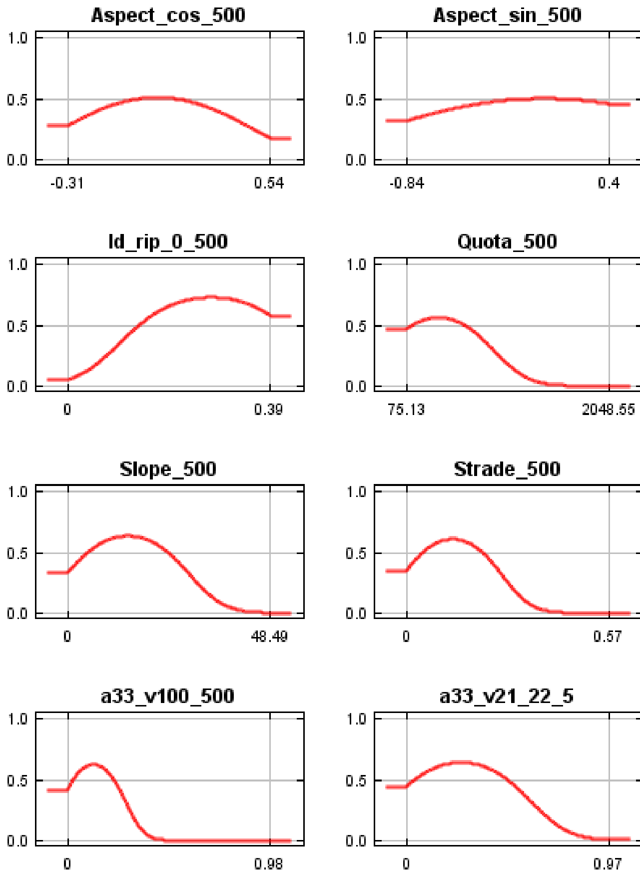

Figure S2b. Adult/dispersal habitat suitability model: MaxEnt response curve (i.e. how the variable affects the MaxEnt prediction) for each variable used in the analyses considering all the other at the mean values.

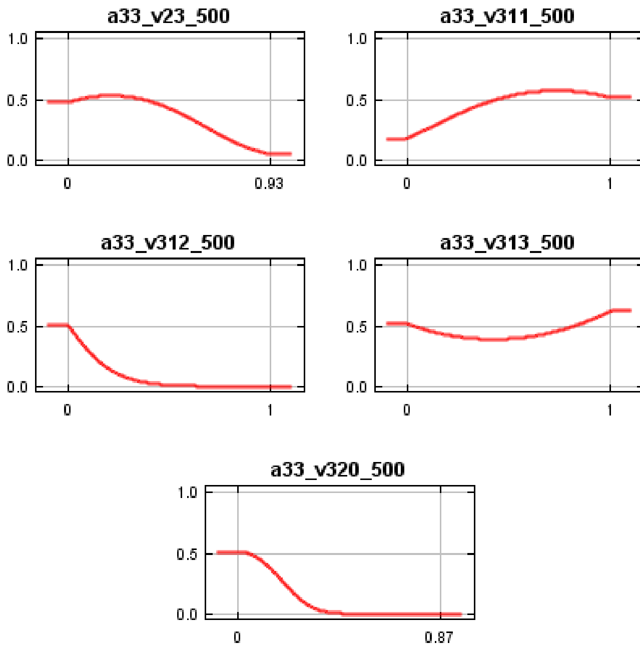

Figure S2b. Adult/dispersal habitat suitability model: MaxEnt response curve (i.e. how the variable affects the MaxEnt prediction) for each variable used in the analyses considering all the other at the mean values.

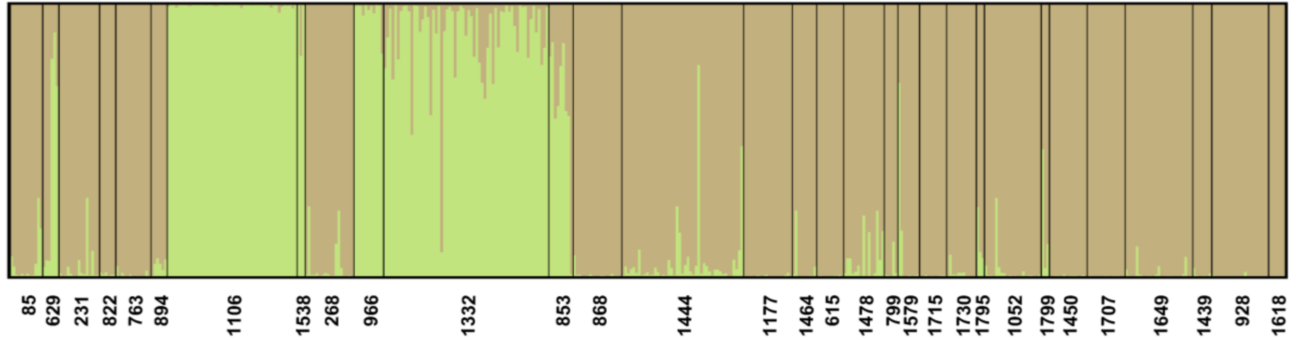

Figure S3a. STRUCTURE analysis of all samples (471 samples, 31 populations). Parameters: NO-ADMIXTURE model, INDEPENDENT ALLELE FREQUENCIES, LOCPRIOR, MCMC length 100,000 with a *burnin* of 10,000. K=2: green, WF, and brown, PEF.

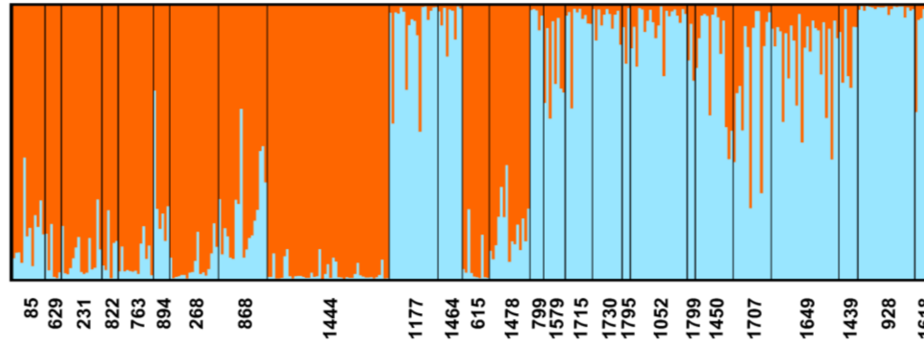

Figure S3b. STRUCTURE analysis of PEF (339 individuals, 26 populations). Parameters: NO-ADMIXTURE model, CORRELATE ALLELE FREQUENCIES, LOCPRIOR, MCMC length 100,000 with a *burnin* of 10,000. K=2.

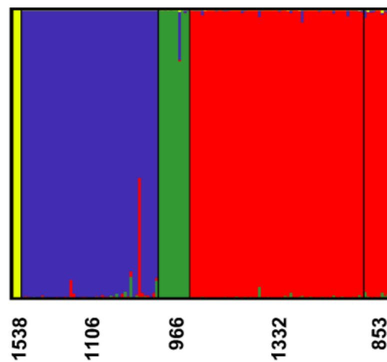

Figure S3c. STRUCTURE analysis of WF (132 individuals, 5 populations). Parameters: NO-ADMIXTURE model, CORRELATE ALLELE FREQUENCIES, LOCPRIOR, MCMC length 100,000 with a *burnin* of 10,000. K=4.

3. Ecological connectivity assessment by using molecular markers in fragmented populations of Fire Salamander (*Salamandra salamandra*)

Bani, L., G. Pisa, G. Spilotros, M. Luppi, E. Fabbri, E. Randi, V. Orioli

ABSTRACT

One of the most important consequences of anthropic pressure is habitat loss, degradation, and fragmentation. These phenomena affect the spatial distribution of animals and may divide big population in small isolated sub-populations that are more prone to extinction. To overcome this problem in fragmented landscapes is essential to enhance the meta-population persistence, conserving the functional connectivity between each fragment of habitat where populations are confined. The genetic distance between populations living in different fragments is a good meter of the genetic flow deriving from dispersal processes and, thus, the ecological connection or distance between fragments of habitat. According to the landscape genetic approach, genetic distances can be determined by geographic (isolation-by-distance) and/or ecological (isolation-by-resistance) distances. The aim of this study is to assess the degree of isolation of fragments of broad-lived forests in Lombardy (Northern Italy) by means of a genetic approach and using the Fire Salamander (*Salamandra salamandra, AMPHIBIA*, *URODELA*) as a target species, particularly affected by fragmentation due to its low dispersal capability. We collected 471 biological samples in 31 populations evenly distributed in the Prealpine and foothill lowland range of the species in the Region. All samples were genotyped at 16 species-specific microsatellites. Ecological distances were calculated by using one of the most promising methodology in landscape genetics studies, the circuit theory, applied to habitat suitability model. We found that the Euclidean distance did not explained genetic distances between sampling populations, while there was a significant correlation (Mantel's r) between genetic and ecological distances. These results highlighted the isolation of the populations living in the most fragmented and urbanised areas, that are actually surrounded by a high resistant matrix. Populations living in these areas showed a high ecological distance, although they are very close each other. This research highlight the usefulness of the application of the circuit theory to functional connectivity analysis for the study of the ecology of fragmented populations.

3.1 INTRODUCTION

Despite of the importance of biodiversity is recognized by a long time, human activities have not stopped altering its components, which are essential to ensure the ecosystem processes, from which the man himself depends (Costanza et al. 1997; Chapin et al. 2000; Hooper et al. 2005). Until now, the rate of biodiversity loss has not been reduced because the five main pressures on natural ecosystems still persist and are even intensified: habitat destruction; climate change; excessive nutrient load and other forms of pollution; over-exploitation and unsustainable use of natural resources; introduction of alien species (Wilcove et al. 1998; Primack, 2010; Groom et al. 2006; Kareiva & Marvier, 2011).

Among these pressures, the habitat destruction is probably one of the most studied. Nevertheless, the effects produced by the overall habitat loss still remain complex to understand. The habitat loss generates many other processes (most of them acting at genetic level) whose actions play cumulatively or interactively with it, determining the dynamics and the fate of populations (Gilpin & Soulé, 1986; Lindenmayer, 1995; Young et al. 1996). For example, the habitat loss (physical loss) and degradation (loss of ecological functionality) produce a reduction of population size; small populations are more prone to extinction because of genetic drift, inbreeding, environmental and demographic stochasticity (Gilpin & Soulé, 1986).

The habitat loss usually determines a fragmentation process of the original population, affecting the spatial distribution of remaining sub-populations, confining them to residual habitat fragments. Sub-populations may constitute a typical meta-population (*sensu* Levins 1969) or several isolated small populations. The isolation prevent the genetic exchange between sub-populations, determining their genetic differentiation and emphasizing the effects previously produced by the habitat loss.

For these reasons, population survival in fragmented landscapes depends on the maintenance of functional (i.e. ecological) connectivity among fragments of residual habitat, which should be guaranteed by the presence of dispersal corridors. They allow

maintaining the ecological connection between sub-populations in a form of meta-population (Levins, 1969). The knowledge of the ecology of fragmented populations is thus essential in order to prevent the habitat isolation and mitigate its effects (Saunders et al. 1987; Burgman & Lindenmayer, 1998). Understanding how landscape features affect dispersal between populations is thus important both for conservation purposes and evolutionary processes (Moore et al. 2011).

Traditional approaches aiming to evaluate the effectiveness of dispersal between populations, like radio-tracking or capture-mark-recapture methods (White & Garrott, 1990; Barrett & Peles, 1999; Fagan & Calabrese, 2006) appear to be not adequate to evaluate the ecological connectivity, because they hardly detect movements from birth sites to reproductive ones. However, these movements can be assessed by using DNA molecular markers that are able to detect gene flow in a meta-population (Cushman, 2006). For this reason, molecular markers are now one of the most efficient and promising tools to verify the ecological connectivity (Avise, 1994; Frankham et al. 2002; Frankham, 2006). Among molecular markers microsatellites are widely used, because they are considered "neutral" compared to selection pressures. Nevertheless, it should be underlined that microsatellites has sometimes been suspected to be non-completely neutral, as some observed variations within and among populations may be attributed to the selection (Kauer et al. 2003). Microsatellites are commonly used in landscape genetic studies for several practical reasons, because they are now available for many *taxa*, they require the collection of small amounts of tissue and a limited field effort.

Modelling genetic pattern over large areas by using environmental spatial data as covariates (i.e. landscape genetics; Manel et al. 2003; Holderegger & Wagner, 2006; Manel & Segelbacher, 2009), may lead to quantify how landscape features shape population genetic variability more accurately than traditional ecological methods do. Landscape genetic approach is ideal for investigating functional connectivity, particularly in species with low vagility or relatively small ranges (Moore et al. 2011).

The correlation of genetic distances between populations with the Euclidean distance (assuming a spatially homogenous landscape) or ecological distance (taking into account the influence of a heterogeneous landscape) is one of the most common approaches for evaluating the importance of organism-environment interaction in regards to gene flow (Dixon et al. 2006; Epps et al. 2007; Spear et al. 2010; Storfer et al. 2010). The isolation-by-distance theory (Wright 1943) predicts that genetic similarity among individuals decreases as the geographic distance between them increases: this pattern results from spatially limited dispersal; individuals living nearby to one another are more likely to interbreed than geographically distant ones. However, recent studies have demonstrated that measures of geographic distance which reflect landscape connectivity often explain a greater proportion of the genetic variability than simple Euclidean distance (Michels et al. 2001; Coulon et al. 2004; Spear et al. 2005; Vignieri 2005; Broquet et al. 2006; Cushman et al. 2006; Stevens et al. 2006; Pérez-Espona et al. 2008; Schwartz et al. 2009; Goldberg & Waits, 2010). In fact, in heterogeneous landscapes, straight-line geographical distances may not adequately reflect the true pattern of dispersal.
Effective geographic distances are often evaluated by means of the ecological resistance approach (e.g. Bani et al 2002; Compton et al. 2007; Tracey 2006; Beier et al. 2006, 2009; Carrol et al. 2011), that assess the different effects played by land-use and landscape features on dispersal movements and thus genetic distances (Sork & Waits 2010; Spear et al. 2010; Cushman et al. 2006; Shirk et al. 2010). These effects are usually quantified by habitat suitability models that allow to calculate the resistance to dispersal between populations. The resistance of potential routes along with dispersal may occur, can be evaluated with several methods. The most traditional method is the least-cost path analysis (Adriaensen et al. 2003), which calculates the pathway between two locations resulting in the least accumulated resistance. Recently McRae (2006) developed the circuit-theory analysis, which calculates a metric analogous to the amount of electrical current that could flow between two locations. This approach improve the LCPA, by identifying multiple pathways

connecting the studied populations.
In this paper we evaluated the effects of Euclidean (isolation-by-distance) and ecological distances (isolation-by-resistance) on genetic distance between 31 sampling populations of Fire Salamander (*Salamandra salamandra, AMPHIBIA*, *URODELA*). The species is mainly linked to broad-leaved forests ecosystems (Lanza et al. 2007), and is characterized by low dispersal capability, estimated in less than 500 m (Pisa et al. submitted; Denoël 1996; Schulte et al. 2007). Sampling populations were distributed along a gradient from continuous to fragmented broad-leaved forests located in the Prealpine and foothill belt of Lombardy (Northern Italy). In this area, according to a genetic population structure analysis, salamander populations were divided in two main sub-populations (Pisa et al. submitted): one inhabits a portion of the Western foothill belt (WF), where urban sprawl almost completely surrounded the residuals forests patches; the second sub-population lives in the continuous forest areas of the Prealps and Eastern foothill lowland (PEF). In the PEF sub-population a Western-Eastern gradient was detected, while in the WF the 5 sampling populations were further split in 4 populations of origin.

