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Shall the Wild Boar Pass? A Genetically Assessed Ecological Corridor in the Geneva Region

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Abstract: Landscape fragmentation caused by road infrastructures represents a major threat to the genetic diversity of a region. The resulting genetic isolation between subpopulations may lead to consanguinity, and consequently to population collapse and extinction. However, the construction of wildlife crossings can help maintain connectivity. In the present paper, we evaluated the genetic spatial structuring of populations of wild boars (*Sus scrofa*) in three areas of the Geneva region connected by an ecological corridor. Those areas are cut off either by a highway that is crossed by a wildlife overpass or by an anthropized sector. Genetic profiling with 9 nuclear microsatellite markers yielded 61 single profiles, which allowed for clustering, parentage, and linkage disequilibrium analyses, uncovering the populations' genetic structure. We also evaluated whether the genetic structure was affected by the sex of individuals. In our analyses, all individuals clustered into a single genetic group, suggesting that no structure limited significantly the gene flow in the region. However, a recent admixture indicated a potential increase in the gene flow between two of the subpopulations due to the wildlife overpass, while the other part of the ecological corridor was not or was only partially functional. Genetic distances between males were significantly higher than between females, although the role of sex remains unclear as to its influence on population genetics. Finally, in order to avoid a subregion becoming fully isolated, urbanization planning should consider this genetic evaluation and proceed with further monitoring, especially by focusing on species more sensitive to landscape fragmentation.

Keywords: clustering; genetic profiling; microsatellites; wild boar; wildlife overpass; functionality

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1. Introduction

Roads, urbanized areas, or areas with a lack of stepping stones in weakly permeable matrices are currently one of the biggest threats to wildlife biodiversity, as the installation of such structures causes fragmentation and loss of natural habitats [1–3] leading to a decrease in available resources (e.g., food, shelter, mate). In order to meet their basic needs, animals will attempt to cross these areas and, if a road is in the way, this may lead to their death. As a result, vehicle–animal collisions increase wildlife mortality rates and reduce the drivers' security [4,5]. Population fragmentation is an issue from a genetic perspective too [6], as it can lead to higher sensitivity to genetic drift or to a genetic bottleneck resulting from a decrease in population size and interconnectivity [7]. Both phenomena can lead to the loss of allelic diversity, eventually leading to inbreeding depression involving fitness and genetic adaptation range diminution, and at the worst stage to the collapse and extinction of population [8].

Connecting biodiversity hotspots and maintaining ecological connectivity at the landscape-scale with wildlife crossings is a concrete measure to counteract the landscape fragmentation caused by roads [9,10]. Wildlife crossings should be placed in pre-existing ecological corridors, defined as axes connecting isolated patches of habitats [11], in order

to improve their functionality. The efficiency of these constructions may vary depending on the characteristics of the targeted species, the structure of the populations, the landscape structure, and the construction features themselves [9]. In order to evaluate them, field observations (e.g., camera traps or radio-tracking), and genetic evaluations, such as measurements of the gene flow before and after their construction, are needed [6].

For the genetic part, small tandem repeat (STR) markers, also named microsatellite markers, are a simple tool that allows for investigating the population structure and the evolutionary processes including migration and genetic drift (i.e., random variation of allelic frequencies) [12]. Furthermore, they present the advantage of being quickly revealed using gel electrophoresis [13] and especially to still be amplifiable with partially degraded DNA (e.g., field-collected samples) [14]. However, they may lack statistical power; therefore, enough STR markers with a sufficient allelic diversity must be selected, especially for small divergence time [15]. To reveal STR loci profiles, non-invasive samples such as hair or feces can be used and present the advantage of not disturbing the animals [14] and avoiding the use of trapping methods [16]. Nevertheless, some samples may not be amplifiable or may have a higher error genotyping rate than invasive samples (e.g., tissue) because of the DNA degradation caused by field conditions and time [16,17]. In order to avoid genotyping errors, several replicates should be extracted and compared [17–19]. In two studies using field-collected feces from mammals, one-third of the samples were successfully amplified [20,21]. Small mammals' hairs, when freshly collected (i.e., regularly visited hair traps), provided accurate genotyping and were as amplifiable and informative as liver samples [22].

Combined with the STR genetic profiling method, wild boar is a good model species to evaluate the impact of habitat fragmentation. Indeed, it is a vagile species that has wide home ranges, even if in this study the home range is among the smaller ones observed in Europe (i.e., ~4 km^2 mean [23]). Small home range size was congruent with the wild boar population density in the Geneva Basin being among the highest in Europe at the time of the study [24]. The species is ubiquitous, and has relevant reproductive characteristics to rapidly initiate population structuration because sexual maturity is reached at 9 months and sows have 5–6 piglets per litter [25]. The species can also travel long dispersal distances as highlighted in a study in Spain [26] (i.e., ~50 km on average for both sexes and ~60 km (min. 600 m, max. 250 km) according to the literature. Furthermore, it is a species with economical, agricultural, public health, and political implications that require a significant management effort such as monitoring and regulating populations, tracking the spread of diseases (e.g., swine fever), providing financial compensation (e.g., for agricultural damages), etc. [27]. Studies yield various results by using the STR genetic profiling method to uncover the effect of urbanization on the population structure of wild boars. On the one hand, it was shown that motorways in Belgium and Lithuania did not represent a significant limitation to gene flow (i.e., migration between regions with successful reproduction) [28,29]. Two studies suggest that most of clustering effects were due to characteristics and recolonization history of the species [30], or to the isolation-by-distance [31], rather than to the landscape features or the urbanization coverage. On the other hand, a Sardinian study highlighted the clustering's effect caused by a quasi-impervious motorway, and to a lesser degree, by urbanized and cultivated areas [32].

In this study, we evaluated the functionality of an ecological corridor in the Geneva region. This corridor, which is ~2 km long, is of supra-regional importance for Switzerland (i.e., object Ge-01-02) [33]. It has been crossed by a 2 × 2 lane road equipped with a wildlife overpass since 2014 and further constrained by an urbanized area with only a narrow agricultural area remaining between villages. Between the road and the urbanized area, a remnant forest patch may act as a stepping stone between the linked core areas. On the one hand, it is interrupted by a road that is equipped with a wildlife overpass, and on the other hand, it is threatened by an urbanized zone. A complementary question consisted of learning if dispersion patterns of males and females could have an influence on the genetic structure of the total considered population. This study is relevant for the region since wild

boar is considered a priority species for ecological corridors [34] and because a wildlife overpass, which was confirmed to be crossed through by wild boars in a camera-trap study from 2020 [35], has been built in response to the political biodiversity objectives of the region (i.e., Geneva Biodiversity Strategy 2030 [11] and European Natura 2000 ecological network [36]).

Our clustering analysis results suggest that all subpopulations of the 3 studied areas form a single, genetically consistent population that could be indicative of sufficient gene flow before and after the wildlife overpass construction. However, a recent admixture indicated a potential increase of the gene flow between two of the subpopulations thanks to the wildlife overpass, while the other part of the ecological corridor was not or was only partially functional. An alternative corridor linking both core areas might however have a sufficient functionality level further to the north. Furthermore, genetic distances between males were significantly higher than between females, suggesting that males were migrants or dispersants from more remote populations, while females were genetically closer to each other. However, the role of sex remains unclear as to its influence on population genetics.

