Patterns of gene flow across multiple anthropogenic infrastructures: Insights from a multi-species approach

Jonathan REMON[a,b], Sylvain MOULHERAT[a], Jérémie H. CORNUAU[a], Lucie GENDRON[a], Murielle RICHARD[b], Michel BAGUETTE[c], Jérôme G. PRUNIER[b]

[a] - TerrOïko, 14 Rue Ferlus, BP 26, 81540 Sorèze, France

[b] - Station d'Ecologie Théorique et Expérimentale, UMR 5321, 2 Route du CNRS, 09200 Moulis, France

[c] - Museum National d'Histoire Naturelle, UMR 7205, Institut de Systématique, Evolution, Biodiversité, Rue St Hillaire, 75005 Paris, France

jonathan.remon@wanadoo.fr ; sylvain.moulherat@terroiko.fr; jeremie.cornuau@terroiko.fr; lucie.gendron@terroiko.fr; Murielle.RICHARD@sete.cnrs.fr; baguette@mnhn.fr; jerome.prunier@gmail.com

Corresponding author:

Jérôme G. PRUNIER

Station d'Ecologie Théorique et Expérimentale, UMR 5321, 2 Route du CNRS, 09200 Moulis, France

+33 5 61 04 03 61

jerome.prunier@gmail.com

Highlights

- Most Large-scale Transportation Infrastructures (LTIs) act as barriers to dispersal
- Not all species respond to LTIs the same
- Some LTIs have no effect while others can promote dispersal in some species.
- Close LTIs can have antagonistic effects on species
- Conservation planning should rely on multi-species studies.

ABSTRACT

Large-scale Transportation Infrastructures (LTIs) are among the main determinants of landscape fragmentation, with strong impacts on animal dispersal movements and metapopulation functioning. Although the detection of LTIs impacts is now facilitated by landscape genetic tools, studies are often conducted on a single species, although different species might react differently to the same obstacle. We surveyed four species (a snake, an amphibian, a butterfly and a ground-beetle) in a landscape fragmented by six LTIs: a motorway, a railway, a country road, a gas pipeline, a power line and a secondary road network. We hypothesized that LTIs carrying vehicles would mostly impact ground-dwelling species, possibly in a cumulative way. We showed that half of the overall explained genetic variability across all species was due to LTIs. While the butterfly was seemingly not impacted by any LTI, the genetic structure of the three other species was mostly influenced by roads and motorway. The power line did not affect any species and the gas pipeline only impacted gene flow in the ground-beetle through forest fragmentation, but roads systematically affected at least two species. Interestingly, we also showed that some LTIs could somehow promote gene flow, embankments probably providing favourable habitats for vertebrate species. Considering the high variability in species response to LTIs, we argue that drawing general conclusions on landscape connectivity from the study of a single species may lead to counterproductive mitigation measures and that multi-species approaches should be more systematically considered in conservation planning.

1. INTRODUCTION

The human-induced fragmentation of natural habitats is one of the main determinants of the global biodiversity collapse (Fahrig, 2003). The most ubiquitous form of habitat fragmentation is due to large-scale transportation infrastructures (LTIs; Forman & Alexander, 1998). LTIs are linear infrastructures allowing the transportation of goods, vehicles or energy, such as roads, motorways, railways, power lines, pipelines and canals. They are expanding considerably, creating dense transportation networks with profound impacts on natural ecosystems. It notably deeply affects metapopulation dynamics through a reduction in population sizes in response to direct habitat degradation. It also affects metapopulation dynamics through a reduction in demographic and genetic exchanges between populations in response to a decrease in the permeability of the landscape matrix to dispersal (Balkenhol & Waits, 2009; Trombulak & Frissell, 2000). As populations become smaller and isolated, they might exhibit higher rates of inbreeding through genetic drift, resulting in an increased risk of population extinction (McCauley, 1991). Understanding the influence of LTIs on wildlife dispersal patterns is thus of critical importance to fuel conservation policies.

The most obvious detrimental effects of LTIs on dispersal success are direct collisions with vehicles and physical crossing impediment when infrastructures are, for instance, fenced (Forman & Alexander, 1998; Trombulak & Frissell, 2000). Most animals are affected, from small invertebrates to large mammals (Balkenhol & Waits, 2009; Fahrig & Rytwinski, 2009). LTIs may also induce behavioral alterations that further affect nearby populations (Trombulak & Frissell, 2000). For example, both breeding migrations and reproductive success of anurans can be perturbed by main roads due to sound interference with males mating calls (Bee & Swanson, 2007), in turn possibly impacting effective dispersal and thus gene flow (Ronce, 2007).

Over the past fifteen years, "molecular road ecology" has emerged as a fully-fledged discipline to thoroughly estimate landscape functional connectivity (Balkenhol & Waits, 2009; Holderegger & Di Giulio, 2010). Building on population genetics, landscape ecology and spatial statistic tools (Manel & Holderegger, 2013), its objective is to elucidate how the genetic variability is influenced by LTIs and other anthropogenic obstacles, with numerous applications in species management and conservation (Segelbacher et al., 2010). However, one major limitation of such studies is that they generally focus on a single species (Balkenhol & Waits, 2009; D. Keller et al., 2015), while different species may actually respond differently to the same type of infrastructure. Furthermore, they also often focus on a single LTI, while multiple LTIs are commonly built next to each other because of technical and economic constraints, notably within valleys or along coastlines: although the impacts of LTIs are then expected to add up and result in a cumulative barrier effect, some LTIs might actually be neutral to movement or even create corridors to dispersal (Bartzke et al., 2015), these antagonistic effects making the whole picture even more complex. For example, Paquet and Callagan (1996) showed that a motorway strongly impeded crossing events in wolves but that a railway and power lines located within the same study area together redirected wolves movements and thus rather acted as corridors. In the same vein, Latch et al. (2011) found that gene flow in the desert tortoise *Gopherus agassizii* was affected by roads but not by power lines. In highly fragmented landscapes, it is thus highly advisable to assess the concomitant influence of all existing LTIs using a multi-species approach to adopt efficient conservation policies (D. Keller et al., 2015; Richardson et al., 2016).

In this study, we assessed the respective and cumulative impacts of six French LTIs in four terrestrial species with contrasted life history traits (two vertebrates and two insects including a flying species) using molecular data. We hypothesized that flying species would be

less affected by LTIs than ground-dwelling ones and that large infrastructures carrying vehicles (roads, motorways, railways) would overall be more impactful than infrastructures carrying energy (power lines, gas pipelines). We also hypothesized that the impacts of some LTIs might accumulate to shape spatial patterns of gene flow in studied species.

2. MATERIAL AND METHODS

2.1. Study area and biological models

The study was carried out in the 'Périgord' region in southwestern France (45°07'31.8"N; 0°58'56.9"E; Fig. 1). It is a 300 km^2 rural landscape composed of limestone plateaus including crops, mowed meadows, deciduous forests and small villages. The hydrology is limited to small streams and ponds. Altitude ranges from 91 to 294 m above sea level. Six types of LTIs are present in this study area (from the widest to the narrowest): a fenced motorway (A89) commissioned in 2004; a low traffic single-track railway built in the 19th century; a high traffic country road (D6089) present since the 18th century; a power line and a gas pipeline constructed in 1962 and 1955, respectively, both associated with breaches in forest cover; a 1370 km dense network of low traffic secondary roads (Fig. 1).

We considered four species with various life history traits in order to span a large amount of biological variability: two vertebrates (the snake *Natrix helvetica* and the midwife toad *Alytes obstetricans*) and two insects (the butterfly *Maniola jurtina* and the ground-beetle *Abax parallelepipedus*). *Alytes obstetricans* is a small toad widely distributed in Western Europe. It is highly sensitive to fragmentation because local populations are known to function as relatively independent entities with strong genetic structuring (Tobler et al., 2013). *Natrix helvetica* is also widely distributed in Western Europe and is considered to exhibit good dispersal abilities, with individuals travelling over more than 1 km in less than a month (Pettersson, 2014). A previous study did not detect any genetic structure in this species in an intensively used agricultural landscape, indeed suggesting good dispersal ability in fragmented environments (Meister et al., 2010). *Maniola jurtina* is a univoltine butterfly which is very common in Europe with locally very high densities. It shows medium dispersal capacity with mean dispersal distances ranging

from 50 to 300 m (Stevens et al., 2013). Previous studies revealed that both land cover (arable lands and forests) and LTIs (motorway and railway) could affect its dispersal (Remon et al., 2018; Villemey et al., 2016). Finally, *Abax parallelepipedus* is an opportunistic carnivorous ground-beetle that inhabits the upper layer of forest litter (Loreau, 1987). It typically shows limited dispersal capacity, avoids open habitats and is highly sensitive to fragmentation by roads (I. Keller et al., 2004).

2.2. Sampling and genotyping

All captures were authorized by Préfecture d'Aquitaine (ref number: AD_AD_150224_arrete_06-2015_terroiko). For all species, tissues were collected between April and September in 2015 and 2016. For the two vertebrate species *N. helvetica* and *A. obstetricans*, we followed an individual-based sampling design due to overall low abundances in the field. Individual-based sampling design has been proved to be a good alternative method to population-based sampling design as less individuals are required per sampling location (1 to 4) and more geographical locations can be sampled over the landscape (Luximon et al., 2014; Prunier et al., 2013). Accordingly, the entire study area was prospected to collect toads and snakes, at night and at day time, respectively. We mainly focused on sampling locations with high probability of presence such as wetlands, ponds, rivers, woodland edges and small villages. To attract snakes and facilitate data collection, 108 artificial shelters were also laid across the study area. In *A. obstetricans*, some sites with high abundances were surveyed more intensely (as part of a companion demographic study), allowing up to 50 individuals to be sampled locally (see details in Appendix A). When an individual was detected, it was hand-captured and manipulated directly in the field. A GPS location (Garmin Etrex20, USA) was recorded for each

captured individual (see Fig. 1 and 2 for sampling locations). Each individual was sexed, measured, weighed, marked (to avoid sampling individual twice) and a genetic sample was collected. Captured toads were marked using 7x1.35 mm FDX-B Passive Integrated Transponder (PIT) tags (Loligo Systems, Denmark) and a non-destructive genetic sample was collected by gently opening mouth with a little metal spoon and swabbing mouth cavity for about 10 seconds (Broquet et al., 2007). We used ventral scales clipping following Brown and Parker (1976) to both mark snakes and collect DNA. We also opportunistically collected genetic samples from snakes and amphibians found dead (road kill or predation) and from snake shed skins.

The two insect species *M. jurtina* and A. *parallelepipedus* were sampled within 30 sites using a classical population-based sampling design. Site locations were obtained by dividing the study area into 30 sectors using a 5x6 regular grid in QGIS (V. 2.8). In each sector and each species, a single sampling site was chosen according to the presence of suitable habitats (woodlands for beetles and grasslands for butterflies). At each sampling location, 30 individuals were sampled, resulting in 900 genetic samples per species (see Fig. 1 and 2 for sampling locations). Butterflies were captured during day time with nets. Beetles were trapped using non-lethal dry pitfalls. Pitfalls were 20 cm in diameter and 15 cm in depth and were arranged in circles at regular intervals of 5 m. They were emptied every day until 30 individuals were captured. For both insect species, we collected the middle right leg of each captured individual, as both a source of DNA and a way to avoid sampling the same individual twice.

All genetic samples were stored in 70 % ethanol until DNA extraction. All material for marking animals and collecting genetic samples was washed and disinfected using absolute ethanol between each individual sampling. Care was taken to minimize animal handling and stress and all individuals were rapidly released at the place of capture after manipulation. We

amplified 13, 14, 15 and 14 polymorphic microsatellite loci in *N. helvetica*, *A. obstetricans*, *M. jurtina* and *A. parallelepipedus*, respectively. For a detailed procedure of DNA extraction, amplification and genotyping, see Appendix B. Some individuals could not be correctly genotyped because of insufficient amounts of DNA: genotypes with more than 2 loci presenting missing values were discarded to allow robust subsequent genetic analyses. We used Genepop 4.2 (Rousset, 2008) to test for linkage disequilibrium among pairs of loci and deviation from Hardy-Weinberg Equilibrium after sequential Bonferroni correction to account for multiple related tests (Rice, 1989). The presence of null alleles was tested using MICROCHECKER 2.2.3 (Van Oosterhout et al., 2004). Loci with null alleles and/or in linkage disequilibrium were discarded, resulting in the final selection of 13, 10, 6 and 10 microsatellite loci in toads, snakes, butterflies and beetles, respectively (Appendix B).

2.3. Genetic structure and genetic distances

The presence of related individuals in data sets may lead to an over-estimate of the number of clusters when assessing population structure and thus bias subsequent genetic analyses (E. C. Anderson & Dunham, 2008). We therefore used COLONY2 (Jones & Wang, 2010) to identify and discard siblings within our individual-based data sets (*N. helvetica* and *A. obstetricans*, Appendix C). In the population data sets, we only retained populations for which at least 15 genotypes were available. The final data sets comprised 848 genotypes (30 populations) in *A. parallelepipedus*, 508 genotypes (21 populations) in *M. jurtina*, 115 genotypes in *N. helvetica* (68 sampling locations) and 358 genotypes in *A. obstetricans* (56 sampling locations).

For each of the four final data sets, genetic structure was investigated using STRUCTURE 2.3.4 (Pritchard et al., 2000) with the admixture and the correlated allele

frequency models and prior sampling location information. We followed a hierarchical genetic clustering procedure (Coulon et al., 2008): it has been shown to allow a better detection of sharp genetic variations locally expected in the vicinity of linear features acting as barriers to gene flow (Prunier et al., 2017). At each hierarchical level, we tested the number K of clusters from 1 to 10 and repeated analyses for each value 5 times. Runs were performed with a minimum burn-in period of 50 000 and 50 000 subsequent Markov chain Monte Carlo (MCMC) iterations, provided the algorithm converged correctly, as indicated by the stability of the estimated α values (i.e. the Dirichlet parameter for the degree of mixing) throughout the runs. Whenever α plots showed substantial fluctuations throughout the run, , we used a burn-in period of 100 000 and 100 000 subsequent MCMC iterations and adjusted the standard deviation of α from 0.025 (default) to 0.5 to enhance parameter space exploration and achieve algorithm convergence (Prunier et al., 2017). We then used STRUCTURE HARVESTER (Earl & vonHoldt, 2012) to obtain ΔK statistics to infer the optimal K-value. We used this optimal K-value to perform 20 runs with a burn-in period of 200 000 and 200 000 MCMC iterations. We finally compiled the ten best runs (highest lnP(D) values; Coulon et al., 2008) using CLUMPP (Jakobsson & Rosenberg, 2007) to obtain individual or population ancestry values (i.e., Q-values), measuring the level of admixture among the inferred genetic clusters. Each individual or population was assigned to the cluster for which the Q-value was higher than 0.6, following Balkenhol et al. (2014). We then repeated the analysis for each inferred cluster separately until no more structure was found in the data (same probability of assignment to each cluster across all individuals), until inferred clusters comprised less than 50 individuals or in situations where convergence could not be achieved even with a total of 2 million MCMC iterations (see Appendix D for details). For each hierarchical level, we used individual- or population-based Q-values to

compute pairwise matrices of ancestry-based hierarchical genetic distances (HGD; Balkenhol et al., 2014). HGD were only calculated for species displaying a significant genetic structure. When more than one hierarchical level was detected, each hierarchical level (HGD1, HGD2) was considered separately. We also computed classical genetic distances (GD), using the Bray-Curtis (bc) percentage dissimilarity index for the individual-based data sets and Fst for the population-based data sets. While these classical genetic distances are well suited to detect surface elements affecting gene flow at a regional scale, HGD have been shown to allow a better detection of sharp genetic variations caused by linear elements such as LTIs (Prunier et al., 2017).