3.2 METHODS

3.2.1 Study area, sampling design and tissue sampling

The study area is a belt about 8000 km^2 wide, corresponding to the range of Fire Salamander within the Prealpine and foothill areas of Lombardy (Northern Italy). Although forests are mainly continuous in the Prealpine area, they have been progressively removed and fragmented by the urban sprawl in the foothill lowland (Figure 1).
In the study area, from 2010 to 2013, we collected 471 tissue samples, cutting the salamander larvae tail (about 3-4 mm): 204 in the Prealpine area and 267 in the foothill lowland. Salamander larvae were captured and handled with permit of the Lombardy regional administration (P. T1.2009.0016990 decreed on 2009/09/16 by D.G. Ambiente, Parchi e aree protette for 2010-2012 and administrative

order 964 decreed on 2013/02/11 by D.G. Agricoltura for 2013-2014). According to a habitat suitability model, individuals were grouped in 31 sampling populations, each one inhabiting a suitable habitat patch (Pisa et al. submitted). Distance between sampling populations varies from 1.5 to 140 kilometres (Figure 1).

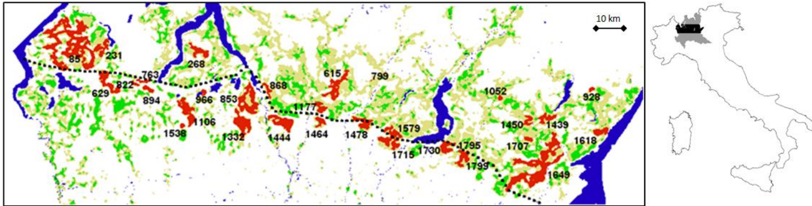

Figure 1. Study area in Lombardy (Northern Italy). In red the 31 sampling populations, where Fire Salamander presence probability, derived from a habitat suitability model, is higher than 25%. In green, suitable areas for the salamander (presence probability >25%), in light brown low suitable areas (5%< presence probability <25%). In white, unsuitable areas and in blue main water bodies. Dashed line represents the boundary between Prealps (in the North) and foothill lowland (in the South).

3.2.2 Genetic analyses and genetic distance

Tissue samples were stored in 95% ethanol and, subsequently, kept in laboratory at −20 °C. DNA was extracted with the Quick-g DNA™ MiniPrep kit (Zymo Research, USA), eluted in 180 µL of TE buffer (10 mM TrisHCl, pH 8; 0.1 mM EDTA) and stored at -20°C until subsequent handlings. All samples were genotyped by Polymerase Chain Reaction (PCR) for 16 species-specific microsatellite markers (Steinfartz et al. 2004; Hendrix et al. 2010): PCR amplifications were carried out in 10-µL mix reactions and PCR conditions were optimized for each primer pair (for laboratory details see Pisa et al. submitted). Amplifications were performed in a 9700 ABI thermal cycler, PCR products were analysed in an Applied Biosystems 3130XL DNA sequencer (Life Technology) and allele sizes were estimated using the software GENEMAPPER 4.0 (Life

Technology). Positive (known genotypes) and negative (no DNA) controls were always used to check for laboratory contaminations, which never occurred. A 10% randomly selected subset of the other samples were PCR-replicated two times to check for allelic drop-out and false alleles. Each *locus* was checked for null alleles (alleles that are present in a sample, yet are not amplified) using MICRO-CHECKER (Van Oosterhout et al. 2004).
To estimate the genetic distance between each pair of populations we calculated the chord distance, a measure based on allele frequencies (D_c, Cavalli-Sforza & Edwards, 1967), using the software Microsatellite Analyzer (Dieringer & Schlötterer, 2002). This measure seems to be more robust than Fst, as it may reflect allele frequencies changes more rapidly (Kalinowsky, 2002) and is more appropriate to account for genetic drift particularly at fine geographic scale (Goldberg & Waits, 2010).

3.2.3 Ecological distance

In this study, we adopted a habitat suitability model, developed in a previous study on Fire Salamander in the same study area (Pisa et al. submitted), that returned the probability of presence of the species for the whole study area. This model was here used in order to realize an ecological resistance surface. The habitat suitability model was developed using presence-only data, accounting for aquatic and terrestrial species needs. Environmental variables included in the model were elevation, slope, aspect, road density and land-use fractional cover. The model was built with MaxEnt software (Phillips et al. 2005).
We identified as core areas all those patches of continuous habitat with a probability of species presence higher than 25%. The 31 sampling populations corresponded to those core areas where at least three sampled individual were collected.
In order to identify the potential connections between core areas, we used the CIRCUITSCAPE software (Shah & McRae, 2008), that can evaluate the ecological distance between populations in heterogeneous landscapes using the electric circuit theory. This

approach allow to identify several routes of connections between populations, in contrast with the single route deriving from the classical least cost path approach. According to the circuit theory, circuit Resistance (R, ohm) can be interpreted as the opposition of each landscape element or feature to the movement of organism along its dispersal route. The isolation between pairs of nodes of the circuit (i.e. the isolation between populations in the landscape) can be measured by means of effective resistance, $\hat{R}$, (i.e. the ecological distance) calculated as the ratio between the voltage and current between pairs of nodes (McRae et al. 2008). As measure of ecological resistance we used the inverse of the habitat suitability.
The ecological distance considered multiple pathways connecting populations, decreasing as more connections are added, and incorporated both the minimum movement distance and the availability of alternative pathways. Distances were calculated considering both all the 31 sampling populations (ALL) as a whole, and the two main sub-populations separately, the 26 sampling populations of the Prealpine and Eastern foothill sub-population (PEF) and the 5 sampling populations of the Western foothill sub-population (WF).

3.2.4 Statistical analyses

We calculated the genetic distance of the 5 WF sampling populations and three groups of 5 sampling populations corresponding to the two extremity of the Western-Eastern gradient and the central area of the PEF sub-population (Pisa et al. submitted), in order to evaluate variation in chord distance between populations with different degrees of fragmentation. Then, we tested the isolation-by-distance hypothesis performing a Mantel test (Mantel, 1967; Mantel & Valand, 1970) between genetic distance (D_c) and Euclidean distance measured for each pair of sampling populations. Finally, we tested the isolation-by-resistance hypothesis performing a partial Mantel test (Smouse et al. 1986) between genetic distance (D_c) and ecological distance, controlling for the Euclidean distance measured for each pair of sampling populations. These analyses were

performed for ALL, PEF and WF populations.
Statistical analysis were performed using the software R (R Core Team, 2012) with the Vegan package (Oksanen et al. 2013).

3.3 RESULTS

The 5 WF sampling populations (853, 966, 1106, 1332, 1538) showed a mean genetic distance (measured as chord distance, D_c=0.322) lower than that calculate for the 5 selected Eastern PEF sampling populations (1052, 1439, 1450, 1649, 1707; D_c=0.230). Conversely, no differences were found between the 5 WF sampling populations and the 5 selected Western PEF ones (85, 231, 629, 763, 822; D_c=0.285) as well as the 5 central PEF ones (615, 868, 1177, 1444, 1464; D_c=0.255; Figure 2). Mean genetic distances were controlled for mean Euclidean distances among each of these four groups and no differences were found: mean Euclidean distance (ED) of WF was 11.8 km,; mean ED of Eastern PEF was 12.0 km; mean ED of Western PEF was 10.7 km and mean ED of central PEF was 13.6 km.
The Figure 3 represents the ecological resistance map derived from the habitat suitability model. The 5 WF sampling populations (853, 966, 1106, 1332, 1538) appeared to be surrounded by high resistant surfaces, while many other sampling populations from foothill areas (e.g. 629, 1444, 1715, 1795, 1799) seemed to be more connected with the Prealpine belt and its populations (e.g. 85, 615, 1579, 1618, 1649). For each pair of sampling populations, genetic, Euclidean and ecological distances are shown in Table S1.
The correlation analysis (Mantel test) between the genetic distance (chord distance, D_c) and the Euclidean distance was not significant analysing both all sampling populations (ALL) and the two main sub-populations (PEF and WF) separately (Table 1). Conversely, the correlation analysis (partial Mantel test) between the chord distance and the ecological distance, calculated by CIRCUITSCAPE, controlling for the Euclidean distance, always resulted significant. Mantel's r values appeared to be particularly high (r=0.618) in the

WF sub-population, while lower, although significant, in the PEF sub-populations (r=0.291).

	N	Euclidean distance		Ecological dist. | Euclidean distance	
		Mantel's r	p-value	Mantel's r	p-value
ALL	31	0.100	0.132	0.247	0.006
PEF	26	0.096	0.171	0.291	0.003
WF	5	0.095	0.351	0.618	0.044

Table 1. Correlation between chord distance (D_c) and Euclidean distance (Mantel test) and correlation between chord distance and ecological distance, controlling for Euclidean distance (partial Mantel test). Analysis were performed for all (ALL) sampling populations, Prealpine and Eastern foothill lowland (PEF) and Western foothill lowland (WF) sub-populations.

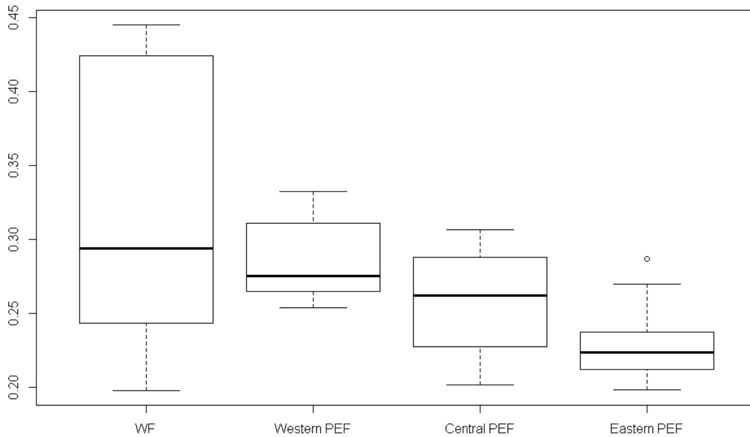

Figure 2. Boxplot of chord distances (Dc) of the WF, Western, central and Eastern PEF sub-populations. All sub-populations included 5 sampling populations (WF: 853, 966, 1106, 1332, 1538; Western PEF: 85, 231, 629, 763, 822; central PEF: 615, 868, 1177, 1444, 1464; Eastern PEF: 1052, 1439, 1450, 1649, 1707).

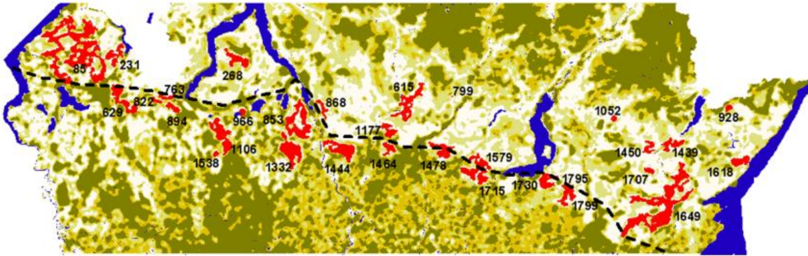

Figure 3. Ecological resistance map: the ramp yellow colour, from light to dark, represent the gradient from more permeable to high resistant surfaces to dispersal movement. In red the 31 sampling populations and in blue main water bodies. Dashed line represents the boundary between Prealps (in the North) and foothill lowland (in the South).

3.4 DISCUSSION

A previous work, that analysed the genetic population structure of Fire Salamander in this study area, showed a sharply separation between Prealpine and Eastern foothill lowland populations (PEF) from those inhabiting a portion of the Western foothill lowland (WF) (Pisa et al. submitted). The study also highlighted that the PEF sampling populations are not separated from each other but they constituted a Western-Eastern gradient along all the Prealps, including a wide portion of the Eastern foothill area. Conversely, the WF sampling populations, beyond being ecologically separated from the PEF sub-populations, tended also to be isolated from each other. This was highlighted by the measure of the mean genetic distance (measured as chord distance) that resulted higher in WF compared to the PEF sub-populations, where mean genetic distances decreased West-Eastward (Figure 2). This may be due to the different degree of fragmentation of the salamander populations: the Eastern PEF sampling populations are all located in the continuous Prealpine belt, while the WF sampling populations are completely included in the foothill lowland, where species habitat is almost completely fragmented and isolated; conversely, the Western PEF and central PEF sampling populations are located over the boundary between the

Prealpine and foothill areas (Figure 3). This genetic population structure is confirmed by the results of the present research that allowed identifying some significant environmental features (e.g. elevation, roads and land-use) affecting the genetic distances (chord distance, D_c) between populations. On the whole, the Euclidean distance was not important in determining the genetic distance between sampling populations (Mantel's r=0.100, p=0.132). Even analysing only the PEF sub-population, where forests are mainly continuous (see habitat suitability map in Figure 1) and permeable (see ecological resistance map in Figure 2), the Euclidean distance was not significant in explaining genetic distances between sampling populations (r=0.096, p=0.171). In fact, the ecological distance was proved to be significant correlated with the genetic distances controlling for the Euclidean distance (r=0.291, p=0.003). The correlation between genetic distance and Euclidean distance in the WF sub-populations was not significant (r=0.095, p=0.351). Nevertheless, in this case the role of ecological distance appeared even important than in PEF sub-population in determining the genetic distances between sampling populations: the partial Mantel test on genetic distance and ecological distance, controlling for Euclidean distance reached the value of 0.681 (p=0.044). This quite high value may explain the genetic population structure of WF sampling populations (Pisa et al. submitted), that resulted highly separated from each other by a the presence of a high resistant matrix (Figure 2), characterized by wide urban surfaces and high road density (Vos & Chardon, 1998; Carr & Faharig, 2001) that separate suitable areas for the Fire Salamander. In fact habitat fragmentation and other anthropic barriers halt dispersal (Gibbs, 1998; deMaynadier & Hunter, 2000), increase mortality (Faharig et al. 1995; Carr & Faharig, 2001), thus concurring to emphasize genetic divergence between populations and genetic diversity (Reh & Seits, 1990).

The results of the present research emphasized the effectiveness of the circuit theory approach to define the effective ecological distances between populations in fragmented landscapes. This approach allowed identifying significant correlations between

genetic and ecological distances and thus the usefulness of ecological resistance maps as a basic tool for conservation purposes (Carroll et al. 2011).

