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Genetic Diversity and Population Structure Derived from Body Remains of the Endangered Flightless Longhorn Beetle *Iberodorcadion fuliginator* in Grassland Fragments in Central Europe

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Abstract: Knowledge of patterns of genetic diversity in populations of threatened species is vital for their effective conservation. However, destructive sampling should be avoided in threatened species so as not to additionally increase the risk of local population extinction. We exclusively used beetle remains and beetles collected after death to analyze local and regional patterns of genetic variation in the endangered flightless longhorn beetle *Iberodorcadion fuliginator* in the border region of Switzerland, France and Germany, in grassland remnants. We extracted DNA from the beetles' remains and genotyped 243 individuals at 6 microsatellite loci. We found moderate genetic differentiation between populations, each belonging to one of two metapopulations situated on either side of the river Rhine, but distinct genetic differentiation between populations across metapopulation. The genetic distance between populations was correlated with the geographic distance between the sites sampled. Genetic structure analysis inferred the presence of two genetic clusters. The populations in the Alsace (France) represent one cluster, together with the Swiss populations near Basel, which is separated by the river Rhine from the cluster composed of the populations in southwestern Germany. Thus, the historical separation by the river Rhine surpasses more recent effects of human-induced habitat fragmentation on the genetic differentiation in *I. fuliginator*.

Keywords: agricultural intensification; Cerambicidae; habitat degradation; habitat fragmentation; insect decline; microsatellites; natural barrier; non-invasive method; semi-natural grassland; species conservation

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1. Introduction

The fragmentation of natural habitats is generally considered to be a major threat to many species [1–3]. Habitat fragmentation reduces the area suitable for organisms, and leads to the isolation and decrease in size of remnant populations of plants and animals, which are exposed to an increased risk of local extinction [4,5]. Human activities are often the main causes of habitat fragmentation, but geographical processes and/or specific habitat requirements may also contribute to natural segregation of populations. The combination of increased random genetic drift, inbreeding, and reduced gene flow can significantly reduce genetic variation in remnant populations [6,7]. Genetic variation is important because it allows populations to adapt to changing environmental conditions [8,9]. Loss of genetic variation in isolated populations is also closely associated with increased inbreeding, which may result in inbreeding depression and reduce population viability [10,11].

The evaluation of the extent of isolation of existing populations and information about their genetic diversity are of great importance for the conservation of endangered insect species that occur in fragmented habitats [12,13]. At the same time, however, the populations should not be further reduced by collection activity. Destructive sampling should,

therefore, be avoided in threatened or legally protected species [14,15]. Even if collecting insect tissue for research purposes (e.g., wing clipping, tarsi or antenna amputation) is not lethal, it could still adversely affect the fitness, behavior, and welfare of the sampled individuals [16–18]. Therefore, using different types of remains or secretions that can be collected without having to capture or disturb the animal as a source of DNA is an appealing approach [15,19].

In our study, we exclusively used individuals found dead, as well as remains (elytra, part of thorax) of the highly endangered longhorn beetle *Iberodorcadion fuliginator* (L., 1758) as a DNA source (Figure 1). These were collected at 25 grassland sites in the border region of Switzerland, France, and Germany, and in the species' wider distribution area. Dead beetles and beetle remains were collected over the course of two long-term monitoring projects on the population dynamics of *I. fuliginator* over two decades [20–22]. The distribution of this grass-feeding flightless beetle extends from the Iberian Peninsula through Central Europe to the eastern part of Germany, and from southern Holland to the northern border of Switzerland [23–26]. In the past decades, significant declines in the number of *I. fuliginator* populations were recorded in Central Europe, mainly due to the destruction and degradation of extensively managed dry grasslands combined with increasing levels of fragmentation [27–29]. The dramatic decrease is reflected in the Red Lists of Switzerland (critically endangered [30]), the Federal States of Rhineland-Palatinate, Germany (critically endangered [31]), and Bavaria (endangered [32]). In the border region of Switzerland, France, and Germany, the overall abundance of *I. fuliginator* individuals in 13 populations decreased by 90% between 1999 and 2018: at one site, the population went extinct; at five sites, the populations were critically decreasing; at four sites, the populations were decreasing to a lesser degree; and at only three sites, the population size remained stable [20]. Progressive habitat deterioration expressed by a change in plant species composition and a decrease in grass cover were the crucial factors for this decline [20].

Figure 1. The highly endangered longhorn beetle *I. fuliginator* inhabits extensively managed dry grasslands (**left**). In our study, we used individuals found dead and beetle remains as DNA sources (**right**). Photos: B. Baur.

We used six microsatellite markers to characterize and compare the genetic diversity and degree of genetic differentiation within and between local populations of *I. fuliginator* on three spatial scales: within metapopulation, between metapopulations (regional scale), and on the scale of a wider distribution range of the beetle. We tested the following hypotheses:

(1) It is generally assumed that populations with an exchange of individuals have high genetic diversity, but a lower degree of genetic differentiation, than isolated populations [33]. In the border region of Switzerland, France, and Germany, populations of *I. fuliginator* are partly connected to form metapopulations [34], while the two populations in northeastern

Switzerland at the southeastern edge of the species' range are completely isolated [22]. We, therefore, expected that the genetic diversity of the populations belonging to the two metapopulations 'Blotzheim' and 'Istein/Huttingen' would be higher than that of the two isolated populations in northeastern Switzerland. Furthermore, we expected that the genetic differentiation of populations within a metapopulation is smaller than across metapopulations.

(2) On the basis of distribution and dispersal data, Baur et al. [33] suggested that the populations near Blotzheim and those around Istein/Huttingen can be considered as belonging to two distinct metapopulations, although an exchange of individuals only exists between a few populations. Using a genetic structure analysis, we tested the hypothesis that the populations belonged to two formerly functioning metapopulations.

(3) The study region was separated by the river Rhine, which may act as a major dispersal barrier for this flightless beetle. We expected that the river Rhine affected the genetic structure of *I. fuliginator* in the border region of Switzerland, France, and Germany. The effect of this geographical barrier might be stronger than the more recent impact of human-induced habitat fragmentation.

(4) The genetic diversity of a population may change with time due to random genetic drift, reduced gene flow, and inbreeding [7]. In a few populations, we collected dead beetles and remains of *I. fuliginator* over a period of two decades. We tested the hypothesis of a potential temporal change in genetic diversity in three populations with relatively large sample sizes by comparing estimates of genetic diversity and genetic differentiation of samples obtained before 2006 with those of samples found after 2012, within the same population.

(5) It has been stated in the literature that *I. fuliginator* has a life cycle of two years [35]. If the beetle has a strict biennial life cycle, then there were two temporarily separated populations with beetles emerging either in odd years or in even years at the same site. Consequently, differences in genetic diversity can be expected between the two temporarily separated populations. We tested this hypothesis by comparing measures of genetic diversity and the extent of genetic differentiation of beetles that emerged in odd years with those of beetles that emerged in even years at the same site.

(6) Genetic effects may influence the population dynamics of *I. fuliginator*. Populations that decrease in size over a longer period may suffer from inbreeding depression or have a low genetic diversity. We used data from the two long-term monitoring projects [20,22] to test the hypothesis that populations with decreasing individual numbers would show a higher level of inbreeding and a lower level of genetic diversity than populations with stable sizes in the past 11–20 years.

