diversity

MDPI

Article

Spatial and Temporal Patterns of Genetic Diversity and Structure in Danish Populations of the Alcon Blue Butterfly *Phengaris alcon* (Denis & Schiffermüller)

Andreas M. B. Boe [1,2], Thomas J. Simonsen [1,2,*], Camilla T. Colding-Jørgensen [3], Philip H. Folman [3], Thomas T. Jensen [3], Kian J. Spencer [3], Philip F. Thomsen [2,4] and David R. Nash [3,*]

1 Natural History Museum Aarhus, Aarhus C, DK-8000 Aarhus, Denmark
2 Department of Biology, Aarhus University, Aarhus C, DK-8000 Aarhus, Denmark
3 Department of Biology, University of Copenhagen, Copenhagen OE, DK-2100 Copenhagen, Denmark
4 Novo Nordisk Challenge Centre for Ecological Genetics, Aarhus University, DK-8000 Aarhus, Denmark
* Correspondence: t.simonsen@nathist.dk (T.J.S.); drnash@bio.ku.dk (D.R.N.)

Abstract: *Phengaris alcon* is an endangered, ant-associated butterfly found, amongst other places, in Denmark, where it has undergone a severe decline during the last century. However, the population genetic consequences of this decline remain unknown. To explore past and current patterns in population structure in relation to the decline, we analyzed DNA microsatellite data from 184 recent and 272 historical *P. alcon* specimens from 44 spatiotemporal locations in Denmark. We thus generated the most temporally and spatially comprehensive population genetic dataset for *P. alcon* in Denmark so far. Our results for the Bayesian population assignment of recent samples revealed three major current genetic clusters: western Jutland, northern Jutland, and the island of Læsø. Estimates of genetic diversity showed signs of inbreeding in several extant populations. When including data from museum specimens, only a single locatSion showed a decline in heterozygosity between 1967 and 2021. We suggest that the two distinct clusters in western and northern Jutland indicate two temporally separated Holocene colonizations of Denmark, the latter of which may have been aided by changes in agricultural practice in the late Neolithic period. The unique genetic signature of the Læsø populations may be a result of the admixture of northern Jutland and western Swedish populations.

Keywords: population genetics; Lycaenidae; post-glacial expansion; isolation by distance

check for updates

Citation: Boe, A.M.B.; Simonsen, T.J.; Colding-Jørgensen, C.T.; Folman, P.H.; Jensen, T.T.; Spencer, K.J.; Thomsen, P.F.; Nash, D.R. Spatial and Temporal Patterns of Genetic Diversity and Structure in Danish Populations of the Alcon Blue Butterfly *Phengaris alcon* (Denis & Schiffermüller). *Diversity* **2022**, *14*, 1098. https://doi.org/10.3390/d14121098

Academic Editor: Andre Victor Lucci Freitas

Received: 31 October 2022
Accepted: 1 December 2022
Published: 11 December 2022

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Copyright: © 2022 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

1. Introduction

Land use has changed dramatically in Denmark over the past few centuries. While forest cover has increased slightly, natural and seminatural open habitats have severely decreased because of intensification of agriculture and the spread of urban areas [1]. Heathland is one of the habitat types that have experienced the most severe decline, particularly in western Jutland [2,3]. This decline has naturally had dramatic effects on insect species dependent on heathland as their habitat. Strong decline and genetic isolation have been documented for the beetle *Carabus arcensis* Herbst [2] and the butterfly *Euphydryas aurinia* (Rottemburg) [4,5], while the iconic and once widespread grasshopper *Bryodemella tuberculata* (Fabricius) went extinct in the 1940s [6]. However, widespread, open heathlands are not a natural habitat type in Denmark, and heathlands as we now know them are a direct result of Neolithic and Bronze-age agricultural practices, followed by more-or-less deliberate land management until the onset of the industrial and agricultural revolutions of the past two to three centuries [7]. These changes in heathland cover must surely have had an immense effect on local fauna. Unfortunately, we have little evidence for trends going back further than public natural history collections, and even anecdotal evidence becomes scant further back than the late 19th century. However, population genetic studies based on both current and extinct populations—the latter from natural history collections—may reveal hidden patterns for

both recent and more ancient nature [8,9] and thus give an insight into past trends in fauna development. Butterflies are one group severely affected by recent land-use changes [10,11] and out of 75 species resident in Denmark over the past century, 12 have gone extinct during the same period, 14 are considered threatened, and another 11 near-threatened [12]. Country-wide extinctions during this period have mainly occurred among low-mobility habitat specialists associated with open forest habitats, but sedentary host plant specialists and species generally associated with heathlands and bogs have also declined dramatically or become locally extinct [10,11].

One butterfly species that is of particular interest with respect to the formation and decline of wet heathlands is *Phengaris alcon* (Denis & Schiffermüller) (the Alcon Blue butterfly) [13,14]. It was once widely distributed in western Denmark but has become rarer over the course of the last century [10,11] (see Figure 1). The species is not only of conservation interest in its own right but is an excellent indicator of the presence of other threatened species associated with the same habitat [15]. *P. alcon* is a butterfly in the family Lycaenidae with a complex life cycle. In Denmark, the female oviposits on the developing flower buds of the perennial forb *Gentiana pneumonanthe* Linnaeus (Marsh Gentian) [16]. The larva then feeds on the flower ovaries for three instars [17]. In the fourth instar, it descends to the ground, where it mimics the composition of the surface hydrocarbons of the host ant species [18], in Denmark either *Myrmica rubra* (Linnaeus) or *M. ruginodis* Nylander [18–20]. Consequently, workers from nearby ant colonies will pick up the larva and transport it to the nest, where it is fed by worker ants [21,22]. As *P. alcon* depends on the co-occurrence of both plants and host ants to complete its lifecycle, its distribution is restricted to wet heathlands and bogs, and it is listed as "Vulnerable" in the Danish Red List and considered in decline [12].

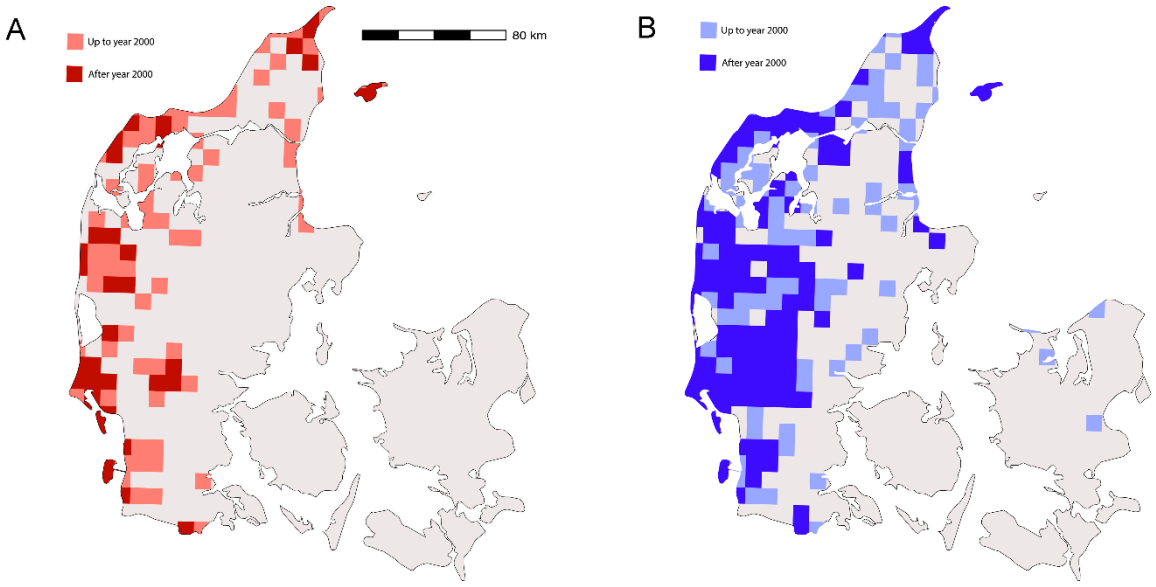

Figure 1. Spatiotemporal distribution maps for *P. alcon* (**A**), and *G. pneumonanthe* (**B**) in Denmark, shown as 10 × 10 UTM grid squares in which each species has been recorded (see Supplementary Methods S1).