2. Materials and Methods

2.1. Studied Areas

This study encompasses 3 main defined areas, two core areas considered biodiversity hotspots (Jussy and Les Voirons [10]), and one patch that is considered a stepping stone area (Foron). These areas are located across the border between France and Switzerland in the Geneva region at an altitude between 500 and 1000 m above sea level and are characterized by a continental climate (Figure 1). According to the objectives of the State of Geneva, those areas should be ecologically connected through the ecological corridor GE-O-01+02 (~2 km long) [10] and offer sufficient resources to serve as partial or complete home-range for the wild boar. The core area of Jussy is a lowland area mainly constituted of deciduous forests: more specifically, oak-hornbeam forests mixed with several plantations of coniferous trees and alder forests. The second core area of Les Voirons is a mountainous area, partially under Natura 2000 protection status, and includes beech forests partly colonized by spruces [10]. These core areas are separated by 2 main obstacles to the movement of wildlife, respectively, by an urbanized area (village of Juvigny) and agricultural areas lacking stepping stones or linear structures likely to serve as guides to moving animals [10,35], and by a 4-lane road subject to heavy traffic [35]. Between these two conflict areas that act as barriers to movements and therefore to gene flow, a small forest patch, the Foron area, may provide food and shelter for wild animals and thus be used as a stepping stone area. Whereas the wild boar was almost absent from the Geneva Canton in the 1970s, the density of wild boars was estimated at more than 10 individuals/km^2 in the 2000s [24]. The growth of their population is notably due to agricultural areas, surrounding both forested hotspots of Les Voirons and Jussy, which can be considered as extension zones providing food resources [25]. Furthermore, the region is also attractive to humans with a population density of ≅1500 residents/km^2 in the center of Geneva Canton that decreases to ≅400 residents/km^2 in Les Voirons massif [37].

Such a dense human population needs transportation infrastructures such as highways. In the sector of this study, there is a 2 × 2 lane departmental road D1206 with each pair of lanes separated by a concrete wall. This road was built at the end of the 19th century, first as a 3-lane road, but it became a real barrier to wildlife movement in the middle-end of the 20th century as the traffic load increased to over 10,000 vehicles a day and as it was upgraded to a 2 × 2 lane road. In 2020, the daily users of the road averaged 30,000 in Les Chasseurs section [38] which is far more than what is considered an impermeable barrier for most of the species (>10,000 users per day) [9]. To avoid this further ecological disconnection and animal–vehicle collisions, a wildlife overpass was inaugurated in 2014 to help the animals cross through the D1206 road [39]. It is 16 m wide and 39.7 m long and is equipped with wooden screens between 2.5 and 3 m high with flared and broadening extremities [40]. Both sides of the road were further secured with the construction of fences.

One other passage that is worth noting, the corridor GE-O-04a-c [33], is located to the north of Machilly (France) when the 2 × 2 lane road becomes a normal 2 lane road that is lined on both sides with forests for ~275 m before entering urbanized areas. According to French hunters, wild boars use this passage even if some of them get killed on the road. This observation was confirmed by the Hunting Federation of Haute-Savoie that studies the ecological connectivity of the region and that considers this single passage as active in the area [41]. Thus, it might facilitate the exchanges between the subpopulations of Jussy and Les Voirons.

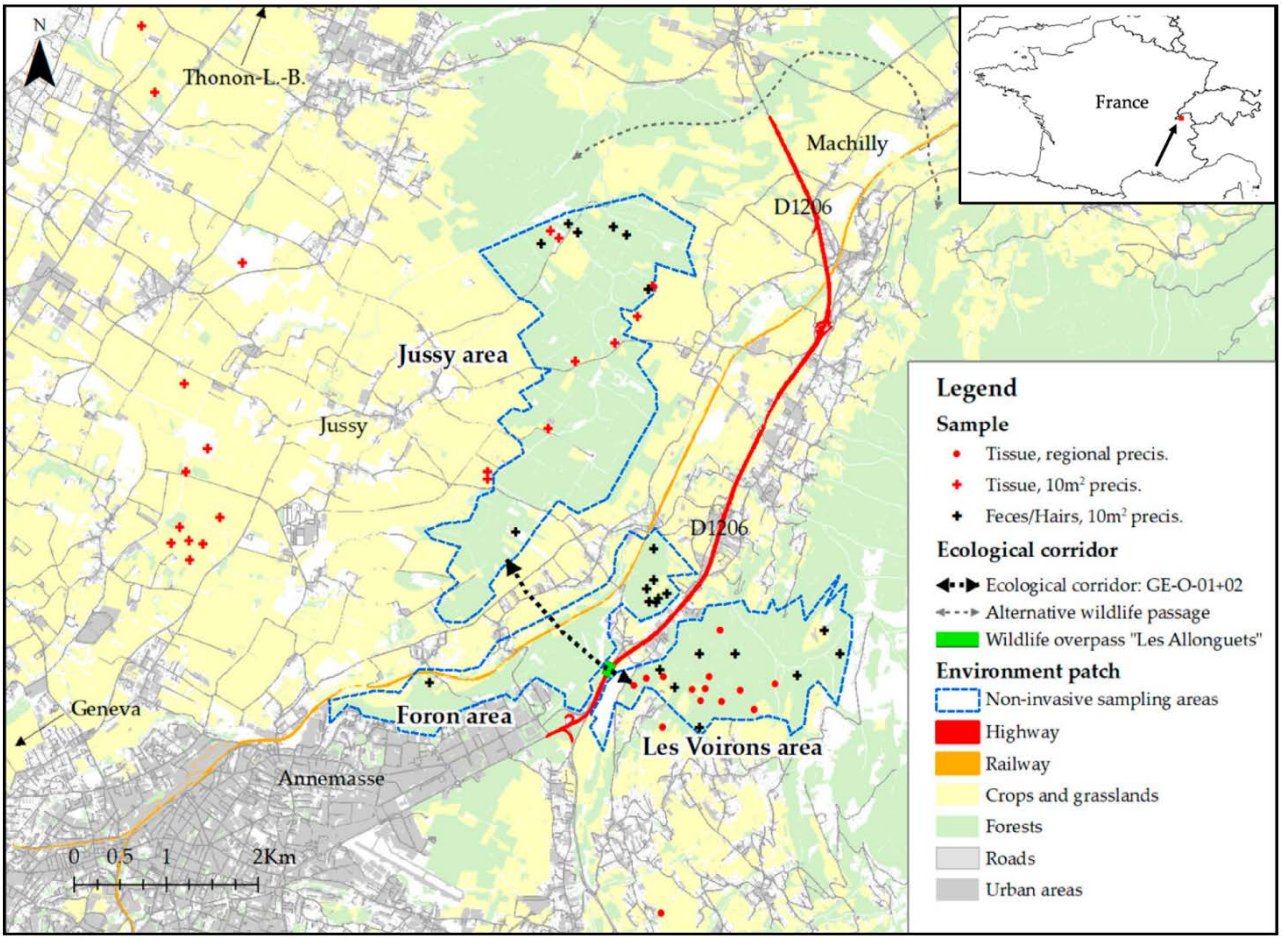

Figure 1. The location of the studied areas, the final samples, and the main landscape features are shown. The environment patches (5 × 5 m resolution) came from www.ge.ch/sitg (accessed on 2 February 2021).