2. Materials and Methods

2.1. Study Species

It is assumed that most individuals of *I. fuliginator* have a life cycle of 2 years [35]. Females deposit their eggs in stems of grass, preferably *Bromus erectus*, their main larval host plant, in late March to May. In the region of Basel, the larvae hatch in May or June, feed on grass roots, and pupate after 13.5–14.5 months (including one hibernation in the late larval stage). Adults (14–17 mm body length) emerge from the pupae after 2–3 weeks in July or August, but rest in the soil until the end of the second hibernation [35]. Depending on weather conditions, adults emerge from the soil in March or April and are sexually active for 2–4 weeks [34]. A mark–release–resight study revealed that individuals move 20–100 m, mainly along habitat edges and verges of field tracks [35]. The maximum distance moved by a marked male was 218 m [34]. Beetles are capable of crossing tarmac roads, but have the risk of being run over by cars [36].

2.2. Study Sites and Sampling

We collected dead beetles and beetle remains in known populations of *I. fuliginator* in the border region of Switzerland, France, and Germany (Figure 2) as part of a long-term monitoring project which began in 1999 [20,21,29]. In this region, *I. fuliginator* occurs in patchily distributed populations in remnant grassland areas surrounded by intensively used cropland, vineyards, and/or settlements [29,35]. Inspired by this long-term project, another two populations were monitored in northeastern Switzerland (population 29 since 2005, population 30 since 2010 [22]) and any dead beetles or beetle remains were sampled (Table 1). These collections were supplemented by a few dead beetles or beetle remains sampled in the wider distribution range of *I. fuliginator* (Table 1, Figure 2).

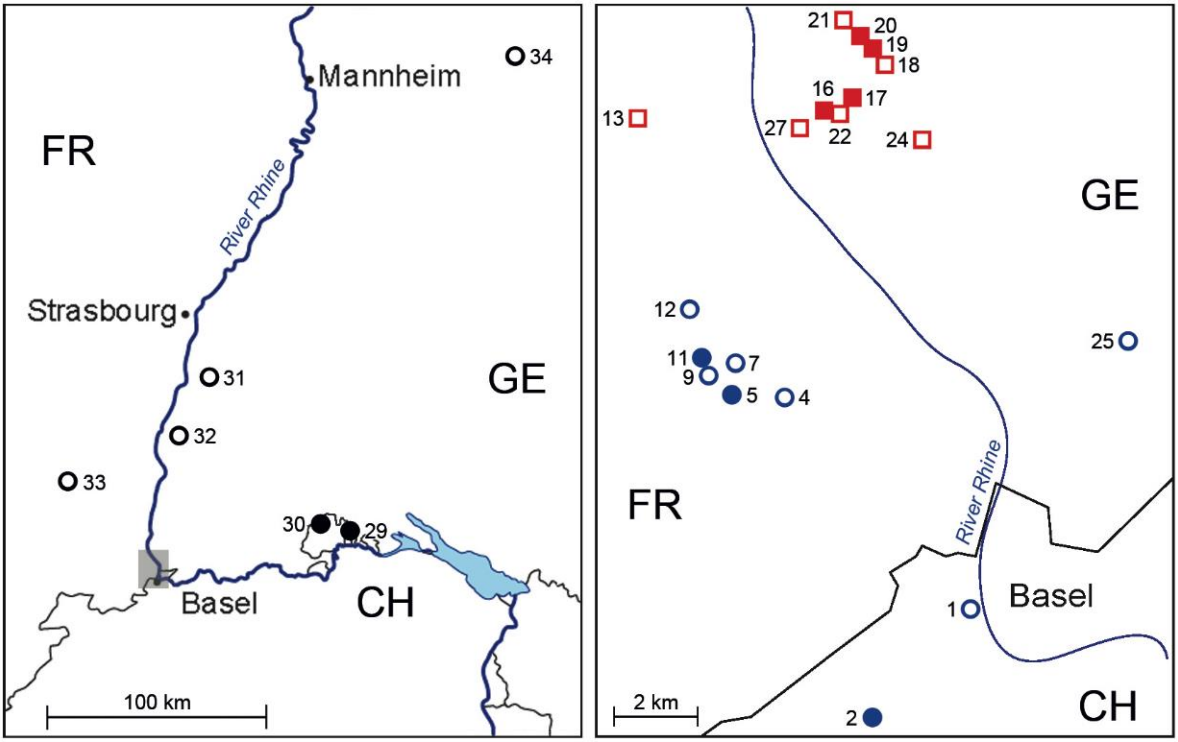

Figure 2. Locations of the *I. fuliginator* populations sampled in Switzerland, eastern France (Alsace) and southwestern Germany (left), with a detailed map of the border region near Basel (right; grey rectangle in the map left). Full symbols represent populations from which $\geq$ 10 individuals were genotyped (Table 2); open symbols represent populations with smaller sample sizes. Blue symbols represent populations assigned to the cluster "metapopulation Blotzheim", red symbols to populations assigned to the cluster "metapopulation Istein/Huttingen". Black symbols represent the populations sampled in the wider distribution area of *I. fuliginator* (the isolated populations 29 and 30 are situated in northeastern Switzerland).

Dead beetles and beetle remains were stored dry for a period ranging from 4 to 23 years. A methodological study showed that neither DNA quantity nor DNA quality was affected by the state of beetle (intact, crushed, or only fragments), storage duration, or the weight of the sample [37].

Table 1. Overview of *I. fuliginator* populations examined in the border region of Switzerland, France, and Germany (populations 1–27) and in the wider distribution range of the species (29–34) with sample size (number of individuals analyzed), state of the specimens, and year(s) when the dead beetles or their remains were found.

Population (Country) [1]	No. of Individuals Genotyped	State of Specimens [2]	Year(s)
1 Basel, embankment of river Rhine (Switz)	3	c (1), f (1), i (1)	2000
2 Allschwil (Switz)	12	c (4), f (2), i (6)	1998–2017
4 St. Louis, E of airport (Fra)	1	i (1)	1999
5 Blotzheim, E (Fra)	17	c (7), f (3), i (7)	2012–2017
7 Blotzheim, NW of airport (Fra)	1	i (1)	1998
9 Blotzheim, E (Fra)	3	c (2), f (1)	2013
11 Blotzheim, Ruti SW (Fra)	21	c (12), f (5), i (4)	1998–2017
12 Blotzheim, Rotfeld-Hattel (Fra)	2	c (2)	2012
13 Sierentz, Hardt (Fra)	4	c (2), f (1), i (1)	1998–1999
16 Istein, NW (Ger)	63	c (44), f (5), i (14)	1998–2000
17 Istein, NE (Ger)	10	c (8), f (1), i (1)	2000–2013
18 Huttingen, E (Ger)	6	c (4), f (1), i (1)	2000
19 Huttingen, NE (Ger)	12	c (7), f (4), i (1)	2000–2014
20 Huttingen, Tischlig (Ger)	19	c (8), f (5), i (6)	1999–2017
21 Huttingen, Tischlig nature reserve (Ger)	2	i (2)	1999–2000
22 Istein, N (Ger)	2	c (2)	1999–2000
24 Efringen-Kirchen, N (Ger)	1	i (1)	2001
25 Ötlingen, Tüllinger Berg (Ger)	1	i (1)	1999
27 Istein, Isteiner Klotz (Ger)	1	c (1)	2000
29 Thayngen, SH (Switz)	14	c (9), f (4), i (1)	2010–2016
30 Altdorf, SH (Switz)	34	c (22), f (4), i (8)	2005–2017
31 Taubergiessen (Ger)	1	c (1)	2004
32 Kaiserstuhl (Ger)	4	c (3), f (1)	1998
33 Westhalten (Fra)	6	c (1), f (2), i (3)	1998
34 Bad Windsheim (Ger)	3	i (3)	2012

[1] Designation of the populations 1–27 follows Coray et al. [28]. [2] c, crushed; f, beetle fragment (an elytra or a single leg); i, more or less intact specimen found dead.