The distribution of *P. alcon* in Denmark today is highly fragmented (Figure 1) and comprises a series of areas along the west coast of the Jutland peninsula, from the German border, including the Wadden Sea islands of Fanø and Rømø, to northern Jutland, where it extends its range to the east coast and reaches the island of Læsø, as well as parts of central Jutland and localities along the Limfjord. In the south, it has had a few isolated populations in southeastern Jutland. It has disappeared from localities in the bogs of southern Jutland and many saline meadows along the Limfjord. This decline is illustrated in Figure 1A, which shows the geographic distribution of extant, potentially recently extinct,

and historical localities of the species in Denmark based on published records. Figure 1B shows that the host plant has undergone a similar, but less severe, decline.

Surprisingly, remarkable phenotypical variation in wing pattern and coloration appears to be present in *P. alcon* across its limited distribution in Denmark. Kaaber [23] described a cline with a significant shift around the Limfjord. He attributed this morphological variation to the presence of the putative species *Maculinea "rebeli"* north of the Limfjord. However, the exact status of this taxon has been debated. Originally described as a variant (*Lycaena alcon* var. *rebeli*) in the Styrian Alps by Hirschke [24], it was later elevated to full species status [25], including the subspecies *Maculinea rebeli xerophila* Berger, applied to the Belgian *P. alcon* populations utilizing *Gentiana cruciata* (cross gentian) as host plant in dry habitats [26]. Several studies on the genus have since included *Maculinea rebeli* as a separate species (reviewed in [26]). However, in a broad geographical analysis of the morphology of the *alcon/rebeli* complex, Kudrna and Fric [26] found no consistency in morphological characteristics to justify that *P. alcon* utilizing *G. cruciata* in dry habitats (see [25,27,28]) should be a separate species [26]. This is further supported by genetic data in regional studies [29–31] and global phylogenetic analyses of the entire genus and its allies [32,33]. We note that Sielezniew et al. [34] found different genetic variability in *P. alcon* and *P. rebeli* ecotypes in Poland and Lithuania but were unable to consistently separate the two types using microsatellite data. Lastly, populations from the high-altitude areas close to the type locality of Hirschke's *Lycaena alcon* var. *rebeli* have a unique biology, utilizing *Gentianella rhaetica* Kern & Kern as host plant and *Myrmica sulcinodis* Nylander as host ant [35], thereby differing themselves from the lower altitude *P. alcon*, which use *G. cruciata* and, generally, *M. sabuleti* Meinert as hosts [36]. These high-altitude populations have furthermore been shown to be genetically distinct from any lower altitude populations, perhaps justifying their independent taxonomic status [37]. In this light, it seems more likely that the phenological variation found in Denmark, including the shift in wing pattern around the Limfjord [23], can be explained by intraspecific variation in *P. alcon*. However, it remains to be investigated to what extent this morphological variation is reflected in the genetic variation of *P. alcon* in Denmark and whether populations north of the Limfjord might constitute an evolutionarily significant unit (ESU) worth specific conservation attention [38,39].

It is not known whether the many stretches of water separating different regions of Denmark and the fragmentation of natural habitats caused by agricultural practices act as barriers to geneflow, as such barriers may depend entirely on the species and its dispersal abilities. Preliminary microsatellite studies on the population genetics of *P. alcon* associated with the establishment of natural parks in Denmark have indicated distinct genetic clusters in the country in southwestern and northwestern Jutland [40] and on the island of Læsø [41]. Furthermore, an earlier study based on allozymes found lower genetic diversity north of the Limfjord than south [42]. There are, however, several populations of *P. alcon*—both extant and extinct—distributed between these clusters, which have so far not been included in any genetic analyses. Including these localities is key to understanding what geographic features influence gene flow and how changes in agricultural practices and climatic fluctuation have influenced the genetic variation in the species in Denmark.

Low genetic diversity and small effective population sizes seem to be common in *P. alcon* in Denmark [40–42] and elsewhere in Europe [29,43], but see [44] for a very different situation in eastern Poland. At the same time, the small populations are often poorly connected, leading to strong genetic differentiation between them. This results in a pattern of isolation by distance, which has been observed for *P. alcon* in Denmark and southern Scandinavia [40,41,45,46]. While it is clear from laboratory experiments that inbreeding can lead to extinction [47], its importance for natural populations is still not well-understood, although for some butterflies, it can contribute to extinction [48]. Despite the negative effects that inbreeding has, it has been suggested that it is an inherent consequence of the high mortality imposed on *Phengaris* spp. by their very specialized life cycle, creating potential bottlenecks at each generation [29,49]. Specimens in natural history collections

offer a unique opportunity to explore how some of these genetic factors have developed over time as long as genetic markers of appropriate length are available [50].

In this study, we used specimens from three different natural history collections, as well as freshly collected samples, to generate the most temporally and spatially comprehensive population genetic dataset for *P. alcon* in Denmark so far. Specifically, we aimed to (i) provide descriptive statistics of genetic diversity for extant populations of *P. alcon*, (ii) investigate how these measures have changed through time, (iii) test for evidence of isolation by distance, and (iv) describe the genetic population structure and discuss how this has been shaped by landscape changes during the Holocene.

2. Materials and Methods

2.1. Sampling

In the following, we use the term "locality" for the geographical place where a sample was collected. We use "location" for a spatial and temporal sample—i.e., a sample collected at a specific place and in a specific year.

2.1.1. Museum Specimen Sampling

Single mid- or hind legs were removed from 297 Danish specimens from the collections of the Natural History Museum Aarhus (NHMA) and the Natural History Museum of Denmark (NHMD) and the private collection of Svend Kaaber, Aarhus, Denmark (SKC). Specimens were selected for DNA extraction based on the criteria that they should be assignable to clear localities with several specimens collected in the same year. We also gave preference to localities identical to or near those where we were also able to collect samples from live individuals, as well as some localities that were thought to have gone extinct but with a relatively large number of historical specimens. Sterile forceps were used for removal of legs, which were then stored at −20 °C in 1 mL Eppendorf tubes, either in 98% ethanol or dry. DNA was successfully extracted and amplified from 272 museum specimens.

2.1.2. Sampling of Live Individuals

Contemporary samples were collected in the field in 2005, 2018, 2019, and 2021. Tissue for DNA extraction was sampled non-lethally by clipping 2 × 2 mm wing fragments from the anal angle of the hind wing, including a section of a wing vein. This method has been shown not to affect the survival of butterflies [51]. When adults could not be found in the field, host plants with eggs were collected instead. The plants were selected by choosing the plant with the second highest number of eggs on it in each group of host plants. Eggs were then reared to larvae by placing each plant in a beaker filled with water to sustain the flower. The top was covered with plastic film to keep descending larvae from drowning. The beaker was then placed inside a petri dish of larger diameter to catch any larvae descending from the flower. After a couple of weeks, all flower stems were dissected to find any larvae that had died while still feeding inside the buds. Living or dead larvae were collected in 98% ethanol, and half of each larva was used for DNA extraction. A total of 184 specimens were amplified for contemporary samples.

2.2. Laboratory Procedures

2.2.1. DNA Extraction

Genomic DNA was extracted using a Qiagen DNeasy Blood and Tissue Kit (Hilden, Germany) following the manufacturer's instructions, except that lysis was done overnight at 56 °C on a rotating device, ensuring thorough mixing.