2.2. Sample Collection

The samples of this study were either collected on dead animals (ear, skin tissue, hair, and/or feces) or in the field (hair or feces). Sampling was conducted in a sterile manner (i.e., disposable sterile gloves, only one packaging/sample, knife disinfection with single-use wipes Meliseptol® followed by water rinsing). Tissue samples (i.e., entire ear, muscle, or skin) were stored in 14 mL tubes or plastic bags. Feces and hair were, respectively, placed in plastic bags and in paper bags. All samples were stored at −20 °C, and bags and tubes were annotated with the following information registered electronically: unique ID and coordinates of each individual with location name, sample type, and how the sampling was carried out (e.g., collected on the field). When available, the sex, as well as other data not pertinent to this study (i.e., age and cause of death of the animals) were added. All collected information is available in Supplementary Material Table S1.

A minimum of 30 non-invasive samples per area (Figure 1) was targeted, and it was considered that a minimum of 3 km^2 would be sufficient to include 30 different wild boars (i.e., estimation of >10 individuals/km^2 [24]). Each non-invasive sampling area covered approximately 4 to 6 km^2 of territory. Hairs and feces were searched in pre-selected locations spotted with the help of orthophoto imagery (clearings surrounded by rejuvenated forest were favored) as well as in locations containing feeders for hunting purposes, as they attract the species [25]. The prospections were conducted between the winter of 2020 and spring of 2021 until the achievement of our minimum number of samples. We maximized the chance to attain samples by following animal paths and by making a longer prospecting effort in areas with presence indices. Hairs were recovered on rubbed trees, on mud, on bedding places, and barbed wires, and feces were recovered directly on the ground.

To complete the non-invasive sampling, tissues were opportunistically collected by hunters and game wardens on road-killed or legally hunted animals during the winters of 2020 and 2021. They were located in and around the non-invasive sampling areas (i.e., maximum 6 km from each area). When possible, hairs and feces were also extracted from the individuals to test for consistency between each sample type.

In total, we obtained 125 collected samples of which 30 were from Jussy (23 of dead animals), 22 from Les Voirons (14 of dead animals), and 9 non-invasive samples from Foron.

2.3. DNA Extraction

The DNA was extracted from the ear, skin, and muscle samples using the following adapted Doyle protocol [42]: 20 mg of tissue was added to 1 mL of hexadecyltrimethylammonium bromide (CTAB) extraction buffer, composed of 2% (*w*/*v*) CTAB, 1.4 M NaCl, 20 mM Na_2EDTA, and 100 mM Tris-HCl (pH 8), in 2 mL Eppendorf tubes. Then, 100 µL of 10% sodium bisulfite solution and 25 µL of proteinase K solution (10 mg/mL, Carl Roth GmbH, Karlsruhe, Germany) were added and the mixture was vortexed and incubated in a thermo-shaker (Hangzhou Allsheng Instruments, Hangzhou, China) at 55 °C for 3 h with gentle agitation every hour. Following incubation, 700 µL of chloroform:isoamyl alcohol 24:1 was added, and samples thoroughly vortexed, prior to centrifugation at 14,000 rpm for 15 min at 20 °C. The resulting aqueous phase was pipetted in a fresh new tube and nucleic acids precipitated by the addition of an equal volume of isopropanol. Nucleic acids were then recovered by centrifugation at 14,000 rpm, washed with 1 mL 70% ethanol, and resuspended in 40 µL warmed up (55 °C) ultrapure sterile water (H_2Oup).

Hair samples between 0.2–0.5 mm thick were prepared by cutting fragments 0.5–2 cm in length from the base of the follicle. When present, the roots were included because they are known to contain more DNA [43]. When hairs were thinner, they were entirely added. For each sample, 1 to 25 unitary hairs, depending on the number at disposal, were used. Hair DNA extraction followed the same protocol as previously mentioned, and the resulting nucleic acids were resuspended in 30 µL H_2Oup.

Concerning feces, each agglomerate was washed up to 10 times with 300 µL of H_2Oup. If possible, at least 50 cm^2 of the surface was treated as it always gave operable results with fresh feces. The outermost part of the feces was targeted as it yields more host-quality DNA [44]. In detail, agglomerates of feces were hydrated with 100 µL to 5 mL of H_2Oup (until no more water was soaked up by the feces) and then washed up to 10 times with the same 300 µL H_2Oup volume. Nucleic acids were then extracted from these 300 µL rinsing liquid using the QIAamp® PowerFecal® Pro DNA Kit following the supplier protocol (Qiagen GmbH, Hilden, Germany) and recovered in 50 µL 10 mM Tris-HCL (pH 8.5, C6 solution).

The quality and quantity of collected samples were then assessed with a NanoDrop® ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Part of the non-invasive samples was also assessed using a Qubit 4 Fluorometer (Qiagen GmbH).

2.4. Amplification of Microsatellite Markers and Sex Gene

A set of 9 microsatellite markers were selected for their high allelic richness and for their location on different chromosomes for the population structure analysis as well as a sex-specific gene primer to determine the sex of the individuals (Table 1). Nucleic acids of extracted tissue were diluted to 50 ng/µL (Nanodrop DNA concentration) and 25 ng of DNA template was used per amplification reaction. For hair and feces DNA samples, because of the impossibility to estimate the real part of host DNA, 1 and 2 µL, respectively, of non-diluted DNA sample were used (raw total nucleic acids concentration ranging from 0 to 55 ng/µL). Triplicates on individuals (i.e., tissue, hair, and feces) were all amplified 1 time and two-thirds of the non-invasive samples collected on the field were amplified 2 to 3 times. Samples such as feces with coprophagous insects were discarded and not considered for DNA extraction.

Table 1. Selected markers with their type and their forward and reverse primer sequences as well as the pooling groups used are given.

Marker Name	Marker Type	Forward Primer	Reverse Primer	Ref.	Pooling Group-Label
SW24	STR, autosomal chr.	CTTTGGGTGGAGTGTGTGC	ATCCAAATGCTGCAAGCG	[45]	P3-FAM
SW122	STR, autosomal chr.	TTGTCTTTTTATTTTGCTTTTGG	CAAAAAAGGCAAAAGATTGACA	[45]	P2-FAM
SW632	STR, autosomal chr.	TGGGTTGAAAGATTTCCCAA	GGAGTCAGTACTTTGGCTTGA	[45]	P2-HEX
SW857	STR, autosomal chr.	TGAGAGGTCAGTTACAGAAGACC	GATCCTCCTCCAAATCCCAT	[45]	P3-HEX
SW911	STR, autosomal chr.	CTCAGTTCTTTGGGACTGAACC	CATCTGTGGAAAAAAAAAGCC	[45]	P1-ROX
SW936	STR, autosomal chr.	TCTGGAGCTAGCATAAGTGCC	GTGCAAGTACACATGCAGGG	[45]	P1-FAM
S0005	STR, autosomal chr.	TCCTTCCCTCCTGGTAACTA	GCACTTCCTGATTCTGGGTA	[46]	P3-ROX
S0097	STR, autosomal chr.	GACCTATCTAATGTCATTATAGT	TTCCTCCTAGAGTTGACAAACTT	[45]	P2-ROX
S0226	STR, autosomal chr.	GGTTAAACTTTTNCCCCAATACA	GCACTTTTAACTTTCATGATACTCC	[47]	P1-HEX
SRYB	sexual gene, sex chr.	TGAACGCTTTCATTGTGTGGTC	GCCAGTAGTCTCTGTGCCTCCT	[48]	-