2.4. Multiple linear regressions and commonality analyses

Both classical and hierarchical genetic distances were tested against the six types of LTIs present in our study area, along with a number of covariates likely to affect patterns of genetic differentiation (isolation-by-distance IBD, difference in altitude and the following landcover features: water, crops, woodlands, grasslands and urban areas), although assessing the respective influence of these non-LTI features was not the main scope of this study (details about the effects of non-LTIs are provided in Appendix K). All LTIs but the secondary road network were coded into binary pairwise matrices, with 0 indicated that individuals/populations of each pair were on the same side and 1 indicated that they were on either side of the LTI. Because of the density of the secondary road network in the study area, this LTI (hereafter simply called Roads) was treated as other landcover features. Landcover features were defined by digitalizing the entire study area at a minimal resolution of 20 m in QGIS (V. 2.8) using national databases (BDTopo and BDCarthage) and 2015 high resolution aerial photographs from the French National Geographic Institute (IGN) with RGF93 / Lambert-93 projected coordinates. Photo-interpretation

additionally allowed digitalizing any landscape feature larger than 25 m². Every element of the landscape was classified into 49 habitat types of the EUNIS Habitat Classification System (Davies & Moss, 1999). To assist the classification of habitats from photo-interpretation based on texture, we used 82 phytosociological quadrats randomly selected across the study area: quadrats were visited in spring and summer 2015 and the classification based on the presence of indicator species listed by the French administration. All GIS analyses were performed by a single investigator. We combined these 49 elements into six main landcover predictors (Appendix E): Water (stagnant water bodies, streams and rivers), Crops (intensive and non-intensive cultures), Woodlands (all types of forests), Grasslands (uncultivated open lands), Urban (villages, industrial sites, *etc.*) and Roads (all roads excluding small trails, motorway and D6089 country road). These six classes were each rasterized at a 1 m resolution using ARCGIS 10.2.2 and its SPATIAL ANALYST extension. Each raster was then used to create a resistance surface based on the spatial density of the corresponding element in the landscape. This procedure hypothesizes that a pixel covered with 100% (respectively 0%) of an unfavorable landscape feature would be 100% resistant (respectively permeable) to gene flow and thus avoids assigning arbitrary resistance values to landscape features. To do so, we overlaid a 20 m grid on each spatial class and calculated the percentage of the element in each grid. For each resistance surface, we rescaled pixel resistance values to range from 1 (null or extremely low densities) to 100 (the element covers the entire pixel) and the final rescaled resistance surface was used in CIRCUITSCAPE 4.0 (McRae et al., 2016) to compute pairwise effective distances between individuals or populations. The IBD pairwise matrix was similarly obtained by running CIRCUITSCAPE on a uniform resistance surface only composed of pixels of value 1. Finally,

altitude pairwise matrices were computed as the absolute values of pairwise differences in altitude between sampling locations.

The local influence of landscape features may go unnoticed if all pairs of genetic distances are retained, as isolation-by-distance might take over the influence of landscape features, with strong implications in terms of biological interpretation of results (C. D. Anderson et al., 2010; D. Keller et al., 2013). We thus considered subsets of pairwise data by defining a maximum Euclidean distance threshold between sampling locations. Following Cayuela et al. (2019), this distance threshold was selected for each species and each metric of genetic distances (GD or HGD) as the neighboring distance maximizing the model fit of a classical multiple linear model including all predictors (see Appendix F for details). For each species, we then explored the relationship between subsets of each type of genetic distances (GD or HGD) and the corresponding predictors using standard multiple linear regressions. In the case of *A. obstetricans*, the corresponding dataset being characterized by a skewed distribution of genotypes across sampling locations, we used a specific Jackknife resampling procedure detailed in Appendix A so as to avoid the risk of biased inferences stemming from an over-representation of some locations over the others (Prunier et al., 2013).

The contributions of predictors to the dependent variables were assessed using commonality analyses (CA). Commonality analysis is a variance partitioning procedure allowing the detection and the withdrawal of statistical suppressors that are responsible for a distortion of model estimates (beta weights β and confidence intervals), thus providing decisive support when trying to assess the reliability of model parameters in face of multicollinearity. It also allows isolating the unique contribution U of each predictor to the variance in the dependent variable (for more details about CA, see Appendix G and Prunier et al., 2015, 2017; Ray-Mukherjee et al.,

2014). We performed model simplification by discarding predictors identified as statistical suppressors in an iterative way following Prunier et al. (2017; see Appendix H for details).

In each final simplified model, we assessed levels of collinearity among predictors using Variance Inflation Factors (Dormann et al., 2013). Because pairwise data are not independent, the p-values inferred from simplified models could not be interpreted: except in the case of *A. obstetricans* (see Appendix A), we thus computed 95 % confidence intervals around regression estimates using a Jackknife procedure, with 1000 replicates based on a random removal of 10 % of individuals/populations without replacement (Peterman et al., 2014). These confidence intervals were used to assess the significance of the predictors' contributions to the variance in the corresponding genetic distances. We considered that a predictor was a robust contributor to the variance in the response variable as soon as the confidence interval about the corresponding β value did not include 0. A predictor with a positive β value was associated with an increase in the genetic distances and was interpreted as impeding gene flow. On the contrary, a predictor with a negative β was associated to a reduction in genetic distances and was thus interpreted as promoting gene flow (Jacquot et al., 2017). In the case of LTIs, this interpretation translates into two categories of LTI effects: LTI+ (impeding gene flow) and LTI- (promoting gene flow). In order to make unique contributions comparable across models, we finally computed the relative unique contribution (i.e., U/R^2) of each predictor to the explained genetic variance in each model.

In order to summarize our main findings across species, we summed the relative contributions of LTIs *versus* non-LTIs predictors as well as the relative contributions of LTI+ *versus* LTI- effects within each model. We then averaged these contributions across models for each species and across species. To summarize our main findings across LTIs, we also averaged

the relative unique contributions of LTI+ *versus* LTI- effects for each LTI and across LTIs. Non-significant predictors or predictors that were absent from final simplified models (including LTI- when only LTI+ was present, and vice versa) were given a relative contribution of 0. Results were plotted in the form of 100% stacked barplots.

3. RESULTS

3.1. Genetic structures

Structure outputs indicated a single genetic cluster in both *N. helvetica* and *M. jurtina*, suggesting high gene flow across the study area in these species (Fig. 1; Appendix D). On the contrary, we found strong hierarchical genetic clustering in both *A. parallelepipedus* and *A. obstetricans* (Fig. 2; Appendix D). We identified two hierarchical clustering levels in beetles (Fig. 2A). At the first level, 19 populations were assigned to cluster A and ten were assigned to cluster B. Cluster A included populations sampled mostly in the western part of the study area and north of the road D6089. One population at the extreme south-west could not be assigned to any of these two clusters (cross-assigned). Cluster B, was further divided into two sub-clusters at the second hierarchical level. Clusters B1 and B2 were separated by the D6089 and the gas pipeline, with B1 in the north comprising five populations and B2 in the south comprising four populations. At the second hierarchical level, only one population could not be assigned to any of these two clusters (cross-assigned). This population was located between the road D6089 and the gas pipeline, exactly in-between clusters B1 and B2.

In toads, we similarly identified two hierarchical genetic levels, though with much more blurred spatial patterns. At the first level, most individuals were assigned to two clusters A and B, with no clear geographical boundaries explaining this pattern (Fig. 2B). Twenty-six individuals

could not be assigned to any of these two clusters (cross-assigned individuals), suggesting some exchanges between these two clusters. At the second hierarchical level, cluster A was further divided into two sub-clusters A1 and A2, while cluster B was further divided into five sub-clusters B1 to B5 (Figure 2C), with a high number of cross-assigned individuals (69), again suggesting frequent exchanges among them. Cluster A1, located in the center of the study area, was surrounded by cluster A2, again with no clear geographical boundaries explaining this pattern. Clusters B1, B2 and B4 corresponded to unique populations, whereas clusters B3 and B5 comprised individuals from different locations. The complexity of these spatial genetic patterns was also recovered when using spatial principal component analysis (sPCA; Jombart et al., 2008; Appendix I).

3.2. Multiple linear regression and commonality analyses

The maximum Euclidean distances between sampling locations that optimized the amount of variance in classical and hierarchical genetic distances (variance explained by full regression models) ranged from 2800 to 5700m in individual-based data sets and from 4500 to 18500m in population-based data sets (Table 1; Appendix F). After simplification (Appendix H) and whatever the model, Variance Inflation Factors ranged from 1.00 to 1.56 (Appendix J), suggesting little collinearity among retained variables (Dormann et al., 2013).

When considering classical genetic distances in toads, the multiple linear regression explained 3.9% of variance (Table 1). Roads (U = 0.006) were associated with an increase in genetic distances (positive β values) in this model, thus suggesting barrier effect. This predictor uniquely contributed to 16.9% of explained variance. When considering the first level of hierarchical genetic distances (HGD1), the model only explained 1.5% of the variance and did not include any LTI predictor. At the second level of the hierarchy, the model explained 6.0% of

variance in HGD2. The D6089 was associated with an increase in genetic distances (β = -0.109, U = 0.015) whereas the Motorway was detected has having a significant positive effect on toads' effective dispersal (β = 0.125, U = 0.021). When relative contributions of LTIs were summed in each model and then averaged across models, LTIs accounted for 19.8% of total explained variance (Fig. 3A). Infrastructures were mostly associated with an increase in genetic distances, with 86.4% of variance explained by LTIs stemming from the barrier effects (Fig. 3B) of the D6089 and Roads. The 13.6% left were explained by a reduction in genetic distances across the Motorway at the second level of the hierarchy (HGD2; Fig. 3B).

In snakes, the simplified model explained a small amount (4.2%) of variance in the dependent variable (Table 1) but only comprised LTIs predictors (Fig. 3A). The Motorway was associated with an increase in genetic distances and uniquely accounted for 48.5% of explained variance (U = 0.021; Fig. 3B). The two other LTIs (Roads and Railway) had unique contributions of 0.015 and 0.008, respectively, and both were associated with a reduction in genetic distances in this species, together accounting for 51.5% of explained variance (Fig. 3B).

In butterflies, the simplified model explained 19.9% of variance in Fst values (Table 1). The only LTI that remained in the final model was the Power line but it did not significantly contribute to the model predictive power. The entire genetic variability in this species was thus explained by IBD and Woodlands, both impeding gene flow (Fig. 3A and Appendix K).

In the ground-beetle, the simplified model explained 25.9% of the variance in Fst values (Table 1). The entire genetic variability was yet here explained by non-LTI features (Fig. 3A; Appendix K). When considering the first and the second level of the inferred hierarchical genetic structure, simplified models explained 17.2% and 26.8% of the variance in HGD1 and HGD2, respectively. In both cases, the D6089 was associated with an increase in genetic distances,

indicating a consistent barrier effect (U = 0.059 in HGD1 and 0.114 in HGD2). In addition, Roads (HGD1) and the Gas pipeline (HGD2) were also detected as having negative effects on gene flow (U = 0.063 and 0.049, respectively). In HGD2, the Motorway did not significantly contribute to the model predictive power. Overall, explained variance in genetic distances was accounted for by both LTIs (53.5%) and non-LTIs elements (46.5%; Fig. 3A), with all LTIs being associated with an increase in genetic distances (Fig. 3B), suggesting a possible cumulative barrier effect across hierarchical levels.

3.3. Assessment of infrastructure effects

Overall, 47.9% of the explained variance in genetic distances across all species was due to LTIs (Fig. 3A), of which 79.6% was associated with an increase in genetic distances, that is, with a barrier effect (Fig. 3B-C). The only LTI that did not contribute to genetic distances in any species was the Power line. The D6089 and the Gas pipeline were both systematically associated with barrier effects, in toads and beetles for the former and in beetles only for the latter. On the contrary, the Railway was only associated with a corridor effect in snakes. The two last LTIs showed more nuanced impacts, with corridor effects detected in some species (20.4% of explained variance by LTIs). While 81% of the overall genetic variability explained by the Motorway across species corresponded to a barrier effect in snakes, the remaining 19% corresponded to a reduction in genetic distances in toads (Fig. 3C). It was the opposite in the case of the Roads, with 60% corresponding to a barrier effect in toads but 40% to a reduction in genetic distances in snakes (Fig. 3C).

4. DISCUSSION

The goal of this study was to assess landscape functional connectivity in four species occupying a landscape fragmented by multiple LTIs. We were particularly interested in the potential cumulative or on the contrary the antagonistic effects of six LTIs. We used individual- and population-based regression analyses along with commonality analyses over restricted spatial scales to thoroughly evaluate the relative contribution of various landscape predictors to the variance in both classical and hierarchical genetic distances. As expected, we found that all ground-dwelling species suffered from the barrier effect of at least one LTI, notably those carrying vehicles (Roads, D6089 and Motorway) whereas the flying species was not affected by any LTI. We also identified a possible cumulative barrier effect of roads and D6089 in *A. parallelepipedus*, the least mobile species, shaping spatial patterns of gene flow across several hierarchical levels. Most importantly, we found that LTIs did not only act as barriers to gene flow but might on the contrary promote gene flow, with some antagonistic effects across species.