2.2.2. PCRs and Microsatellite Loci

A PCR master mix was prepared using 7 µL Invitrogen Platinum II Taq Hot-Start DNA Polymerase and primers according to Table S1. Three microliters of DNA from elution 1 was mixed and run as multiplexes according to the mix groups in S1. PCR conditions were: 5 min of denaturation at 95 °C, a touchdown of 14 cycles for 30 s at 60–55 °C, 30 cycles of 55 °C, and

final extension of 72 °C for 30 min. Allele length was determined by capillary electrophoresis using an ABI 3031xl automated sequencer with the GeneScan-500 LIZ size standard.

2.3. Analysis

2.3.1. Distribution Maps

We prepared maps of Danish *P. alcon* and *G. pneumonanthe* using 10 × 10 km UTM grid squares (Figure 1) based on records from various public data sources, supplemented with our own records and data extracted from both the preserved specimens of *P. alcon* that we examined and specimens of *G. pneumonanthe* held at the Danish national herbarium (see Supplementary Methods S1 for more details).

2.3.2. Genetic Data Preparation

We examined the ABI 3031xl chromatograms in Geneious R10 (https://www.geneious.com, accessed on 30 October 2022) using the microsatellite plugin. If the GeneScan-500 LIZ ladder was not automatically detected by the program, we fixed it manually by adjusting the trimmed region and manually selecting peaks. Microsatellite peaks were first automatically identified by the program and subsequently checked for questionable interpretations of peaks; e.g., in stutter-bands. Initially, we attempted to include all 14 loci for both historical and contemporary samples (Table 1). Following initial evaluation, we excluded Macu15 as it produced very variable stutter-band patterns that were impossible to score in a consistent way. Malc169 was omitted by error during preparation of the PCR master mix. For historical samples, we were only able to consistently amplify loci with alleles shorter than 160 bp. Similar results have been reported for the congener *Phengaris arion* [50]. To address this, we constructed two datasets: one comprising the 15 localities sampled in the 21st century based on all 13 loci, excluding individuals with missing data for more than 7 of the loci; and one dataset comprising all 44 locations based on the four relatively short loci (Macu20, Macu26, Macu30, and Macu31), excluding individuals missing data for more than two of these loci. In the following, the two datasets are referred to as the contemporary and combined datasets. To evaluate the effect of only using four loci in the genetic structuring analysis, we constructed a third dataset comprising the 15 contemporary locations and including only the loci Macu20, Macu26, Macu30, and Macu31. All 520 locality x year x location combinations were tested for Hardy–Weinberg equilibrium in GenoDive 3.05 [52] using the least squares method and 999 permutations. Only three showed statistically significant deviations from equilibrium, so no corrections were made.

Table 1. Summary of samples, genetic diversity (N_A = number of alleles, N_E = effective number of alleles, H_O = observed heterozygosity, H_S = expected heterozygosity), and estimated inbreeding (G_{IS}) for the contemporary dataset.

Locality	Sampling Year	# Sequenced	# Included	N_A	N_E	H_O	H_S	G_{IS}
Læsø: WGC	2018	20	18	3.2	2.31	0.36	0.52	0.3
Læsø: ALS	2018	10	9	3.3	2.52	0.48	0.58	0.17
Læsø: LN	2018	20	17	3.7	2.64	0.47	0.56	0.16
Læsø: LS	2018	10	7	3.2	2.36	0.53	0.57	0.07
Tversted Rimmer	2021	16	15	1.3	1.25	0.15	0.14	−0.11
Hansted	2019	18	14	2.6	1.84	0.46	0.41	−0.12
Vestergård	2019	8	8	2.7	2.08	0.26	0.49	0.48
Klosterheden	2021	7	7	2.6	2.07	0.39	0.49	0.21
Sørup Sande	2021	2	2	2.1	1.89	0.35	0.58	0.39
Lønborg Hede	2021	9	9	2.6	2.13	0.417	0.5	0.17
Vorbasse	2019	4	4	2.8	2.24	0.46	0.54	0.15
Vejers	2019	1	1	-	-	-	-	-
Fanø	2019	30	30	3.3	2.08	0.43	0.47	0.09
Rømø	2019	15	15	4.1	2.54	0.54	0.59	0.09
Ålbæk Stampemølle	2021	16	16	2.4	1.83	0.29	0.35	0.17
Frøslev Mose	2005	16	12	3	2.35	0.26	0.53	0.51
Total	-	202	184					

2.3.3. Genetic Structuring

We performed Bayesian population assignment analyses on all three datasets using Structure v. 2.3.4 [53], testing for k (number of inferred populations) = 2 to 10 with 10 repeats per value of k, a burn-in period of 100,000, and 400,000 steps after burn-in, allowing admixture and using the sampling location as prior. The best overall value of k for each dataset was selected using the ΔK approach [54]. To illustrate the results, we calculated average membership proportions for the sample values of each cluster in R and plotted them as pie charts using the R packages ggplot2 [55], ggtree [56], and ggpubr [57].

2.3.4. Distribution of Genetic Variance and Private Alleles

Based on the ΔK analysis of the Structure output, we defined three genetic regions: (1) Læsø, including all locations on the island; (2) north Jutland, including all locations north of the Limfjord plus Klosterheden; and (3) west Jutland, including all remaining locations in Jutland. Based on these regions, we also carried out analysis of molecular variance (AMOVA [58]) using GenoDive to quantify the amount of genetic variance within and between regions based on an infinite alleles model and constructed a Venn diagram of private alleles between regions using the R package VennDiagram [59].

2.3.5. Isolation by Distance

To test for isolation by distance, we calculated F_{ST} in GenoDive for both the contemporary and combined datasets based on analysis of molecular variance [10,60]. We used the resulting matrix of genetic distances and a matrix of geographical distances to perform Mantel's test [61] for isolation by distance in R with the package ade4 [62]. Furthermore, we tested the effect of time on genetic variation by including a matrix of time (in years) between the collection dates of pairs of samples. Variables were transformed to achieve normality when possible (see Figure S1). We also did a post hoc test of isolation by distance within each of the three regions (Læsø, north Jutland, and west Jutland) defined based on the Structure output.

2.3.6. Genetic Diversity

We calculated heterozygosity-based statistics of genetic diversity in GenoDive 3.05, [52] including number of alleles (N_A), effective number of alleles (N_E), observed heterozygosity (H_O), expected heterozygosity (H_S), and inbreeding coefficient (G_{IS}), for both datasets. For the contemporary dataset, we estimated these statistics for each of the 12 loci separately and imported the values into R v4.1.0 [63], where they were used as replicates to calculate means and confidence intervals with the function mean_cl_boot in the package ggplot2, based on jackknife resampling [55]. For the six localities Fanø, Frøslev Mose, Hammer Bakker, Hansted, Klosterheden, and Læsø, we were able to calculate changes in genetic diversity over time. These were compared by plotting statistics of genetic diversity against time for all locations at each locality and using jackknife resampling over the four sequenced loci to provide confidence intervals. We combined the contemporary dataset with the data published by Vanden Broeck et al. [43] to compare levels of genetic diversity to other European populations. Their study used the same microsatellite markers but excluding Macu08 and including Malc169. Locations with less than seven samples were excluded. Measures of genetic diversity were then estimated again, and the mean N_E and H_O were compared between countries in one-way ANOVA tests. When the results were statistically significant, we performed Tukey's range test to evaluate pairwise differences between countries.

2.3.7. Effective Population Size

We estimated effective population sizes and associated 95% parametric confidence intervals for the six localities with multiple samples over time using a multiple sample method [64] based on F-statistics [65] as implemented in N_eEstimator v2.1 [66]. The contemporary localities in Læsø (WGC, ALS, LN, and LS) were treated as a single locality

for comparison with earlier samples from the island (which mostly lacked any information as to where on the island they were collected).