PCR reactions were run in 25 µL mixtures made of 5 µL MyTaq Reaction Buffer (Meridian Bioscience, Cincinnati, OH, USA), 2.5 µL of 5′ fluorescently labeled forward primer, and non-fluorescent reverse primer (5 µM each), 0.4 µL (0.2 µL for the SRYB sexual marker) of MyTaq HS DNA Polymerases (5 U/µL, Meridian Bioscience), 0.5 to 2 µL of extracted DNA (following previous description above) and completed with H_2Oup. Reactions were run in a TProfessional BASIC GRADIENT Thermocycler (Biometra, Göttingen, Germany) with the following cycling program: 3 min at 95 °C, 36 cycles of 20 s at 95 °C (35 cycles for SRYB sexual marker), 20 s at 55 °C (60 °C for SW24 marker), and 10 s at 72 °C, terminated by a final elongation of 20 s at 72 °C. A negative control was added to each line.

PCR products for microsatellite markers were then pooled with 3 markers' combinations according to Table 1. Negative controls were checked with a Fragment Analyzer™ (Advanced Analytical, Santa Rosa, CA, USA) using 18 µL of Dilution Buffer 1× TE (Agilent, dsDNA905 (1–500 bp), Santa Clara, CA, USA) mixed with 6 µL of the PCR product. Fragment length reading was carried out at Microsynth AG (Switzerland). Dilutions of 1:30 for hair and tissue samples and of 1:3 for feces were employed, and the selected size standard and filter set were GS500 LIZ and DS-33_G5, respectively. For its part, the PCR product of SRY sexual gene amplification was revealed on a 1% agarose electrophoresis gel, loading a mix of 12 µL (6 µL for tissue) from each PCR product with 6 µL (3 µL for tissue) of GelRed® (biotium) loading buffer. Migration was performed using the MyRun device (COSMOBIO Co., Ltd., IMR-201, Tokyo, Japan) for 35 min at 135 volts. The results were visualized using the U:GENIUS 3 machine (Syngene, Cambridge, UK). All amplified microsatellite data are provided in Supplementary Material Table S2, and the determined sex data are provided in Supplementary Material Table S1.

2.5. Statistical Analyses

2.5.1. Data Quality Check

As the study contained non-invasive samples collected in the field, we checked for double sampling (i.e., several samples of the same individual) and for the uniqueness of

the hair sample (i.e., no hair sample containing several individuals). We also checked the coherence between the 3 main types of samples (i.e., tissue, hair, and feces) for the same individual and between replicates of samples collected on the field. Finally, we completed the evaluation with a multi-step screening process to assess the microsatellites' quality (i.e., genotyping error rate, alleles dropout, linkage equilibrium, and selective neutrality checks) [13].

During the double sampling check, 3 pairs of samples with identical genetic profiles were identified. When projected on a map, the samples in each pair were always located within 500 m of each other, with no barrier of movement between them. We considered only one individual per duplicate. The uniqueness of each hair sample collected from the field was evaluated by comparing its allelic richness to the expected allelic richness of unique samples (i.e., collected directly on individuals) and by checking the ploidy (i.e., max 2 variants by locus by individual). The distribution of the expected allelic richness and one of the non-invasive samples were visually compared. Due to their triploidy on a marker, 2 samples were not considered for future analyses.

The genotyping error rate was evaluated using replicates by dividing the number of misamplified alleles by the total number of alleles for each locus and with all loci [49]. We only used effectively twice- or thrice-replicated alleles to make the calculation. To provide our analyses with data of quality (i.e., avoid allele dropout due to low quality DNA concentration), only 66 samples with 7 to 9 loci amplified were considered.

Then, null alleles were sought using the Chakraborty method [50] implemented in the PopGenReport package and using the adegenet package [51,52] on R. The linkage equilibrium was not tested for the purpose of quality control because each marker was located on a different chromosome (i.e., no physical linkage) and because functional linkage was unlikely [53]. The microsatellite data were also checked for selective neutrality using BayeScan with the multinomial Dirichlet model [54]. Out of 125 collected samples, we successfully genotyped 7–9 loci out of 9 for 66 individuals, and 61 samples were effectively kept for the study.

2.5.2. Data Analysis

Allelic richness was estimated using the rarefaction method [55] implemented in the hierfstat package [56]. Then, the private alleles were counted for each region and locus using PopGenReport package [52]. We also calculated the observed heterozygosity (Ho) and the expected heterozygosity (Hs) within each region, and the overall gene diversity (Ht) [57], as well as the Fst and Fis values [58] for each region and overall with the help of the hierfstat package [56]. Pairwise Fst between regions (pwFst) as well as Fis were tested using a bootstrap confidence interval (10,000 bootstrap samples). Then, the linkage disequilibrium was calculated by computing a likelihood-ratio test (10,000 permutations and 5 initial conditions for the expectation–maximization (EM) algorithm) in each region and overall using Arlequin software [59]. To feed Arlequin, we converted the data into an arp format using PGDSpider [60].

A Bayesian inference approach was used to test for clusters of genetically differentiated populations using STRUCTURE software [61]. It was combined with the ΔK predictor that gives the most probable number of clusters (K) [62]. A total of 8 runs were processed using a combination of several models, parameters, and datasets. We launched the admixture model that allows working without samples of well-separated ancestry populations. We also used the model using prior population information that is particularly adapted with weak Fst values. We used all samples and all microsatellite loci except the SW24 locus, as null alleles do not fit the basic assumptions of STRUCTURE. A second dataset was tested with no full-sibship individuals that were established using COLONY software (it will be described below), as multiple family members sampling may bias the results. The parameter Freqscoor, which is notably used for closely related populations by considering identical allelic frequencies [63], was either active or inactive. The Popalphas option was always activated and allowed the admixture to vary for each population. Twenty repetitions

for each K from 1 to 10 were performed. The burn-in length was 80,000 and the iteration number of the Markov chain Monte Carlo (MCMC) was 180,000. The best K was estimated using the Evanno method [62] and visualized with the pophelper package [64] on R.

To confirm the outputs of STRUCTURE, we used GENELAND software [65] which presents the advantage of dealing with spatial coordinates. We used their admixture model, which is analogous to the STRUCTURE admixture model. The number of MCMC iterations was 100,000. The spatial localizations were transformed from WSG84 World Geodetic System to the Swiss CH1903+/LV95 Projected System using the ArcGIS Project Tool [66].