Overall, LTIs were found to have a strong influence (either positive or negative) on gene flow, accounting for 47.9% of the total explained genetic variability across species and genetic distances. All ground-dwelling species were affected by LTIs, with contributions to the variance by LTIs ranging from 37% in toads to 100% in snakes, contrary to the flying species *M. jurtina* whose genetic variability was only affected by distance and woodlands, as expected from a previous study (Villemey et al., 2016). Although butterflies have a lower probability to be impacted by vehicles than ground-dwelling species, previous studies showed that roads and motorways could hinder crossing events in this species (Polic et al., 2014; Remon et al., 2018)). A direct Mark-Release-Recapture survey conducted in the same study area notably found that the motorway was responsible for a six-fold decrease in crossing events when compared to adjacent habitats (Remon et al., 2018). It is possible that large population sizes in *M. jurtina* are

responsible for a temporal inertia in the setting-up of genetic differentiation since the creation of the motorway in 2004 (Landguth et al., 2010), but this study showed that some butterflies were able to cross it, thus possibly ensuring sufficient gene exchange across the landscape. Although we could not ascertain the negative aftermaths of human-induced fragmentation in *M. jurtina* from our genetic data, our study highlights the potential benefits of combining landscape genetics and Mark-Release-Recapture surveys (Cayuela et al., 2018).

As expected, LTIs were mainly associated with a reduction in gene flow, barrier effects accounting for 79.6% of the variance explained by LTIs across ground-dwelling species. LTIs carrying vehicles (roads, D6089 and motorway) were more impacting than infrastructures carrying energy (Gas pipeline and Power line). Roads and D6089 were responsible for most of inferred barrier effects in this landscape, with negative effects on gene flow in both toads and beetles. The motorway also accounted for non-negligible amounts of explained genetic variability but to a lesser extent than roads, only negatively affecting snakes. In contrast, the contributions of LTIs carrying energy were less important. The gas pipeline negatively affected gene flow in the ground-beetle only, probably in response to associated breaches in forest cover (Charrier, 1997), and the power line did not affect any studied species. These results suggest that conservation measures should primarily focus on infrastructures carrying vehicles rather than on infrastructures carrying energy (Bartzke et al., 2015), although we acknowledge that some taxa not considered in this study, for instance birds, might be negatively affected by LTIs such as power lines (Loss et al., 2015).

Despite these general negative impacts of LTIs on gene flow, we found that species showed very different responses to the same LTI, which perfectly highlights the importance of considering functional rather than just structural landscape connectivity in empirical studies

(Taylor et al., 2006). Two of the six studied LTIs were associated with an increase in genetic distances in toads, these barrier effects together accounting for 86.4% of genetic variance explained by LTIs. Roads and D6089 were the main barriers to dispersal in *A. obstetricans*, affecting classical (GD) and second-order hierarchical genetic distances (HGD2), respectively. Garcia-Gonzalez et al. (2012) similarly found that all roads, including small secondary roads, acted as barriers to gene flow in *A. obstetricans* in northern Spain. Amphibians are particularly vulnerable to road kills because of their numerous movements during dispersal but also during seasonal migrations between breeding water bodies and shelters (Fahrig & Rytwinski, 2009). Although these results advocate for effective mitigation measures to limit road kills of amphibians (Beebee, 2013), it is important to keep in mind that other road features such as traffic noise may also affect amphibians population dynamics (Bee & Swanson, 2007).

In addition to toads, we found that roads also deeply impacted the ground-beetle *A. parallelepipedus* across all hierarchical levels a result congruent with Keller et al. (2004). Roads and D6089 explained the whole genetic variance at the first hierarchical level (HGD1) resulting in clusters A and B (Fig. 2A). At the second hierarchical level (HGD2), the D6089 (but also the gas pipeline) was associated with the split of cluster B into two sub-clusters (Fig. 2A) and thus probably further impacted gene flow. Roads may act as barrier to gene flow because of road kills but also because ground-beetles may be reluctant to cross roads due to behavioral changes (Holderegger & Di Giulio, 2010).

Contrary to roads, we found that the motorway and the railway showed limited barrier effects. The only species that was negatively affected by the motorway was the snake *N. helvetica*. We here revealed that half of the explained genetic variability in snakes resulted from the negative impacts of the motorway. Because it is fenced with fine mesh, snakes might only be

able to reach the other side by using crossing structures (bridges, underpasses, culverts, etc.). These crossing structures may yet be seldom used by snakes due to inadequate placement, architectural design and snakes' behavior (Woltz et al., 2008). Thermoregulatory behavior of reptiles is probably the main reason why individuals would not use underpasses, as a 50 m-length underpass would provide inadequate thermal conditions due to the absence of sunlight. In addition, Baxter-Gilbert et al. (2015) evaluated the effectiveness of different mitigation measures implemented to reduce reptile road mortality (including underneath culverts) and found that these structures were seldom used by reptiles. Underpasses may yet be used by other taxa such as amphibians and insects (Georgii et al., 2011), which may explain why the motorway was only found as acting as a barrier in a single species.

Our most striking finding is that, instead of acting as barriers, some LTIs might somehow promote dispersal. This corridor effect accounted for 20.4% of the overall genetic variance explained by LTIs across species and concerned both vertebrates. We first found that, at the second level of the hierarchy (that is, at a more local scale), gene flow in toads was promoted by the motorway. This counter-intuitive genetic pattern could stem from the availability of new habitats provided by the LTI. Adults and tadpoles of *A. obstetricans* were indeed detected in eight out of the ten storm-water retention ponds present along the studied motorway (data not shown). These ponds may provide favorable breeding habitats, free of predatory fish and surrounded by sand or gravel, the ideal substrates to build their burrows. Furthermore, the motorway is crossed by underneath culverts and tracks which are good dispersal corridors for amphibians (Georgii et al., 2011), especially when they are filled with water. This is not the first study showing a potential positive effect of a motorway on amphibian gene flow. Prunier et al. (2014) revealed that a 40-years old motorway was not a barrier for the alpine newt (*Ichthyosaura*

alpestris) and could even serve as a longitudinal dispersal corridor when the surrounding landscape matrix is highly unfavorable. Interestingly, they even found negative relationships between genetic distances and presence of the motorway, indicating that, as in our study, gene flow across the motorway was probably enhanced; but because they analyzed their data using one-tailed Mantel tests, they did not discuss this possibility. These results might yet be interpreted with caution due to the recent age of the motorway (<15 years old): this genetic pattern could also stem from ancestral landscape configurations and direct monitoring surveys are now necessary to confirm that the motorway is indeed not an obstacle for toads.

Despite limited explained variance in snakes, we also identified two LTIs possibly acting as corridors in this species, Roads and Railway, together accounting for 51.5% of genetic variance explained by LTIs. Roads are known to be responsible for a high mortality in snakes (Rosen & Lowe, 1994): they bask on road surfaces to absorb radiant heat but this behavior increases the probability of collisions and can result in a reduction in gene flow across roads (Clark et al., 2010). However, we found the exact reverse pattern, with roads associated with a reduction in genetic distances in *N. helvetica*. This conflicting result may be explained by an attractive effect of roads and road verges that provide basking surfaces, reinforced by a limited traffic volume in our study area. In addition, the distribution of grass snakes being strongly dependent on wetlands for foraging, water-filled ditches often found alongside secondary roads may provide rich feeding areas, resulting in a local increase in snake abundance that favors road crossings and gene flow: a similar explanation was proposed by Johansson et al. (2005) who found a positive effect of gravel roads and associated ditches in the common frog (*Rana arvalis*). The railway was probably as attractive as roads for snakes, which may similarly explain gene flow enhancement observed in snakes. Railway embankments provide important alternative

habitats for reptiles with optimal thermal conditions for basking (Borda-de-Água et al., 2017), especially when traffic density -and associated disturbance- is low, as it is the case here, with approximately 10 trains/day. Additionally, even active lines can harbor particularly high diversity in reptile species, notably because human presence is scarce and because reptiles can perceive vibrations transmitted through the rail tracks and the ballast when a train approaches, allowing them to reach a shelter before collision (Borda-de-Água et al., 2017).

Although we made our best to ensure optimal detection of the impacts of LTIs on gene flow in considered species, this study is not without some limitations. Inferring the impact on dispersal of linear features such as LTIs using genetic data requires a proper spatial sampling scheme, ideally with many sampling sites located in the direct vicinity of LTIs and few locations left unsampled (Burgess & Garrick, 2021; Prunier et al., 2013; Richardson et al., 2016). This could not be achieved in all species, especially in *A. parallelepipedus* and *M. jurtina* because of an uneven spatial distribution of populations within the study area. Furthermore, the total number of sampled individuals and the number of loci should be high enough to allow correct inferences (Oyler-McCance et al., 2013): with only 115 genotypes available in *N. helvetica* and only six (though highly polymorphic) loci available in *M. jurtina*, we may have lacked statistical power to detect the actual influence of some LTIs on gene flow in these two species. Finally, the setting-up of genetic structures is a process characterized with high temporal inertia, which could somehow prevent the detection of barrier effects for the most recent LTIs (C. D. Anderson et al., 2010; Prunier et al., 2014): the railway was more than 150 years old, which seems to be of sufficient duration for the detection of a putative barrier effect from genetic data (Landguth et al., 2010; Prunier et al., 2014), but others were not so old (the motorway was for instance less than 15 years old), which may have prevented us from detecting actual barrier effects. Nevertheless, and

although we were probably not able to detect all LTIs effects, we consider reasonable our main findings that barrier effects of different LTIs may accumulate to shape spatial patterns of gene flow in some species (notably in *A. parallelepipedus*, with a cumulative effect of roads and gas pipeline) and that some LTIs may have antagonistic effects (i.e., acting a barrier for some species but somehow promote gene flow in some others).

5. CONCLUSION

The accumulation of LTIs within landscapes is emerging as an important concern and local conservation policies are to be fueled by a thorough assessment of landscape functional connectivity. Although focusing on a single species may help corridor planning (Baguette et al., 2013), we here illustrated how important it is to assess landscape connectivity from a multi-species perspective. Considering the high variability in species response to LTIs, we argue that considering a single species may lead to counterproductive mitigation measures and that integrative approaches based on multiple species are to be more systematically considered. This work must necessarily be carried out on a case-by-case basis, ideally using both genetic and demographic tools, depending on the species present and the topographic characteristics of the existing or planned LTIs in the landscape under study. Although functional connectivity directly underpins the design of green infrastructures and drives biodiversity offsetting measures through the use of equivalence assessment methods (Boileau et al., 2022), it is the hardest mitigation currency to assess on the field based on expert opinion: in a context where target-based mitigation measures are developing (Simmonds et al., 2020), the use of genetic tools to monitor functional connectivity for a set of targeted species could improve biodiversity conservation through a better implementation of the mitigation hierarchy and the design of conservation site

networks (Boileau et al., 2022), especially when considering the management of cumulative barrier effects (Blakley & Franks, 2021).

As it obviously seems impossible to assess functional connectivity in all existing species in a given landscape, it is also necessary to determine the extent to which species-specific mitigation measures can benefit the largest number of species, and, more generally, to investigate which life-history traits drive the taxonomic-specific response of organisms to the presence of LTIs.

Authors' contributions

Jonathan Remon: Conceptualization, Methodology, Formal analysis, Investigation, Writing - Original Draft, Visualization; **Sylvain Moulherat:** Conceptualization, Methodology, Investigation, Supervision, Project administration, Funding acquisition; **Jérémie H. Cornuau:** Investigation; **Lucie Gendron:** Formal analysis; **Murielle Richard:** Formal analysis, Resources; **Michel Baguette:** Conceptualization, Methodology, Supervision; **Jérôme G. Prunier:** Conceptualization, Methodology, Formal analysis, Writing - Original Draft, Visualization, Supervision

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TABLES

Table 1: Outputs of multiple linear regressions and additional parameters from commonality analyses (CA) for each species and for each type of data set. DV represents the dependent variable: classical genetic distances (GD; calculated either with the Bray-Curtis dissimilarity index (bc) or with Fst) and hierarchical genetic distances (HGD1 and HGD2 for first and second level of hierarchy, respectively). For each model, the model fit (R^2) was estimated from reduced scale analyses, with a maximum distance threshold between pairs of individuals or populations (Dist.) ranging from 2800 to 18500m. In each model and for each retained predictor, we estimated the structure coefficient (rs), the beta weight (β), as well as unique (U), common (C) and total (T) contributions. The relative contribution of each predictor (% of R^2) was computed as U/R^2. Significance of a predictor's contribution to the dependent variable was estimated using confidence intervals (CI-inf and CI-sup). A CI that included 0 was considered as a non-informative predictor (indicated in italic). The putative effect of significant LTI predictors on gene flow (Barrier or Corridor) is also provided. Note that results in A. obstetricans were obtained following a specific Jackknife procedure (see Appendix A for details).

Species	DV	Dist. (m)	R^2	Predictor	rs	β	CI-inf	CI-sup	U	C	T	% of R^2	Effect
A. obstetricans	GD(bc)	3300	3.9%	Urban	0.336	0.070	0.008	0.129	0.005	0.000	0.005	13.2	
				Grassland	0.476	0.073	0.021	0.128	0.005	0.004	0.010	13.3	
				Roads	0.758	0.092	0.040	0.145	0.006	0.016	0.022	16.9	Barrier
				IBD	0.714	0.081	0.039	0.120	0.005	0.015	0.019	12.1	
	HGD1	3300	1.5%	Urban	0.819	0.037	0.012	0.064	0.001	0.000	0.001	79.2	
				IBD	*0.364*	*0.019*	*0.000*	*0.041*	*0.000*	*0.000*	*0.000*	*29.4*	
	HGD2	5700	6.0%	Motorway	-0.358	-0.084	-0.106	-0.060	0.011	0.002	0.012	11.9	Corridor
				D6089	0.737	0.187	0.158	0.215	0.046	-0.002	0.044	58.6	Barrier
				Urban	0.557	0.133	0.085	0.178	0.031	0.002	0.033	29.7	
N. helvetica	GD(bc)	2800	4.2%	Roads	-0.109	-0.125	-0.193	-0.062	0.015	-0.003	0.012	35.4	Corridor
				Motorway	0.125	0.148	0.078	0.221	0.021	-0.005	0.016	50.8	Barrier
N. helvetica *M. jurtina*				Railway	-0.106	-0.088	-0.155	-0.022	0.008	0.004	0.011	18.6	Corridor
	GD(Fst)	5500	19.9%	IBD	0.209	0.264	0.001	0.490	0.066	-0.023	0.044	33.3	
				Woodlands	0.306	0.315	0.077	0.519	0.089	0.004	0.093	44.8	
				Power line	*-0.266*	*-0.180*	*-0.388*	*0.046*	*0.030*	*0.040*	*0.071*	*15.3*	
A. parallelepipedus	GD(Fst)	6500	25.9%	*Altitude*	*0.103*	*0.121*	*-0.023*	*0.251*	*0.015*	*-0.004*	*0.011*	*5.6*	
				Grasslands	0.494	0.498	0.372	0.610	0.248	-0.004	0.244	95.9	
	HGD1	18500	17.2%	Roads	0.337	0.262	0.170	0.350	0.063	0.051	0.114	36.5	Barrier
				D6089	0.331	0.254	0.159	0.338	0.059	0.051	0.110	34.1	Barrier
	HGD2	4500	26.8%	Altitude	0.230	0.223	0.056	0.397	0.049	0.004	0.053	18.3	
				D6089	0.393	0.350	0.184	0.500	0.114	0.040	0.154	42.7	Barrier
				Motorway	*-0.163*	*-0.114*	*-0.273*	*0.041*	*0.012*	*0.015*	*0.027*	*4.5*	
				Gas pipeline	0.268	0.225	0.070	0.368	0.049	0.022	0.071	18.4	Barrier

FIGURES

Figure 1: Study area in southwestern France and sampling locations of *Natrix helvetica* (115 individuals) and *Maniola jurtina* (21 populations of about 30 individuals each). For these two species, no genetic structure was identified (see result section).