3. Results

3.1. Distribution of P. alcon and G. pneumonanthe in Denmark

The marsh gentian, *G. pneumonanthe*, is still widespread across the peninsular of Jutland, particularly in the western half; it occurs patchily in central and eastern Jutland. It has not, however, been reliably recorded from the island of Zealand since the turn of the century and has also apparently disappeared from many of its former inland sites in Jutland. The Alcon blue butterfly, *P. alcon*, has shown a massive decrease in records over the last decades, with only 34% of the 10 × 10 km squares from which it was previously recorded having records after the year 2000.

3.2. Samples and Data

We included a total of 456 DNA samples for *P. alcon* from 34 Danish localities (Figure 2). A total of 272 samples from 22 localities were from historical collections, while 184 samples from 16 localities were collected in the field. For four of the localities, we were able to include both historical and recent material, and for five localities, we were also able to include historical samples from different decades. Sampling localities and years are provided in Tables 1 and 2, and locality coordinates are provided in Table S2. The 456 successfully genotyped individuals were organized into three datasets (Table S2). The combined dataset comprised all 456 individuals from 44 locations (34 localities) with four loci sequenced, and the two contemporary datasets each comprised the same 184 individuals from 16 localities but with 12 and 4 loci analyzed, respectively.

Figure 2. Geographic distribution of sampled localities included in this study. The position of the Limfjord is also marked. Note that the three localities on the southern part of the island of Læsø are within 200 m of each other, so cannot be separated at this resolution.

Table 2. Summary of samples, genetic diversity (N_A = number of alleles, N_E = effective number of alleles, H_O = observed heterozygosity, H_S = expected heterozygosity), and estimated inbreeding (G_{IS}) for the combined dataset.

Locality	Sampling Year	# Sequenced	# Included	N_A	N_E	H_O	H_S	G_{IS}
Læsø: WGC	2018	20	20	3.25	2.52	0.39	0.59	0.33
Læsø: ALS	2018	10	10	2.5	2.23	0.49	0.58	0.14
Læsø: LN	2018	20	20	3.25	2.24	0.4	0.53	0.25
Læsø: LS	2018	10	10	3.25	2.29	0.45	0.58	0.22
Læsø	1934	6	6	1.67	1.64	0.6	0.33	−0.8
Læsø	1956	16	6	2	1.89	0.41	0.53	0.22
Læsø	1964	13	13	2.33	1.43	0.42	0.27	−0.69
Læsø	1976	13	13	2	1.59	0.29	0.36	0.19
Lille Vildmose	1941	1	1	-	-	-	-	-
Udbyhøj	1949	1	1	-	-	-	-	-
Hammer Bakker	1925	14	12	2.25	1.64	0.22	0.36	0.37
Hammer Bakker	1946	8	8	2.5	2.03	0.26	0.38	0.31
Tversted Rimmer	2021	16	15	1.25	1.25	0.1	0.13	0.23
Hvims	1961	15	15	2.75	2.22	0.32	0.49	0.34
Kandestederne	1971	16	15	3.25	2.29	0.55	0.52	−0.07
Slettestrand	1941	15	15	2.5	1.82	0.21	0.46	0.54
Hune	1916	1	1	-	-	-	-	-
Hune	1932	1	0	-	-	-	-	-
Lendrup	1973	1	1	-	-	-	-	-
Østerild	1930	1	1	-	-	-	-	-
Hansted	1954	16	16	3.25	2.3	0.37	0.46	0.2
Hansted	1961	2	2	1.67	1.67	0	0.67	1
Hansted	2019	18	18	2.25	1.57	0.36	0.3	−0.2
Vestergård	2019	8	8	2.25	1.89	0.13	0.4	0.69
Legind Bjerge	1961	14	14	2	1.91	0.55	0.49	−0.13
Lem Hede	1961	12	11	2	1.69	0.39	0.43	0.09
Havris Hede	1961	8	6	3	2.07	0.58	0.53	−0.09
Venø	1948	16	16	2.33	1.75	0.44	0.43	−0.02
Klosterheden	1967	14	12	4	2.26	0.63	0.58	−0.1
Klosterheden	2021	7	7	2.5	1.88	0.25	0.5	0.5
Husby	1980	16	15	3.5	2.76	0.43	0.63	0.32
Sørup Sande	2021	2	2	2.5	2.13	0.63	0.69	0.09
Lønborg Hede	2021	9	9	3	2.71	0.5	0.64	0.22
Vorbasse	2019	4	4	2.5	2.28	0.38	0.54	0.31
Vejers	2019	1	1	-	-	-	-	-
Oksby	1961	8	7	2.75	2.27	0.67	0.58	−0.14
Fanø	1923	13	13	3.75	2.59	0.61	0.6	−0.01
Fanø	1949	16	15	4.25	3.02	0.56	0.65	0.14
Fanø	2019	30	30	3.5	2.17	0.5	0.49	−0.01
Gånsager	1938	3	3	2.67	2.31	0.67	0.67	0
Rømø	2019	15	15	4.25	2.39	0.47	0.6	0.23
Ålbæk Stampemølle	2021	16	16	3	2.43	0.44	0.56	0.2
Frøslev Mose	1934	11	11	2.75	2.56	0.38	0.59	0.36
Frøslev Mose	2005	16	16	3	2.04	0.24	0.52	0.55
Visø Mose	1968	7	6	3	2.26	0.46	0.58	0.21
Total	-	481	456	-	-	-	-	-

3.3. Genetic Structuring

For all three datasets, k = 3 was chosen as the optimal number of clusters (see Figure S2). The contemporary dataset (Figure 3) showed a clear pattern, with Læsø being dominated by cluster 2 and a transition zone in western Jutland from cluster 1 in the south to cluster 3 in the north. The largest shift occurred between Sørup Sande and Klosterheden. Cluster 2 was more-or-less absent north of the Limfjord, while cluster 3 was present in southern Jutland; most notably, at Ålbæk Stampemølle. The results of the clustering analysis on the combined dataset showed a similar, but slightly different, picture (Figure 4). Cluster 1 still dominated the southern half of Jutland, reaching north to Husby Klit and Havris Hede, and was almost absent in more northern localities. Cluster 2, however, was more randomly distributed geographically but was most common in the eight locations sampled in 2018–2019. Læsø did not appear as a separate cluster but, instead, the historical locations showed high membership for cluster 1, and those sampled in 2018–2019 were dominated by cluster 2. The results of the clustering analysis of the contemporary dataset using only four

loci (Figure 5) were rather similar to the analysis of the combined dataset (using the same four loci), with the exception that Læsø then clustered entirely with northern Jutland.

Figure 3. Results of genetic clustering analysis of the contemporary dataset in Structure for k = 3. The dataset included 12 loci. The bar plots show an individual's probability of belonging to each of the three clusters and the pie charts on the map show the means of all individuals of each population.