Then, we performed a Mantel test [67] to verify if any correlation between geographical distance and genetic distance existed. The Reynold genetic distance was calculated for each pair of individuals using the sum of the squared size differences [68] with Arlequin software [59] and all values were converted in absolute values. To feed Arlequin, we converted the data into an arp format using PGDSpider [60]. The geographical distance between each pair of individuals was calculated with the distHaversine method assuming a spherical earth using the enmSdm package [69] on R. Then, the Mantel test was performed and the significance and strength of the correlation tested using the Pearson method thanks to the vegan package [70] on R.

We conducted a sibship reconstruction analysis (i.e., without *a priori* known relationships) using COLONY software [71]. It allows one to infer if there are members of a same parentage cluster with the help of a full-pedigree likelihood analysis which consider parentage and sibships jointly [71], or if there are members of a full-sibship family, in which individuals are considered to share parentage or have sibship relationships, while having no kinship with other defined families [72]. All individuals were considered as potential offspring data and male or female genotypes were not indicated. Both sexes of the model were indicated as polygamous. As COLONY needs an estimation of allelic dropout and genotyping error rate for each microsatellite characteristic, the value of 0.123 was given for the microsatellite SW24 null allele rate. The value of 0.063 was indicated for the microsatellite SW936 genotyping error rate and all the other values were set to 0. The options long run, high likelihood precision, and no sibship prior were selected, and the full-likelihood model, which relies on a simulated annealing algorithm and uses Mendelian segregation laws [72], was launched. Only results with more than 75% probability of being true were kept for the full-sibship families. The attributed family members were then projected on a map to find out their distribution between the 3 areas of our study.

A supplementary analysis on 54 individuals was conducted to determine if part of the genetic structure of population could be explained by the sex, using data with the required quality (cf. Data quality check) and from successfully sexed individuals. First, the Reynold genetic distance was calculated for each individual pairwise using the sum of the squared size differences [68] on Arlequin software [59]. To feed Arlequin with, we converted the data into an arp format using PGDSpider [60]. Then, the pairwise individuals were categorized as female pairs, male pairs, or female–male pairs, and the data distribution was visualized with boxplots using the ggplot2 package [73] on R. Then, each group was tested for the homoscedasticity of the variance using a Bartlett test [74] with the stats package [75] on R. A significant deviation from the homogeneity of variance was highlighted (K-squared = 15.256, df = 2, p-value = 0.000). Classical analysis of variance (ANOVA) cannot be used, as it requires parametric data, and non-parametric analysis was conducted instead using the Dunn Kruskal–Wallis multiple comparison [76] test with the FSA package [77] on R. The p-values were adjusted for multiple testing using the implemented Holm method.

3. Results

3.1. Descriptive Statistics and General Genetic Characteristics

Out of the 125 collected samples, 118 were extracted and subsequently amplified. Duplicate or triple amplifications were carried out for 67.79% of the total number of samples. Values of the read fragment lengths were always unique between the fresh

triplicate samples on the same individual (i.e., hair, tissue, and feces) and between the non-invasive replicate samples from the field (i.e., feces or hair). We successfully genotyped 7–9 loci out of 9 for 66 individuals (all tissue samples and triplicate samples, 22% of the hair collected in the field, and 25% of the feces collected in the field), of which 32 belonged to males and 29 to females. Five other samples were removed because they belonged to the same individual or because they originated from more than one individual. A description of the 61 final samples is given in Table 2.

Table 2. Count of genotyped samples presented by type and area.

Sample Type	# Jussy	# Les Voirons	# Foron
triplicate	15	0	0
tissue	8	14	0
feces	6	5	0
hair	1	3	9
total	**30**	**22**	**9**

#, number of samples; triplicate, tissue/feces/hair for one wild boar.

The characteristics of the microsatellite loci and the allele distribution at each microsatellite locus for each area are presented in Tables 3 and 4, respectively. The S0097 microsatellite locus has the highest allelic diversity with 15.867 estimated alleles. No other specificity is noted for the loci. No selection was detected for the microsatellite markers using the multinomial Dirichlet model [54] of BayeScan. Then, the marker SW24 differed significantly from a no null allele frequency (2.5 percentile: 0.027; 97.5 percentile: 0.236) using the Chakraborty method [50]. The more PCR-stringent conditions for this locus may have led to allelic dropout. The overall error rate that was only due to the SW936 marker, comparing amplified alleles against misamplified alleles, was estimated at 0.68%.

Table 3. Genetic characteristics summarized for each microsatellite locus.

Locus Name	Ho	Hs	Fis	# Est. Alleles	Length Range
SW24	0.636	0.685	0.072	5.967	115–127
SW122	0.761	0.664	−0.146	4.998	97–115
SW632	0.842	0.807	−0.044	7.902	126–160
SW857	0.635	0.681	0.069	4.998	160–168
SW911	0.458	0.514	0.109	3.902	160–166
SW936	0.537	0.486	−0.104	4.902	91–107
S0005	0.808	0.756	−0.069	8.000	218–242
S0097	0.849	0.839	−0.012	15.867	219–264
S0226	0.743	0.687	−0.081	4.000	177–187

Ho, observed heterozygosity; Hs, expected heterozygosity; Fis values were calculated after [58]; # est. alleles, number of estimated alleles.

Table 4. Allele frequencies distributions presented at each microsatellite locus for each area.

Locus Name	Allele Length	Freq. Jussy	Freq. Les Voirons	Freq. Foron
SW936	91	0.08	0.16	0.22
	93	0.82	0.68	0.56
	101			0.06
	105		0.02	
	107	0.10	0.14	0.17
S0226	177	0.17	0.16	0.11
	179	0.33	0.32	0.44
	185	0.41	0.34	0.44
	187	0.09	0.18	

Table 4. *Cont.*

Locus Name	Allele Length	Freq. Jussy	Freq. Les Voirons	Freq. Foron
SW911	160		0.02	
	162	0.65	0.52	0.44
	164		0.02	
	166	0.35	0.43	0.56
SW24	115	0.03	0.02	
	117	0.15	0.27	0.50
	119	0.10	0.18	0.13
	123	0.58	0.41	0.13
	125	0.13	0.09	0.25
	127		0.02	
SW857	160	0.02	0.02	
	162	0.15	0.07	
	164	0.10	0.27	0.28
	166	0.23	0.14	0.33
	168	0.50	0.50	0.39
S0005	218	0.03		
	222	0.41	0.29	0.31
	232	0.21	0.31	0.38
	234		0.02	
	236	0.03		
	238	0.16	0.26	0.19
	240	0.10		
	242	0.05	0.12	0.13
SW122	97	0.28	0.23	0.33
	99	0.40	0.52	0.50
	109	0.28	0.07	0.17
	111	0.03	0.14	
	115		0.05	
SW632	126	0.18	0.05	0.06
	148	0.10	0.09	0.11
	150	0.08	0.07	0.06
	152	0.20	0.41	0.22
	154	0.18	0.23	0.28
	156	0.02		
	158	0.22	0.16	0.28
	160	0.02		
S0097	219	0.12	0.07	
	224	0.10	0.25	0.38
	230	0.05	0.02	
	232	0.13	0.02	
	234	0.17	0.11	0.25
	236	0.23	0.23	0.25
	240	0.02		
	242	0.05	0.05	
	244	0.02	0.05	
	250	0.02	0.05	
	252	0.08		
	254		0.02	0.13
	256		0.02	
	260		0.02	
	262	0.02		
	264		0.09	

There is no significant Fis within the subpopulations and overall (Table 5), and the subpopulations are therefore considered in Hardy–Weineberg equilibrium. The Fst value

calculated within each region is below 0.1, which is low considering that is it a measure of the correlation between genes of a subpopulation compared to the total population (i.e., Fst may vary from 0 to 1 and 1 is the highest differentiation). PwFst indicates significant differentiation between Les Voirons and Jussy subpopulations with 95 CI [0.006,0.022], and the Foron and Jussy subpopulations with 95 CI [0.001,0.085] using confidence intervals from the bootstrap method.