REFERENCES

Adriaensen F, Chardon JP, De Blust G, Swinnen E, Villalba S, Gulinck H & Matthysen E (2003) The application of 'least-cost' modelling as a functional landscape model. *Landscape and Urban Planning*, 64(4), 233-247. DOI: 10.1016/S0169-2046(02)00242-6

Avise JC (1994) *Molecular Markers, Natural History and Evolution*. New York, Chapman & Hall.

Bani L, Baietto M, Bottoni L & Massa R (2002) The use of focal species in designing a habitat network for a lowland area of Lombardy, Italy. *Conservation Biology* 16 (3), 826-831. DOI: 10.1046/j.1523-1739.2002.01082.x

Barrett GW & Peles JD (1999) *Landscape Ecology of Small Mammals*. New York , Springer-Verlag Inc.

Beier P, Penrod KL, Luke C, Spencer WD & Cabañero C (2006) South Coast Missing Linkages: connectivity to wildlands in the largest metropolitan areas in the USA. In K.R. Crooks, M. Sanjayan (red.), *Connectivity Conservation* (pp. 555-586). Cambridge, Cambridge University Press.

Beier P, Majka DR & Newell SL (2009) Uncertainty analysis of least-cost modeling for designing wildlife linkages. *Ecological Applications*, 19 (8), 2067-2077. DOI: 10.1890/08-1898.1

Broquet T, Ray N, Petit E, Fryxell JM & Burel F (2006) Genetic isolation by distance and landscape connectivity in the American marten (*Martes americana*). *Landscape Ecology*, 21 (6), 877-889. DOI: 10.1007/s10980-005-5956-y

Burgman MA & Lindenmayer DB (1998) *Conservation biology for the Australian environment.* NSW: Surrey Beatty, Chipping Norton.

Carr LW & Fahrig L (2001) Effect of road traffic on two amphibian species of different vagility. *Conservation Biology*, 15(4),1071-1078. DOI: 10.1046/j.1523-1739.2001.0150041071.x

Carroll C, McRae BH & Brookes A (2011) Use of Linkage Mapping and Centrality Analysis Across Habitat Gradients to Conserve Connectivity of Gray Wolf Populations in Western North America. *Conservation Biology*, 26(1), 78-87. DOI: 10.1111/j.1523-1739.2011.01753.x

Cavalli-Sforza LL & Edwards AWF (1967) Phylogenetic analysis: models and estimation procedures. *American Journal of Human Genetics*, 19, 233-257.

Chapin FS, Zavaleta ES, Eviner VT, Naylor RL, Vitousek PM, Reynolds HL, Hooper DU, Lavorel S, Sala OE, Hobbie SE, Mack MC & Diaz S (2000) Consequences of changing biodiversity. *Nature*, 405, 234-242. DOI: 10.1038/35012241

Compton BW, McGarigal K, Cushman SA & Gamble LR (2007) A Resistant-Kernel Model of Connectivity for Amphibians that Breed in Vernal Pools. *Conservation Biology*, 21(3), 788-799. DOI: 10.1111/j.1523-1739.2007.00674.x

Costanza R, dArge R, deGroot R, Farber S, Grasso M, Hannon B, Limburg K, Naeem S, ONeill RV, Paruelo J, Raskin RG, Sutton P & vandenBelt M (1997) The value of the world's ecosystem services and natural capital. *Nature*, 387, 253-260.

Coulon A, Cosson JF, Angibault JM, Cargnelutti B, Galan M, Morellet N, Petit E, Aulagnier S & Hewison AJM (2004) Landscape connectivity influences gene flow in a roe deer population inhabiting a fragmented landscape: an individual-based approach. *Molecular Ecology*, 13(9), 2841-2850. DOI: 10.1111/j.1365-294X.2004.02253.x

Cushman SA (2006) Effects of habitat loss and fragmentation on amphibians: A review and prospectus. *Biological Conservation*, 128(2), 231-240. DOI: 10.1016/j.biocon.2005.09.031

Cushman SA, McKelvey KS, Hayden J & Schwartz MK (2006) Gene flow in complex landscapes: testing multiple hypotheses with causal modeling. *American Naturalist*, 168(3), 486-499.

deMaynadier PG & Hunter Jr. ML (2000) Road effects on amphibian movements in a forested landscape. *Natural Areas Journal*, 20, 56–65.

Denoël M (1996) Phénologie et domaine vital de la salamandre terrestre *Salamandra salamandra* terrestris (*Amphibia, Caudata*) dans un bois du Pays de Herve (Belgique). *Chaiers d'Ethologie*, 16, 291-306.

Dieringer D & Schlötterer C (2002) Microsatellite Analyzer (MSA): a platform independent analysis tool for large microsatellite datasets. *Molecular Ecology Notes*, 3(1), 167-169. DOI: 10.1046/j.1471-8286.2003.00351.x

Dixon JD, Oli MK, Wooten MC, Eason TH, McCown JW & Paetkua D (2006) Effectiveness of a Regional Corridor in Connecting Two Florida Black Bear Populations. *Conservation Biology*, 20(1), 155-162. DOI: 10.1111/j.1523-1739.2005.00292.x

Epps CW, Wehausen JD, Bleich VC, Torres SG & Brashares JS (2007) Optimizing dispersal and corridor models using landscape genetics. *Journal of Applied Ecology*, 44(4), 714-724. DOI: 10.1111/j.1365-2664.2007.01325.x

Fagan WF & Calabrese JM (2006) Quantifying connectivity: balancing metric performance with data requirements In K.R. Crooks, M. Sanjayan (red.), *Connectivity Conservation* (pp. 297-317). Cambridge , Cambridge University Press.

Fahrig, L, Pedlar JH, Pope SE, Taylor PD & Wegner JF (1995) Effect of road traffic on amphibian density. *Biological Conservation*, 73(3), 177–182. DOI: 10.1016/0006-3207(94)00102-V

Frankham, R (2006) Genetics and landscape connectivity. In K.R. Crooks, M. Sanjayan (red.), *Connectivity Conservation* (pp. 72-96). Cambridge, Cambridge University Press.

Frankham R, Ballou JD & Briscoe DA (2002) *Introduction to Conservation Genetics*. Cambridge, Cambridge University Press.

Gibbs JP (1998) Amphibian movements in response to forest edges, roads, and streambeds in Southern New England. *Journal of Wildlife Management*, 62, 584-589.

Giplin ME & Soulé ME (1986) Minimum viable populations: processes of species extinctions. In: *Conservation biology: the science of scarcity and diversity* (pp. 19-34) Soulé, M.E. (ed.). Sunderland, MA: Sinauer Associates.

Goldberg CS & Waits LP (2010) Comparative landscape genetics of two pond-breeding amphibian species in a highly modified agricultural landscape. *Molecular Ecology*, 19(17), 3650-3663. DOI: 10.1111/j.1365-294X.2010.04673.x

Groom MJ, Meffe GK & Carroll RC (2006) *Principles of Conservation Biology, Third Edition.* Sunderland (USA), Sinauer Associates.

Hendrix R, Hauswaldt SJ, Veith M & Steinfartz S (2010) Strong correlation between cross-amplification success and genetic distance across all members of 'True Salamanders' (*Amphibia: Salamandridae*) revealed by *Salamandra salamandra*-specific microsatellite *loci*. *Molecular Ecology Resources*, 10(6), 1038-1047. DOI: 10.1111/j.1755-0998.2010.02861.x

Holderegger R & Wagner HH (2006) A brief guide to landscape genetics. *Journal Landscape Ecology*, 21(6), 793-796. DOI: 10.1007/s10980-005-6058-6

Hooper DU, Chapin FS, Ewel JJ, Hector A, Inchausti P, Lavorel S, Lawton JH, Lodge DM, Loreau M, Naeem S, Schmid B, Setala H, Symstad AJ, Vandermeer J & Wardle DA (2005) Effects of biodiversity on ecosystem functioning: A consensus of current knowledge. *Ecological Monographs*, 75(1), 3-35. DOI: 10.1890/04-0922

Kalinowski ST (2002), Evolutionary and statistical properties of three genetic distances. *Molecular Ecology*, 11(8), 1263-1273. DOI: 10.1046/j.1365-294X.2002.01520.x

Kareiva P & Marvier M (2011) *Conservation Science: Balancing the Needs of People and Nature.* Colorado (USA), Roberts and Company Publishers.

Kauer M, Dieringer D & Schlotterer C (2003) Nonneutral admixture of immigrant genotypes in african *Drosophila melanogaster* populations from Zimbabwe. *Molecular Biology and Evolution*, 20, 1329-1337.

Lanza B, Andreone F, Bologna MA, Corti C & Razzetti E (2007) Fauna d'Italia. AMPHIBIA. Bologna (Italy), Ed. Calderini.

Levins R (1969) Some demographic and genetic consequences of environmental heterogeneity for biological control. *Bulletin of the Entomological Society of America*, 15, 237-240.

Lindenmayer DB (1995) Disturbance, forest wildlife conservation and a conservative basis for forest management in the mountain ash forests of Victoria. *Forest Ecology and Management*, 74, 223-231.

Manel S & Segelbacher G (2009) Perspectives and challenges in landscape genetics. *Molecular Ecology*, 18(9), 1821-1822. DOI: 10.1111/j.1365-294X.2009.04151.x

Manel S, Schwartz MK, Luikart G & Taberlet P (2003) Landscape genetics: combining landscape ecology and population genetics. *Trends in Ecology & Evolution*, 18(4), 189-197. DOI: 10.1016/S0169-5347(03)00008-9

Mantel N (1967) The detection of disease clustering and a generalized regression approach. *Cancer Research*, 27(2 Part 1), 209-220.

Mantel N & Valand RS (1970) A technique of nonparametric multivariate analysis. *Biometrics*, 26, 547-558.

McRae BH (2006) Isolation by resistance. *Evolution*, 60(8), 1551-1561. DOI: 10.1111/j.0014-3820.2006.tb00500.x

McRae BH, Dickson BG, Keitt TH & Viral B (2008) Using Circuit Theory to Model Connectivity in Ecology, Evolution, and Conservation. *Ecology*, 89(10), 2712-2724. DOI: 10.1890/07-1861.1

Michels E, Cottenie K, Neys L, De Gelas K, Coppin P & De Meester L (2001) Geographical and genetic distances among zooplankton populations in a set of interconnected ponds: a plea for using GIS modelling of the effective geographical distance. *Molecular Ecology*, 10(8), 1929-1938. DOI: 10.1046/j.1365-294X.2001.01340.x

Moore JA, Tallmon DA, Nielsen J & Pyare S (2011) Effects of the landscape on boreal toad gene flow: does the pattern–process relationship hold true across distinct landscapes at the Northern range margin? *Molecular Ecology*, 20(23), 4858–4869. DOI: 10.1111/j.1365-294X.2011.05313.x

Oksanen J, Guillaume Blanchet F, Kindt R, Legendre P, Minchin PR, O'Hara RB, Simpson GL, Solymos P, Henry M, Stevens H & Wagner H (2013) vegan: Community Ecology Package. R package version 2.0-9.

Pérez-Espona S, Pérez-Barbería FJ, McLeod JE, Jiggins CD, Gordon IJ & Pemberton JM (2008) Landscape features affect gene flow of Scottish Highland red deer (*Cervus elaphus*). *Molecular Ecology*, 17(4), 981-996. DOI: 10.1111/j.1365-294X.2007.03629.x

Phillips SJ, Anderson RP & Schapire RE (2005) Maximum entropy modeling of species geographic distributions. *Ecological Modelling*, 190, 231-259.

Pisa G, Orioli V, Spilotros G, Fabbri E, Randi E & Bani L (2014) Fire Salamander population structure in fragmented landscape in Northern Italy. *Submitted.*

Primack RB (2010) *Essentials of Conservation Biology, Fifth Edition.* Sunderland (USA), Sinauer Associates.

Reh W & Seitz A (1990) The influence of land use on the genetic structure of populations of the common frog (*Rana temporaria*). *Biological Conservation*, 54(3), 239-249.

Saunders DA, Arnold GW, Burbridge AA & Hopkins AJ (1987) *Nature conservation: the role of remnants of native vegetation.* NSW: Surrey Beatty , Chipping Norton.

Schulte U, Küsters D & Steinfartz S (2007) A PIT tag based analysis of annual movement patterns of adult Fire Salamanders (*Salamandra salamandra*) in a Middle European habitat. *Amphibia-Reptilia*, 28(4), 531-536. DOI: 10.1163/156853807782152543

Schwartz MK, Copeland JP, Anderson NJ, Squires JR, Inman RM, McKelvey KS, Pilgrim KL, Waits LP & Cushman SA (2009) Wolverine gene flow across a narrow climatic niche. *Ecology*, 90(11), 3222-3232. DOI: 10.1890/08-1287.1

Shirk AJ, Wallin DO, Cushman SA, Rice CG & Warheit KI (2010) Inferring landscape effects on gene flow: a new model selection framework. *Molecular Ecology*, 19(17), 3603-3619. DOI: 10.1111/j.1365-294X.2010.04745.x

Shah VB & McRae BH (2008) Circuitscape: a tool for landscape ecology. Proceedings of the 7th Python in Science Conference 2008 (pp.62-66) ,SciPy.

Smouse PE, Long JC & Sokal RR (1986) Multiple regression and correlation extensions of the Mantel test of matrix correspondence. *Systematic zoology*, 35, 627-632.

Sork VL & Waits L (2010) Contributions of landscape genetics - approaches, insights, and future potential. *Molecular Ecology*, 19 (17), 3489-3495. DOI: 10.1111/j.1365-294X.2010.04786.x

Spear SF, Peterson CR, Matocq MD & Storfer A (2005) Landscape genetics of the blotched tiger salamander (*Ambystoma tigrinum melanostictum*). *Molecular Ecology*, 14(8), 2553-2564. DOI: 10.1111/j.1365-294X.2005.02573.x

Spear SF, Balkenhol N, Fortin MJ, McRae BH, Scribner K (2010) Use of resistance surfaces for landscape genetic studies: considerations for parameterization and analysis. *Molecular Ecology*, 19(17), 3576-3591. DOI: 10.1111/j.1365-294X.2010.04657.x

Steinfartz S, Küsters D & Tautz D (2004) Isolation and characterization of polymorphic tetranucleotide microsatellite *loci* in the Fire Salamander *Salamandra salamandra* (Amphibia: Caudata). *Molecular Ecology Notes*, 4(4), 626-628. DOI: 10.1111/j.1471-8286.2004.00716.x

Stevens VM, Verkenne C, Vandewoestijne S, Wesselingh RA & Baguette M (2006) Gene flow and functional connectivity in the natterjack toad. *Molecular Ecology*, 15(9), 2333-2344. DOI: 10.1111/j.1365-294X.2006.02936.x

Storfer A, Murphy MA, Spear SF, Holderegger R & Waits LP (2010) Landscape genetics: where are we now? *Molecular Ecology*, 19(17), 3496-3514. DOI: 10.1111/j.1365-294X.2010.04691.x

Tracey JA (2006) Individual-based modeling as a tool for conserving connectivity. In K.R. Crooks, M. Sanjayan (red.), *Connectivity Conservation* (pp. 343-368). Cambridge, Cambridge University Press.