Table 2. Genetic diversity in nine populations of the highly endangered longhorn beetle *I. fuliginator*.

Population (Country) Long-Term Dynamics [1]	*N*	*A*	*Ar*	*%P*	*I*	*PA*	H_O	H_E	F_{IS}
2 Allschwil (Switz) s	12	2.833	2.226	100.0	0.618	3	0.208	0.382 ***	0.395
5 Blotzheim, E (Fra) d	17	3.000	2.550	100.0	0.698	1	0.275	0.435 ***	0.359
11 Blotzheim, Ruti SW (Fra) s	21	2.667	2.386	100.0	0.752	0	0.340	0.507 ***	0.231
16 Istein, NW (Ger) e	63	2.667	1.995	100.0	0.497	2	0.241	0.310 ***	0.144
17 Istein, NE (Ger) s	10	2.000	2.000	100.0	0.523	0	0.360	0.368	0.021
19 Huttingen, NE (Ger) d	12	1.833	1.832	83.3	0.481	0	0.194	0.345 ***	0.336
20 Huttingen, Tischlig (Ger) d	21	1.833	1.829	83.3	0.468	0	0.216	0.324 ***	0.229
29 Thayngen, SH (Switz) s	14	2.000	1.812	66.7	0.290	0	0.095	0.168 ***	0.356
30 Altdorf, SH (Switz) s	34	2.333	1.946	83.3	0.438	0	0.098	0.270 ***	0.609

[1] Populations 2–20 are situated in the border region of Switzerland, France, and Germany, while populations 29 and 30 represent the two other populations still occurring in Switzerland (Figure 1). Data on the long-term dynamics of the populations were obtained from Baur et al. [20] and Weibel [22]: *s*, stable; *d*, decreasing, *e*, extinct. *N* corresponds to the number of individuals genotyped. Mean observed allelic richness (*A*) of six loci relates to all individuals genotyped within a population, while rarefied allelic richness (*Ar*) was estimated for 10 individuals per population based on all six loci. Percentage of polymorphic loci (*%P*), Shannon Index (*I*), number of private alleles (*PA*), observed (H_O) and expected (H_E) heterozygosity, and inbreeding coefficient (F_{IS}) were estimated on the basis of the six loci. *** indicates significant deviation from Hardy–Weinberg equilibrium ($p < 0.001$).

2.3. DNA Extraction and Microsatellite Markers

We extracted DNA from dead beetles and beetle remains using a modified CTAB extraction method (described in detail in Rusterholz et al. [37]). Each sample was ground

using a pestle in a mixture of 525 µL CTAB puffer, 15 µL Proteinase K (10 mg/mL), and 10 µL RNase (10 mg/mL). After incubation at 65 °C for 90 min, the suspension was extracted with 500 µL chloroform/isoamyl alcohol (25:1) and centrifuged at 12,000 *g* for 10 min. We transferred the supernatant into a new 1.5 mL tube and added 450 µL isopropyl alcohol to precipitate the DNA. After 30 min incubation at 4 °C, the sample was centrifuged at 12,000 *g* for 10 min and the supernatant was removed. The pellet was washed with 300 µL of 70% ethanol and centrifuged at 12,000 *g* for 10 min. After removing the supernatant, the pellet was dried in an Eppendorf Vacfuge™ (Vaudaux-Eppendorf AG, Schönenbuch, Switzerland) at 37 °C for 15 min, and resuspended in 100 µL sterile water. We assessed both the DNA quantity and quality using a NanoDrop ND-1000 spectrometer (NanoDrop Technologies, Inc., Washington, DC, USA).

Nine microsatellite markers (Dorful_000213, Dorful_001410, Dorful_010423, Dorful_014284, Dorful_024913, Dorful_025921, Dorful_029315, Dorful_031273, and Dorful_032392), developed by Rusterholz et al. [37], were amplified in two multiplex of 10 µL volume using the Type-It Microsatellite PCR kit (Qiagen, Hombrechtikon, Switzerland) following the protocol provided by the manufacturer (pre-incubation at 95 °C for 5 min, followed by 28 cycles of 95 °C for 30 s, 56 °C for 90 s, 72 °C for 30 s, and, finally, 60 °C for 30 min). The first multiplex comprised Dorful_000213, Dorful_010423, Dorful_031273, and Dorful_032392, the second consisted of Dorful_001410, Dorful_014284, Dorful_024913, Dorful_02592, and Dorful_029315). The F-primers were dyed, allowing for the detection of the amplification product on an ABI 3730xl sequencer (Applied Biosystems, Zug, Switzerland). We used a PeakScanner v 1.0 (Applied Biosystems, Zug, Switzerland) to visualize the extent of amplification and to record the height of the peaks.

2.4. Genetic Analyses

We used FSTAT, version 2.9.4 [38], to check for genotypic disequilibrium. The linkage disequilibrium analysis demonstrated significant links between Dorful_029315, Dorful_024913, and Dorful_025921. These three markers were, therefore, excluded in the data analyses. We checked microsatellite results of each population for null alleles and mis-scoring using MICRO-CHECKER version 2.2.3 [39]. Null alleles were detected only in one population for one marker (Dorful_001410). Thus, all further analyses were based on six microsatellite markers (Appendix S1).

Populations with heterozygote deficiency were further analyzed with INEST 2.0 [40], which applies a Bayesian approach for estimating both null alleles and inbreeding simultaneously. Three parameters (n: null alleles; f: inbreeding; b: genotyping failure) were used for the comparison of six models (n, b, nf, nb, bf, and nfb). For example, the n-model considers null alleles. All models were run with 50,000 burn-ins and 500,000 cycles for each population. Model selection was performed using the lowest Deviance Information Criterion (DIC) of the six models. The procedure revealed the best fit for the nf-model in five populations, and for the nfb-model in three populations.

We applied FSTAT to calculate the following estimates of genetic diversity for populations with a sample size of at least ten individuals: observed allelic richness (*A*), related to all individuals genotyped in a population; rarefied allelic richness (*Ar*), estimated for 10 individuals per population, considering all six loci; percentage of polymorphic loci (%*P*); Shannon Index (*I*); number of private alleles (*PA*); and observed (H_O) and expected (H_E) heterozygosity, all based on six loci. We calculated the inbreeding coefficient F_{IS} using INEST 2.0 [40]. None of these measures of genetic diversity were correlated with the number of beetles examined (sample size; Spearman correlation, $P > 0.61$ in all cases).

Divergence from the Hardy–Weinberg (HW) equilibrium was tested for each populations using GenoDive, version 3.0.6 [41], with 9999 permutations. Significant deviations from the Hardy–Weinberg equilibrium ($P < 0.001$) were observed in eight of the nine populations due to a deficiency of heterozygotes (Table 2).