Table 5. Genetic structure of population summarized for each area.

Areas	Ho	Hs	Fst	Fis *	# Priv. All.	# Allel. Richness.
Jussy	0.672	0.660	0.063	−0.018	8	39.457
Foron	0.773	0.682	−0.017	−0.114	1	32.654
Les Voirons	0.645	0.695	0.007	0.075	9	40.460
overall	0.697	0.680	0.017	−0.019	-	-

* not significant (bootstrap with 10,000 permutations); Ho, observed heterozygosity; Hs, expected heterozygosity; # priv.all., number of private alleles; # allel. richness., total estimated allelic richness; Fst and Fis values were calculated after Weir and Goudet (2017).

The significant linkage disequilibrium (p-value < 0.05), which is considered as a measure of the joint transmission of two loci (i.e., non-random association within a population [53]), was discovered between several microsatellite loci in the Foron and Les Voirons subpopulations (Table 6). More loci are in linkage disequilibrium (LD) in Les Voirons subpopulation than in the Foron subpopulation.

Table 6. Matrix that shows the linkage disequilibrium for Les Voirons and Foron areas.

Locus Name	SW936	S0226	SW911	SW24	SW857	S0005	SW122	SW632	S0097
SW936		-	-	-	-	-	-	-	-
S0226	-		+	+	-	-	-	-	+
SW911	-	-		-	-	-	-	-	+
SW24	-	-	-		-	-	-	-	+
SW857	-	-	+	-		+	-	-	+
S0005	-	-	-	-	-		-	+	+
SW122	-	-	-	+	-	-		+	+
SW632	-	+	+	-	+	+	-		-
S0097	-	-	-	+	+	-	+	-	

The upper limit above diagonal represents Les Voirons subpopulation and the lower limit below diagonal is for the Foron subpopulation. The "+" sign represents significant LD (p-value < 0.05), while the "-" sign means that it is not significant.

3.2. Population Genetic Structure Analysis

The cluster analysis using Bayesian inferences with STRUCTURE indicated only one population (Table 7). The ΔK, which allows one to infer the better K by selecting the first more likely peak, cannot be used for K = 1 and should be completed with other criteria in this case [62]. The mean likelihood (LK), which is higher when the number of estimated clusters better fit the data, is always higher with K = 1 than with other K values for all runs.

Second, the visualization of the clusters with the better K using ΔK for each individual and by region always shows nearly identical individuals (i.e., the mix of clusters, see Figure 2), indicating a probable unique population [63]. The dataset "without kin" was obtained by keeping only one member of each full-sibship family of COLONY run. GENELAND was used to confirm STRUCTURE-interpreted result of K = 1 by using all the individuals and by adding spatial coordinates. Along the chain after burn-in, the MCMC indicated 1 population with 65% of the clusters aligned to K = 1.

Table 7. STRUCTURE runs and results summarized.

Model	Dataset	N	Freqscoor	Best K (ΔK)	Prob. K
admixture	with kin	61	on	4	1
			off	4	1
	without kin	50	on	2	1
			off	2	1
prior information (region)	with kin	61	on	5	1
			off	2	1
	without kin	50	on	2	1
			off	2	1

N, number of individuals used; freqscoor, an option in STRUCTURE software that allows to analysis closely related populations (may overestimate the number of clusters); best K (ΔK), visual estimation of the best-estimated number of clusters (K) using ΔK; prob. K, visual estimation of the best K using Mean L(K), ΔK, and visual graph clustering.

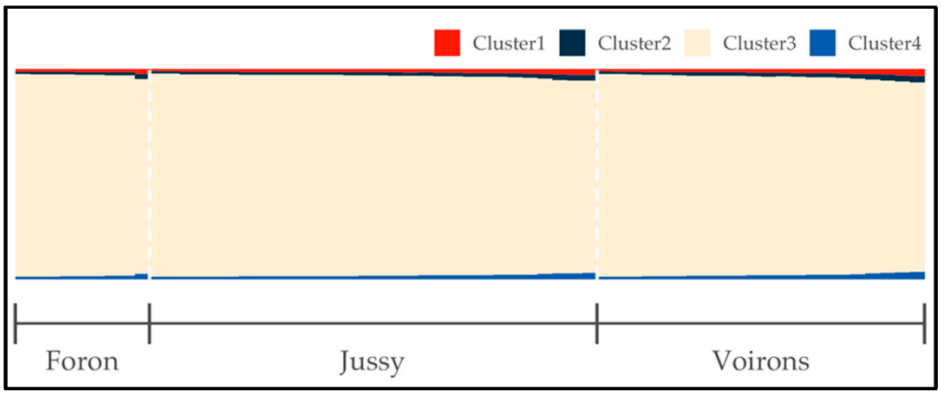

Figure 2. Visualization of a cluster analysis with STRUCTURE, using admixture model, dataset with kin, freqscoor = 0 and with best K by ΔK = 4. The individuals are distributed along the x-axis and were ordered by region and by cluster distribution. The best K was estimated using the Evanno method [63] and visualized with the pophelper package 2.3.1. [64] on R.

We obtained the data without kin and the full-sibship family members with COLONY using the full-likelihood method and by only keeping full-sibship family results with >75% probability of being correct (Table 8). Furthermore, the inferred parentage clusters were projected in Figure 3, even if the probability that the parentage clusters are representative of the reality is low (Table 9).

To verify if there may be detectable isolation by distance, a Mantel test was conducted using the Reynold genetic distance and the geographic distance between each pair of individuals. No significant relationship was found with the Pearson correlation test (r = 0.008, *p*-value = 0.344).

3.3. Sexual Genetic Structure Analysis

The comparison of the Reynold genetic distance between 1485 pairs (i.e., Gauss summation) of sexed animals for 26 females and 28 males was conducted using a Dunn Kruskal–Wallis multiple comparison [76]. The genetic distance was significantly different between female–female versus male–male groups (*p*-value = 0.001) and between male–female versus male–male groups (*p*-value = 0.038). However, the difference was rather small when we compared the median value of each group: 0.221 for the female–female group, 0.256 for the male–female group, and 0.306 for the male–male group (Figure 4). While the members of each full-sibship family were projected on a map, no sex pattern could be highlighted.