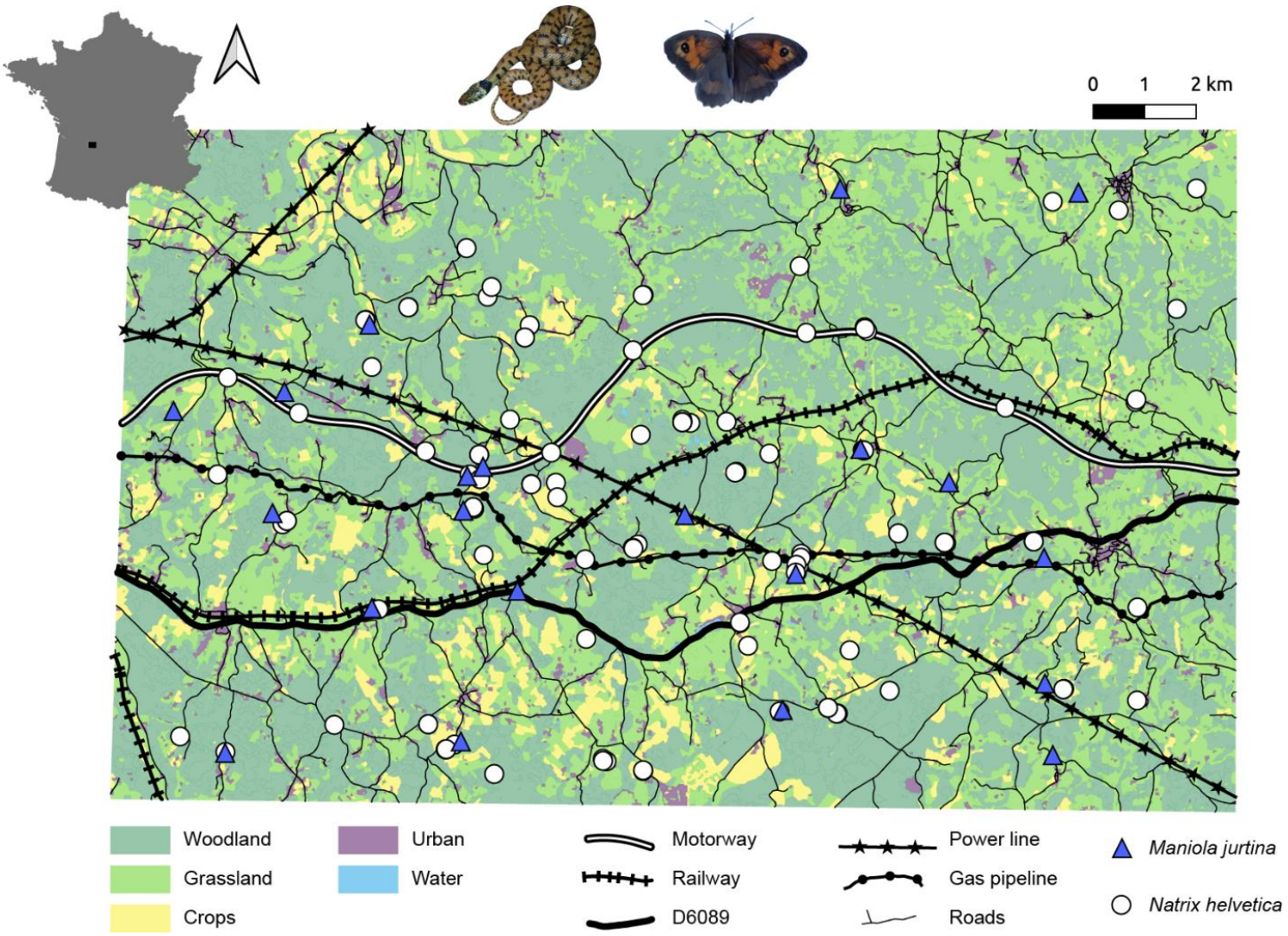


Figure 2: Left panels: STRUCTURE outputs for *A. parallelepipedus* (30 populations of about 30 individuals each; panel A) and *A. obstetricans* (358 individuals in 56 sampling locations; first hierarchical level in panel B, second hierarchical level on panel C) plotted over the study area. Right panels: hierarchical splits of inferred clusters from the first to the second hierarchical level. Each box represents a cluster, with n the corresponding number of assigned samples. The number of cross-assigned samples at each hierarchical level (Q-values < 0.6) is also indicated.

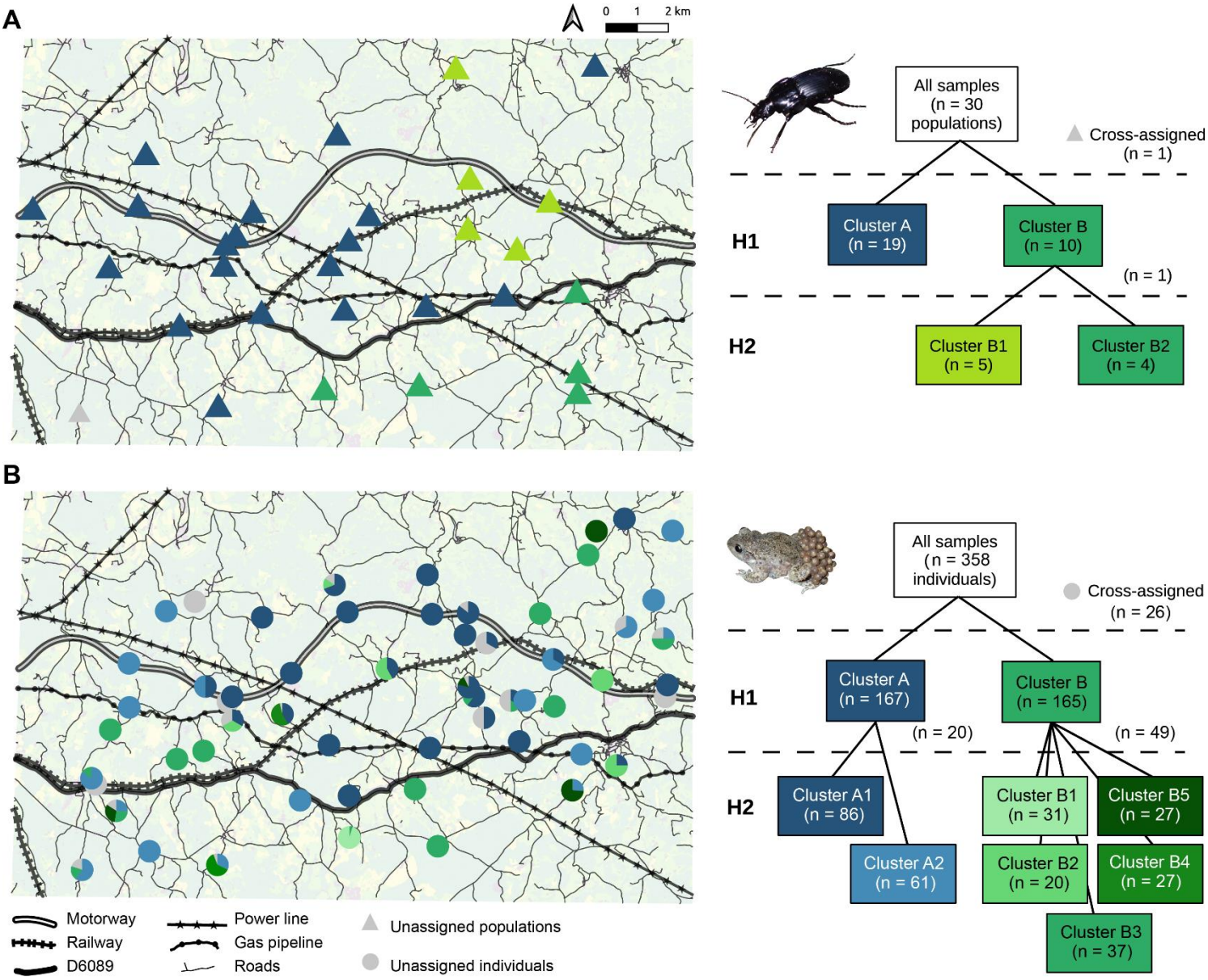
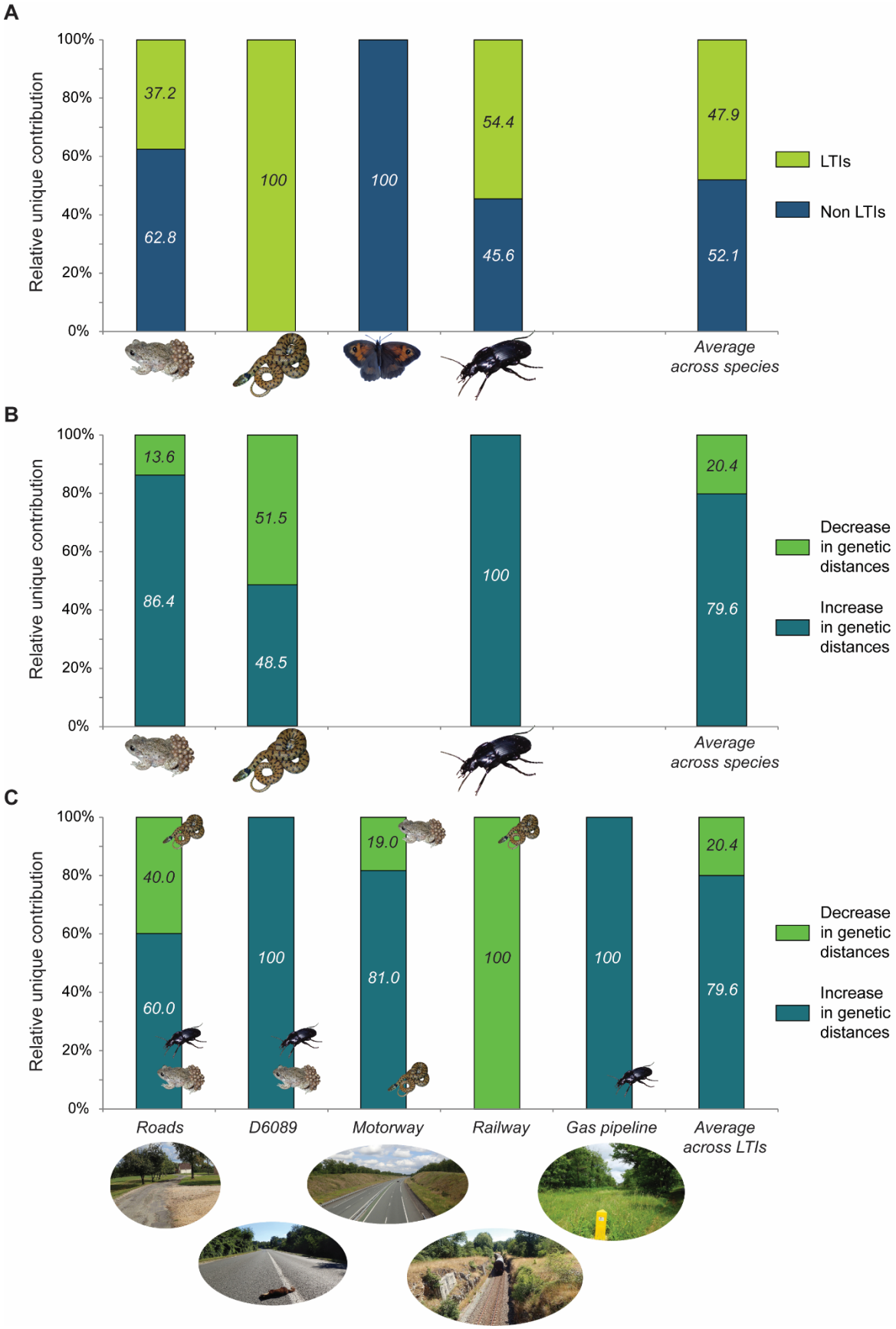


Figure 3: Stacked barplots of the averaged relative contributions of various predictors to the explained genetic variance. Panel A: For each species and for all species combined, averaged relative contributions of LTIs (all infrastructures combined) versus non-LTI predictors. Panel B: For each species (except *M. jurtina*) and for all species combined, averaged relative contributions of LTIs associated with an increase (barrier effect) versus a decrease (corridor effect) in genetic distances. Panel C: For each LTI (except the power line) and for all LTIs combined, averaged relative contributions of LTIs associated with an increase (barrier effect) versus a decrease (corridor effect) in genetic distances.