We collected dead beetles and remains of *I. fuliginator* over a period of two decades. It is possible that the genetic diversity of a population changes with time. We tested this

hypothesis in populations with relatively large sample sizes (populations 2, 11, and 30) by comparing estimates of genetic diversity (*Ar*, H_O, H_E, and inbreeding coefficient F_{IS}), calculated separately, for each of the six loci of individuals collected before 2006 with those of individuals found after 2012 within the same population, using the paired *t*-test. We also applied paired *t*-tests to evaluate potential differences in the genetic diversity between beetles that emerged in odd years and those that emerged in even years in three populations. Furthermore, we examined potential genetic differentiation between individuals collected earlier (before 2006) and later (after 2012) within population by calculating F_{ST}-values for each of the six loci separately for both subsamples (separate analyses for the populations 2, 11, and 30). We used *t*-tests to examine whether the mean F_{ST} of the six loci differed from zero. There is genetic differentiation between the two subsamples of a population if F_{ST} differs from zero. We conducted the same analysis to examine potential genetic differentiation between individuals that emerged in odd years and those that emerged in even years in the same populations (for populations 5, 11, and 30).

To examine the genetic population structure of *I. fuliginator*, we analyzed the data on three different spatial scales. Firstly, a previous field study on the fine-scale spatial distribution and dispersal of *I. fuliginator* indicated that the populations around Blotzheim and those near Istein/Huttingen might be remnants of two formerly functioning metapopulations separated by the river Rhine (hypothesis 2; [34]). To test this hypothesis, we considered the populations 2, 5, and 11 to belong to the metapopulation Blotzheim, and the populations 16, 17, 19, and 20 to the metapopulation Istein/Huttingen (Figure 2, Table 1). We investigated the genetic population structure at the metapopulation scale (the isolated populations 29 and 30 in northeastern Switzerland were not considered in this analysis). Then, we assessed genetic differentiation among populations within and between the two metapopulations using an analysis of molecular variance (AMOVA) in GenAlEX, version 6.5.02 [42], with 10,000 permutations, and calculated pairwise F_{ST}-values as a measure of the degree of genetic differentiation among populations. At the metapopulation scale (seven populations), we also tested isolation by distance using Mantel's test [43] by comparing pairwise $(F_{ST}/(1 - F_{ST}))$-values with the corresponding geographical distance (log-transformed) following Rousset [44], with 10,000 permutations. We also examined the population structure at this spatial scale using the Bayesian individual assignment approach, as in STRUCTURE, version 2.3.2 [45]. STRUCTURE identifies population clusters or groupings. We tested a model of admixture with numbers of populations (*K*) ranging from 1 to 18. Likelihood values for ten replicates of each *K*-value were estimated after 1,000,000 iterations (with the first 100,000 iterations discarded as burn-in). The best *K*-value was chosen by applying the method of Evanno et al. [46], which considered a second order rate of change to determine the most likely value of *K*.

Secondly, we examined the population structure at the regional scale (border region of Switzerland, France, and Germany) by considering data from 18 populations (some of them with small sample sizes; Table 1) using STRUCTURE as described above. Data from the populations 20 and 21 were combined because of recorded dispersal between the populations [34]. With this analysis, it was possible to check whether the genetic differentiation was maintained beyond the level of the metapopulations.

Thirdly, to compare the allele frequencies of *I. fuliginator* in the border region of Switzerland, France, and Germany with those of the species' wider distribution area, we constructed phylogenetic trees both of the populations that belonged to the border region ($n = 18$) and, in an extended version, of all sites sampled in this study (Table 1). We analyzed the allele frequencies using a neighbor-joining (NJ) method in POPTREE2 [47], with 10,000 permutations. Due to large differences in the sample size of the populations (Table 1), we used Nei's standard genetic distance with sample size correction as genetic measure (D_{ST}) and adjusted the constructed NJ trees using MEGA version 6.0 [48]. Analyses with other indices (D_{ST} and F_{ST}) yielded very similar results (data not shown). We also applied Principal Coordinate Analysis (PCoA) to evaluate the potential separation of populations using the R-package *ecodist* [49].

Trends in the long-term dynamics of the *I. fuliginator* populations are known from long-term monitoring projects (Table 2). In the border region near Basel, population 16 went extinct (due to habitat destruction); populations 5, 19, and 20 decreased in size between 1999 and 2018; and populations 2 and 11 were considered to be stable [20]. In northeastern Switzerland, populations 29 and 30 are considered to be stable over the period of 2010–2021 [22]. We used *t*-tests to examine whether populations showing a decreasing size differ in genetic measures (*Ar*, H_O, H_E, and inbreeding coefficient F_{IS}) from populations with stable individual numbers (population 16 was excluded from this analysis).

3. Results

3.1. Genetic Diversity and Population Structure at the Regional Scale

The average number of rarefied alleles in a population ranged from 1.81 in population 29 to 2.55 in population 5 (Table 2). The AMOVA revealed that the highest amount of genetic variation occurred within individuals (52%), followed by variation among individuals (25%) and variation between metapopulations (17%; Table 3). This indicates different genetic structures between the assumed metapopulations Blotzheim and Istein/Huttingen, which are separated by the river Rhine. The genetic structure analysis (see below) confirmed the presence of the two distinct metapopulations. Populations 2, 5, and 11, belonging to the metapopulation Blotzheim, had a higher rarefied allelic richness on average (2.39 ± 0.09; mean ± SE) than the four populations (16, 17, 19, and 20) belonging to the metapopulation Istein/Huttingen (1.91 ± 0.009; t = 4.887, d.f. = 5, P = 0.0045).

Table 3. Analysis of molecular variance (AMOVA) considering 156 individuals of *I. fuliginator,* from seven populations belonging to two metapopulations, in the border region of Switzerland, France, and Germany [1].

Source of Variation	d.f.	Sum of Squares	Estimated Variance	Percentage of Variation	*F*	*P*
Between metapopulations	1	39.25	0.241	17	0.165	<0.001
Among populations	5	25.69	0.097	7	0.079	<0.001
Among individuals	149	221.50	0.363	25	0.231	<0.001
Within individuals	156	118.50	0.760	52	0.324	<0.001

[1] The populations 2, 5, and 11 belong to the metapopulation 'Blotzheim'; populations 16, 17, 19, and 20 belong to the metapopulation 'Istein/Huttingen'; see Figure 1.

Observed heterozygosity (H_O) ranged from 0.095 (population 29) to 0.360 (population 17; Table 2). The two isolated populations in northeastern Switzerland (populations 29 and 30 at the southeastern edge of the species' distribution range) exhibited a lower H_O than the populations belonging to the two metapopulations (0.097 ± 0.002; t = 3.390, d.f. = 7, P = 0.0116). Genetic diversity (H_E) varied from 0.168 (population 29) to 0.507 (population 11; Table 2). The populations belonging to the metapopulation Blotzheim showed a higher H_E-value than those of the metapopulation Istein/Huttingen (0.441 ± 0.036 vs. 0.337 ± 0.013; t = 3.094, d.f. = 5, P = 0.027). The two isolated populations in northeastern Switzerland (populations 29 and 30) had even a lower H_E than the populations belonging to the two metapopulations (0.219 ± 0.051; t = 2.920, d.f = 7, P = 0.023).