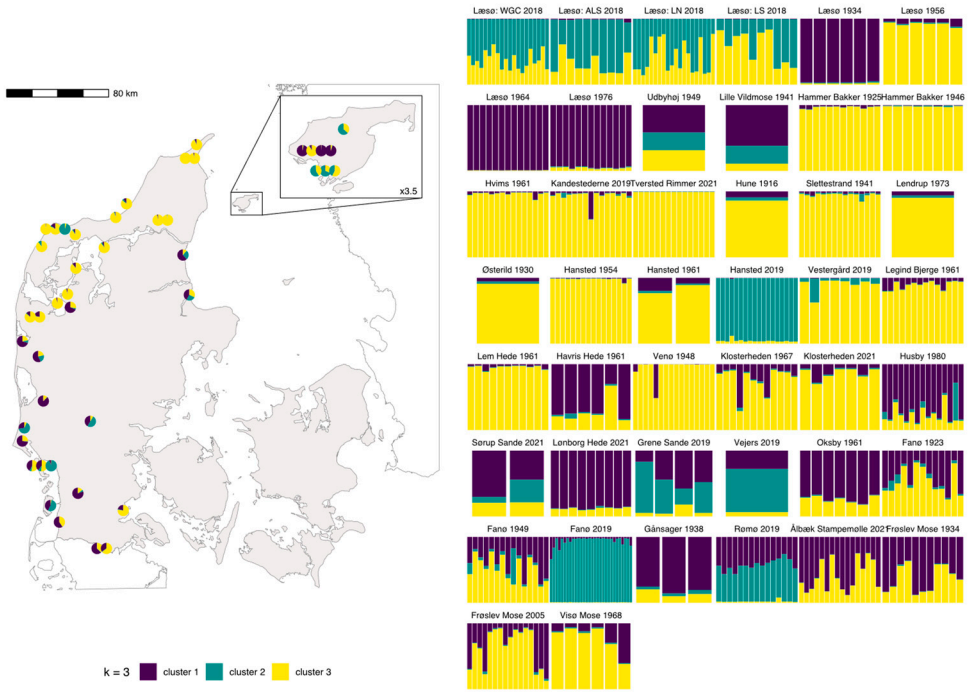

Figure 4. Results of genetic clustering analysis of the combined dataset in Structure for k = 3. The dataset included four loci. The bar plots show an individual's probability of belonging to each of the three clusters and the pie charts on the map show the means of all individuals of each population. Populations placed on a row on the map show the separate points in time at which they were sampled, except for Læsø, where the bottom row shows the localities ALS, LN, and LS, which were in proximity to each other.

Figure 5. Results of genetic clustering analysis of the contemporary dataset in Structure for k = 3. The dataset included four loci. The bar plots show an individual's probability of belonging to each of the three clusters and the pie charts on the map show the means of all individuals of each population.

3.4. AMOVA and Distribution of Private Alleles

Analysis of molecular variance (Table 3) showed that the variation among the three regions explained a significant amount of the genetic variance, approximately half as much as that among locations within regions, for each dataset.

Table 3. Summary of analysis of molecular variance for each dataset. The percentage of molecular variance (%var) assignable to each hierarchical population level is given, together with the appropriate F-statistic. For variance above the individual level, the statistical significance of the difference of the value from zero is given based on 9999 permutations.

Dataset	Source	Within	%var	F-Statistic	Value	p
Combined	Within individuals	-	63.5	F_{IT}	0.365	-
	Among individuals	Locations	12.9	F_{IS}	0.169	<0.001
	Among locations	Region	15.1	F_{SC}	0.165	<0.001
	Among regions	-	8.5	F_{CT}	0.085	<0.001
Contemporary (12 loci)	Within individuals	-	56.3	F_{IT}	0.437	-
	Among individuals	Locations	13.1	F_{IS}	0.189	<0.001
	Among locations	Region	17.8	F_{SC}	0.204	<0.001
	Among regions	-	12.9	F_{CT}	0.129	<0.001
Contemporary (4 loci)	Within individuals	-	57.1	F_{IT}	0.429	-
	Among individuals	Locations	15.2	F_{IS}	0.210	<0.001
	Among locations	Region	18.1	F_{SC}	0.200	<0.001
	Among regions	-	9.7	F_{CT}	0.097	0.002

All three regions possessed exclusive alleles: six on Læsø, five in north Jutland, and 25 in west Jutland (Figure 6). Furthermore, Læsø shared 12 alleles exclusively with west Jutland, while north Jutland shared 10 alleles exclusively with west Jutland.

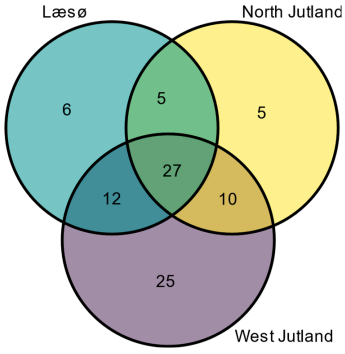

Figure 6. Venn diagram of the distribution of alleles between regions for all loci in the contemporary dataset.

3.5. Genetic Diversity

An overview of the calculated measures of genetic diversity for the contemporary and combined datasets is presented in Tables 1 and 2, and the full data are provided in Table S2. The localities WGC, Vestergård, Klosterheden, and Frøslev Mose showed a significantly positive inbreeding coefficient (Figure 7A). The effective number of alleles ranged from one to three and was particularly low at Tversted Rimmer (Figure 7B). There was little change over time in the localities sampled at multiple points in time when the confidence intervals were considered (Figure 8). Only Klosterheden showed a statistically significant decline in the observed heterozygosity between 1967 and 2021. There were no significant differences in the effective number of alleles between countries (ANOVA: $F_{2,23} = 1.34$, $p = 0.281$). However, the test of differences in observed heterozygosity was significant (ANOVA: $F_{2,23} = 5.00$, $p = 0.016$). A subsequent Tukey's range test revealed that the Dutch populations had significantly higher observed heterozygosity than the Danish populations (difference = 0.16, $p = 0.012$). Belgian populations were not significantly different from the two others due to higher intrinsic variance (Figure S3).