A
Relative unique contribution
100%
80%
60%
40%
20%
0%
37.2
62.8
100
100
54.4
45.6
47.9
52.1
LTIs
Non LTIs
Average across species
B
Relative unique contribution
13.6
86.4
51.5
48.5
100
20.4
79.6
Decrease in genetic distances
Increase in genetic distances
Average across species
C
Relative unique contribution
40.0
60.0
100
19.0
81.0
100
100
20.4
79.6
Decrease in genetic distances
Increase in genetic distances
Roads
D6089
Motorway
Railway
Gas pipeline
Average across LTIs

APPENDICES

A. Sampling scheme and specific analytical framework in *Alytes obstetricans*

A total of 434 individuals were sampled across 56 locations, with a highly skewed spatial distribution of genotypes: a maximum of three samples were collected in most locations except in nine locations where up to 50 samples could be collected. From these 434 individuals, 76 were discarded as siblings (see Appendix C). The remaining 358 genotypes were used to compute BC genetic distances and HGD hierarchical genetic distances following STRUCTURE analyses, as explained in main text. However, because subsequent statistical analyses might be flawed by the overrepresentation of some heavily sampled locations compared to locations with a maximum of three genotypes, we used a Jackknife subsampling procedure: we created 1000 subsets from the 358 original genotypes (without replacement) so as to only retain a maximum of three genotypes per location in each subset. The statistical analysis described in the main text was applied to each subset to produce a specific Jackknife distribution for each parameter of interest (beta weights, commonalities, etc.). Final estimates (with 95% confidence intervals) were calculated as the mean (along with the 2.5% and 97.5% quantiles) of each distribution. By doing so, we exploited all available genetic data but limited any possible bias stemming from the over-representation of some sampled locations.

B. Laboratory procedures and microsatellite markers

For all species, we used a Qiagen Type-it Microsatellite kit. We extracted total DNA from insect legs, scales and swabs using the DNeasy Blood and Tissue kit (Qiagen, Valencia, CA). Before enzymatic digestion, each insect leg and scale was cut in 4-6 pieces to facilitate DNA extraction. Buccal swabs were used as is. For *Natrix helvetica* and *Alytes obstetricans*, we amplified 13 (Pokrant et al. 2016) and 14 (Tobler et al. 2013, Maia-Carvalho et al. 2014) polymorphic microsatellite loci, respectively. For both species, loci were amplified in 10 µl reaction volumes containing 2 µl multiplex PCR Master Mix, 1.2 to 1.6 µl of primer mix (between 0.13 and 0.25 µM of each primer), 5.4 to 5.8 µl of purified water and 1 µl of template DNA (10-20 ng µl-1). For *Maniola jurtina*, we amplified 15 polymorphic microsatellite loci (Richard et al. 2015) in three Multiplexes, in 10 µl reaction volumes containing 2 µl multiplex PCR Master Mix, 0.7 µl of primer mix (between 0.03 and 0.08 µM of each primer), 4.3 µl of purified water and 3 µl of template DNA (1-10 ng µl-1). For *Abax parallelepipedus*, we amplified 14 polymorphic microsatellite loci (Marcus et al. 2013) in three Multiplexes, in 5 µl reaction volumes containing 1 µl multiplex PCR Master Mix, 0.7 µl of primer mix (between 0.04 and 0.11 µM of each primer), 2.3 µl of purified water and 1 µl of template DNA (approx. 10 ng µl-1).

Polymerase Chain Reaction (PCR) conditions were set on an Applied Biosystems thermal cycler. For the two vertebrate species, conditions were set as follows: initial denaturation 10 min at 95°C; 30 cycles of 30 s at 95°C, 90 s at 51 to 60°C (depending on the multiplex) and 30 s at 72°C; final elongation of 5 min at 72°C. For the two insect species, conditions were set as follows: initial denaturation 10 min at 94°C; 40 cycles of 30 s at 94°C, 90 s (for the 10 first) or 30 s (for the 30 following) at 61°C (*A. parallelepipedus*) or 56°C (*M. jurtina*) and 30 s at 72°C; final elongation of 5 min at 72°C. All PCR products were ten times diluted and were run on an ABI 3730 DNA Analyser (Applied Biosystems) with the GeneScan-600 LIZ size standard. Genotyping was performed with GENEMAPPER 5.0 (Applied Biosystems) and all peaks were manually confirmed.

In the *A. obstetricans* data set, there was no evidence of linkage disequilibrium among loci. We found evidence of null alleles for locus Aly7. Accordingly, we retained 13 loci for subsequent analyses (Aly28, Aly3, Aly4, Aly17, Aly19, Aly20, Aly23, Aly24, Aly25, Aobst14, Aobst15, Aobst16 and Aobst17).

In the *N. helvetica* data set, two loci could not be amplified (Nsµ3 and 3TS) either in multiplex or in standalone PCR. There was no evidence of null alleles, but we found evidence of linkage disequilibrium between loci Natnat05 and µNt8new and between loci Natnat05 and TbuA09. Therefore, we only retained 10 loci for subsequent analysis (Natnat09, µNt8new, µNt3, µNt7, µt06, Natnat11, Eobµ1, Eobµ13, TbuA09 and 30).

In the *M. jurtina* data set, the locus Mj2410 was discarded as it showed sex linkage (Richard et al. 2015, Villemey et al. 2016). As Villemey et al. (2016), we found evidence of frequent null alleles for loci: Mj5522, Mj5287, Mj5647, Mj3956, Mj5563, Mj0272, Mj0283 and Mj3637. Thus, we only retained six loci for subsequent analysis (Mj0008, Mj7132, Mj0247, Mj7232, Mj4870 and Mj5331).

In the *A. parallelepipedus* data set, there was no evidence of linkage disequilibrium among loci. We found evidence of null alleles for loci: apar14, apar44, apar46 and apar50. Then, we retained 10 loci for subsequent analysis (apar20, apar50, apar27, apar34, apar32, apar12, apar23, apar25, apar02, apar46, apar05, apar44, apar14, apar06).

The following tables describe the specificity of the microsatellite markers tested for the four species followed in this study. Gray colours represent markers that were not used in the landscape genetic analyses either because they could not be amplified, showed sex-linkage, presence of null alleles or linkage disequilibrium.

Locus	Primer sequences (5'- 3')	Repeat motif	Allele size range (bp)	Number of alleles	Multiplex set	Annealing temperature	Fluorescent label	Original reference
Alyobs3	f – CCAACATGTTCACTTTATAGAGCAG r – GGAACCTTGAATCTCGAAAGC	(TATC)28	168-276	27	1	52	FAM	Tobler *et al.* (2013)
Alyobs4	f – TTTTCCCTTGCTAAATCCTCAG r – AAAGTGTTGATGCACATTTTCC	(CTGT)11	117-161	9	1	52	NED	Tobler *et al.* (2013)
Alyobs7	f – AAGGACGTGCTTCTATCTGC r – AGTTCGCACACATTACATTGC	(TATC)16(TG)3(TA)3(TC)(TA)4			1	52	PET	Tobler *et al.* (2013)
Alyobs28	f – CCAGTGCTGTGGTTTTCTCA r – AAATATCAAGAGCCTTAGCTAACATTT	(GT)13(GA)3(GTGA)3	96-106	4	1	52	VIC	Tobler *et al.* (2013)
Alyobs17	f – TTCTCTTCAGCTGGGCAATC r – TGGAACTGAAGAGCGAGGAC	(GT)13	146-160	8	2	56	VIC	Tobler *et al.* (2013)
Alyobs19	f – TGAATGTGCCGGTGAAGAC r – AAACACATATGAACAGGTGAAAAGAG	(GT)12	72-112	13	2	56	NED	Tobler *et al.* (2013)
Alyobs20	f – GATGCAGCACATTTCTGAGC r – GGTGCATCTGCCATAGTGTG	(GT)12	105-115	5	2	56	PET	Tobler *et al.* (2013)
Alyobs23	f – TGCAGAGCTCAGCCACTTAG r – TGACCAATCCAATCATCCAG	(GT)13	208-238	6	2	56	PET	Tobler *et al.* (2013)
Alyobs24	f – TCCTCAAAATCTTGTGATGTGC r – ATGGCCAGATGTCCCAATAC	(CA)28	75-139	23	2	56	FAM	Tobler *et al.* (2013)
Alyobs25	f – CCTTCTGTCTACCTTGTCATATTTCC r – AAAGCGACTAATACAGAACACTGC	(GT)16	141-163	9	2	56	NED	Tobler *et al.* (2013)
Aobst14	f – TGTGGGAACCTTTACATCATAA r – CCCTCCTCTAAGCCGTCA	(ACT)n	102-158	11	3	52	VIC	Maia-Carvalho *et al.* (2013)
Aobst15	f – TTGGATGGTGGGTACAATCA r – TGAGGACAAATGCCTGACAA	(AGAT)n	251-401	21	3	52	NED	Maia-Carvalho *et al.* (2013)
Aobst16	f – TCAGAATAAACAAGAGCTGCAAA r – GGAGATCCACGCTCAGGATA	(AGAT)n	445-497	8	3	52	FAM	Maia-Carvalho *et al.* (2013)
Aobst17	f – CGGTGTCCCCATCTTATCAA r – CCCAGTGCTCAAACCTCAAT	(ACC)n	244-268	6	3	52	PET	Maia-Carvalho *et al.* (2013)

Locus	Primer sequences (5'- 3')	Repeat motif	Allele size range (bp)	Number of alleles	Multiplex set	Annealing temperature	Fluorescent label	Original reference
Natnat09	f – TGTAAATAACACTGTACCATTTTGG r - TGACTGGGCAACAGAAAAGC	$(AC)_{22}$	96-132	15	1	55	FAM	Meister *et al.* (2009)
Natnat05	f – TCTGCACTGGGGATAGGAAG r - GTCCCTTTTTCAGTGCTGTTG	$(GT)_{16}$			1	55	VIC	Meister *et al.* (2009)
µNt8new	f – GTATCGTCCTTCCAGACAAG r - GCAAATCAAATAAATCTCACTGG	$(AC)_{15}$	81-123	14	1	55	NED	Meister *et al.* (2009)
Nsµ3	f – CTGACTCACTTCTGACCCTAAT r - AATATTTGCTTGGCTCAAAC	$(ATCT)_{14}$ $ATC(CA)_{20}$			1	55	PET	Prosser *et al.* (1999)
µNt3	f – GGCAGGCTATTGGAGAAATG r – GGCAAAACTCCAGGTGCTAC	$(AC)_{16}$	127-145	5	2	60	FAM	Gautschi, Widmer & Koella (2000)
µNt7	f – TTTGAAAGGAGAATGAATCGTG r – CGCGAGGAATCAGAATGAAC	$(AC)_{17}$	177-185	3	2	60	VIC	Gautschi *et al.* (2000)
Natnat11	f – GGCTGTTTTCCCAGTGAAGC r - GGTCTGGGGAAAAAGAAAGG	$(GA)_{13}$	106-118	4	3	55	FAM	Meister *et al.* (2009)
Natnat06	f – AATGGCATTCTCTCCAGCTC r - ACCCATATCCGTATCCATATCC	$(GT)_{21}$	159-187	13	3	55	VIC	Meister *et al.* (2009)
3TS	f – GGTCACTTAAATACAACGAAATTGGTTAGCT r - CGGACAGCTCTGGCTCCCTTG	$(GATA)_{19}$			3	55	PET	Garner *et al.* (2002)
30	f – CCCACTGGCTCATTTCAAGT r – CCACATTTGCATCGGAGTG	$(CA)_{14}$	250-274	13	—	60	NED	Burns & Houlden (1999)
Tbu A09	f – CATCTCAACCAAAGTCGCTTC r – GGATGTTGTGGGGTGTTTTC	$(AC)_{7}$	110-140	14	—	55	NED	Sloss *et al.* (2012)
Eobµ1	f – ATCAGTAGGAGTGAGAGCAACT r – CTGCATACTCTTCCAGAACC	$(TG)_{21}$	128-134	3	—	51	NED	Blouin-Demers & Gibbs (2003)
Eobµ13	f – TGATCTGAGTCTCTTTCTGG r - CAATTCAAATCCATTGGTTT	$(AC)_{20}$	138-162	9	—	51	PET	Blouin-Demers & Gibbs (2003)

Locus	Primer sequences (5'- 3')	Repeat motif	Allele size range (bp)	Number of alleles	Multiplex set	Annealing temperature	Fluorescent label	Original reference
Mj0008	f – CGTGTCGCCTAAACCACATC r – TGGCAACCCTAAACCCTACG	(ACAT)7	91-149	5	1	56	PET	Richard *et al.* 2015
Mj5287	f – GCTAGCTCGTGGGTACTCTG r – CTCCAAGCAATAAGACCGCC	(GATA)11			1	56	FAM	Richard *et al.* 2015
Mj7132	f – ATCTGCGGATTTGCAGTTGG r – CACTATTGAGCACGTGTGTCC	(TATG)13	165-213	19	1	56	NED	Richard *et al.* 2015
Mj5647	f – GCGTTCTGATTACCACCCTG r – GCGACAGTCCCCTAAGATCG	(TATG)13			1	56	PET	Richard *et al.* 2015
Mj5563	f – CGGTTTTGCCGATAGCGTAG r – CGCAAGGCAATAGACCACTC	(ATCT)7			1	56	VIC	Richard *et al.* 2015
Mj3956	f – CAACATCGGGAGTCGAAACG r – CTCAGCCAGGATACCCACTC	(GATA)7			2	56	PET	Richard *et al.* 2015
Mj7232	f – AAGTTACAAGAGCGTTGGCG r – GCGGGAACTCTTGGGTTTTC	(CTGT)7	144-214	19	2	56	FAM	Richard *et al.* 2015
Mj5522	f – TGATCTTTGCCAGCAGGAAC r – AGTGTAAGCTGGCCCTAAAC	(GATA)8			2	56	NED	Richard *et al.* 2015
Mj0247	f – ATTCCACAAACGAGCCAACG r – ACTCCGATGGTAAGAGGTGC	(GATG)8	182-328	53	2	56	PET	Richard *et al.* 2015
Mj0272	f – GTTGCATTGGCACACTCCTC r – CAGCTGCACACTACGACAAG	(AGAT)7			2	56	VIC	Richard *et al.* 2015
Mj5331	f – TTAGACCGTGATCCCACTGC r – ATTTCGATAGGCAACGAGGC	(TATC)10	100-204	25	3	56	PET	Richard *et al.* 2015
Mj4870	f – ATGATCCATAGCTGCGTTGC r – CTCCTTAGCGCTTACACGTC	(ATGT)7	156-184	13	3	56	FAM	Richard *et al.* 2015
Mj3637	f – CTTCCGCAAAATAACGTCTGC r – AGATACTCCATTGACCCGGC	(TCTA)7			3	56	NED	Richard *et al.* 2015
Mj2410	f – TAATTAGAGTTTGCGCGGGG r – CGCACACCGCAGTATAAGTG	(TGTA)7			3	56	PET	Richard *et al.* 2015
Mj0283	f – CCCTTAGAATAAGAACTCGGCTC r – TGTTCGCACATGCTTAGTCC	(AGAT)9			3	56	VIC	Richard *et al.* 2015