Significant positive F_{IS}-values were observed for almost all populations examined, an exception being population 17 (Table 2). F_{IS}-values ranged from 0.021 (population 17) to 0.609 (population 30; Table 2). The mean F_{IS} of the two metapopulations did not differ (0.328 ± 0.050 vs. 0.182 ± 0.067; t = 1.636, d.f. = 5, P = 0.163; Table 2). The two isolated populations in northeastern Switzerland (populations 29 and 30) tended to show a higher F_{IS} (0.482 ± 0.126) than the average F_{IS} of the two metapopulations (t = 2.120, d.f. = 7, P = 0.072; Table 2).

Populations belonging to the metapopulation Blotzheim had the highest mean number of private alleles (1.3), followed by populations of the metapopulation Istein/Huttingen

(0.5; Table 2). The two isolated populations in northeastern Switzerland had no private alleles (Table 2).

None of the genetic diversity estimates changed over time in the populations from which we collected beetles over a long period of time ($P > 0.3$ in all cases; Table S1). Furthermore, F_{ST}-analysis revealed no genetic differentiation between the two subsamples of individuals collected in a population at different periods ($P > 0.1$ in all cases; Table S1). Similarly, within populations, beetles that emerged in even years did not differ in any measures of genetic diversity from individuals that emerged in odd years. F_{ST}-analysis revealed no genetic differentiation between beetles that emerged in even or odd years (Table S2).

Pairwise F_{ST}-values, representing the degree of genetic differentiation between populations, indicate moderate to strong differentiation between most populations (Table 4). Pairwise F_{ST}-values ranged from 0.011 (between populations 17 and 19 in the metapopulation Istein/Huttingen) to 0.404 (populations 2 and 20) in individual population pairs (Table 4). Average genetic differentiation between populations within metapopulations was lower (Blotzheim: 0.103; Istein/Huttingen: 0.050) than that between populations across metapopulations (0.229; Table 4). A Mantel test showed that the genetic distance between populations is significantly associated with geographic distance of the sites sampled on the metapopulation scale (Figure 3).

Table 4. Pairwise F_{ST}-estimates (below the diagonal) and *P*-values (above the diagonal) of seven populations of *I. fuliginator,* which belong to two metapopulations in the border region of Switzerland, France, and Germany [1].

Population	2	5	11	16	17	19	20
2	–	**	***	***	***	***	***
5	0.104	–	*	***	***	***	***
11	0.171	0.035	–	***	**	**	***
16	0.391	0.235	0.200	–	**	**	**
17	0.334	0.138	0.110	0.084	–	ns	ns
19	0.313	0.139	0.141	0.074	0.011	–	ns
20	0.404	0.205	0.137	0.060	0.020	0.052	–

[1] The populations 2, 5, and 11 belong to the metapopulation 'Blotzheim'; the populations 16, 17, 19, and 20 belong to the metapopulation 'Istein/Huttingen'; see Figure 1. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; ns = not significant.

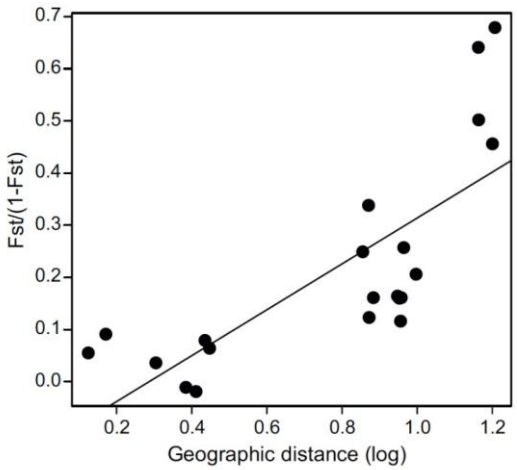

Figure 3. Isolation by distance (IBD) analysis of *I. fuliginator* populations. IBD was calculated using genetic differentiation ($(F_{ST}/(1 - F_{ST})$ based on six microsatellite loci) and geographic distance (log) across seven populations in the border region of Switzerland, France, and Germany (Mantel test: $R^2 = 0.585$; $F_{ST}/(1 - F_{ST}) = -0.126 + 0.440 * \log(\text{geographic distance})$, $P = 0.008$).

3.2. Spatial Genetic Structure

Considering the populations with large sample sizes in the border region of Switzerland, France, and Germany, STRUCTURE inferred the presence of two genetic clusters (optimum $K = 2$) as being the most likely (Figure S1a,b), which represent the two metapopulations Blotzheim and Istein/Huttingen (see above). Of the 156 beetles examined, 153 individuals (98.1%) were correctly assigned to the cluster of geographical origin. Extending the data set by including all 18 *I. fuliginator* populations sampled in the border region (populations 1–27; Table 1), STRUCTURE confirmed the presence of two genetic clusters (optimum $K = 2$) as being the most likely across the sampled sites (Figure 4a,b). The populations in the Alsace (France) represent one cluster, together with the Swiss populations near Basel, which is separated by the river Rhine from the cluster composed of the populations in southwestern Germany. Two populations (population 13 and 25) on either side of the river Rhine were assigned to the opposite cluster (Figure 2). In population 13, represented by only four beetles, each two individuals were assigned to either cluster (Figure 4b), while population 25 was represented by a single individual in the analysis (Table 1). Both populations were rather isolated.