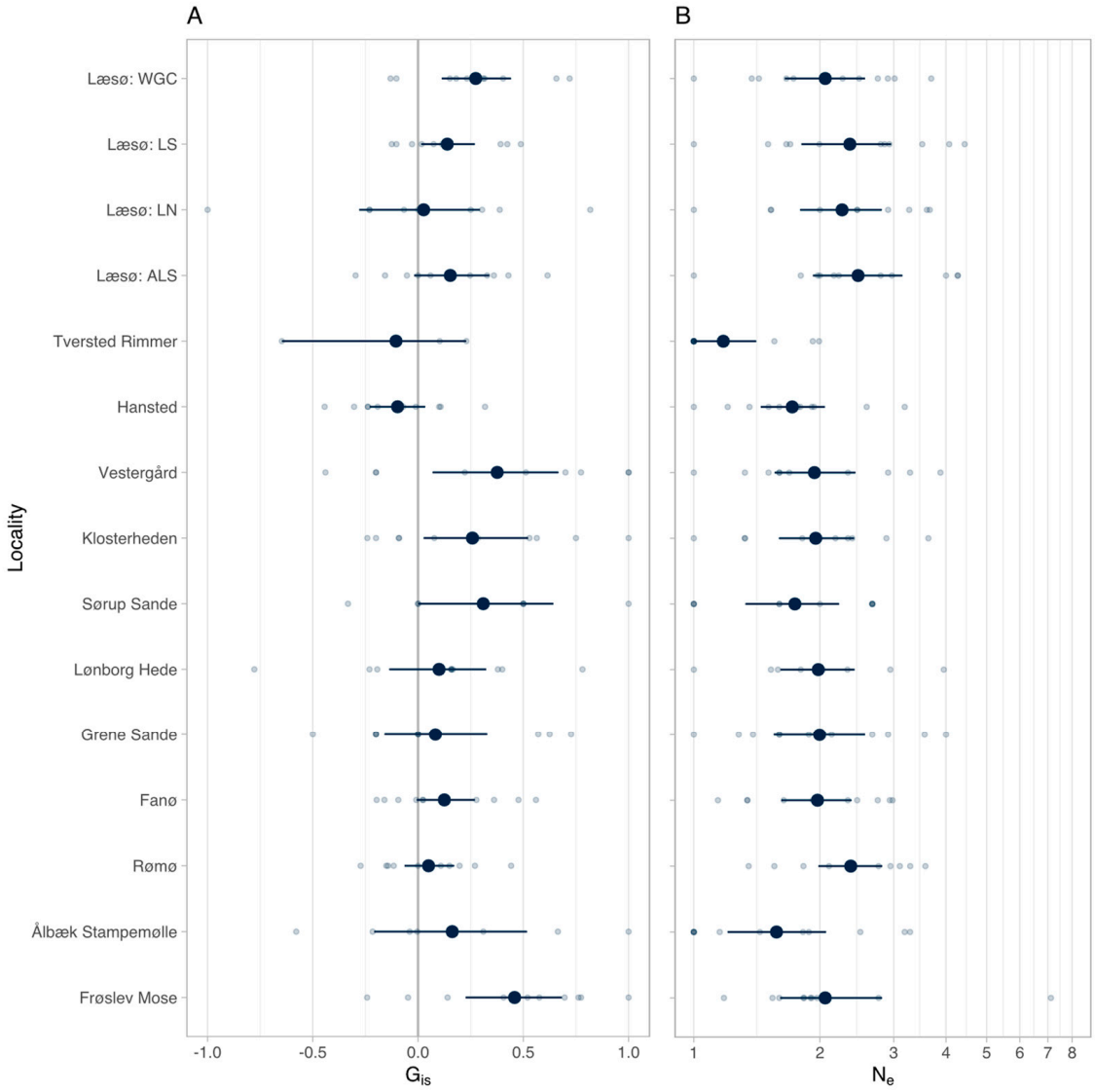

Figure 7. Estimates of genetic diversity of contemporary populations with 95% jackknife confidence intervals. Populations are ordered to approximately reflect their occurrence next to each other geographically. (**A**) Coefficient of inbreeding, (**B**) effective number of alleles; note the $\log_2$-transformation of the x-axis.

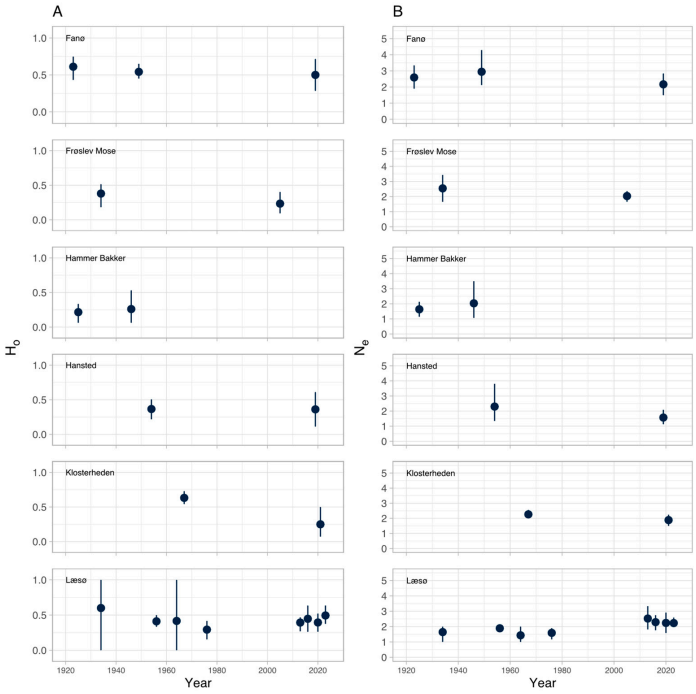

Figure 8. Change in measures of genetic diversity over time in six populations. The four contemporary Læsø populations were all sampled in 2018 but were scattered on the x-axis for visibility. The species is extinct at both Hammer Bakker and Frøslev Mose. (**A**) Observed heterozygosity, (**B**) effective number of alleles.

3.6. Effective Population Size

Estimates of effective population size ranged from 5.8 on Læsø to 397 in Klosterheden. Læsø, however, seemed to have a stable number of around 50 in the most recent samples. None of the localities showed significant changes in effective population size through time (Figure 9).

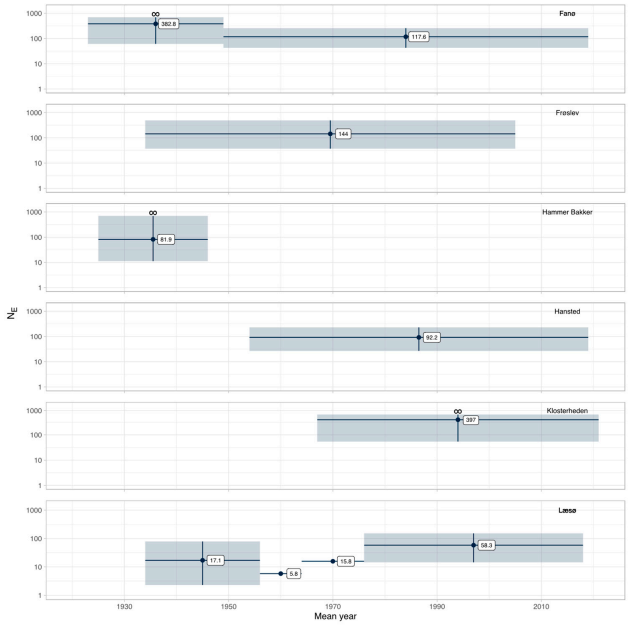

Figure 9. Estimates of effective population sizes in six populations. Vertical whiskers show 95% confidence intervals where infinite upper limits are labeled with "∞". Points and lateral whiskers show the mean and range of the pair of years used for the estimate. The species is extinct at both Hammer Bakker and Frøslev Mose.

3.7. Isolation by Distance

There was a strong positive correlation between genetic differentiation and geographic distance in the contemporary dataset (Figure 10A, Table 4). However, when each of the three regions were tested separately for isolation by distance, the correlation was only significant for Læsø (Table 4). There was also a positive correlation between genetic differentiation and temporal distance in years, but this did not reach statistical significance (Figure 10B, Table 4). The relationships between both temporal and geographic distance and genetic differentiation were positive for the combined dataset but neither reached statistical significance (Figure 11, Table 4).

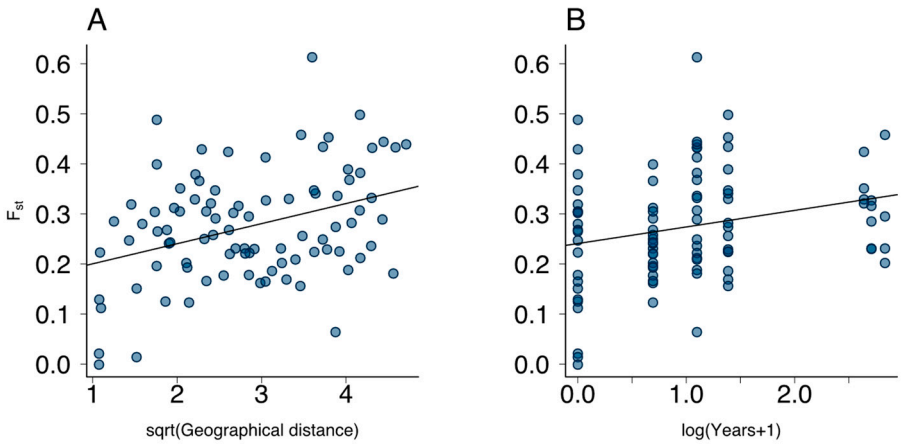

Figure 10. Plots showing isolation by distance (**A**) and year (**B**) for the contemporary dataset.

Table 4. Summary of Mantel tests of isolation by distance and isolation by time for the different datasets. The highly significant results for isolation by distance for the contemporary dataset are also broken down by region. Statistically significant associations are shown in bold.