Van Oosterhout C, Hutchinson WF, Wills DPM & Shipley P (2004) Microchecker: software for identifying and correcting genotyping errors in microsatellite data. *Molecular Ecology Notes*, 4(3), 535-538. DOI: 10.1111/j.1471-8286.2004.00684.x

Vignieri SN (2005) Streams over mountains: influence of riparian connectivity on gene flow in the Pacific jumping mouse (*Zapus trinotatus*). *Molecular Ecology*, 14 (7), 1925-1937. D OI: 10.1111/j.1365-294X.2005.02568.x

Vos CC & Chardon JP (1998) Effects of habitat fragmentation and road density on the distribution pattern of the moor frog *Rana arvalis*. *Journal of Applied Ecology*, 35(1), 44-56. DOI: 10.1046/j.1365-2664.1998.00284.x

White GC & Garrott RA (1990) *Analysis of Wildlife Radiotracking Data*. London, Academic Press.

Wilcove DS, Rothstein D, Dubow J, Philips A & Losos E (1998) Quantifying threats to imperiled species in the United States -- assessing the relative importance of habitat destruction, alien species, pollution, overexploitation and disease. *Bioscience*, 48, 607-615.

Wright S (1943) Isolation by distance. *Genetics*, 28(2), 114-138.

Young AG, Boyle T & Brown AHD (1996) The population genetic consequences of habitat fragmentation for plants. *Trend in Ecology and Evolution*, 11(10): 413-418. DOI: 10.1016/0169-5347(96)10045-8

SUPPLEMENTARY MATERIAL

p1	p2	Euc	Eco	D_c	p1	p2	Euc	Eco	D_c	p1	p2	Euc	Eco	D_c
85	231	5.2	1.8	0.255	231	1618	137.0	4003.8	0.321	615	1478	15.5	852.3	0.287
85	268	35.2	1464.7	0.231	231	1649	119.5	2586.0	0.241	615	1538	47.3	314.6	0.246
85	615	72.0	926.2	0.243	231	1707	120.7	3712.5	0.222	615	1579	21.6	470.9	0.269
85	629	11.9	34.2	0.328	231	1715	85.4	1480.6	0.281	615	1618	68.8	495.0	0.338
85	763	16.8	212.8	0.270	231	1730	99.3	2351.9	0.286	615	1649	53.6	169.6	0.284
85	799	78.8	2416.0	0.326	231	1795	103.3	3737.4	0.354	615	1707	53.0	844.9	0.300
85	822	16.5	213.4	0.280	231	1799	105.8	2920.6	0.342	615	1715	23.2	557.2	0.340
85	853	47.7	558.4	0.289	268	615	37.1	481.9	0.244	615	1730	34.1	838.9	0.365
85	868	51.1	1035.4	0.206	268	629	27.4	888.1	0.306	615	1795	37.7	1840.9	0.478
85	894	19.7	272.2	0.298	268	763	20.5	993.4	0.249	615	1799	40.7	369.5	0.247
85	928	139.9	4354.7	0.268	268	799	44.0	1452.2	0.330	629	763	6.9	2674.0	0.213
85	966	33.9	1083.6	0.325	268	822	21.6	1284.6	0.290	629	799	69.5	969.5	0.274
85	1052	113.8	2606.8	0.243	268	853	18.4	667.7	0.334	629	822	6.0	1718.0	0.243
85	1106	33.0	313.9	0.246	268	868	17.5	699.5	0.333	629	853	37.1	445.8	0.331
85	1177	67.6	950.3	0.259	268	894	19.8	200.6	0.279	629	868	41.6	150.0	0.290
85	1332	46.9	587.8	0.245	268	928	104.9	2304.3	0.260	629	894	8.8	746.5	0.251
85	1439	127.4	2974.8	0.292	268	966	11.2	1126.8	0.299	629	928	130.5	1304.1	0.293

Table S1. Euclidean (Euc), ecological (Eco) and genetic chord (D_c) distances for each pair of sampling populations (p1, p2).

p1	p2	Euc	Eco	D_c	p1	p2	Euc	Eco	D_c	p1	p2	Euc	Eco	D_c
85	1444	58.1	796.5	0.250	268	1052	79.0	533.6	0.206	629	966	23.4	793.7	0.339
85	1450	121.4	2749.8	0.241	268	1106	17.6	1097.4	0.273	629	1052	104.3	3706.1	0.283
85	1464	66.9	3187.2	0.293	268	1177	34.3	2990.6	0.252	629	1106	21.6	484.6	0.234
85	1478	78.6	1589.8	0.314	268	1332	20.4	344.8	0.289	629	1177	57.4	552.3	0.234
85	1538	32.3	588.5	0.482	268	1439	93.0	1742.1	0.229	629	1332	35.8	187.2	0.244
85	1579	86.1	1423.9	0.254	268	1444	27.3	285.9	0.268	629	1439	117.6	2675.8	0.341
85	1618	140.3	4263.1	0.315	268	1450	86.9	495.1	0.246	629	1444	47.4	2305.7	0.329
85	1649	122.3	2810.8	0.233	268	1464	34.4	268.8	0.204	629	1450	111.6	2111.1	0.352
85	1707	123.8	3942.4	0.244	268	1478	45.7	2004.2	0.265	629	1464	56.5	2622.5	0.462
85	1715	88.2	1611.6	0.261	268	1538	20.9	390.2	0.216	629	1478	68.1	288.0	0.445
85	1730	102.2	2570.8	0.270	268	1579	53.4	1084.3	0.309	629	1538	20.5	161.4	0.279
85	1795	106.1	3961.8	0.330	268	1618	105.8	669.5	0.327	629	1579	75.5	26.0	0.425
85	1799	108.5	3141.3	0.338	268	1649	89.1	1846.3	0.245	629	1618	130.4	690.7	0.265
231	268	31.4	1324.1	0.225	268	1707	89.7	1976.7	0.289	629	1649	111.7	227.1	0.385
231	615	68.5	834.2	0.284	268	1715	55.5	763.7	0.351	629	1707	113.7	509.4	0.315
231	629	12.3	29.0	0.286	268	1730	69.0	949.7	0.274	629	1715	77.5	1552.3	0.279
231	763	15.1	169.9	0.254	268	1795	72.8	701.8	0.459	629	1730	91.7	24.5	0.265
231	799	75.4	2289.0	0.293	268	1799	75.5	847.7	0.278	629	1795	95.6	610.3	0.328
231	822	15.2	174.3	0.265	615	629	62.9	1890.7	0.239	629	1799	97.9	71.7	0.233

Table S1. Euclidean (Euc), ecological (Eco) and genetic chord (D_c) distances for each pair of sampling populations (p1, p2).

p1	p2	Euc	Eco	D_c	p1	p2	Euc	Eco	D_c	p1	p2	Euc	Eco	D_c
231	853	45.3	495.9	0.277	615	763	56.2	725.2	0.293	763	799	62.8	82.9	0.342
231	868	48.0	947.9	0.203	615	799	7.0	2918.9	0.276	763	822	1.3	879.5	0.327
231	894	18.4	224.0	0.276	615	822	57.0	73.3	0.316	763	853	31.0	327.9	0.267
231	928	136.3	4083.5	0.262	615	853	28.1	696.9	0.297	763	868	34.9	531.7	0.326
231	966	31.5	1012.0	0.290	615	868	21.4	1598.2	0.282	763	894	3.4	448.1	0.254
231	1052	110.3	2388.4	0.236	615	894	54.3	237.8	0.286	763	928	123.8	2091.1	0.279
231	1106	31.6	273.1	0.254	615	928	67.8	3328.2	0.254	763	966	17.1	1108.6	0.393
231	1177	64.6	858.6	0.251	615	966	39.9	232.0	0.335	763	1052	97.7	1522.5	0.331
231	1332	44.8	525.9	0.215	615	1052	41.8	2682.5	0.311	763	1106	16.5	85.5	0.244
231	1439	124.2	2748.6	0.289	615	1106	44.2	197.2	0.314	763	1177	51.0	939.2	0.254
231	1444	55.6	713.7	0.240	615	1177	11.1	1011.3	0.331	763	1332	30.1	490.5	0.440
231	1450	118.1	2527.0	0.228	615	1332	31.5	378.4	0.259	763	1439	111.1	3481.1	0.265
231	1464	64.2	2999.4	0.288	615	1439	56.0	689.3	0.304	763	1444	41.4	565.8	0.289
231	1478	75.8	1462.4	0.255	615	1444	20.8	411.9	0.276	763	1450	105.1	861.7	0.269
231	1538	31.4	536.6	0.407	615	1450	49.8	61.2	0.265	763	1464	50.2	629.6	0.290
231	1579	83.4	1294.8	0.273	615	1464	14.1	761.4	0.281	763	1478	61.9	774.0	0.330
763	1538	16.4	40.6	0.226	822	1715	71.9	341.1	0.230	894	1332	27.2	2643.1	0.298
763	1579	69.4	382.9	0.296	822	1730	86.0	650.8	0.263	894	1439	108.8	1287.2	0.244
763	1618	123.9	59.4	0.197	822	1795	90.0	0.8	0.214	894	1444	38.7	1204.7	0.279

Table S1. Euclidean (Euc), ecological (Eco) and genetic chord (D_c) distances for each pair of sampling populations (p1, p2).

p1	p2	Euc	Eco	D_c	p1	p2	Euc	Eco	D_c	p1	p2	Euc	Eco	D_c
763	1649	105.6	154.8	0.350	822	1799	92.3	578.1	0.266	894	1450	102.8	526.5	0.430
763	1707	107.3	477.0	0.194	853	868	8.7	91.0	0.285	894	1464	47.7	176.1	0.227
763	1715	71.4	422.6	0.266	853	894	28.4	411.5	0.294	894	1478	59.4	2379.8	0.315
763	1730	85.5	735.6	0.332	853	928	94.1	31.5	0.265	894	1538	13.1	171.5	0.241
763	1795	89.4	343.4	0.248	853	966	13.9	363.3	0.260	894	1579	66.8	1593.4	0.275
763	1799	91.8	125.8	0.322	853	1052	68.0	152.0	0.302	894	1618	121.6	252.3	0.233
799	822	63.6	37.7	0.233	853	1106	16.5	88.5	0.308	894	1649	103.0	1138.0	0.282
799	853	33.9	206.5	0.317	853	1177	20.6	437.6	0.483	894	1707	104.9	3589.8	0.314
799	868	28.0	310.6	0.293	853	1332	4.1	79.0	0.282	894	1715	68.8	72.1	0.301
799	894	60.8	2451.9	0.279	853	1439	80.7	682.9	0.337	894	1730	82.9	36.0	0.258
799	928	61.0	3349.2	0.372	853	1444	10.4	379.0	0.248	894	1795	86.9	250.2	0.280
799	966	46.3	923.2	0.378	853	1450	74.8	620.1	0.224	894	1799	89.2	196.7	0.250
799	1052	35.0	2460.5	0.320	853	1464	19.4	96.9	0.303	928	966	107.2	369.3	0.315
799	1106	50.3	76.0	0.278	853	1478	31.1	325.9	0.300	928	1052	26.2	232.2	0.285
799	1177	14.5	223.6	0.291	853	1538	19.3	610.1	0.357	928	1106	110.6	1932.8	0.322
799	1332	37.0	172.7	0.231	853	1579	38.4	444.4	0.356	928	1177	73.5	1359.7	0.299
799	1439	49.0	790.8	0.265	853	1618	93.5	48.7	0.311	928	1332	96.6	1240.5	0.337
799	1444	25.4	2107.8	0.315	853	1649	74.7	1715.6	0.353	928	1439	15.9	772.8	0.282
799	1450	42.9	83.3	0.245	853	1707	76.7	48.5	0.332	928	1444	84.4	875.8	0.321

Table S1. Euclidean (Euc), ecological (Eco) and genetic chord (D_c) distances for each pair of sampling populations (p1, p2).

p1	p2	Euc	Eco	D_c	p1	p2	Euc	Eco	D_c	p1	p2	Euc	Eco	D_c
799	1464	17.0	64.6	0.275	853	1715	40.5	284.5	0.308	928	1450	20.4	124.4	0.298
799	1478	12.9	2605.9	0.381	853	1730	54.6	619.3	0.294	928	1464	75.1	410.8	0.312
799	1538	53.2	63.8	0.198	853	1795	58.5	71.3	0.363	928	1478	64.1	891.8	0.364
799	1579	17.0	1699.0	0.357	853	1799	60.9	3002.5	0.338	928	1538	113.2	206.3	0.328
799	1618	61.8	614.3	0.219	868	894	32.9	423.8	0.291	928	1579	58.1	827.7	0.314
799	1649	46.7	460.3	0.234	868	928	88.9	1713.5	0.308	928	1618	11.1	224.0	0.424
799	1707	46.0	772.3	0.226	868	966	18.5	2190.0	0.322	928	1649	30.4	870.7	0.303
799	1715	18.2	1588.8	0.453	868	1052	62.8	982.8	0.329	928	1707	21.8	284.4	0.312
799	1730	27.6	433.2	0.273	868	1106	23.1	318.9	0.432	928	1715	56.4	1308.7	0.464
799	1795	31.0	547.1	0.249	868	1177	16.9	830.0	0.351	928	1730	43.4	202.2	0.343
799	1799	34.1	64.2	0.324	868	1332	12.8	2960.6	0.357	928	1795	40.0	1301.9	0.351
822	853	31.4	8.7	0.288	868	1439	76.3	1761.7	0.332	928	1799	39.6	855.5	0.299
822	868	35.6	1905.7	0.304	868	1444	11.1	2770.6	0.326	966	1052	81.0	23.9	0.278
822	894	3.2	617.4	0.238	868	1450	70.2	990.9	0.358	966	1106	6.5	4109.7	0.301
822	928	124.6	2607.7	0.378	868	1464	17.0	1563.2	0.341	966	1177	34.0	938.2	0.362
822	966	17.7	525.5	0.287	868	1478	28.3	2804.4	0.427	966	1332	13.5	2492.8	0.293
822	1052	98.4	3716.8	0.375	868	1538	26.3	2073.7	0.338	966	1439	94.1	283.5	0.288
822	1106	16.6	3.6	0.222	868	1579	36.0	2020.1	0.333	966	1444	24.3	900.0	0.302
822	1177	51.6	470.3	0.236	868	1618	89.2	6.9	0.266	966	1450	88.1	550.5	0.277

Table S1. Euclidean (Euc), ecological (Eco) and genetic chord (D_c) distances for each pair of sampling populations (p1, p2).