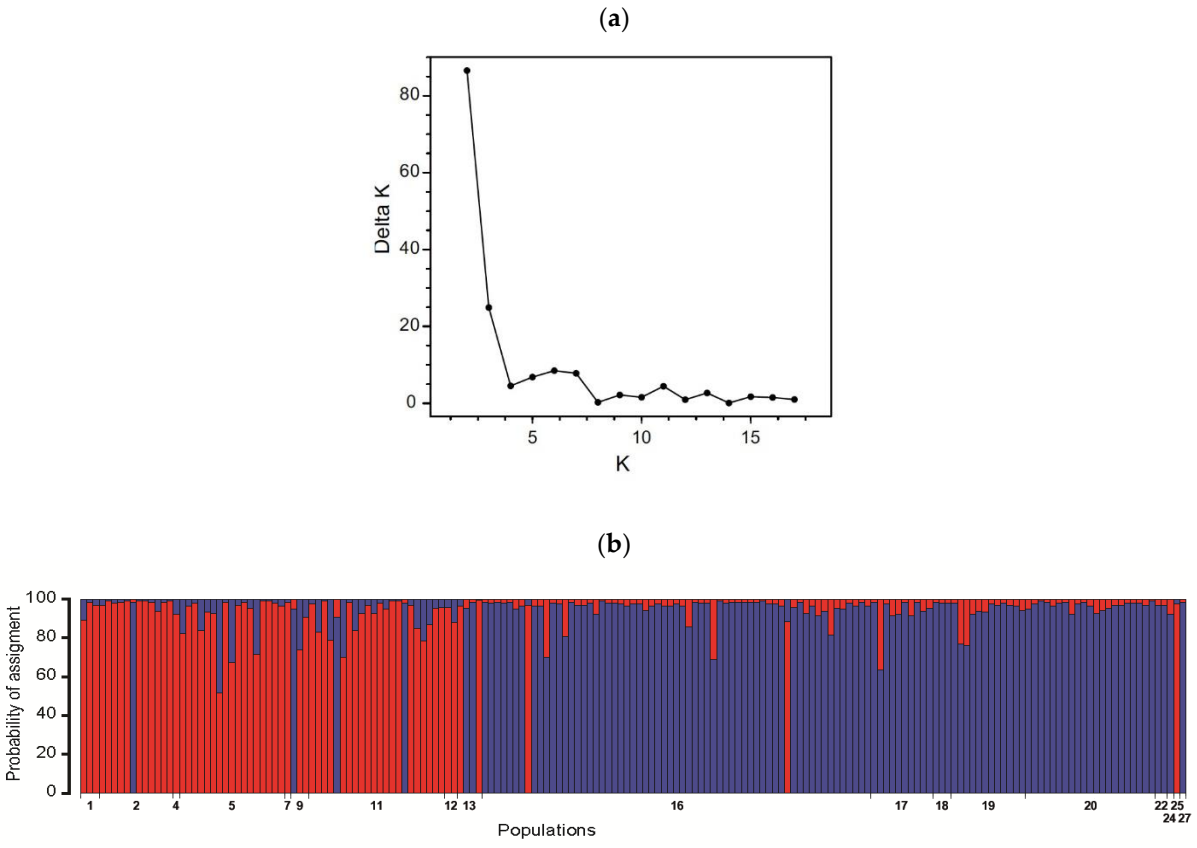

Figure 4. Results of STRUCTURE analysis identifying population clusters of *I. fuliginator* in the border region of Switzerland, France, and Germany (regional scale, 18 populations). (**a**) Delta*K* with cluster number *K* from 1 to 18. (**b**) Barplot of admixture assignment for the 179 individuals of 18 populations with $K = 2$. Each individual is represented by a vertical bar, and its likely assignment to a specific genetic cluster is encoded by different colors (blue: cluster "metapopulation Blotzheim"; red: cluster "metapopulation Istein/Huttingen").

The neighbor-joining (NJ) tree of the 18 *I. fuliginator* sampled sites showed that the beetle populations were divided into two main clusters (Figure 5a), parallel to the results of the STRUCTURE analysis. However, several bootstrap values were relatively low (Figure 5a). The Principle Coordinate Analysis (PCoA) confirmed the spatial structure of

two groups of populations (Figure 5b). The first and second principal coordinates explained 17.0% and 10.6% of the total variation.

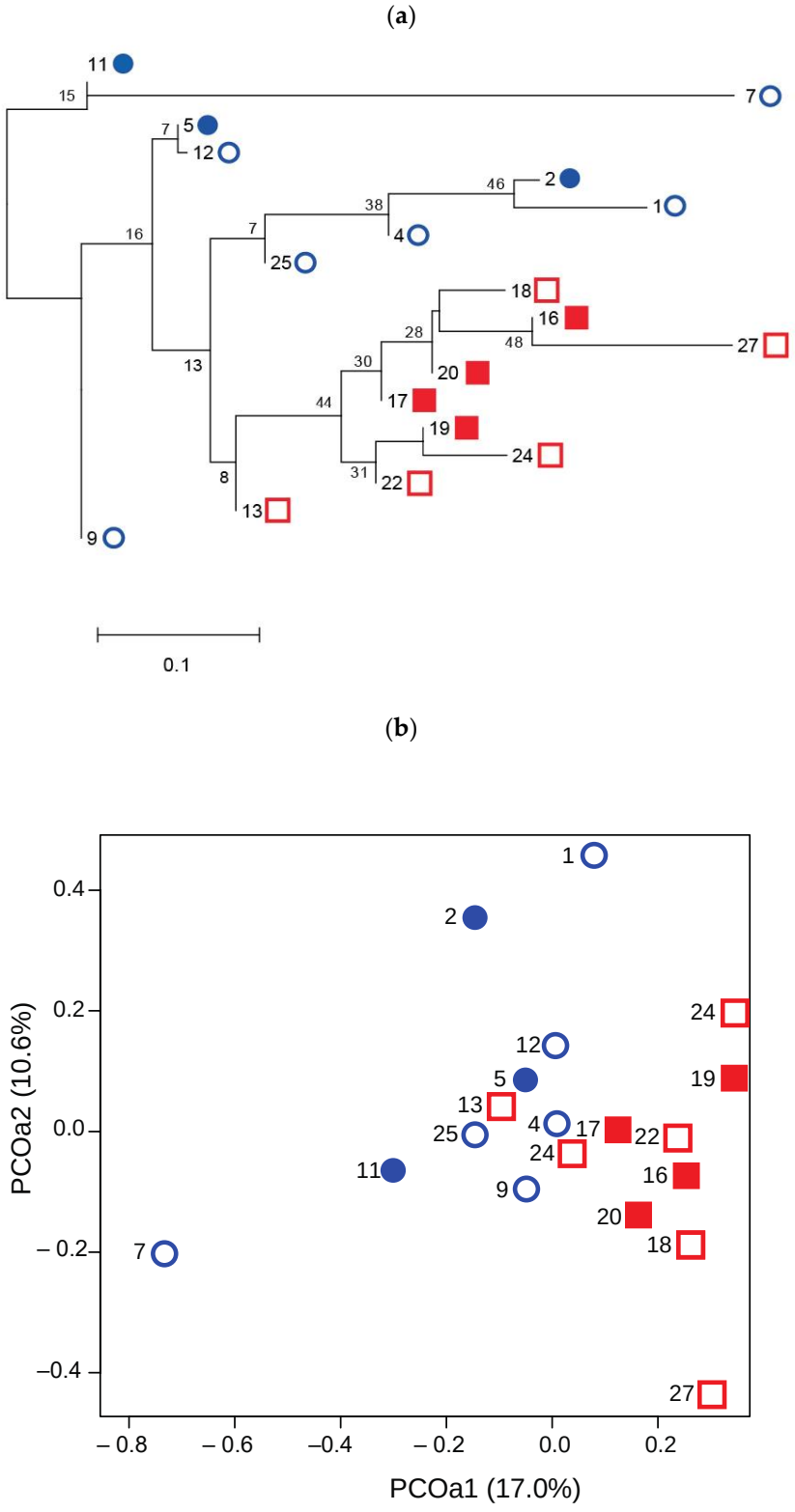

Figure 5. (**a**) Neighbor-joining (NJ) tree using Nei's genetic distance of 18 *I. fuliginator* populations in the border region of Switzerland, France and Germany (regional scale). Values at the nodes (in green) are bootstrapping percentages from 10,000 replicates. (**b**) Principal Coordinate Analysis (PCoA) based on pairwise genetic distances (DST) of the same populations.

The pattern of two main clusters remained when additional sites, sampled in the wider distribution area of *I. fuliginator* (populations 29–34; Figure 2), were included in the neighbor-joining tree analysis (Figure S2a). Populations that were more geographically distant formed their own branches in the tree. Again, most bootstrap values were relatively low (Figure S2a). PCoA confirmed the spatial structure of the populations (Figure S2b). The first and second principal coordinates explained 13.7% and 7.9% of the total variation.

3.3. Genetic Diversity and Long-Term Population Dynamics

The long-term dynamics of the *I. fuliginator* populations appear not to be affected by their genetic diversity (Table 2). Populations that decreased in size in the past 15 years did not differ in allelic richness (*Ar*), H_O, H_E, or inbreeding coefficient F_{IS} from populations that remained stable in this period (*t*-test, $P > 0.72$ in all cases).