Locus	Primer sequences (5'- 3')	Repeat motif	Allele size range (bp)	Number of alleles	Multiplex set	Annealing temperature	Fluorescent label	Original reference
apar_20	f – ACACTCCACTCAAAGTTGCG r – AAACGGTCAACTTTCCACCC	(AC)	185-189	3	1	61	PET	Marcus *et al.* 2013
apar_50	f – GCTGGACTATTACAGAAGTCTTTTGC r – ATGTGGAGGAAGCACGTGTT	(CATA)			1	61	FAM	Marcus *et al.* 2013
apar_27	f – CCTCCTTACCAAGTAACGGG r – GTTTGGAAGCGACAGTCAACGTG	(AC)	251-255	2	1	61	NED	Marcus *et al.* 2013
apar_34	f – GTTTGCCATACTAGGTGCTCTGG r – ATCTCCCGTGAAATCAACGC	(AC)	103-111	4	1	61	PET	Marcus *et al.* 2013
apar_32	f – TTTACCAACACACGCAGGC r – GTTTGGACCACAACACGTTAGCAC	(AG)	92-94	2	2	61	NED	Marcus *et al.* 2013
apar_12	f – GACCGTCGAGTGTAATGACG r – CAATCTGCTCCTCAAGTTCAAG	(AG)	123-133	4	2	61	VIC	Marcus *et al.* 2013
apar_23	f – GTGCCTATCGTTCTTTGTCAC r – GTTTGCGATATTGTCTCTTGGCGG	(AC)	156-162	4	2	61	NED	Marcus *et al.* 2013
apar_25	f – GTTTCGTAGCGAAACAGTGCCTTG r – ATACTCCGGCGCTACTTTGG	(AC)	198-204	5	2	61	NED	Marcus *et al.* 2013
apar_02	f – GCCGCACGATATTAGCGAC r – TTGGGAGTAAGTCTGTCCGG	(AC)	165-169	3	2	61	PET	Marcus *et al.* 2013
apar_46	f – CAGTTCAGTTCATCACGGGC r – GTTTGGAACCCAACGCAGAAAGTC	(AAC)			2	61	PET	Marcus *et al.* 2013
apar_05	f – CAACAACATTACCGGCGGAG r – GCCGAGTCACTTGTTACGTG	(AG)	150-156	4	3	61	FAM	Marcus *et al.* 2013
apar_44	f – GTTTCTTAATGTTCCATGCCGCG r – TCTTCTTCGGCAAGCGTTAC	(AG)			3	61	VIC	Marcus *et al.* 2013
apar_14	f – GACATCTCGACTGCACCTAC r – CCCTGTCTTTCCAACATCGC	(AG)			3	61	NED	Marcus *et al.* 2013
apar_06	f – AAACATTCTGCGGTGACACC r – CTGCTGCCCTCTTGTAAACG	(AG)	284-308	5	3	61	PET	Marcus *et al.* 2013

C. Sibship reconstruction

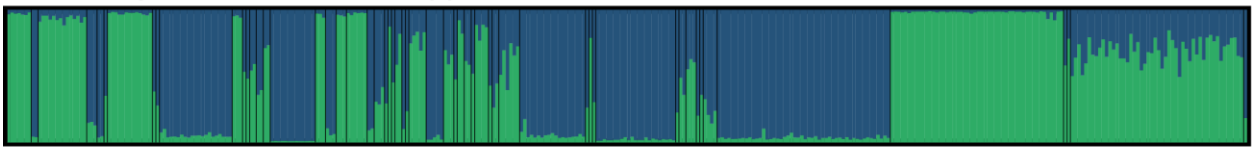
We used COLONY2 (Jones and Wang 2010) to identify full-sib and parent-offspring groups among our individual-based data sets (*N. Helvetica*; n= 116) and (*A. obstetricans*; n = 434, see Appendix A). We used the full-likelihood approach based on the individual multilocus genotypes. For both species, we assumed that males and females were polygamous (for the snake, see Meister at al. 2012a). All individuals were considered as potential offspring and no a priori candidate parental genotype was defined. Allele frequencies were determined directly from genetic datasets. We ran three independent long runs with various seed numbers to test for congruence among results. Only relationships with an associated inclusion probability higher than 95% were considered as significant. In *A. obstetricans*, we identified 67 different groups (composed of full-sib and/or parent-offspring dyads; 2 to 4 individuals per group). All groups were clustered within the same location (suggesting actual siblings), at the exceptions of two dyads, whose members were split in different locations and distant from about 4 km and 10 km, respectively (suggesting possible misclassification in these two cases). In *N. Helvetica*, we identified one full-sib dyad, corresponding to the shed skin of an already sampled individual. We randomly selected one genotype from each group of related individuals, resulting in the removal of 76 genotypes in *A. obstetricans* and one genotype in *N. helvetica*.

D. STRUCTURE outputs

For each species and each hierarchical level (if any), the following figures provide plots of individual ancestry Q-values. Each bar stands for an individual and thick vertical lines delineate different sampling locations.

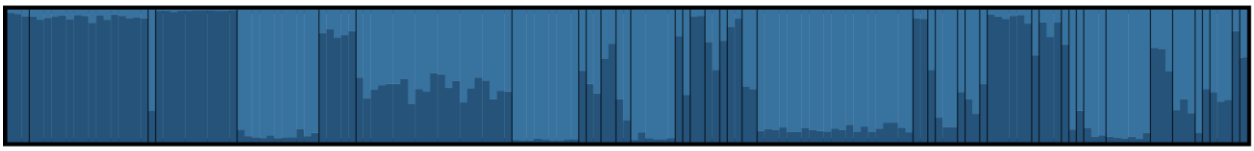
In *A. obstetricans*, the uppermost hierarchical level allowed identifying two main clusters A and B. Cluster A was further split into two subclusters A1 and A2. However, these subclusters could not be split further, since we failed to achieve correct convergence criteria for the STRUCTURE algorithm despite up to 2 million MCMC iterations (large fluctuations of the α parameter along runs). Cluster B was further split into five subclusters B1 to B5. Lower hierarchical levels were not explored since subclusters comprised less than 50 individuals each.

K=2 in *Alytes obstetricans* - Uppermost hierarchical level (358 individuals)



A B

K=2 in *Alyte obstetricans* - First hierarchical level A (167 individuals)

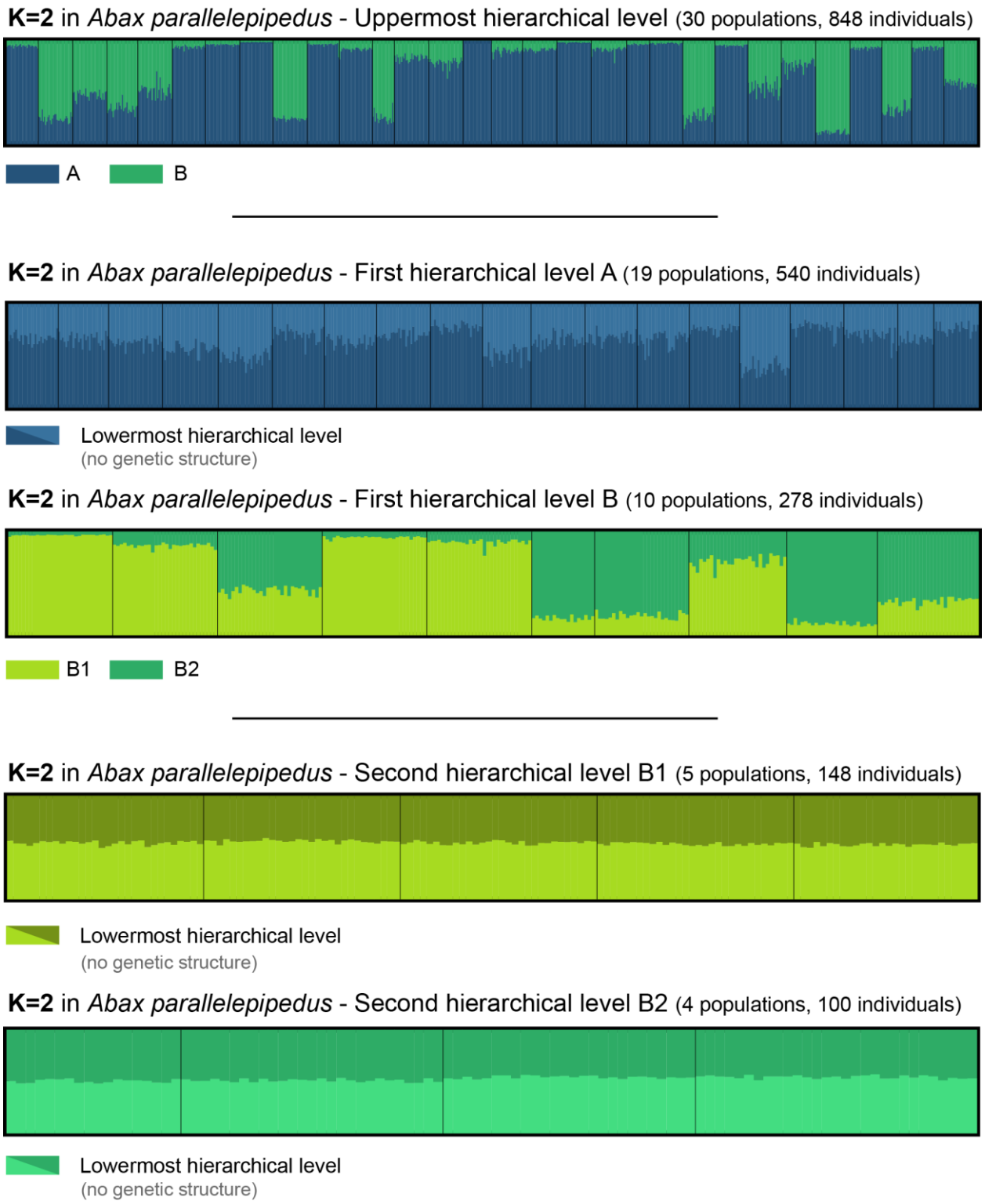


A1 A2 Lowermost hierarchical level
(No convergence at lower hierarchical levels despite 2 million MCMC iterations)

K=5 in *Alytes obstetricans* - First hierarchical level B (165 individuals)

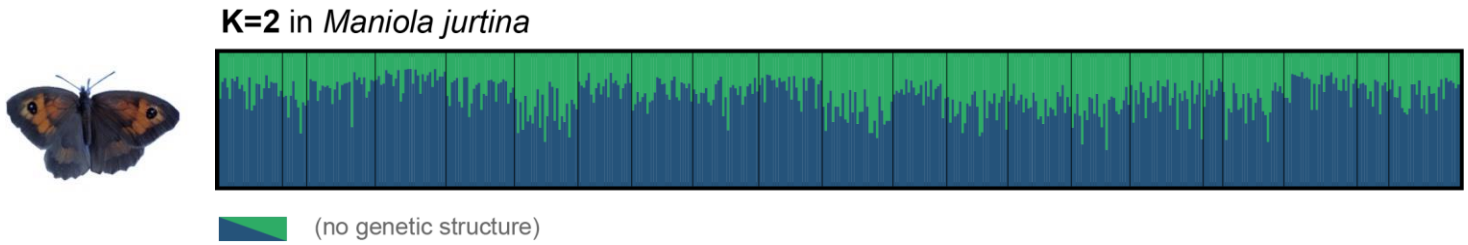
B1 B2 B3 B4 B5 Lowermost hierarchical level
(<50 genotypes per cluster)

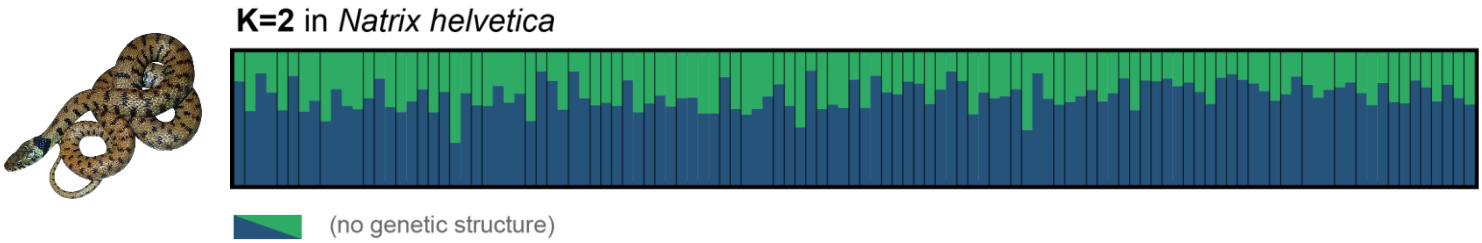
In *A. parallelepipedus*, the uppermost hierarchical level allowed identifying two main clusters A and B. Cluster A could not be split further, since all individuals had the same probability of assignment to any subcluster (illustration for K=2). Cluster B was further split into two



subclusters B1 and B2. These subclusters could not be split further, since all individuals had the same probability of assignment to any subcluster (illustrations for K=2).

We were finally unable to detect any genetic structure in *M. jurtina* or *N. helvetica*: whatever the number of clusters K, all individuals had the same probability of assignment to each cluster (illustrations for K=2).





E. Landscape features defining the six main retained landscape

- Water: Stagnant water; Streams; Ditches; Rivers
- Crops: Intensive monocultures; Gardens; Orchards; Vineyards; Vegetable gardens or horticultures
- Woodlands: Recent logged forests; Coniferous forests; Decideous forests; Riparian forests; Mixed woodlands; Heathlands; Hedgerows; Tree plantations; Bushlands
- Grasslands: Grass stripes; Forest clearings; Openings; Grazed pastures; Dry grasslands; Hayed meadows; Meadows; Trails and paths; Rocky lands; Abandoned arable lands
- Urban: Agricultural buildings; Residential Buildings; Waste disposals; Electric pylons; Water tanks; Artificial gardens; Domestic gardens; Cemeteries; Sport equipment such football fields; Surroundings of agricultural buildings; Camp sites; Car parks; Greenhouses; Open cast mines; Stone quarry; Industrial sites; Urban paved areas; Windmills
- Roads: Gravelled roads; Paved roads

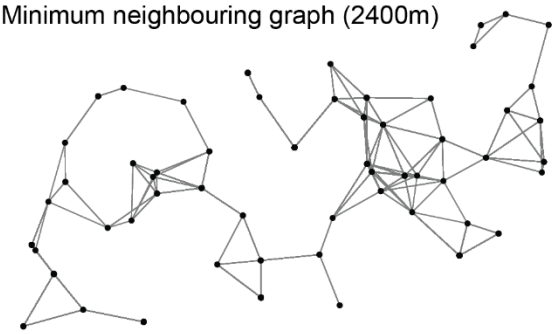
F. Spatial scale of analyses

The spatial scale retained in landscape genetic analyses can deeply influence the conclusions of studies (Keller et al. 2013, Schregel et al. 2018). The local influence of landscape elements on genetic distances can remain unnoticed if spatial scale retained is wide in comparison to dispersal capacities of individuals (Anderson et al. 2010). Accordingly, we did not use all possible pairs of populations or individuals in our data sets. For each dataset, we retained a subset of pairwise data by defining a maximum Euclidean distance between pairs, following Cayuela et al. (2019). The maximum Euclidean distance was selected as the neighboring distance maximizing the R^2 of our full model including all predictors in a classical multiple linear regression. This retained distance was higher than the minimum distance in a neighboring graph which ensured that no individual was excluded from the network (Jombart et al. 2008). It was estimated using Gabriel graphs with the "adegenet" package (Jombart 2008) in R 3.3.2 (R Core Team, 2015). Subsequent analyses were only run with pairwise data associated with Euclidean distances lower than the computed maximum neighboring distance.