p1	p2	Euc	Eco	D_c
822	1332	30.4	1007.6	0.279
822	1439	111.7	1984.8	0.260
822	1444	41.8	2516.2	0.343
822	1450	105.7	2523.0	0.298
822	1464	50.8	55.3	0.255
822	1478	62.5	1720.3	0.304
822	1538	16.2	346.6	0.246
822	1579	69.9	940.5	0.259
822	1618	124.6	509.6	0.292
822	1649	106.1	250.6	0.306
822	1707	107.9	155.9	0.263
1052	1106	84.5	48.2	0.220
1052	1177	47.4	663.5	0.332
1052	1332	70.5	615.8	0.226
1052	1439	15.2	2975.6	0.376
1052	1444	58.3	390.5	0.418
1052	1450	9.5	78.4	0.260
1052	1464	49.0	669.4	0.266
1052	1478	38.4	873.0	0.324
868	1649	71.8	420.5	0.265
868	1707	72.8	823.6	0.235
868	1715	38.0	18.3	0.261
868	1730	51.6	3660.7	0.282
868	1795	55.5	840.7	0.299
868	1799	58.1	2124.6	0.246
894	928	121.8	2223.0	0.345
894	966	14.7	968.6	0.276
894	1052	95.6	2250.8	0.258
894	1106	13.3	606.9	0.311
894	1177	48.7	937.6	0.321
1332	1478	32.9	1899.1	0.395
1332	1538	16.7	217.9	0.258
1332	1579	40.0	93.8	0.228
1332	1618	95.5	261.8	0.255
1332	1649	76.1	547.2	0.249
1332	1707	78.6	890.7	0.308
1332	1715	42.0	183.8	0.339
1332	1730	56.2	468.5	0.351
966	1464	33.1	2831.3	0.299
966	1478	44.8	756.4	0.303
966	1538	10.0	2625.7	0.299
966	1579	52.2	476.6	0.296
966	1618	106.9	215.4	0.260
966	1649	88.5	2947.8	0.340
966	1707	90.3	1499.3	0.303
966	1715	54.3	9.5	0.218
966	1730	68.4	529.2	0.419
966	1795	72.3	1354.6	0.321
966	1799	74.7	4018.1	0.341
1464	1715	21.3	523.4	0.278
1464	1730	35.2	361.9	0.308
1464	1795	39.2	1681.6	0.318
1464	1799	41.6	1694.0	0.365
1478	1538	49.6	42.0	0.270
1478	1579	7.7	102.4	0.248
1478	1618	62.6	6.6	0.223
1478	1649	43.7	419.1	0.225

Table S1. Euclidean (Euc), ecological (Eco) and genetic chord (D_c) distances for each pair of sampling populations (p1, p2).

p1	p2	Euc	Eco	D_c	p1	p2	Euc	Eco	D_c	p1	p2	Euc	Eco	D_c
1052	1538	87.1	233.6	0.301	1332	1795	60.1	31.0	0.304	1478	1707	45.7	27.4	0.224
1052	1579	33.1	811.4	0.279	1332	1799	62.3	260.8	0.267	1478	1715	9.7	2434.7	0.334
1052	1618	27.6	1115.3	0.296	1439	1444	70.6	3174.4	0.287	1478	1730	23.6	1461.7	0.268
1052	1649	22.0	386.3	0.249	1439	1450	6.1	195.4	0.281	1478	1795	27.5	388.0	0.258
1052	1707	15.0	1025.1	0.267	1439	1464	61.3	1590.2	0.300	1478	1799	30.0	1494.3	0.432
1052	1715	31.6	11.2	0.203	1439	1478	50.0	835.3	0.245	1538	1579	56.5	450.2	0.439
1052	1730	21.0	357.1	0.296	1439	1538	99.5	95.5	0.198	1538	1618	112.2	1676.2	0.294
1052	1795	19.0	499.3	0.358	1439	1579	43.4	462.6	0.252	1538	1649	92.5	159.6	0.231
1052	1799	20.5	241.7	0.336	1439	1618	12.9	548.9	0.245	1538	1707	95.2	910.1	0.250
1106	1177	37.1	768.3	0.272	1439	1649	15.3	145.6	0.249	1538	1715	58.5	1591.1	0.298
1106	1332	14.4	533.0	0.264	1439	1707	6.0	689.1	0.285	1538	1730	72.7	1628.4	0.453
1106	1439	97.1	439.3	0.343	1439	1715	41.6	117.4	0.308	1538	1795	76.7	270.5	0.277
1106	1444	26.5	1055.6	0.303	1439	1730	28.0	742.0	0.424	1538	1799	78.8	16.0	0.306
1106	1450	91.2	215.9	0.245	1439	1795	24.4	124.5	0.286	1579	1618	55.9	467.5	0.302
1106	1464	35.7	300.9	0.258	1439	1799	23.8	516.6	0.353	1579	1649	36.2	95.7	0.239
1106	1478	47.2	1569.2	0.331	1444	1450	64.8	1219.4	0.318	1579	1707	38.8	751.8	0.260
1106	1538	3.5	153.6	0.307	1444	1464	9.3	1951.1	0.274	1579	1715	2.0	428.0	0.220
1106	1579	54.4	1610.1	0.337	1444	1478	20.8	3198.1	0.350	1579	1730	16.2	680.8	0.224
1106	1618	109.8	634.6	0.217	1444	1538	28.9	2463.3	0.413	1579	1795	20.2	115.7	0.247

Table S1. Euclidean (Euc), ecological (Eco) and genetic chord (D_c) distances for each pair of sampling populations (p1, p2).

p1	p2	Euc	Eco	D_c	p1	p2	Euc	Eco	D_c	p1	p2	Euc	Eco	D_c
1106	1649	90.5	212.7	0.417	1444	1579	28.0	30.1	0.227	1579	1799	22.4	370.6	0.256
1106	1707	93.0	179.2	0.287	1444	1618	83.3	1026.1	0.320	1618	1649	23.1	1735.0	0.470
1106	1715	56.4	369.1	0.267	1444	1649	64.3	2674.1	0.327	1618	1707	17.0	380.1	0.285
1106	1730	70.6	619.5	0.347	1444	1707	66.5	177.6	0.226	1618	1715	53.9	59.4	0.306
1106	1795	74.5	356.6	0.348	1444	1715	30.1	558.6	0.307	1618	1730	40.0	9.7	0.286
1106	1799	76.7	169.9	0.331	1444	1730	44.2	1717.9	0.298	1618	1795	36.2	33.9	0.237
1177	1332	23.1	1036.7	0.216	1444	1795	48.2	768.9	0.256	1618	1799	34.9	408.8	0.277
1177	1439	60.2	477.4	0.301	1444	1799	50.5	1134.4	0.252	1649	1707	9.7	128.1	0.244
1177	1444	11.1	3417.6	0.320	1450	1464	55.5	95.1	0.258	1649	1715	34.2	2166.3	0.277
1177	1450	54.2	551.8	0.220	1450	1478	44.2	374.3	0.220	1649	1730	20.2	2367.3	0.451
1177	1464	2.9	458.5	0.343	1450	1538	93.6	1033.0	0.296	1649	1795	16.3	394.8	0.370
1177	1478	11.4	2373.0	0.403	1450	1579	37.9	411.8	0.215	1649	1799	13.8	12.4	0.328
1177	1538	39.7	260.5	0.243	1450	1618	19.0	746.0	0.322	1707	1715	36.9	608.7	0.270
1177	1579	19.1	1140.7	0.327	1450	1649	15.0	358.8	0.443	1707	1730	23.0	854.6	0.411
1177	1618	73.0	1029.6	0.236	1450	1707	6.0	411.1	0.247	1707	1795	19.2	252.7	0.339
1177	1649	54.9	40.2	0.201	1450	1715	36.1	1731.4	0.462	1707	1799	18.2	0.9	0.344
1177	1707	56.3	96.4	0.194	1450	1730	23.0	2567.5	0.511	1715	1730	14.2	1447.1	0.311
1177	1715	21.1	133.9	0.259	1450	1795	19.7	647.2	0.319	1715	1795	18.1	2144.9	0.403
1177	1730	34.7	656.5	0.257	1450	1799	19.6	370.4	0.366	1715	1799	20.4	1733.0	0.360

Table S1. Euclidean (Euc), ecological (Eco) and genetic chord (D_c) distances for each pair of sampling populations (p1, p2).

p1	p2	Euc	Eco	D_c
1177	1795	38.6	29.9	0.202
1177	1799	41.2	596.7	0.217
1332	1439	82.8	904.9	0.205
1332	1444	12.2	2159.7	0.282
1332	1450	77.0	200.2	0.250
1332	1464	21.5	285.5	0.336
1464	1478	11.7	563.3	0.225
1464	1538	38.2	455.1	0.295
1464	1579	19.2	99.9	0.268
1464	1618	74.1	518.5	0.278
1464	1649	55.4	199.8	0.274
1464	1707	57.3	1816.4	0.343
1730	1795	4.0	1469.6	0.432
1730	1799	6.6	2373.2	0.479
1795	1799	3.3	8.7	0.212

Table S1. Euclidean (Euc), ecological (Eco) and genetic chord (D_c) distances for each pair of sampling populations (p1, p2).

4. Hazel Dormouse (*Muscardinus avellanarius*) population structure and differentiation in two landscapes of Latium (central Italy)

In alphabetic order: Bani L., Fabbri E., Fagiani S., Mortelliti A., Orioli V., Pisa G., Randi E., Sozio G. *et al.*

ABSTRACT

The aim of this project was to evaluated the effects of fragmentation on the genetic population structure of the Hazel Dormouse (*Muscardinus avellanarius, RODENTIA, GLIRIDAE*) in Latium (central Italy). Sampling populations were located in two different landscapes, one with continuous ("Selva del Lamone" Regional Reserve) and another with fragmented forests (Viterbo landscape). Individuals were captured using nest box. Biological samples were collected by cutting the ear tip of adult non-breeding individual. We genotyped all biological samples at 7 specie-specific microsatellites, highly variable genetic markers, and we analysed genotype in order to describe and quantify genetic differentiation and population structure. Our result showed that there is a strongly differentiation between the two studied landscapes. Moreover we found that the populations living in the continuous landscape appeared to be panmictic, while those living in fragmented forests resulted heavily structured. Finally, one of the sampling population inhabiting the fragmented landscape was unexpectedly strongly isolated from the surrounding ones. This result highlighted the importance of genetic studies respect to traditional ecological approaches that can hardly detect the effective dispersal processes.

4.1 INTRODUCTION

Spatial variation in landscape structure arises from naturally occurring ecological processes and/or from anthropogenic causes producing habitat loss and fragmentation (Fischer & Lindenmayer 2007). Anthropic pressure plays an important role in this phenomenon but the effects produced on populations are often complex to understand because many impacting factors do not act separately, but rather they act cumulatively or interactively, affecting the dynamics of populations from local to landscape scale (Gilpin & Soulé, 1986; Lindenmayer, 1995; Young et al. 1996).

The fragmentation process generates metapopulations, a network of spatially discrete sub-populations linked by dispersal (Hanski & Simberloff, 1997). The amount of dispersal between sub-populations represents the degree of their ecological connectivity. The disruption of dispersal movements leads to isolation and, consequently, the halting of the gene flow between sub-populations, whose lack further emphasize the negative effects produced by the habitat loss on long-term population persistence.
The knowledge of the ecology of fragmented populations is essential in order to prevent their isolation or even restoring the ecological connectivity between them (Saunders et al. 1987; Burgman & Lindenmayer, 1998).
The traditional methods used to evaluate animal movements in the field, such as radio telemetry or capture–mark–recapture are poorly effective in study ecological connectivity at landscape level (White & Garrott, 1990; Barrett & Peles, 1999; Tracey, 2006, Moore et al. 2011). These methods are particularly time-expensive and they supply information about individuals movement only. Conversely, molecular-markers, i.e. polymorphic proteins or DNA sequences, are increasingly used for evaluating the effective ecological connectivity, since they can account for breeding events, and allow estimating migration rates and gene-flow between sub-populations (Avise, 1994; Frankham et al. 2002; Frankham, 2006). In addition, molecular techniques require a less intensive sampling effort, as they usually rely on biological samples collected in a single time (Neville et al. 2006). Ecological connectivity between populations, linked by dispersal processes, may thus be evaluated using a genetic approach (Neigel 1997; Manel & Holderegger, 2013). Although these findings may be hidden by historical conditions or events, combining population genetic structure with landscape features (Manel et al. 2003; Holderegger & Wagner 2006), it is possible to better understand of the effects played by recent habitat fragmentation processes.
Microsatellites are non-coding DNA part of genome, with no known function. This “neutral” region of DNA is thus particularly useful because it could change over time without bias induced by selection

pressures. We anyway stress that microsatellites has sometimes been suspected to be non-completely neutral, meaning that at least some of the variation observed within and among populations may be attributed to the selection (Kauer et al. 2003). Nevertheless, microsatellites markers generally have high-mutation rates resulting in high standing allelic diversity (Selkoe & Toonen, 2006), and when they are used for evaluating the ecological connection between populations, they should be identified in sequences with low mutation rates in comparison to the migration rates of individuals (Beebee & Rowe, 2004).

Most of species are affected by habitat fragmentation, but arboreal mammals, such as tree squirrels and dormice, are often more threatened than others due to their low dispersal capability in absence of structural connection between habitat patches (Mortelliti et al. 2009). In European regions, various single-species studies have been carried out, suggesting similar responses in this guild of mammals (e.g. Bright et al. 1994; Rodriguez & Andrèn, 1999; Koprowski, 2005; Mortelliti, 2013). Among them, the Hazel Dormouse (*Muscardinus avellanarius, RODENTIA, GLIRIDAE*) is an arboreal rodent living in woodland and hedgerows, whose presence is negatively affected by fragmentation (Bright et al. 1994; Mortelliti et al. 2011). For its poor conservation status and negative long-term trend in almost all Europe, the species is included in the Annex IV of the 92/43/ECC Directive (Habitat Directive), concerning species that require a strict protection regime that must be applied across their entire natural range within the EU.

The scope of this study was thus investigating the genetic population structure and differentiation of Hazel Dormouse in a continuous ("Selva del Lamone" Regional Reserve) and in a fragmented landscape (Viterbo landscape) in Latium Region (central Italy).

4.2 METHODS

4.2.1 General description of study area and sampling design

The study was conducted in the Viterbo province of Latium Region (central Italy, Figure 1), about 100 km apart from Rome. In the study area two sampling areas were identified: the "Selva del Lamone" Regional Reserve (SLR), ranging between 200-428 m a.s.l, is a large continuous forest block (about 2700 ha) mainly composed by *Quercus cerris* and secondly by *Acer monspessulanum*, *A. campestre*, *Fraxinus ornus*, *Ostrya carpinifolia* and *Q. pubescens*. This forest is subjected to periodic coppicing practices. The second sampling area is an hilly landscape (Viterbo Landscape, VTL) ranging between 300-500 m a.s.l., where woodland fragments are surrounded by agricultural and, to a lesser extent, urban matrix. A relatively simplified network of linear structures, as hedgerows and tree lines, connect the system of woodland fragments. The agricultural matrix is mainly composed by arable fields, olive groves and orchards. The fragments may be considered as mixed broadleaved oak woodlands with termophilous and mesophilous species. The dominant arboreal species are *Quercus pubescens* and *Quercus cerris*.

Dormice were captured by using wooden nest-boxes (average size 18x18x21 cm) placed on trees at a height of 1.5-2 m, with the entrance hole (3 cm of diameter) pointed towards the trunk.