4. Discussion

Anthropogenic pressures on connectivity and population size are increasingly affecting many plant and animal species. Habitat fragmentation and population decline can significantly modify the levels and patterns of genetic variation in natural populations [50–52]. Hence, it becomes crucial to understand the scale and genetic consequences of small population sizes, as well as population fragmentation in the wild [53,54]. Species with limited dispersal ability, such as the flightless *I. fuliginator,* particularly suffer from isolation, which may lead to a marked genetic differentiation among populations [55–59].

4.1. Genetic Diversity and Inbreeding

The *I. fuliginator* populations examined in our study are characterized by a relatively low genetic diversity and a high level of inbreeding. Similar levels of inbreeding have been reported in other beetles (e.g., in the palm-seed borer *Coccotrypes dactyliperda* Fabricius, with F_{IS} ranging from –0.156 to 0.664 [60]; in the bark beetle *Xylosandrus germanus* (Blandford) 0.88–0.94 [61]; and in the hermit beetle *Osmoderma barnabita* Motschulsky 0.25–0.37 [52].

Genetic drift is inversely related to the effective population size [9], and typically occurs in small populations, where rare and private alleles face a greater chance of being lost. Reduced genetic diversity due to drift is not expected to cause a reduction in fitness in the short term, but in the long term, it might lower the rate of adaptive evolution and thereby increase the risk of extinction in a changing environment [62]. At present, however, major environmental changes are so rapid that the distinction between 'short-term' and 'long-term' loses significance [63].

It has been proposed that inbreeding contributes to the decline and eventual extinction of small and isolated populations [64]. In a large metapopulation of the Glanville fritillary butterfly (*Melitaea cinxia* (L.)), the risk of local extinction increased with decreasing heterozygosity of the population (an indicator of inbreeding), even after accounting for the effects of relevant ecological factors such as population size and isolation [4]. Larval survival, adult longevity, and egg-hatching rate were adversely affected by inbreeding, and appear to be the fitness components underlying the relationship between inbreeding and local extinction [4]. We found that the long-term dynamics of the *I. fuliginator* populations were not influenced by their genetic diversity and their level of inbreeding. Populations that decreased in size in the past 15 years did not differ in any measures of genetic diversity, nor in the inbreeding coefficient F_{IS}, from populations that remained stable in this period. Indeed, progressive habitat degradation expressed by a change in plant species composition and a decrease in grass cover has been demonstrated to be the main reason for the decrease in population size in the border region of Switzerland, France, and Germany [20]. Our long-term monitoring of *I. fuliginator* habitats revealed that several populations survived with very few individuals for decades [20]. This may at least partly explain the high level of inbreeding recorded in the populations examined. However, it is not known whether individuals in highly inbred populations have a reduced level of fitness. Interestingly, the two *I. fuliginator* populations in northeastern Switzerland (populations 29 and 30) showed

the lowest genetic diversity and the highest levels of inbreeding. Both populations are completely isolated (distance to the nearest known *I. fuliginator* population > 5 km), but have relatively large numbers of individuals and are currently well protected by nature conservation measures [22].

The sampling of beetle remains and dead individuals over a period of almost 20 years did not appear to influence the findings. In three populations, the sample sizes were large enough to allow an analysis of temporal changes in measures of genetic diversity and in the inbreeding coefficient. However, neither the different measures of genetic diversity nor the inbreeding coefficient differed between individuals sampled earlier (before 2006) and those collected later (after 2012). Similarly, Lozier and Cameron [65] found no temporal change in the genetic diversity of two bumblebee species between 1969/1972 and 2008 in the USA, nor did Maebe et al. [66] in the bumblebee *Bombus morio* (Swederus) in South Brazil between 1946 and 2012. In contrast, however, Maebe et al. [66] reported, in the same study, a decrease in the genetic variability in *Bombus pauloensis* Friese from 1946 to 2012.

4.2. Spatial Genetic Structure

Considering the seven populations examined in the border region of Switzerland, France, and Germany, STRUCTURE analysis revealed two genetic clusters, evidenced by a weak differentiation among populations belonging to either of two formerly functioning metapopulations and relatively small differences in Ar, H_O, and H_E among populations within the same metapopulation, supporting our hypothesis 2. The two genetically distinct clusters (metapopulations) are separated by the river Rhine. This indicates that the river Rhine has functioned as a natural barrier for a long time, and that the probability of crossing the river might be extremely low for *I. fuliginator*. Extending the data set by including all populations sampled in the border region (18 populations) confirmed the finding of two clusters separated by the river Rhine. Further analyses (neighbor-joining tree and Principal Coordinate Analysis) also revealed a spatial structure of two groups of populations, mirroring the results of the STRUCTURE analysis (Figure 5b).

At the metapopulation level, the population near Basel (population 2) was genetically close to the populations around Blotzheim in the Alsace (populations 5 and 11; Figure 2). This finding can be explained when we compare a historical map (1920) with the locations of known *I. fuliginator* populations at that time with a map from 2020 showing the still-extant beetle populations (Figure 6). Several *I. fuliginator* populations went extinct in the region between 1900 and 2020. In this period, the environment was severely modified: residential and industrial areas expanded, agricultural land use was intensified, and natural habitats were marginalized (Figure 6). Formerly, the flat area between Basel and Blotzheim was inhabited by numerous *I. fuliginator* populations, and many of them were connected by dispersing individuals. Sufficient dispersal among habitat patches (populations) is a necessary condition for metapopulation persistence [60]. The functioning metapopulation was then thinned out by the extinction of individual populations. The populations of *I. fuliginator* that still exist constitute the remnants of a past metapopulation, and are currently isolated [34]. It is sad to note that during the 20-year collection period for this study, 3 of the 25 populations listed in Table 1 became extinct due to human activities.

Genetic isolation between populations frequently increases with geographical distance and with time, and can result in genetic differentiation [69]. 'Isolation by distance' results from spatially limited gene flow, and is a commonly observed phenomenon in natural populations [70]. We found a positive relationship between genetic and geographical distance among the seven *I. fuliginator* populations in the border region of Switzerland, France, and Germany. Isolation by distance has been reported in some beetles (e.g., in the great silver beetle *Hydrophilus piceus* L. [71] and in the weevil *Geochus politus* Broun [59]), but not in others (e.g., in *Bolitophagus reticulatus* (L.) [72] and the carabids *Abax ater* (Piller and Mitterpacher) and *Pterostichus madidus* (Fabricius) [73]). The presence/absence of an isolation-by-distance pattern has been explained by species characteristics (unable to fly,

otherwise limited dispersal ability, specialized habitat requirements), habitat characteristics, and the presence of spatial distribution between suitable habitat patches in a landscape.