Dataset	Region	Mantel's r	p
Contemporary (geographical)	**Denmark**	**+0.36**	**<0.001**
	West Jutland	+0.07	0.42
	North Jutland	+0.22	0.24
	Læsø	**+0.61**	**0.035**
Contemporary (temporal)	Denmark	+0.25	0.08
Combined (geographical)	Denmark	+0.10	0.09
Combined (temporal)	Denmark	+0.06	0.15

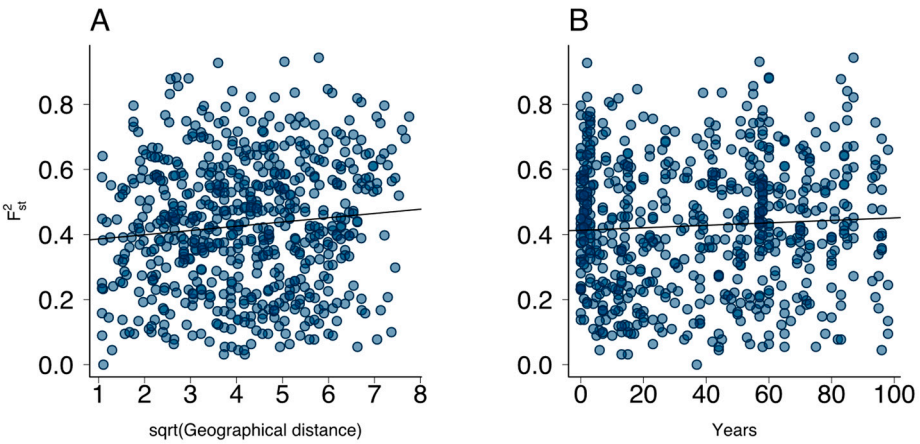

Figure 11. Plots showing isolation by distance (**A**) and year (**B**) for the combined dataset.

4. Discussion

4.1. Genetic Structuring and Biogeographical Patterns

The genetic structuring analyses of both the combined and contemporary datasets showed a clear genetic separation in western Jutland between a southern and a northern cluster (Figures 3 and 4). This is in accordance with Kaaber [23], who demonstrated that there is a similar shift in phenotypical appearance in adults around the Limfjord. The pattern is also corroborated by a previous genetic study that identified two separate clusters in northwestern and southwestern Jutland [40]. One suggestion for the variation in population genetic structure that has previously been advanced is the use of two different host ant species in Denmark [42], but this is not supported by the patterns of host ant use known across Denmark, which do not match the genetic structure of *P. alcon* [18–20].

Our results, however, provide a higher spatial resolution than those previously reported and show that the genetic shift does not occur across the Limfjord, the major waterway in Jutland and a presumed biogeographic barrier. Instead, it occurs along two important lines in the landscape, which both run perpendicular to the west coast and may aid in explaining the genetic shift: the river Storå and the main stationary line of the Weichselian glaciation [67]. The river Storå runs south of Klosterheden, while the glacial boundary runs just north of it. The latter turns south about halfway into Jutland and continues down through the peninsula [67]. The locality Visø Mose in southern Jutland from our historical dataset falls east of this line and shared high membership for cluster 3 with the locations found north of the line. As such, both in northern and southern Jutland, cluster 3 was the dominating cluster on the glaciated side of the line. However, the locality Havris Hede was situated north of the line but shared most of cluster 1 with the southern locations. Another general feature from both datasets was that cluster 3 was more common south of the shift than cluster 1 was north of it. As such, the hypothesis of two immigrations originally suggested by Kaaber [23] seems the most likely explanation. In this scenario, the first immigration would have come from the south sometime after the ice retreated, eventually extending all the way to northernmost Jutland and—later—Læsø. Then, a second immigration followed, which, to a large degree, has displaced the first immigration as far north as the Storå river. Why the second wave has not extended further is unclear. The river itself can hardly act as a barrier to dispersal, as the species has colonized islands such as Læsø over much greater and more hostile water barriers. There are, however, differences in soil types across the glacial boundary (e.g., [7]), which could affect the availability of suitable habitats—e.g., the host plant *G. pneumonanthe* is almost absent from the outwash plain around the river, known as Karup Hedeslette (Figure 1B). Furthermore, a map of the host plant distribution more than half a century ago produced by Hansen [68] (see also Supplementary Methods S1) shows that the host plant is common throughout western Jutland up until the main stationary line, after which only scattered observations can be found between the line and the Limfjord. Another possible explanation is that cluster 1 is still spreading north but has not reached farther north yet. The first immigration could have happened shortly after the postglacial retreat in the Preboreal period (11.700–10.300 BP), as Jutland at this time was dominated by open habitat. Pollen of *G. pneumonanthe* has been found in lake sediments in northwestern Jutland from 11.100–10.350 BP [69]. In the Boreal (10.300–9000 BP) and Atlantic (9000–6000 BP) periods that followed, temperatures rose and forest developed [67], not favoring the open bog-land preferred by *P. alcon*. During this period, the species might have been restricted to western Jutland where the poor soils allowed for a less densely forested landscape, and northern Jutland, which at this time would have been an archipelago due to rising sea levels [67]. An opportunity for the second immigration may have come in 4800–4400 BP, when Corded Ware cultures started clearing forests in western Jutland [7], making the land more suitable for *G. pneumonanthe*. Cluster 2 only appeared on Læsø in the analysis of the contemporary dataset. Another explanation for why a separate Læsø cluster was only detected in the contemporary dataset could be the inclusion of more loci. Læsø was formed around 4900 BP [70], when northern Jutland was probably already inhabited by the first immigration of *P. alcon*. The origin of a

separate cluster on Læsø could be explained by its isolation in the Kattegat strait, limiting geneflow from the mainland and allowing for significant genetic differentiation on the island. Hansen et al. [71], in their study of *C. arcensis*, suggest that *Carabus arcensis* mainly spread to Læsø from mainland Denmark due to the shorter stretch of sea and dominant westerly winds, which would probably also apply to *P. alcon*. However, preliminary work comparing populations from Denmark and Sweden [46] suggests that *P. alcon* on Læsø could have potentially received immigrants from mainland Sweden as well, which would have aided genetic differentiation from Danish mainland populations. The *P. alcon/P. rebeli* complex is phylogenetically young (ca 0.77 MYA [32,33]) and it seems reasonable to assume that its entire diversity has been shaped by Pleistocene glacial cycles.

An alternative explanation to the two clusters in Jutland could be that immigration of *P. alcon* has consisted of one long sequence of founder events, becoming more and more genetically impoverished towards the edge of its distribution in northern Jutland, as also discussed by [40]. However, in this case, we would expect lower genetic diversity in the locations with high membership for cluster 3, which we did not find (Figure 7). Furthermore, five alleles turned out to be exclusive to the locations with a high membership for cluster 3 (Figure 6), strongly indicating that cluster 3 is not just a genetic subset of cluster 1.

Interestingly, Rasmussen et al. [72] showed that Danish populations of the European hedgehog *Erinaceus europeaus* Linnaeus are divided into three clusters: one on the major islands Funen, Zealand, Lolland, and Falster; one on the island of Bornholm in the far eastern part of the country; and one in the peninsula of Jutland with no separation across the Limfjord. In contrast to this, the marsh fritillary butterfly *E. aurinia* shows significant differentiation across the Limfjord [73], while in *C. arcensis* [2], the Limfjord acts as a more significant barrier in the western part of Jutland than in the east.

4.2. Genetic Diversity

Our study presents the most comprehensive overview of the genetic diversity of *P. alcon* in Denmark. In contrast to previous studies [40,42], our estimates of effective numbers of alleles did not indicate lower genetic diversity north of the Limfjord. Comparing our data with that from Belgium and the Netherlands [43] shows that Danish populations have about the same effective number of alleles as Belgian and Dutch populations, but the heterozygosity of Danish populations is lower than of Dutch populations (Figure S3). Although comparing genetic diversity between studies using different genetic markers is problematic [74,75], this fits in with other population genetic studies on *P. alcon* across Europe, where populations more central in the distribution range have higher genetic diversity and more peripheral and isolated populations have lower diversity [31,34,44,76]. This pattern is common for many butterfly species [77], including others in the family Lycaenidae [78,79], although it is not so clear in some other *Phengaris* species, such as *P. arion* and *P. teleius*, where effective population sizes in each generation are smaller [49,76,80,81]. Consequently, one would expect to find a lower number of effective alleles in Danish populations compared to Belgian and Dutch populations, as the more northern populations are probably further away from potential glacial refugia south of the Alps. The analysis of changes of genetic diversity through time showed very few significant results. This may have several explanations. One is the null hypothesis: that there simply has not been much change in heterozygosity and the effective number of alleles in these populations. A second explanation could be that generation-to-generation bottlenecks due to ant association mean that low genetic diversity is inherent to the species, independent of census population sizes and demographic bottlenecks, as has been suggested for *P. arion* [49,50,81]. A third explanation could be that the four loci used simply do not provide the resolution for detecting any statistical changes. The clear pattern of isolation by distance detected in the contemporary datasets confirms what earlier studies of the species in Denmark have also found [40]. Our estimates of effective population sizes with finite confidence intervals were generally higher than those of Vanden Broeck et al. [43]. who used the same software with the linkage disequilibrium method.