Dormice sampling was carried out in 10 squared grids (namely sampling populations) in the SLR, and in 8 grids in as many woodland patches in the VTL. In the first study area, nest-boxes were placed in 4 ha grids, composed by 6 x 6 lines of nest-boxes spaced 40 m. In the second study area, the size and shape of nest-boxes grids depended on fragment size. The distance between nest-boxes was comparable to other studies on this species (Juškaitis 1999, 2007; Chanin & Gubert 2011). A total of 570 nest-boxes were placed and were checked monthly from May 2010 to December 2012. Nest-boxes were not checked in January-March due to hibernation of the Hazel Dormouse (Walhovd & Jensen, 1976; Walhovd, 1976; Chanin & Gubert, 2011).

Tissue samples were collected by cutting a part of the ear from adults individuals to perform laboratory genetic analysis. In order to minimize disturbance on reproductive success, samples were not collected from females with litter. Dormice were captured and handled with permit number PNM 0024822 granted to A.M. by the Ministry of Environment, Rome, Italy.

4.2.2 Tissue collection and DNA analyses of microsatellite markers

DNA was extracted from tissues collected in the field, which were stored in 95% ethanol and, subsequently, at −20 °C. DNA was extracted with the DNeasy Blood & Tissue Kit (QIAGEN), eluted in 180 μL of TE buffer (10 mM TrisHCl, pH 8; 0.1 mM EDTA) and stored at -20°C until subsequent handlings.
All samples were genotyped by polymerase chain reaction (PCR) for 8 species-specific microsatellite markers (Alice Mouton modified from Naim et al. 2009); *locus* designation, primer sequences, label, repeat motif, annealing temperature are summarized in Table 1.
Amplifications were carried out in 10-μL mix reactions with: 1 μL genomic DNA solutions from tissue extractions, 1 μL of 10x PCR buffer with 2,5 mM Mg^{2+}, 2 μL of Bovine Serum Albumin (2%), 0,4 μM of dNTPs, and 0,3 μL of primer mix 10 μM (forward and reverse) plus 0,05 units of Taq polymerase (5 PRIME Inc., Gaithersburg, USA) and purified water. PCR conditions were optimized for each primer pair, amplifications were performed in a 9700 ABI thermal cycler using the following protocol: (94°C x 2’), a number of cycles between 55 and 63 at (94°C x 30’’) (annealing temperature x 30’’) (72°C x 30’’), and a final extension at 72°C for 10’; all primer pair were amplified by using a touchdown PCR.
PCR products were analysed in an Applied Biosystems 3130XL DNA sequencer (Life Technology) and allele sizes were estimated using the software GENEMAPPER 4.0 (Life Technology). Positive (known genotypes) and negative (no DNA) controls were used to check for laboratory contaminations, which never occurred. A 10% randomly selected subset of the other samples were PCR-replicated two times to check for allelic drop-out and false alleles. Each *locus*

was checked for null alleles using MICRO-CHECKER (Van Oosterhout et al. 2004). The microsatellite *loci* Mav6 was excluded from the analysis because we were not able to obtain PCR products that can be clearly interpreted.

4.2.3 Dormice population structure

Allele frequencies, average number of observed (Na) and private (Np) alleles, observed and expected heterozygosity (Ho, He), Hardy-Weinberg equilibrium (HWE), probability-of-identity (Waits et al. 2001) among unrelated individuals (PI) and among full sibs (PIsibs) were computed with GenAlEx v. 6.501 (Peakall & Smouse, 2006, Peakall & Smouse, 2012)

Genetic population structure was performed analysing the biparental multilocus genotypes using a Bayesian clustering procedure implemented in STRUCTURE 2.3.4 (Pritchard et al. 2000; Falush et al. 2003), which was designed to identify the populations of origin (K) of the sampled individuals. This analysis gave the assignment probability of each individual (Q) to pertain to each of the identified populations of origin (cluster). Populations were constructed by minimizing the departures from Hardy–Weinberg equilibrium (HWE) and linkage equilibrium (LE), which could result from recent admixtures, migration or hybridisation.

We ran all the STRUCTURE analyses using the "admixture" model with correlated frequencies and the LOCPRIOR information (sampling population) in order to help clustering procedure (Pritchard et al. 2010). We used a two-step procedure, in order to increase the probability of identifying a weak population structure, that cannot be detected in one step. Indeed, in presence of highly divergent groups of populations, the Bayesian clustering procedure is just able to identify the main clusters, avoiding the internal weaker structure. In this case, it is more appropriate to re-analyse the resulting groups of sampling populations separately, in order to search for a detailed internal structure (Pisa et al. submitted). In the first step we analysed all 265 individuals sampled in the study area, genotyped at 7 microsatellites. In the second step we re-ran

STRUCTURE for each of the main clusters of sampling populations identified in the first step separately.
All simulations were run with a *burnin* period of 10,000 and 100,000 MCMC (Hastings, 1970; Green, 1995), replicated 30 times and setting K from 1 to 6. The optimal number of clusters (K) were selected by means of STRUCTURE HARVESTER (Earl & vonHoldt 2012) following the Evanno method (Evanno et al. 2005), based on the second order rate of change in the log probability of data between successive K values.
In order to evaluate the loss of genetic diversity, we then calculated standard genetic parameters for all populations of origin identified in structure analyses and we tested them for Hardy-Weinberg equilibrium across all *loci*.

Figure 1. Study area (black) within Latium (dark grey) in central Italy.

Locus	**Primer sequence (5' → 3')**	**Label**	**Ta (°C)**
Mav1	(Mouton unpublished data)	HEX	(TD) 55 °C
Mav2	(Mouton unpublished data)	FAM	(TD) 63 °C
Mav3	(Mouton unpublished data)	HEX	(TD) 60 °C
Mav4	(Mouton unpublished data)	FAM	(TD) 60 °C
Mav5	(Mouton unpublished data)	HEX	(TD) 60 °C
Mav6	(Mouton unpublished data)	FAM	(TD) 55 °C
Mav7	(Mouton unpublished data)	HEX	(TD) 63 °C
Mav8	(Mouton unpublished data)	FAM	(TD) 63 °C

Table 1. Hazel Dormouse species-specific microsatellite markers (Alice Mouton modified from Naim et al. 2009).

4.3 RESULTS

4.3.1 Genetic variability

Although the size of our samples was not very high (265 individuals) and some samples were collected in close areas, all *loci* resulted polymorphic, with the number of different alleles (Na) ranging from 4 to 10, and the effective number of alleles (Ne) varying between 1.64 and 6.94. All these parameters, with allelic range (AR), observed (Ho) and expected (He) heterozigosity and fixation index (F), for each *locus*, are shown in Table 2. The Probability of Identity (PI) of all sampled individuals for increasing *locus* combinations (7 *loci*) and the Probability of Identity among full sibs (PIsibs) resulted close to zero, respectively equals to $3.4*10^{-8}$ and 0.0016. We were, thus, confident that the seven microsatellites supported reliable individual genotype identification. The Chi-squared test for Hardy-Weinberg equilibrium showed that 4 out of 7 *loci* were not at equilibrium, after Bonferroni correction ($p<0.001$) with observed Heterozigosity lower than expected (Table 3).

4.3.2. Dormouse population structure

The first step of population structure analysis, performed on all samples (265 individuals), evidenced two distinct clusters of sampling populations (K=2), Ln Pr (X|K) = -3499.4) (Figure 2a; Figure 3a). The first cluster corresponded to the “Selva del Lamone” Regional Reserve (SLR, 157 individuals, in 10 sampling populations), while the second one included all the woodland fragments in the Viterbo landscape (VTL, 108 individuals, in 8 sampling populations). In the second step, when we re-ran the analysis on the two clusters separately, we observed different sub-structures. The SLR sampling populations appeared as a unique cluster, suggesting the presence of a panmictic population (k=1, Ln Pr (X|K) = -5616.0), while the VTL sampling populations were further assigned to three different populations of origin (k=3, Ln Pr

(X|K) = -1836.5, Figure 2b). A first cluster included sampling population (11, 12 and 13) inhabiting the three Northern fragments; a second one included the sampling populations (17 and 18) in the central part of the VTL, whose apparently maintained a link with quite far Easternmost sampling population (15). A separate cluster resulted for the sampling population 16 (Figure 3b).
The mean number of Effective alleles (Ne) was similar between the SLR population (4.25 ± 0.54 S.E.) and the VTL population (3.75 ± 0.48 S.E.). Conversely, in the SLR cluster mean observed and expected Heterozigosity equalled (Ho: 0.706 ± 0.053 S.E.; He: 0.723 ± 0.058 S.E.), while in VTL mean observed Heterozigosity (0.572 ± 0.043 S.E.) was significantly lower than expected (0.694 ± 0.055 S.E.) (Table 4).
The Probability of Identity for increasing *locus* combinations was close to zero in both two main clusters, $7.1*10^{-8}$ for the SLR population and $4.6*10^{-7}$ for the VTL. The Probability of Identity among full sibs (PIsibs) for increasing *locus* combinations was very low as well in both areas, 0.002 for the SLR and 0.0029 for the VTL population.
The SLR population resulted at Hardy-Weinberg equilibrium for all *loci*, according to Chi-squared test after Bonferroni correction, while in the VTL population three *loci* (Mav1, Mav5, Mav7) were not at equilibrium after Bonferroni correction (99.9% significance level) with observed Heterozigosity lower than expected (Table 5).

Locus	N	Na	Ne	AR	Ho	He	F	PI	PIsibs
Mav1	265	10	4.58	114-150	0.736	0.781	0.058	0.077	0.379
Mav2	261	8	4.58	208-222	0.670	0.782	0.142	0.082	0.380
Mav3	263	8	3.94	202-232	0.684	0.746	0.083	0.108	0.404
Mav4	262	10	6.84	218-236	0.782	0.854	0.084	0.037	0.332
Mav5	254	9	6.94	296-320	0.638	0.856	0.255	0.037	0.331
Mav7	265	9	4.20	211-229	0.660	0.762	0.133	0.089	0.391
Mav8	261	4	1.64	252-262	0.395	0.388	-0.016	0.410	0.658
Mean	261.57	8.29	4.67	-	0.652	0.738	0.106	-	-
SE	1.41	0.78	0.69	-	0.047	0.061	0.032	-	-

Table2. Population genetic parameters for the 7 analysed *loci* and mean values across all *loci*: number of genotyped individuals (N), number of different alleles (Na), number of effective alleles (Ne), allelic range (AR), observed (Ho) and expected heterozigosity (He), fixation index (F), Probability of Identity (PI) and Probability of Identity among Sibs (PIsibs).

Locus	DF	ChiSq	Prob	$B_{0.05}$	$B_{0.001}$	Ho/He
Mav1	45	592.42	<0.001	*	***	Ho<He
Mav2	28	77.02	<0.001	*	***	Ho<He
Mav3	28	25.87	0.580	ns	ns	
Mav4	45	81.81	0.001	*	ns	
Mav5	36	179.08	<0.001	*	***	Ho<He
Mav7	36	82.68	<0.001	*	***	Ho<He
Mav8	6	5.89	0.436	ns	ns	

Table 3. Chi-squared test for Hardy-Weinberg equilibrium for the 7 analysed *loci*. $B_{0.05}$: 95% significance after Bonferroni correction. $B_{0.001}$: 99.9% significance after Bonferroni correction; ns= not significant. Ho/He: relation between observed and expected heterozigosity of *loci* significantly far from Hardy-Weinberg equilibrium after 99.9% Bonferroni correction.

Population	*Locus*	N	Na	Ne	Ho	He	F	PI	PIsibs
SLR	**Mav1**	157	7	3.69	0.771	0.729	-0.058	0.106	0.412
	Mav2	157	6	4.30	0.726	0.768	0.054	0.087	0.388
	Mav3	156	8	4.00	0.756	0.750	-0.009	0.107	0.402
	Mav4	156	10	5.86	0.814	0.829	0.018	0.045	0.347
	Mav5	157	9	5.74	0.720	0.826	0.128	0.052	0.350
	Mav7	157	8	4.53	0.758	0.779	0.027	0.075	0.379
	Mav8	155	4	1.62	0.394	0.384	-0.026	0.406	0.660
	Mean	156.43	7.43	4.25	0.706	0.723	0.019	-	-
	SE	0.30	0.75	0.54	0.053	0.058	0.023	-	-

Table 4. Single *locus* population genetic parameters for the two main populations identified by population structure analysis (SLR: “Selva del Lamone” Regional Reserve; VTL: Viterbo landscape) and mean values across all *loci*: number of genotyped individuals (N), number of different alleles (Na), number of effective alleles (Ne), allelic range (AR), observed (Ho) and expected heterozigosity (He), fixation index (F), Probability of Identity (PI) and Probability of Identity among Sibs (PIsibs).

Population	*Locus*	N	Na	Ne	Ho	He	F	PI	PIsibs
VTL	**Mav1**	108	9	4.99	0.685	0.799	0.143	0.069	0.368
	Mav2	104	6	3.20	0.587	0.687	0.147	0.154	0.445
	Mav3	107	5	3.05	0.579	0.673	0.138	0.172	0.457
	Mav4	106	9	4.96	0.736	0.799	0.078	0.064	0.367
	Mav5	97	9	4.97	0.505	0.799	0.368	0.069	0.368
	Mav7	108	8	3.47	0.519	0.712	0.272	0.133	0.427
	Mav8	106	3	1.63	0.396	0.388	-0.022	0.431	0.664
	Mean	105.143	7	3.75	0.572	0.694	0.160	-	-
	SE	1.455	0.900	0.48	0.043	0.055	0.048	-	-

Table 4. Single *locus* population genetic parameters for the two main populations identified by population structure analysis (SLR: "Selva del Lamone" Regional Reserve; VTL: Viterbo landscape) and mean values across all *loci*: number of genotyped individuals (N), number of different alleles (Na), number of effective alleles (Ne), allelic range (AR), observed (Ho) and expected heterozigosity (He), fixation index (F), Probability of Identity (PI) and Probability of Identity among Sibs (PIsibs).

Pop	*Locus*	DF	ChiSq	Prob	$B_{0.05}$	$B_{0.001}$	Ho/He
SLR	Mav1	21	25,655	0,220	ns	ns	
	Mav2	15	27,541	0,025	ns	ns	
	Mav3	28	19,283	0,889	ns	ns	
	Mav4	45	43,215	0,548	ns	ns	
	Mav5	36	53,077	0,033	ns	ns	
	Mav7	28	30,308	0,349	ns	ns	
	Mav8	6	8,941	0,177	ns	ns	
VTL	Mav1	36	256,438	0,000	*	***	Ho<He
	Mav2	15	34,680	0,003	*	ns	
	Mav3	10	14,357	0,157	ns	ns	
	Mav4	36	72,043	0,000	*	ns	
	Mav5	36	158,459	0,000	*	***	Ho<He
	Mav7	28	70,363	0,000	*	***	Ho<He
	Mav8	3	0,253	0,969	ns	ns	

Table 5. Chi-squared test for Hardy-Weinberg equilibrium for the 7 analysed *loci* in the two main populations identified by population structure analysis. SLR: “Selva del Lamone” Regional Reserve. VTL: Viterbo landscape. $B_{0.05}$: 95% significance after Bonferroni correction. $B_{0.001}$: 99.9% significance after Bonferroni correction; ns= not significant. Ho/He: relation between observed and expected heterozigosity of *loci* significantly far from Hardy-Weinberg equilibrium after 99.9% Bonferroni correction.