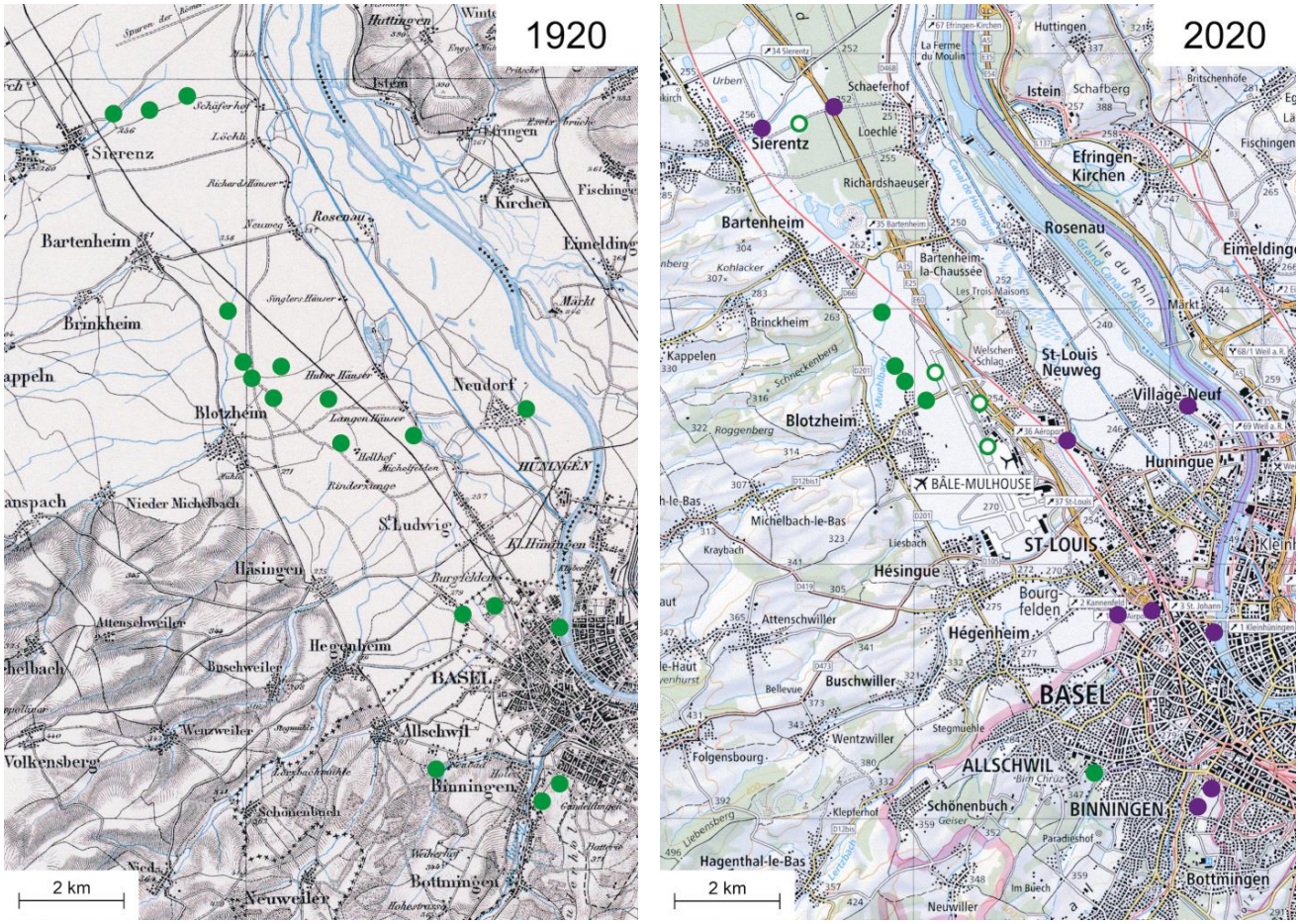

Figure 6. Historical (1920; **left**) and recent (2020; **right**) distribution of *I. fuliginator* populations in the area between Basel (Switzerland) and Blotzheim (France). Green dots: populations existing 1920 and 2020; green open dots: populations existing in 1920 and probably still existing in 2020; violet dots: populations that went extinct in the past decades. Three of the populations that probably still exist are situated in the area of the Basel-Mulhouse airport, with no access for researchers. Data on beetle distribution were extracted from Life Science [67], Baur et al. [35], Buser et al. [68], and Coray et al. [28]. Maps ©swisstopo.

4.3. Variation in Duration of Larval Development

The analyses that we conducted suggested a similar genetic diversity and no significant genetic differentiation between *I. fuliginator* populations emerging in odd and in even years. This indicates that individuals may achieve eclosion within one, two (in most cases), or three years, depending on the varying environmental conditions. Consequently, there might be gene flow between the two populations assumed to be temporally separated at the same site. This aspect is important because a strict biennial life cycle would increase the population fragmentation at a yearly level, in addition to the geographical isolation. Similarly, no clear temporal pattern in genetic diversity or genetic structure has been found in the European stag beetle (*Lucanus cervus* L.) in suburban landscapes, which could be attributed to the varying duration of larval development [19].

4.4. Non-Invasive Approach

Our study is among the first to investigate genetic diversity and differentiation in a relatively small, but highly endangered insect species based entirely on beetle remains (however, see Cox et al. [19], who used remains of the much larger European stag beetle for assessment of the genetic structure). Our approach avoids a further reduction in the already small populations (frequently less than 50 individuals [20,21]). In a previous study, we described the successful isolation of DNA from *I. fuliginator* remains stored dry for long periods [37]. The procedure could be adjusted to other rare and endangered insect species to obtain key information for appropriate conservation actions. However, this non-invasive approach has also disadvantages. Finding dead beetles or beetle remains mostly occurs accidentally. Beetles' remains (especially crushed individuals) can be more easily found on paved roads bordering embankments inhabited by *I. fuliginator* than in grassland patches entirely surrounded by cropland. Furthermore, the density of active beetles is extremely low in most populations, with an encounter rate of fewer than 0.25 beetles per hour searching [21]. It follows that the sample size (number of individuals per population) can hardly be planned, which makes it difficult to adhere to a stringent study design. Large sample sizes of beetle remains or individuals found dead for genetic study can thus only be obtained within the framework of long-term monitoring projects.

4.5. Implications for Conservation

We demonstrated that non-invasive samples of beetle remains can provide satisfactory data for conservation genetic studies in an endangered insect species. With one exception, the *I. fuliginator* populations examined showed a relatively low genetic diversity and a high level of inbreeding. Some populations are remnants of formerly functioning metapopulations, but are currently rather isolated. Other populations are completely isolated. These small, recently isolated populations are at risk of reduced viability owing to demographic and genetic (inbreeding) effects, which can lead to extinction. However, the landscape in the study region continues to change at a rapid rate due to settlement expansion and further agricultural intensification. Conservation efforts should, therefore, focus on increasing suitable habitats for *I. fuliginator* (see [29]), creating dispersal corridors and reducing drifting insecticides and fertilizers from the surrounding agricultural fields.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/d15010016/s1, Figure S1: Results of STRUCTURE analysis identifying population clusters of *I. fuliginator* in the border region of Switzerland, France, and Germany; Figure S2: (A) Neighbor-joining (NJ) tree using Nei's genetic distance of 24 *I. fuliginator* populations, (B) Principal Coordinate Analysis (PCoA) based on pairwise genetic distances; Table S1: Comparison of different measures of genetic diversity in *I. fuliginator* individuals from three populations sampled before 2006 and after 2012; Table S2: Comparison of different measures of genetic diversity in *I. fuliginator* individuals that emerged in odd years or even years in three populations; Appendix S1: Microsatellite data of *I. fuliginator* individuals examined.

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