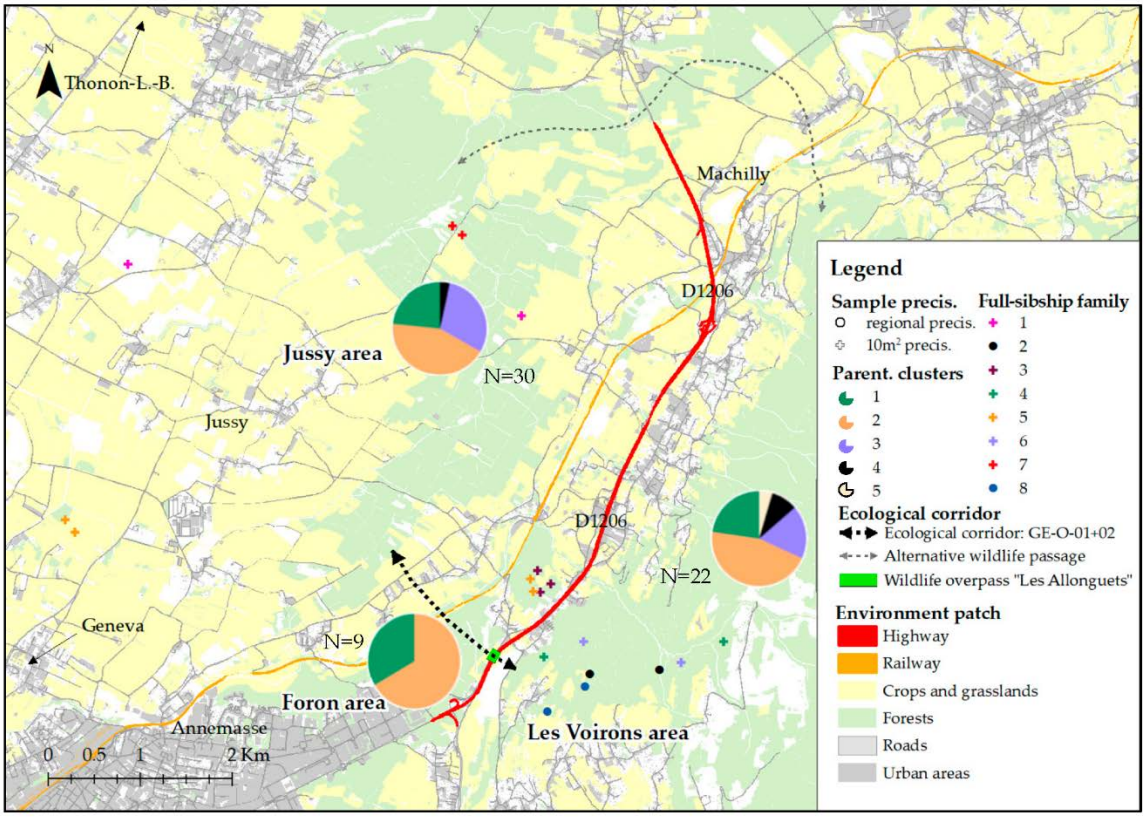

Figure 3. Proportion of parentage clusters and full-sibship family members are projected by regions.

Table 8. Full-sibship families with only >75% probability of being true are presented.

f. s. Family ID	Prob (Inc.)	Prob (Exc.)	# Memb. Jussy	# Memb. Voirons	# Memb. Foron
1	0.960	0.570	2	0	0
2	0.972	0.610	0	2	0
3	0.819	0.431	0	0	3
4	0.913	0.371	0	2	0
5	0.940	0.940	2	0	2
6	0.992	0.183	0	2	0
7	0.868	0.222	2	0	0
8	0.998	0.641	0	2	0

f. s. family ID, full-sibship family ID; prob (Inc.), probability that the composed family is splitable, the higher the value, the less probable; prob (Exc.), probability that all members of the full-sibship family are well included; # memb., number of family members.

Table 9. Parentage clusters shown according to COLONY run, with all individuals.

Parentage Cluster	Prob	# Memb. Jussy	# Memb. Voirons	# Memb. Foron
1	0.427	7	5	3
2	0.211	13	10	6
3	0.519	9	4	0
4	0.279	1	2	0
5	0.728	0	1	0

Prob, probability that the composed parentage cluster is true; # memb., number of parentage cluster members.

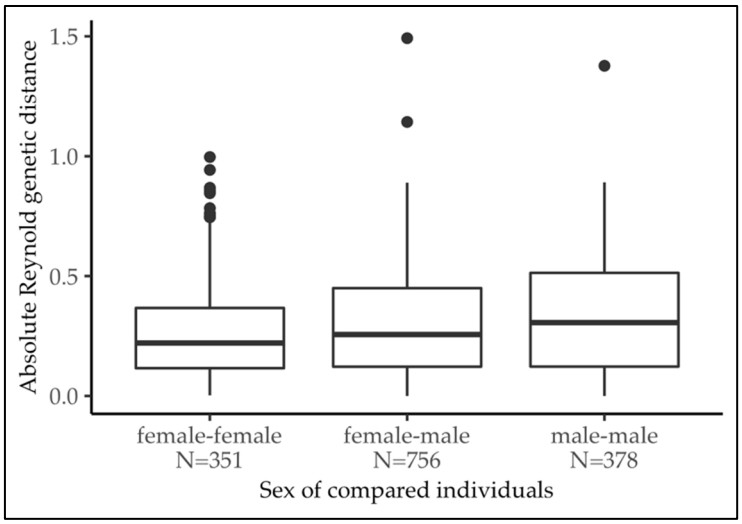

Figure 4. Boxplots with absolute Reynold genetic distance classified by paired sexes.

4. Discussion

4.1. Population Genetic Structure

4.1.1. Population Differentiation

The results of STRUCTURE and GENELAND genetic clustering suggest that, along the ecological corridor GE-O-01+02 and within the connected habitat patches, there is only one genetic population. The Fst values highlighted only low differentiation between Jussy, Les Voirons, and Foron (Table 5). However, the pwFst was significant between Les Voirons and Jussy and between Foron and Jussy, although this differentiation is not enough to consider the wild boar individuals of the area of Jussy as a differentiated population. We suggest that the subpopulations of wild boars experienced sufficient gene flow to maintain a homogeneous population structure either with or without the wildlife overpass. Moreover, if there were ~65 effective years (i.e., since the intensive use of the D1206 departmental road) of complete separation between the regions followed by a recent 6 years of reconnection, the subpopulations would still show a genetic clustering structure due to ancient genetic drift signature. Indeed, this genetic phenomenon randomly changes the allelic frequencies, leading to differentiated clusters. Four decades of elapsed time with a quasi-impermeable barrier to movement (4 lanes motorway over 200 km) were suggested sufficient to end up with distinct genetic clusters in a Sardinian study [32]. Even with a recent reconnection, the two hypothetic clusters would still be visible, and we would have three main types of individuals distributed along the ecological corridor: individuals from the first cluster, individuals from the second cluster, and admixed individuals due to reproduction between the two previously separated clusters. In congruence with our results, analogous studies on wild boar population structures at the landscape scale found no genetic differentiation resulting from motorway separation [29,78,79], with fencing since 2004 [29] and with >20,000 average users/day [29,78]. It was suggested that genetic clustering effects were rather due to isolation-by-distance [30], or to the species' characteristics or recolonization history [31].