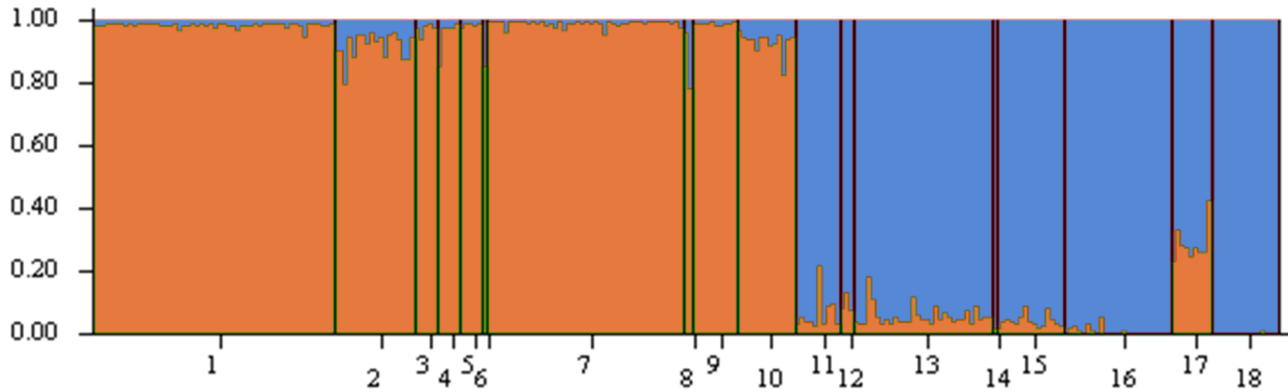

Figure 2a. STRUCTURE analysis of all samples (265 individuals, 18 populations). Parameters: ADMIXTURE model, CORRELATED FREQUENCIES, LOCPRIOR, MCMC length 100,000 with a *burnin* of 10,000. K=2: populations from 1 to 10 pertaining to SLR; population from 11 to 18 pertaining to VTL.

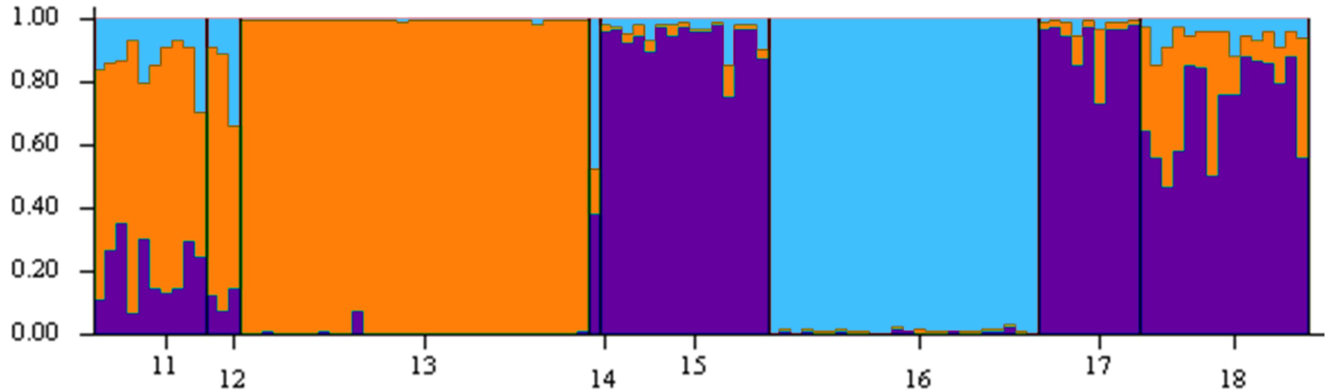

Figure 2b. STRUCTURE analysis of 108 individuals pertaining to the 8 VTL populations. Parameters: ADMIXTURE model, CORRELATED FREQUENCIES, LOCPRIOR, MCMC length 100,000 with a *burnin* of 10,000. K=5.

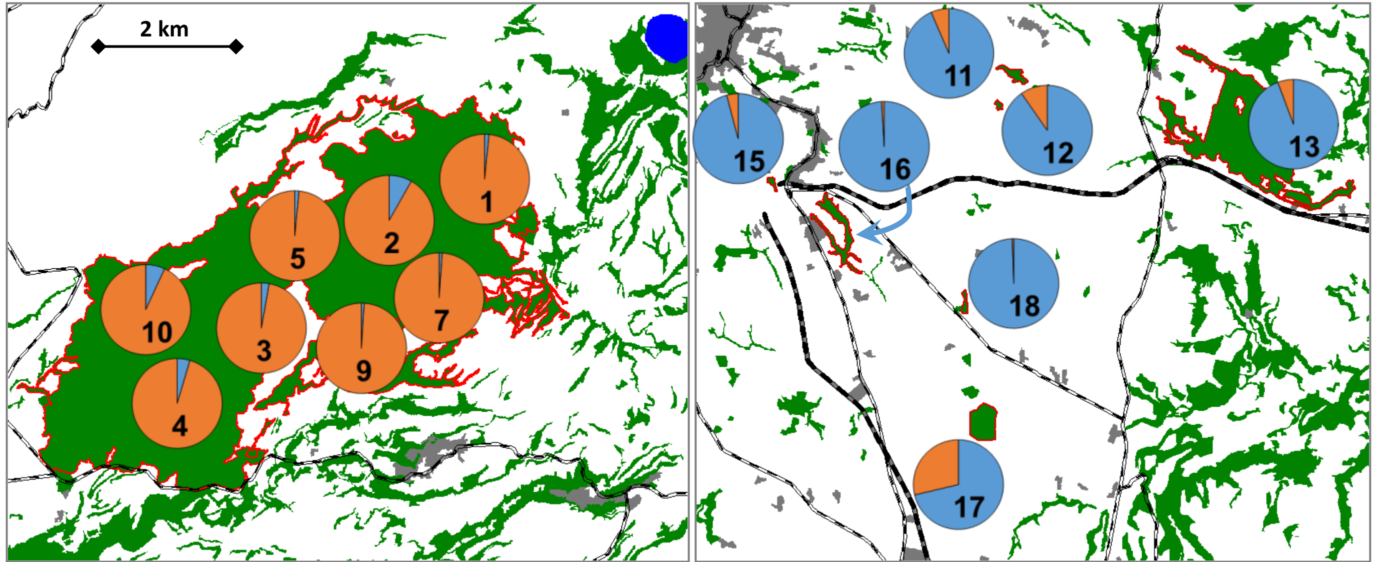

Figure 3a. Hazel Dormice population structure in the two study areas. Pie charts show the assignment probability (Q) to clusters (K) identified by STRUCTURE 2.3.4 (only populations whit at least three samples are represented). Two main clusters were identified in the first step: on the left, "Selva del Lamone" Regional Reserve (SLR) and, on the right, the Viterbo landscape (VTL). Green: forest areas (sampled forests with red boundaries); grey: urban areas. Thick black dashes lines: railway; thin black dashes lines: main roads.

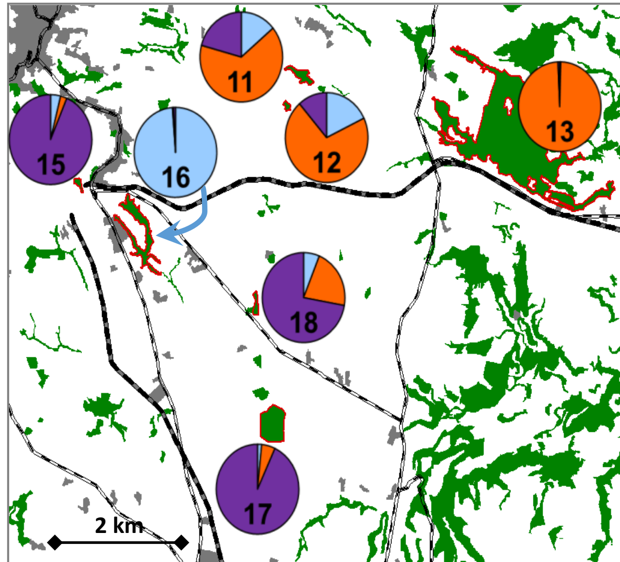

Figure 3b. Dormice population structure in the Viterbo landscape (VTL). Pie charts show the assignment probability (Q) to clusters (K) identified by STRUCTURE 2.3.4 (only populations whit at least three samples are represented). Three main clusters were identified. Green: forest areas (sampled forests with red boundaries); grey: urban areas. Thick black dashes lines: railway; thin black dashes lines: main roads.

4.4 DISCUSSION

This study allowed us to analyse the population structure of the Hazel Dormouse in Latium Region, comparing two populations, one living in a continuous forest ("Selva del Lamone" Regional Reserve, SLR) with another living in fragmented forest (Viterbo landscape, VTL).

The first step of the analysis confirmed what we could have reasonably predict: there was a strong genetic differentiation between populations inhabiting the two different landscapes (Figure 3a). The reason of this pattern should be due to the geographic distance between the two investigated areas, separated linearly by about 25 km. Conversely, the most interesting results derived from the local analysis of the genetic population structure. In fact, although the geographic distances between pairs of sampling populations were similar in the two areas (SLR: minimum distance 0.7 km, maximum distance 5.5 km; VTL: minimum distance 0.5, maximum distance 8km), a panmictic population of Hazel Dormouse was found for the continuous landscape of the "Selva del Lamone" Regional Reserve, while a significant genetic structure was found for the population inhabiting the Viterbo landscape (Figure 3b).

All the SLR sampling populations were actually grouped in a single cluster, validating the hypothesis that a continuous habitat allowed the dormice dispersal process. This result was also confirmed by all *loci* being in Hardy-Weinberg equilibrium after Bonferroni correction (99.9% significance level; Table 3).

On the other hand, the VTL populations where grouped in three populations of origin (clusters) with the evidence of relatively high gene flow within each of them, while a less effective dispersal between them. Two of these clusters reflected a geographical arrangement of sampling populations: one represented the North-Eastern sampling populations group (sampling population 11, 12 and 13), confined by the railway at South and by a main road at West. Another cluster in the Viterbo landscape was represented by South-Western sampling populations (15, 17 and 18): in this case the road network seemed not to be particularly important in limiting the

dispersal process between sampling populations (should be noticed that the railway is in underground in its North-Westernmost part, near sampling population 15). Finally, genetic structure analysis evidenced a cluster formed by the sampling population 16 only, that was genetically divergent from surrounding populations. This result is quite unexpected, because this sampling population was located among the South-Western group and it seemed not as much separated as the others by the infrastructure network.
In conclusion, the population genetic structure analysis allowed us to underline some conservation concern respect to the ecological connectivity between populations in fragmented landscapes (i.e. the isolation of sampling population 16), that can hardly emerge by using traditional ecological approaches, ranging from capture-mark-recapture methods (often used to evaluate dispersal) to census data (used to draw habitat suitability models for the potential ecological networks design).

REFERENCES

Avise JC (1994) *Molecular Markers, Natural History and Evolution*. New York , Chapman & Hall.

Barrett GW, Peles JD (1999) *Landscape Ecology of Small Mammals*. Springer-Verlag Inc., New York.

Beebee T, Rowe G (2004) *An introduction to molecular ecology*. Oxford University Press, New York.

Bright PW, Mitchell P, Morris PA (1994) Dormouse distribution: survey techniques insular ecology and selection of sites for conservation. *Journal of Applied Ecology*, **31**, 329 339.

Burgman MA, Lindenmayer DB (1998) *Conservation biology for the Australian environment*. Chipping Norton, NSW: Surrey Beatty.

Chanin P, Gubert L (2011) Surveying Hazel Dormice (*Muscardinus avellanarius*) with tubes and boxes: a comparison. Mammal notes, The Mammal Society.

Earl DA, vonHoldt BM (2012) STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output and implementing the Evanno method. *Conservation Genetics Resources*, **4**, 359 361.

Evanno G, Regnaut S, Goudet J (2005) Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. *Molecular Ecology*, **14**, 2611 2620.

Falush D, Stephens M, Pritchard JK (2003) Inference of population structure using multilocus genotype data: linked *loci* and correlated allele frequencies. *Genetics*, **164**, 1567 1587.

Fischer J, Lindenmayer DB (2007) Landscape modification and habitat fragmentation: a synthesis. *Glob Ecol Biogeogr*, **16**, 265 280.

Frankham R (2006) Genetics and landscape connectivity. In K.R. Crooks, M. Sanjayan (red.), *Connectivity Conservation*, pp. 72-96. Cambridge, Cambridge University Press.

Frankham R, Briscoe DA, Ballou JD (2002) *Introduction to conservation genetics*. New York, Cambridge University Press.

Giplin ME, Soulé ME (1986) Minimum viable populations: processes of species extinctions. In: *Conservation biology: the science of scarcity and diversity*, pp. 19-34. Soulé, M.E. (ed.). Sunderland, MA: Sinauer Associates.

Green PJ (1995) Reversible jump Markov chain Monte Carlo computation and Bayesian model determination. *Biometrika*, **82**, 711 732.

Hastings WK (1970) Monte Carlo sampling methods using Markov chains and their applications. *Biometrikam*, **57**, 97 109.

Hanski I, Simberloff D (1997) The metapopulation approach, its history, conceptual domain, and application to conservation. In: I. A. Hanski & M. E. Gilpin (eds.), *Metapopulation Biology*, pp. 5-26. Academic Press, San Diego, Californina.

Holderegger R, Wagner HH (2006) A brief guide to landscape genetics. *Landsc. Ecol.*, **21**, 793 796.

Kauer M, Dieringer D, Schlotterer C (2003) Nonneutral admixture of immigrant genotypes in african *Drosophila melanogaster* populations from Zimbabwe. *Molecular Biology and Evolution*, **20**, 1329 1337.

Koprowski JL (2005) The response of tree squirrels to fragmentation a review and synthesis. *Anim Conserv*, **8**, 369 376.

Juškaitis R (1999) Life tables for the common dormouse *Muscardinus avellanarius* in Lithuania. *Acta Theriologica*, **44**, 465 470.

Juškaitis R (2007) Habitat Selection in the Common Dormouse *Muscardinus avellanarius* in Lithuania. *Baltic Forestry*, **13**, 89 95.

Lindenmayer DB (1995) Disturbance, forest wildlife conservation and a conservative basis for forest management in the mountain ash forests of Victoria. *Forest Ecology and Management*, **74**, 223 231.

Manel S, Schwartz MK, Luikart G, Taberlet P (2003) Landscape genetics: Combining landscape ecology and population genetics. *Trends in Ecol. Evol.*, **18**, 189 197.

Manel S, Holderegger R (2013) Ten years of landscape genetics. *Trends in Ecology & Evolution*, **28**, 614 621.