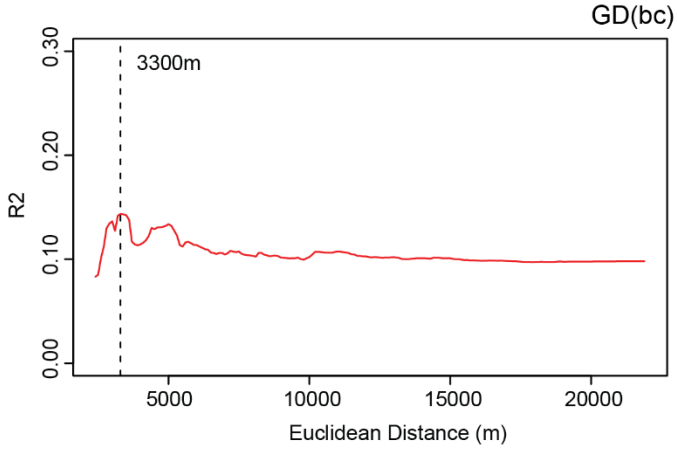
In the four data sets, the minimum neighboring distances detected with the Gabriel graphs were 2400 m, 2700 m, 5100 m and 4500 m for the species *A. obstetricans*, *N. helvetica*, *M. jurtina* and *A. parallelepipedus*, respectively. In the *A. obstetricans* data set (n = 358 individuals), the spatial scales maximizing the R^2 between pairs were 3300 m, 3300 m and 5700 m for the Bray-Curtis genetic distance, HGD1 and HGD2, respectively. In the *N. helvetica* data set, the spatial scale maximizing the R^2 was 2800 m. In the *M. jurtina* data set, the spatial scale maximizing the R^2 was 5500 m. In the *A. parallelepipedus* data set, the spatial scales maximizing the R^2 were 6500 m, 18500 m and 4500 m for the Fst genetic distance, HGD1 and HGD2, respectively.

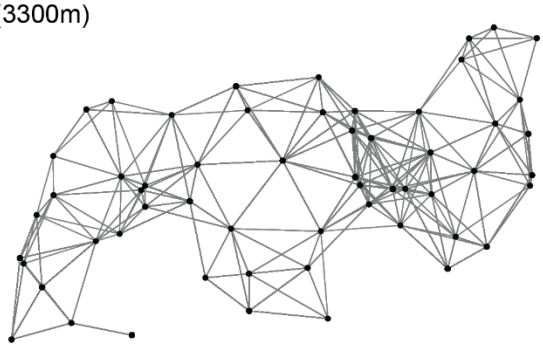
The following figure provides an illustration of the approach for *A. obstetricans*. The inferred minimum neighboring graph is at top right of the figure. For each measure of genetic distances, the left panel is the plot of R^2 with increasing spatial scales (Euclidean distance) indicating with a dashed line the optimal spatial scale of analysis (i.e., maximizing R^2); the right panel represents the corresponding retained neighboring network.

Minimum neighbouring graph (2400m)

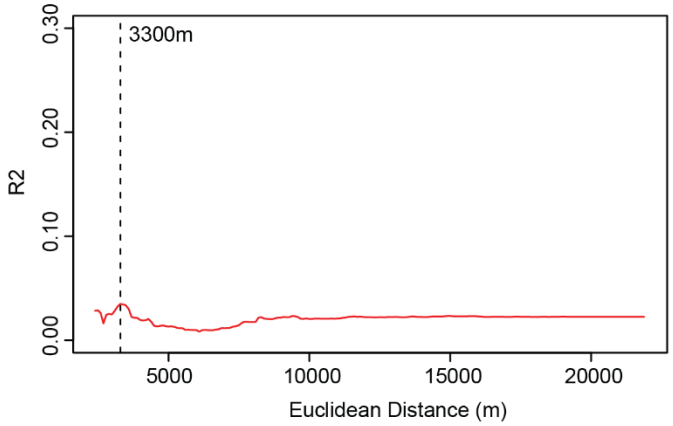


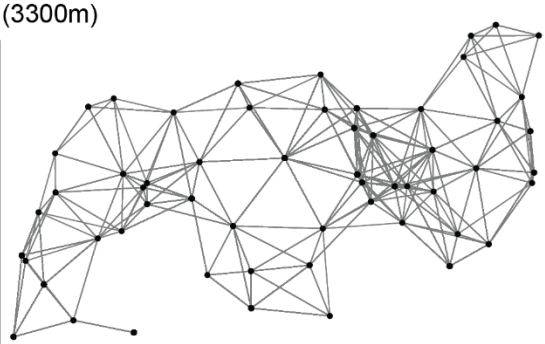
GD(bc) (3300m)



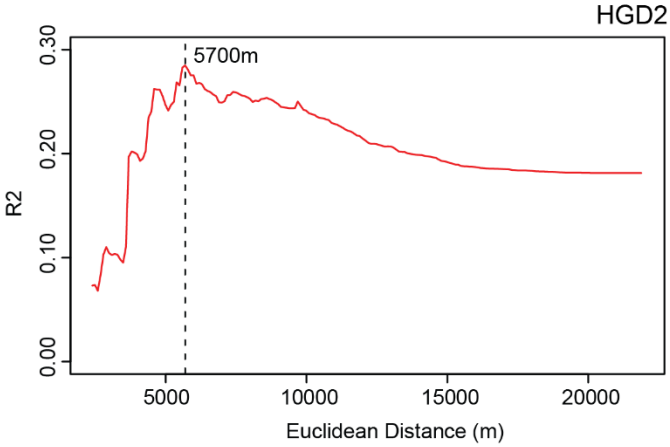


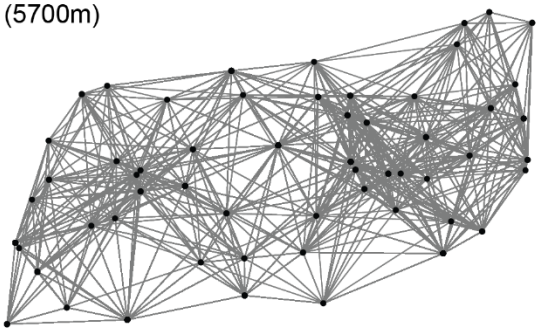
HGD1 (3300m)





HGD2 (5700m)





G. Commonality analyses

In commonality analyses, the effect of each predictor can be decomposed into a unique (U) and common (C; shared with other predictors) effect. For a given predictor, the sum of unique and common effects corresponds to the total contribution (T), equal to its squared zero-order correlation with the dependent variable ($U + C = T = r^2$). Therefore, commonality analyss represents a good opportunity to assess the reliability of predictors to explain the dependent variable face to collinearity. The magnitude of suppression among predictors is indicated by negative commonalities. Negative commonalities represent the amount of predictive power that would be lost by other predictors if the suppressor variable was not included in the regression model.

Accordingly, we can distinguish three specific types of suppressors (Conger 1974). (i) A classical suppressor corresponds to a predictor whose unique contribution is totally counterbalanced by its negative common contribution ($U + C = 0$). (ii) A reciprocal suppressor, also described as a partial suppressor, is a predictor with a negative common effect but that does not counterbalance its unique contribution to the variance in the dependent variable ($T = U + C > 0$). Finally, (iii) a cross-over suppressor is similar to a partial suppressor but with reversal sign. Cross-over suppressors are detected by a sign inversion between the structure coefficients rs and the beta weights (Prunier et al. 2017).

We performed multiple linear regressions and commonality analyses using packages ecodist (Goslee and Urban, 2007) and yhat (Nimon et al. 2008) in R 3.3.2 (R Core Team, 2015). To remove classical suppressors, we discarded predictors presenting low univariate squared correlation against the genetic dependent variables (r^2 lower than 0.01). Low correlated predictors are likely to act as classical suppressors leading to the distortion of regression coefficients (Prunier et al. 2015). When we discarded those non-informative predictors, we ended up with simplified models containing a reduced number of predictors which were likely to explain the variance in the genetic dependent variables. Predictors that were identified as cross-over (CO) and reciprocal suppressors were discarded from our model and subsequent models were ran without these suppressors until no more suppressors could reasonably be discarded from the model (that is, we kept reciprocal suppressors showing a non-negligible unique contribution). We also removed predictors with synergistic (S) association with other predictors, which have a unique contribution to the dependent variable equal to zero but presenting synergistic association with other predictors ($C > 0$).

H. Intermediate steps of commonality analyses on vectors

To explain the dependent variable based on the Bray-Curtis genetic distance in *A. obstetricans*, the predictors with a squared correlation (r^2) with the dependent variable higher than 0.01 were Isolation-by-Distance (IBD), Roads, Grasslands, Urban and Power line. Among these predictors, Power line acted as a cross-over (CO) suppressor and was discarded from subsequent analysis. To explain the first level of hierarchical genetic distances (HGD1) in *A. obstetricans,* the predictors with a r^2 higher than 0.01 were IBD and Urban. None of them acting as a suppressor, they were both kept for subsequent analysis. To explain the second level of hierarchical genetic distances (HGD2) in *A. obstetricans*, ten predictors showed a r^2 higher than 0.01. IBD acted as a cross-over suppressor, both Railway and Crops acted as reciprocal suppressors and both elevation and Grassland showed no significant contribution to the dependent variable across Jackknife subsets (null lower bound of the 95% confidence interval about U): all these predictors were discarded from subsequent analyses.

In the *N. helvetica* data set, only three predictors had a r^2 higher than 0.01: Roads, Motorway and Railway. There was no suppressor among these three predictors and all were used in the final model.

In *M. jurtina*, five predictors had a r^2 higher than 0.01: IBD, Woodlands, Grasslands, D6089 and Power line. Grasslands was a cross-over suppressor and the roads D6089 was a partial suppressor. These two predictors were discarded from subsequent analysis resulting in a final model with three predictors: IBD, Woodlands and Power line.

To explain Fst in *A. parallelepipedus*, six predictors had a r^2 higher than 0.01: Altitude, Grasslands, Water, Urban, Roads and Motorway. Water, Urban, Roads and Motorway were cross-over suppressors. All were discarded from subsequent analysis. Only two predictors remained in the final model: Altitude and Grasslands. To explain the first level of hierarchical genetic distances (HGD1) in *A. parallelepipedus*, we retained the predictors: Grasslands, Water, Crops, Urban, Roads and D6089 ($r^2 > 0.01$). Grasslands, Crops and Urban were cross-over suppressors and Water was a suppressor with synergistic association with other predictors. Therefore, we retained only Roads and D6089 to explain the dependent variable in the final data set. To explain the second level of hierarchical genetic distances (HGD2) in *A. parallelepipedus*, we retained the predictors: Altitude, Roads, D6089, Motorway and Gas pipeline ($r^2 > 0.01$). The predictor Roads was a suppressor with synergistic association with other predictors and was discarded from subsequent analysis.

The following figures provide the runs of identification of unnecessary predictors for each species and each genetic dependent variable DV (GD: genetic distance either calculated with the Bray-Curtis (bc) dissimilarity index for individual-based method or Fst for population-based method; HGD1 and HGD2 for hierarchical genetic distance based on first and second level of STRUCTURE outputs, respectively). Distance stands for the spatial scale retained in our analyses (Appendix F). Results of the different runs of multiple linear regressions (predictors, structure coefficient rs and standardized coefficient β), in addition to parameters derived from CA: unique (U), common (C) and total (T) contributions of predictors to the variance in the genetic dependent

variable. The rationale for withdrawal of predictors (Ra) is the following: CO: cross-over suppression; S: synergistic association with other predictors; PS: partial suppression (or reciprocal suppression); NC: no contribution to the dependent variable (as inferred from the null lower bound of the Jackknife 95% intervals about U in the *A. obstetricans*; see Appendix A for details). All predictors (IBD: isolation by distance; D6089: a large country road; Urban: urban areas) were coded as resistance. In bold: parameters allowing the identification of unnecessary predictors and suppressors. Note that situations of classical suppression were avoided by discarding any predictor with a squared zero-order correlation < 0.01.