4.1.2. Clue of Genetic Reconnection

At the scale of population differentiation (i.e., STRUCTURE or GENELAND runs), no evidence of disconnection was found (see Section 3.1). However, when we look at the genetic features of each studied area, signs of recent reconnection may be visible and could be explained by a theoretical hypothesis (Figure 5), in which we consider the situation before (i.e., in 2014) and some years after the building of the wildlife overpass. In our hypothesis, Les Voirons and Jussy were always connected by the alternative corridor in the north of Machilly (Figure 1), while Foron was isolated from both Les Voirons and

Jussy, leading to slightly differentiated allelic frequencies between the 2 core areas and the potential stepping stone. Then, reconnection was made possible between Foron and Les Voirons due to the wildlife overpass. It allowed regular exchanges of individuals that led to an admixture signature detectable with the measured LD (Table 6). Interestingly, no LD was detected in Jussy and we hypothesize that the anthropized part of the Foron area still represented a partial or complete barrier to movement for the wild boars, while the connexion with Machilly maintain only low genetic exchanges. Finally, the pwFst was significant between Jussy and the other areas. It gives further arguments for our hypothesis because Foron and Les Voirons became homogenized, while Jussy remained more isolated.

It emphasized that the alternative passage of Machilly may allow maintaining connectivity with Jussy. The result is congruent with the study of Dellicour et al. (2019) in that, even if no clustering genetic structure could be highlighted, other indicators, resulting from capture–mark–recapture in their study or from LD measures in our case, still show that wild boars are affected by landscape fragmentation [79]. Moreover, our results are constituent with the Sardinian study that showed that urbanized areas, roads, and intensively cultivated areas were likely to drive the species to distinct genetic clusters [32]. Nevertheless, our approach remains hypothetical and should be confirmed by a further study (e.g., formal modelization and prediction of scenarios). We may also lack sufficient statistical power to find low genetic differentiation (e.g., due to brief separation time [78,79]) that needs a high statistical power to be discovered and this could explain our single population result.

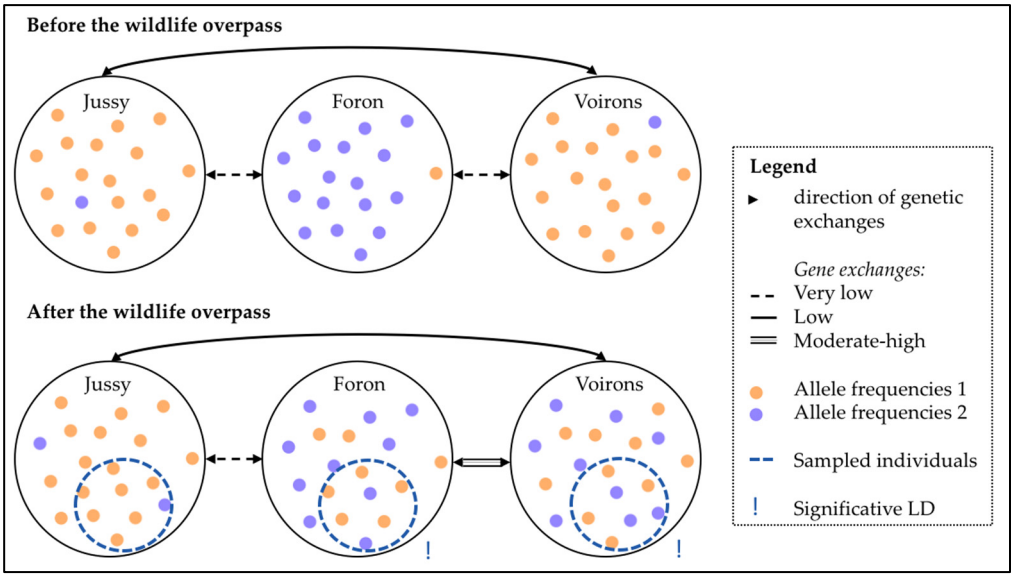

Figure 5. The hypothesis of recent genetic reconnection between areas is shown.

4.2. Sexual Genetic Structure

Our results are slightly different from what would be expected according to previous studies on the dispersion of the wild boars. On the one hand, our results would suggest that the gender of the animals does not greatly influence genetic population structure because Kruskall–Wallis tests (see Section 3.3) showed only low, even if significant, genetic differentiation between sexes. On the other hand, ecological studies on the dispersion of wild boars showed that males tended to disperse further than females [80,81]. From a genetic point of view, we would have expected more genetic contrast between males' pairs and females' pairs. However, our sampling size and quality might explain this result, as it included yearlings and piglets that are genetically not representative of the animals in age to disperse.

4.3. Perspective

We could improve the study by adding samples from the north of Machilly, as this region could be used as an alternative passage, and by adding highly polymorphic microsatellites (i.e., several microsatellites only had an allelic richness between 4–6, Table 3) or by using an SNP BeadChips approach. Furthermore, the Mantel test used in this study, which gave no significant results, was limited because we used an isolation-by-distance approach that only takes the Euclidean distance between individuals into account. One way to improve that would be to use a least-cost path approach that considers real ecological costs of crossing through specific environments [82]. The cumulated costs could then be compared to the Reynold genetic distance between each pairwise individuals using the Mantel test. This approach could be used once with the ecological corridor GE-O-01+02 and once with the alternative Machilly passage by allowing only one specific passage on a resistance map. Then, the results should be compared considering the percentage of explained variance and the *p*-value to find out if the individuals prefer to use one passage over the other.

4.4. Management Recommandations

At the scale of the studied sector, we recommend, as a precautionary principle, reconnecting both the Jussy and Foron areas and maintaining the alternative passage north to Machilly, as the functional connectivity between the Jussy and Foron areas was not confirmed. First, the most valuable parts of the ecological corridor GE-O-01+02 should be defined and protected (i.e., allocated for only wildlife passage). Then, an integration of guiding structures between the Foron and Jussy areas should be carefully planned, according to regional constraints and limitations, and designed to help migrant and dispersing animals find their way. Finally, a 16.5 km further section highway between Machilly and Thonon is being planned and may cut off the alternative wildlife passage north to Machilly. This potential disruption of connectivity should be considered in the planning of the future highway, because if this northern corridor is interrupted, and as long as the functionality of the corridor between the overpass and Jussy is not assured, there will be potentially no longer sufficient gene flow between Les Voirons and Jussy subpopulations. This would lead to the separation of the subpopulations into distinct genetic clusters and to the genetic weakening of the Jussy subpopulation.

This study demonstrated that even short landscape stretches lacking guiding structures like hedgerows for instance and with stochastic barriers to movement, such as cultivations, roads, fences, or houses, could already trigger a genetic signature. The connection between the Jussy and Foron areas seems to be impeded by the anthropized part of the Foron area even if it is less than 500 m wide.

Finally, at the scale of our study, we suggest that the wild boar would not a be species too sensitive to barriers to movement, such as highways, as it seems able to find alternative passages and maintain a consistent genetic single population. We expect that species more sensitive to landscape fragmentation such as roe deer [83] or red deer [79] would show different results. Such monitoring should be repeated after further structuring time to confirm the results of this study and to evaluate any new measures (e.g., construction of linear guides such as favorable hedgerows in the Foron area). It would be instrumental to the goals of the local government in confirming this evaluation with a more sensitive species to fragmentation.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/su14127463/s1, Table S1: samples data; Table S2: microsatellite data.

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