Moore JA, Tallmon DA, Nielsen J, Pyare S (2011) Effects of the landscape on boreal toad gene flow: does the pattern–process relationship hold true across distinct landscapes at the Northern range margin? *Molecular Ecology*, **20**, 4858 4869.

Mortelliti A, Santulli Sanzo G,·Boitani L (2009) Species' surrogacy for conservation planning: caveats from comparing the response of three arboreal rodents to habitat loss and fragmentation. *Biodiversity Conservation*, **18**, 1131 1145.

Mortelliti A, Amori G, Capizzi D, Cervone C, Fagiani S, Pollini B, Boitani L (2011) Independent effects of habitat loss, habitat fragmentation and structural connectivity on the distribution of two arboreal rodents. *Journal of Applied Ecology*, **48**, 153 162.

Mortelliti A (2013) Targeting habitat management in fragmented landscapes: a case study with forest vertebrates. *Biodiversity And Conservation*, **22,** 187 207.

Naim DMD, Kemp SJ, Telfer S, Watts PC (2009) Isolation and characterization of 10 microsatellite *loci* in the common dormouse *Muscardinus avellanarius*. *Molecular Ecology Resources*, **9**, 1010 1012.

Neigel JE (1997) A comparison of alternative strategies for estimating dispersal and gene flow from genetic markers. *Ann. Rev. Ecol. Systemat*, **28**, 105 128.

Neville H, Dunham J, Peacock M (2006) Assessing connectivity in salmonid fishes with DNA microsatellite markers. Pages 318-342 in K. Crooks and M. A. Sanjayan, editors. *Connectivity Conservation*. Cambridge University Press, Cambridge.

Peakall R, Smouse PE (2006) GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. *Molecular Ecology Notes*, **6**, 288 295.

Peakall R, Smouse PE (2012) GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research-an update. *Bioinformatics*, **28**, 2537 2539.

Pisa G, Orioli V, Spilotros G, Fabbri E, Randi E, Bani L (2014) Fire Salamander population structure in fragmented landscape in Northern Italy. *Submitted.*

Pritchard JK, Stephens M, Donnelly PJ (2000) Inference of population Structure using multilocus genotype data. *Genetics*, **155**, 945 959.

Pritchard JK, Wen X, Falush D (2010) Documentation for STRUCTURE software. [Documentation file]. Available with the program at http://pritch.bsd.uchicago.edu/structure.html.

Rodriguez A, Andrèn H (1999) A comparison of Eurasian red squirrel distribution in different fragmented landscapes. *Journal Of Applied Ecology*, **36**, 649 662.

Saunders DA, Arnold GW, Burbridge AA, Hopkins AJ (1987) *Nature conservation: the role of remnants of native vegetation*. Chipping Norton, NSW: Surrey Beatty.

Selkoe KA, Toonen RJ (2006) Microsatellites for ecologists: a practical guide to using and evaluating microsatellite markers. *Ecology Letters*, **9**, 615 629.

Tracey JA (2006) Individual-based modeling as a tool for conserving connectivity. In K.R. Crooks, M. Sanjayan (red.), *Connectivity Conservation*, pp. 343-368. Cambridge University Press, Cambridge.

Van Oosterhout C, Hutchinson WF, Wills DPM, Shipley P (2004) Microchecker: software for identifying and correcting genotyping errors in microsatellite data. *Mol Ecol Notes*, **4**, 535 538.

Waits LP, Luikart G, Taberlet P (2001) Estimating the probability of identity among genotypes in natural populations: cautions and guidelines. *Mol Ecol*, **10**, 249 256.

Walhovd H (1976) Partial Arousals from Hibernation in a Pair of Common Dormice, *Muscardinus avellanarius (Rodentia, Gliridae*). Their Natural Hibernaculum. *Oecologia*, **25**, 321 330.

Walhovd H, Jensen JV (1976) Some Aspects of the Metabolism of Hibernating and Recently Aroused Common Dormouse *Muscardinus avellanarius* L. (*Rodentia, Gliridae*). *Oecologia*, **22**, 425 429.

White GC, Garrott RA (1990) *Analysis of Wildlife Radiotracking Data*. Academic Press, London.

Young AG, Boyle T, Brown AHD (1996) The population genetic consequences of habitat fragmentation for plants. *Trend in Ecology and Evolution*, **11**, 413 418.

5. General conclusions

The aim of this project was to study the effects of habitat fragmentation in broad-leaved forests of two Italian Region: Lombardy (Northern Italy) and Latium (central Italy).
In Lombardy the research was focused on the analysis of the population structure of the Fire Salamander (*Salamandra salamandra*), comparing sub-populations living in continuous forests with those living in fragmented ones. First of all, in the first paper, it was necessary to estimate the individual dispersal distance, in order to develop a habitat suitability model at an appropriate spatial scale. According to the Fire Salamander ecology (Griffiths, 1995; Lanza et al. 2007), the habitat suitability model showed that elevation and slope of breeding sites and the presence of forests and urban areas around them, play an important role in determining the presence probability of the species. Then the population structure analysis underlined a strong differentiation of Fire Salamander populations in the Prealpine/foothill lowland of Lombardy.
Genetic structure analysis showed that only the sampling populations of the Western-central foothill lowland were grouped in a clearly different cluster (separated from the other by wide urban areas in its Northern and Western boundaries and Adda river in the East) while the populations of the Prealpine area, extremely Western and Eastern foothill were linked to each other and represented the continuous habitat.

Moreover, while the latter was further divided in two populations of origin connected along an Western-Eastern gradient, showing good ecological connection, the fragmented cluster was further subdivided in four isolated populations of origin or clusters. These four clusters almost reproduce the spatial configuration of the original five sampling populations, in fact three out of five of these were strongly assigned at three different clusters, while the other two sampling populations were grouped in a unique cluster. Results of genetic analysis suggesting that currently there was not a loss of genetic diversity and this may suggest the hypothesis of a relative short-term fragmentation. In this case, we cannot currently detect a loss of alleles, but in the long-term, lacking gene flow it may arise, mainly in small and/or isolated populations, due to genetic drift, inbreeding or local extinctions.
One of the most common approaches for evaluating the importance of the interactions organism-environment in regards to gene flow (Spear et al. 2010; Storfer et al. 2010), is the isolation-by-distance theory (Wright 1943). This theory predicts that genetic similarity among individuals decreases as the geographic distance between them increases as result of spatially limited dispersal. However, recent studies have demonstrated that measures of geographic distance (which reflect landscape connectivity) often explain a greater proportion of the genetic variability than simple Euclidean distance (Michels et al. 2001; Coulon et al. 2004; Spear et al. 2005; Vignieri, 2005; Broquet et al. 2006; Cushman et al. 2006; Stevens et al. 2006; Pérez-Espona et al. 2008; Schwartz et al. 2009; Goldberg & Waits, 2010) because, in heterogeneous landscapes, straight-line geographical distances may not adequately reflect the true pattern of dispersal. Effective geographic distances are often evaluated by means of the ecological resistance approach that evaluate the different effects played by land-use and landscape features on dispersal movements and thus genetic distances by using a habitat suitability models that allow to calculate the resistance to dispersal between populations.
The second paper take up again results about fragmentation of the Western-central foothill compared with the continuous area. This

situation was re-analysed by the evaluation of the effects of Euclidean (isolation-by-distance) and ecological distances (isolation-by-resistance) on the mean genetic distances.
The mean genetic distances resulted higher in the Western-central foothill in respect to the continuous forests, probably because the first area grouped sampling populations that are almost completely fragmented and isolated. Moreover, in continuous habitat, mean genetic distances decreased West-Eastward probably because Eastern sampling population were all located in the continuous Prealpine belt conversely, the Western and central sampling populations are located over the boundary between the Prealpine and foothill areas. This analysis confirmed the population structure analysis we found before and allowed identifying some significant environmental features (e.g. elevation, roads and land-use) affecting the genetic distances between populations. On the whole, the Euclidean distance (assuming a spatially homogenous landscape) was not important in determining the genetic distance between sampling populations.
In the Prealpine and foothill areas this happened because the ecological distance was proved to be significant correlated with the genetic distances controlling for the Euclidean distance. Instead, in the Western-central foothill the correlation between genetic distance and Euclidean distance was not significant. This results may explain the genetic population structure of this area where the sampling populations resulted highly separated from each other by a the presence of a high resistant matrix, characterized by wide urban surfaces and high road density that separate suitable areas for the Fire Salamander. In fact habitat fragmentation and other anthropic barriers halt dispersal (Gibbs, 1998; deMaynadier & Hunter, 2000), increase mortality (Faharig et al. 1995; Carr & Faharig, 2001), thus concurring to emphasize genetic divergence between populations and genetic diversity (Reh & Seits, 1990).
The third paper propose again the population structure analysis of a threatened species linked to broad-lived forest: in this case the study area was in Latium region and the species that was sampled is the Hazel Dormouse (*Muscardinus avellanarius)*. Also in this analysis two areas were compared: a continuous forest (“Selva del Lamone”

Regional Reserve) and a fragmented landscape (Viterbo landscape). The genetic structure analysis confirmed what we could have reasonably predict: there was a strong genetic differentiation between populations inhabiting the two different landscapes. The reason of this pattern should be due to the geographic distance between the two investigated areas, separated linearly by about 25 km. Conversely, it was really interesting to notice that, although the geographic distances between pairs of sampling populations were similar in the two areas, a panmictic population of Hazel Dormouse is found for the continuous landscape of the "Selva del Lamone" Regional Reserve (validating the hypothesis that a continuous habitat allowed the dormice dispersal process), while a significant genetic structure was found for the population inhabiting the Viterbo landscape. This one was divided in three populations of origin (clusters) with the evidence of relatively high gene flow within each of them, while a less effective dispersal between them. Two of these clusters reflected a geographical arrangement of sampling populations: one (the North-Eastern sampling populations group)delimited by the railway at South and by a main road at West, the other (the South-Western sampling populations) that appeared to be not particularly limited by the road network in the dispersal process between sampling populations (should be noticed that the railway is in underground). The third cluster is formed only by a single sampling population located among the South-Western group and it seemed not as much separated as the others by the infrastructure network.
The results found in this thesis seems to confirm the value of molecular markers as useful tool to detect the functional connectivity (the movement of individuals among patches) among fragmented patches. In particular, the second paper, emphasized the effectiveness of the circuit theory approach to define the effective ecological distances between populations in fragmented landscapes. This approach allowed identifying significant correlations between genetic and ecological distances and thus the usefulness of ecological resistance maps as a basic tool for conservation purposes (Carroll et al. 2011).

In conclusion this research allowed to underline some conservation concern respect to the ecological connectivity between populations in fragmented landscapes that can hardly emerge by using traditional ecological approaches, ranging from capture-mark-recapture methods (often used to evaluate dispersal) to census data (used to draw habitat suitability models for the potential ecological networks design). This results also validates the importance to evaluate the functional connectivity at the same time of the structural connectivity (the measure of how spatially connected the elements of a landscape are, without any reference to any particular ecological process) in order to maintain viable populations that will be able to respond to environmental change.

REFERENCES

Broquet T, Ray N, Petit E, Fryxell JM, Burel F (2006) Genetic isolation by distance and landscape connectivity in the American marten (*Martes americana*). *Landscape Ecology*, **21**, 877 889.

Carr LW, Fahrig L (2001) Effect of road traffic on two amphibian species of different vagility. *Conservation Biology*, **15**, 1071 1078.

Carroll C, McRae BH, Brookes A (2011) Use of Linkage Mapping and Centrality Analysis Across Habitat Gradients to Conserve Connectivity of Gray Wolf Populations in Western North America. *Conservation Biology*, **26**, 78 87.

Coulon A, Cosson JF, Angibault JM, Cargnelutti B, Galan M, Morellet N, Petit E, Aulagnier S, Hewison AJM (2004) Landscape connectivity influences gene flow in a roe deer population inhabiting a fragmented landscape: an individual-based approach. *Molecular Ecology*, **13**, 2841 2850.

Cushman SA, McKelvey KS, Hayden J, Schwartz MK (2006) Gene flow in complex landscapes: testing multiple hypotheses with causal modeling. *American Naturalist*, **168**, 486 499.

deMaynadier PG, Hunter Jr. ML (2000) Road effects on amphibian movements in a forested landscape. *Natural Areas Journal*, **20**, 56 65.

Fahrig L, Pedlar JH, Pope SE, Taylor PD, Wegner JF (1995) Effect of road traffic on amphibian density. *Biological Conservation*, **73**, 177 182.

Gibbs JP (1998) Amphibian movements in response to forest edges, roads, and streambeds in Southern New England. *Journal of Wildlife Management*, **62**, 584 589.

Goldberg CS, Waits LP (2010) Comparative landscape genetics of two pond-breeding amphibian species in a highly modified agricultural landscape. *Molecular Ecology*, **19**, 3650 3663.

Griffiths R (1996) *Newts and salamanders of Europe*. T & AD Poyser Natural History, London.

Lanza B, Andreone F, Bologna MA, Corti C, Razzetti E (2007) *Fauna d'Italia. AMPHIBIA*. Ed. Calderini, Bologna, Italy.

Michels E, Cottenie K, Neys L, De Gelas K, Coppin P, De Meester L (2001) Geographical and genetic distances among zooplankton populations in a set of interconnected ponds: a plea for using GIS modelling of the effective geographical distance. *Molecular Ecology*, **10**, 1929 1938.

Pérez-Espona S, Pérez-Barbería FJ, McLeod JE, Jiggins CD, Gordon IJ, Pemberton JM (2008) Landscape features affect gene flow of Scottish Highland red deer (*Cervus elaphus*). *Molecular Ecology*, **17**, 981 996.

Reh W, Seitz A (1990) The influence of land use on the genetic structure of populations of the common frog (*Rana temporaria*). *Biological Conservation*, **54**, 239 249.

Schwartz MK, Copeland JP, Anderson NJ, Squires JR, Inman RM, McKelvey KS, Pilgrim KL, Waits LP, Cushman SA (2009) Wolverine gene flow across a narrow climatic niche. *Ecology*, **90**, 3222 3232.

Spear SF, Peterson CR, Matocq MD, Storfer A (2005) Landscape genetics of the blotched tiger salamander (*Ambystoma tigrinum melanostictum*). *Molecular Ecology*, **14**, 2553 2564.

Spear SF, Balkenhol N, Fortin MJ, McRae BH, Scribner K (2010) Use of resistance surfaces for landscape genetic studies: considerations for parameterization and analysis. *Molecular Ecology*, **19**, 3576 3591.

Stevens VM, Verkenne C, Vandewoestijne S, Wesselingh RA, Baguette M (2006) Gene flow and functional connectivity in the natterjack toad. *Molecular Ecology*, **15**, 2333 2344.
Storfer A, Murphy MA, Spear SF, Holderegger R, Waits LP (2010) Landscape genetics: where are we now? *Molecular Ecology*, **19**, 3496 3514.
Vignieri SN (2005) Streams over mountains: influence of riparian connectivity on gene flow in the Pacific jumping mouse (*Zapus trinotatus*). *Molecular Ecology*, **14**, 1925 1937.
Wright S (1943) Isolation by distance. *Genetics*, **28**, 114 138.