DV	Species	Distance	Run	Pred	rs	B	U	C	T	Ra
GD(bc)	*A. obstetricans*	3300 m	1	Power Line	**0.241**	**-0.044**	0.001	0.001	0.002	CO
				Urban	0.329	0.079	0.006	-0.001	0.005	
				Grassland	0.469	0.069	0.005	0.005	0.010	
				Roads	0.745	0.087	0.006	0.016	0.022	
				IBD	0.702	0.109	0.006	0.014	0.020	
HGD2	*A. obstetricans*	5700 m	1	Motorway	-0.320	-0.088	0.007	0.001	0.008	
				D6089	0.663	0.173	0.019	0.014	0.033	
				Gas pipeline	0.530	0.046	0.002	0.020	0.021	
				Elevation	0.144	0.044	**0.002**	0.000	0.002	NC
				Railway	-0.025	-0.052	**0.002**	**-0.002**	0.000	PS
				Crops	-0.006	-0.046	**0.002**	**-0.002**	0.000	PS
				Urban	0.500	0.121	0.013	0.007	0.019	
				Grassland	0.095	0.029	**0.001**	0.000	0.001	NC
				Roads	0.418	0.066	0.003	0.011	0.013	
				IBD	**0.176**	**-0.001**	0.000	0.002	0.003	CO
HGD2	*A. obstetricans*	5700 m	2	Motorway	-0.341	-0.097	0.009	-0.001	0.008	
				D6089	0.699	0.144	0.015	0.018	0.033	
				Gas pipeline	0.560	0.056	**0.003**	**0.019**	0.021	S
				Urban	0.526	0.115	0.013	0.006	0.019	
				Roads	0.437	0.062	**0.004**	0.009	0.013	NC

DV	Species	Distance	Run	Pred	rs	B	U	C	T	Ra
				Roads	-0.533	-0.125	0.015	-0.003	0.012	
GD(bc)	*N. hevetica*	2800 m	1	Motorway	0.616	0.148	0.021	-0.005	0.016	
				Railway	-0.520	-0.088	0.008	0.004	0.011	

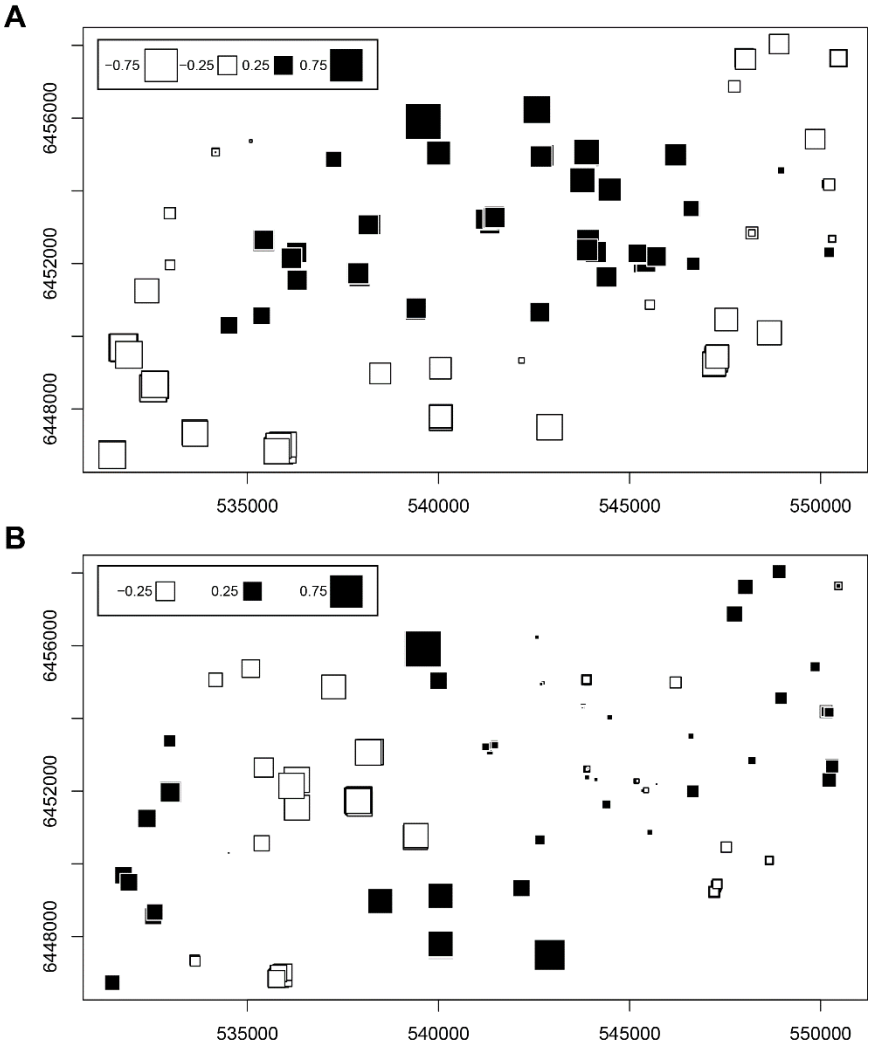
DV	Species	Distance	Run	Pred	rs	B	U	C	T	Ra
				IBD	0.434	0.257	0.062	-0.019	0.044	
				Woodlands	0.636	0.349	0.072	0.022	0.093	
GD(Fst)	*M. jurtina*	5500 m	1	Meadow	**-0.386**	**0.060**	0.002	0.032	0.035	CO
				D6089	-0.255	-0.178	0.030	-0.015	0.015	
				Power line	-0.553	-0.226	0.045	0.026	0.071	

DV	Species	Distance	Run	Pred	rs	B	U	C	T	Ra
				Altitude	0.192	0.144	0.019	-0.008	0.011	
				Grasslands	0.918	0.683	0.213	0.031	0.244	
GD(Fst)	*A. parallelepipedus*	6500 m	1	Water	**0.360**	**-0.156**	0.010	0.028	0.038	CO
				Urban	**0.443**	**-0.023**	0.000	0.057	0.057	CO
				Roads	**0.445**	**-0.089**	0.001	0.056	0.057	CO
				Motorway	**0.226**	**-0.049**	0.002	0.013	0.015	CO
				Grasslands	**0.283**	**-0.140**	0.010	0.006	0.016	CO
				Water	0.595	0.189	0.015	0.055	0.070	
HGD1	*A. parallelepipedus*	18500 m	1	Crops	**0.546**	**-0.043**	0.001	0.058	0.059	CO
				Urban	**0.628**	**-0.172**	0.005	0.073	0.078	CO
				Roads	0.759	0.401	0.031	0.082	0.114	
				D6089	0.745	0.265	0.059	0.051	0.110	
				Water	0.628	0.094	**0.005**	**0.065**	0.070	S
HGD1	*A. parallelepipedus*	18500 m	2	Roads	0.801	0.194	0.018	0.096	0.114	
				D6089	0.787	0.261	0.062	0.048	0.110	
				Altitude	0.440	0.212	0.044	0.009	0.053	
				Roads	0.404	0.085	**0.007**	**0.038**	0.045	S
HGD2	*A. parallelepipedus*	4500 m	1	D6089	0.750	0.324	0.089	0.065	0.154	
				Motorway	-0.312	-0.119	0.013	0.014	0.027	
				Gas pipeline	0.511	0.226	0.050	0.022	0.072	

I. Spatial principal component analysis (sPCA) in *Alytes obstetricans*

To further explore the genetic structure in *Alytes obstetricans*, we ran sPCA (Jombart et al., 2008) using the whole genetic dataset (n = 358 individuals) and the minimum neighboring graph as shown in Appendix F. This method seeks principal components that optimize the variance of individual allelic frequencies while taking spatial autocorrelation of data into account. It provides maps of individual sPCA scores (white and black squares), allowing a visual assessment of spatial genetic structures.

Scores of individuals along the first sPCA axis (panel A in figure below) distinguished a group of individuals located in the center of the study area (in black), surrounded by a second group in the eastern, southern and western part of the study area (in white): this pattern evokes what was found at the second hierarchical level between clusters A1 and A2 using STRUCTURE outputs (Figure 2C in main text). Scores of individuals along the second sPCA axis (panel B in figure below) further suggest a somehow complex spatial pattern, again in accordance with overall STRUCTURE outputs in this species (Figure 2B and C in main text).



J. Correlations among final predictors

Matrices of Pearson's correlation coefficients among final predictors depending on the genetic dependent variables. The genetic dependent variables are genetic distances (GD) based on the Bray-Curtis dissimilarity index (bc), Fst or hierarchical genetic distances at the first and second levels of STRUCTURE outputs (HGD1 and HGD2). The variance inflation factors (VIF) are presented for each predictor.

Species	DV	Pearson's correlation coefficients				VIF
Alytes obstetricans	GD(bc)	**Predictor**	Urban	Grassland	Roads	
		Urban				1.102
		Grasslands	-0.111			1.114
		Roads	0.165	0.052		1.449
		IBD	-0.136	0.297	0.498	1.556
	HGD1	**Predictor**	Urban			
		Urban				1.019
		IBD	-0.137			1.019
	HGD2	**Predictor**	Motorway	D6089		
		Motorway				1.010
		D6089	0.045			1.002
		Urban	-0.091	-0.021		1.009
Natrix helvetica 	GD(bc)	**Predictor**	Roads	Motorway		
		Roads				1.056
		Motorway	0.188			1.038
		Railway	0.131	-0.008		1.019
Maniola jurtina 	GD(Fst)	**Predictor**	IBD	Woodlands		
		IBD				1.049
		Woodlands	-0.197			1.111
		Power line	-0.039	-0.239		1.069
Abax parallelepipedus 	GD(Fst)	**Predictor**	Altitude			
		Altitude				1.001
		Grasslands	-0.034			1.001
	HGD1	**Predictor**	Roads			
		Roads				1.095
		D6089	0.295			1.095
	HGD2	**Predictor**	Altitude	D6089	Motorway	
		Altitude				1.010
		D6089	-0.019			1.071
		Motorway	0.046	-0.229		1.071
		Gas pipeline	0.087	0.095	0.090	1.030

K. Supplementary results and discussion

Alytes obstetricans:

With the classical genetic distances, natural predictors (IBD, Grasslands and Urban) explained most of the dependent variable's variance (63% of the averaged unique contributions).

At the first level of hierarchical genetic distances (HGD1), Urban was the only significant predictor, but the model explained a very limited amount of variance (1.5%), suggesting that the observed spatial pattern at this hierarchical level stems from other non-considered (possibly historical) processes.

Nevertheless, Urban was also found as a significant contributor to the genetic variance at the second hierarchical level (HGD2), suggesting its role in shaping dispersal patterns in this species, along the D6089 (hindering gene flow) and the motorway (enhancing it).

Maniola jurtina:

In this species, woodlands were associated with an increase of genetic distances indicating a barrier effect (positive beta values) and explained most of the variance (U = 0.089). The rest of the explained variance was due to isolation by distance (IBD, U = 0.066). Therefore, the entire variability detected in the butterfly genetic distances was explained by natural predictors.

Abax parallelepipedus:

With the classical genetic distances, two final predictors explained the dependent variable: Altitude and Grasslands. Altitude did not significantly explain genetic distances (95% confidence intervals included 0). Therefore the variance explained by our model was only due to grasslands associated to an increase of genetic distances indicating a strong barrier effect (U = 0.248).

When using the first level of hierarchical genetic distance (HGD1), the linear regression explained 17% of the dependent variable's variance. HGD1 was fully explained by predictors associated with an increase of genetic distances in the ground-beetle (positive beta values): the secondary road network (U = 0.063) and the country road D6089 (U = 0.059).

When using the second level of hierarchical genetic distance (HGD2), the linear regression explained 27% of the dependent variable's variance. Four predictors remained in the final model: the altitude, the road D6089, the motorway and the gas pipeline. The 95% confidence interval around the beta value of the motorway included 0 indicating that the motorway was not significantly explaining HGD2. The three remaining predictors were all associated with an increase of genetic distances (positive beta values). The road D6089 was explaining the highest part of the variability (U = 0.114) suggesting a strong barrier effect of this infrastructure on gene flow. The gas pipeline and Altitude had both a unique contribution to the dependent variable of 0.049.

Infrastructures were not the only landscape elements affecting gene flow in the studied species. Half of the explained genetic variability was, in fact, due to non-LTIs features (Fig. 3A in main text). The non-LTIs features influencing gene flow in *A. obstetricans* were isolation by

distance (IBD), grasslands and urban areas (Table 1). We were able to detect IBD in this study area that was not detected for the same species in Spain (Garcia-Gonzalez et al. 2012) probably because they used mitochondrial DNA instead of microsatellites which are less variable at small geographical scales (Kohn et al. 2006). The negative influence of grasslands on toad dispersal is difficult to interpret, and may stem from the specific arrangement of habitats in the study area. On the contrary, urban areas were found as responsible for an increase in all considered genetic distances (Bc, HGD1 and HGD2), suggesting their important role in shaping spatial genetic patterns in this species: urban areas are indeed inappropriate habitats for amphibians, limiting gene flow in many species (Goldberg et al. 2010, VanBuskirk 2012).

In our study area, the genetic structure of *N. helvetica* was weak. The software STRUCTURE detected only one cluster (interpreted as a single population) indicating that gene flow through this landscape was important. This result may explain the low proportion of the genetic variance explained by landscape features (4% of the variance). In a comparable landscape in Switzerland, (Meister et al. 2010) also found that grass snakes belong to a single population. In this study, we found that *N. helvetica* gene flow was affecting only by infrastructures (roads, motorway A89 and the railway). In seems that, at the local scale, grass snake dispersal is not affected by intensively used landscape features such as crops or urban areas (Wisler et al. 2008, Meister et al. 2010, Meister et al. 2012b). Isolation by distance explains the genetic variance at the regional level (Meister et al. 2012) and genetic structuring can probably only be detected at large spatial scales (Kindler et al. 2013, Pokrant et al. 2016, Kindler et al. 2017, Kindler et al. 2018).

Compared to a previous individual-based study that explained less than 5 % of the genetic variance in three sites across France in the butterfly *M. jurtina* (Villemey et al. 2016), we were able to explain about 20% of the variance when using a population-based method and a restricted spatial scale (maximum neighboring distance = 5500 m). STRUCTURE was not able to find any genetic structure in the data, probably because of high abundance, low specialization and great dispersal capacity in this butterfly (Villemey et al. 2016). Interestingly, we were able to detect an isolation-by-distance effect. This IBD effect was not detected in (Villemey et al. 2016) with pairwise distances up to 60 km apart. We found that woodlands were impeding gene flow in *M. jurtina*, a result similar to (Villemey et al. 2016). The absence of sunlight and the dense vegetation may limit the movements through woodlands.

Unlike Marcus et al. (2015), we found a strong genetic structure in the ground-beetle *A. parallelepipedus* within the studied landscape. The explained proportion of the classical Fst genetic distance was due to grasslands acting as barrier to gene flow. This result is linked to previous studies showing that this species intentionally avoids open fields such as grasslands (Charrier et al. 1997, Petit et al. 1998). This encourages the maintenance of hedges in agricultural environments to favor landscape connectivity between woodland patches (Charrier et al. 1997, Petit et al. 1998, Fournier and Loreau, 1999). Altitude affected gene flow at the second hierarchical level (HGD2), but its effect was modest (Table 1 in main text). In any case, the fragmentation of woodlands due to land conversion, roads or other kind of LTIs could lead to strong isolation of ground-beetles populations. Population abundance are high in this species (Loreau and Wolf, 1993,

Keller et al. 2004) but its dispersal capacity is very limited (Charrier et al. 1997, Brouwers and Newton, 2009). Therefore, populations which are not linked by dispersal may suffer from genetic isolation (Fahrig and Rytwinski, 2009, Beyer et al. 2016).

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