4.3. Museum Specimens and Historical Population Genetics Assessments

Museum specimens provide an invaluable source of genetic material for population and conservation genetic studies. However, the use of such material also presents several problems. Pinned butterfly specimens are stored together in boxes and, therefore, there is a risk that these specimens may over time become cross-contaminated with each other's DNA. Most of the usable DNA is, however, found inside the exoskeleton and, therefore, this might not present a major issue. Rather, the focus should be on not cross-contaminating samples when working with the extractions and PCR, especially because of the very low endogenous DNA concentrations in these samples. Using museum specimens also resulted in limitations in this study due to the degradation of DNA over time. First, the number of loci available was limited to four in the combined dataset, as markers with alleles longer than 160 bp did not amplify consistently. The quantity of DNA extracted from the museum samples was also very low. This could also have been, at least in part, caused by the practice of "relaxing" dry specimens before setting them. In a pilot study, we obtained DNA concentrations ranging from −1.09 to 63.14 ng/µL, which were highly variable but did seem to be associated with both collection and collector, suggesting that treatments during both preparation and storage are both important (TTJ and DRN, unpublished data). Low concentrations of template DNA for PCR can be a problem, as they increase the risk of allelic dropout [82]. This is especially a problem when evaluating changes in genetic diversity over time, as allelic dropout in heterozygotes would cause an increase in homozygotes in older populations and possibly leave more rare alleles undetected. In this study, it could have caused an underestimate of the heterozygosity and number of effective alleles in the historical populations, thus not allowing the analysis to detect the expected decrease. However, if this was a significant problem, we would also have expected these historical locations to show significant deviations from HWE, which they did not.

4.4. Conservation Implications

The presence of alleles exclusive to locations with high membership for cluster 3 in northern Jutland could indicate that these present an ESU. At least all three clusters include genetic diversity not found elsewhere in Denmark and so they are all important for conserving the genetic diversity of *P. alcon* in Denmark. Several of the locations in the combined dataset with high membership for cluster 3 are thought to have since become extinct, with extant localities limited to Klosterheden, Thy (in this study, Hansted and Vestergård), and Tversted Rimmer. Both Klosterheden and Vestergård show signs of inbreeding. The heterozygosity of the former has decreased since 1967 and the latter also showed signs of inbreeding in the study by Kelager [40]. Lastly, Tversted Rimmer has a very low number of effective alleles. This raises concerns about the conservation status of this cluster. Conservation actions could include reintroduction, as the region still has large natural areas where the butterfly has formerly been present and the host plant *G. pneumonanthe* is still abundant; e.g., on the Skagen peninsula. The low genetic diversity at Tversted Rimmer could be a sign of a recent founder event. This is curious, as the localities it could have immigrated from in the area have all gone extinct in the last two decades. If reintroductions on the Skagen peninsula are ever considered, it could prove important to choose individuals from another locality belonging to this cluster with a higher genetic diversity than Tversted Rimmer to ensure sufficient adaptive potential. The locations with high membership for cluster 1 only showed significant signs of inbreeding at Frøslev Mose, which had the highest G_{IS} of all locations. Intriguingly, this location is also extinct today.

5. Conclusions

In this study, we found support for three genetic clusters of populations of the butterfly *P. alcon* in Denmark. If future studies are able to sample genetic material of *P. alcon* throughout Europe at the same spatial resolution as this study, it might be possible to determine if the geographical origins of cluster 1 and cluster 3 were two different glacial refugia and identify the extent of geneflow between Sweden and cluster 2 on Læsø. Furthermore, this

could shed light on whether Danish, Dutch, and Belgian populations all share the same effective numbers of alleles because they are all populations at the margin of the species' distribution, and whether low genetic diversity is present in all populations of *P. alcon* throughout its range because of the high mortality imposed on eggs and larvae.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/d14121098/s1, Supplementary Methods S1: Preparation of distribution maps for *P. alcon* and *G. pneumonanthe* in Denmark; Table S1: Summary of microsatellite markers used in this study; Table S2: Genotypes and metadata of every individual included in the study; Figure S1: Graphs showing genetic distance plotted against geographic and temporal distances; Figure S2: Selection of optimal values of k for Structure analysis using the ΔK method; Figure S3: Comparison of genetic diversity and inbreeding in Danish, Belgian, and Dutch populations of *P. alcon*.

Author Contributions: Conceptualization, D.R.N. and T.J.S.; methodology, D.R.N., A.M.B.B., T.J.S. and P.H.F.; validation, A.M.B.B., D.R.N., T.J.S. and P.F.T.; formal analysis, A.M.B.B., D.R.N., C.T.C.-J., K.J.S. and T.T.J.; investigation, A.M.B.B., D.R.N., C.T.C.-J., K.J.S., T.J.S. and T.T.J.; resources, D.R.N., T.J.S. and P.F.T.; data curation, A.M.B.B., D.R.N., C.T.C.-J., K.J.S., T.J.S., P.H.F. and T.T.J.; writing—original draft preparation, A.M.B.B., T.J.S. and D.R.N.; writing—review and editing, A.M.B.B., T.J.S., D.R.N. and P.F.T.; visualization, A.M.B.B., D.R.N. and K.J.S.; supervision, D.R.N., T.J.S. and P.F.T.; project administration, T.J.S. and D.R.N.; funding acquisition, T.J.S. and D.R.N. All authors have read and agreed to the published version of the manuscript.

Funding: The project was supported financially by the Research Fund under the Danish Ministry for Culture (KFU) (grant FORM.2017-0006). Funds for fieldwork were provided by Schjøtz-Christensen Memorial Fund, Aarhus, Denmark.

Institutional Review Board Statement: Not applicable.

Data Availability Statement: The data used for this study are available in the Supplementary Materials (Table S2).

Acknowledgments: We thank Sylvia Mathiasen (University of Copenhagen, Denmark) and Annie Brandstrup (Aarhus University, Denmark) for help with DNA lab work. We thank Jan A. Rasmussen (Museum Mors, Denmark), René L. Sylvestersen (Museum Salling, Denmark), Nikolaj K. Larsen (Aarhus University, Denmark), and Anne B. Niselsen (Lund University, Sweden) for assistance with geological terms and interpretations, and palaeobotanical information and interpretations. We thank Eline Lorentzen (University of Copenhagen, Denmark) for elaborate and constructive comments on an earlier version of the manuscript. We are indebted to naturbasen.dk and arter.dk for data on past and present distributions used for the distribution maps. We thank the Danish Ministry for Environment and the Danish Ministry of Defense for permission to conduct fieldwork on government land. We thank Thomas Pape (Natural History Museum of Denmark) for access to the museum's collections of Danish material and permission to extract DNA. We are especially indebted to Svend Kaaber (Aarhus, Denmark) for inspiring the project with his earlier research, access to his important collection of Danish material, permission to extract DNA, and for kindly sharing his considerable knowledge with us.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Levin, G.; Normander, B. *Arealanvendelse i Danmark Siden Slutningen af 1800-Tallet*; Faglig rapport fra DMU 682; Danmarks Miljøundersøgelser: Aarhus, Denmark, 2008.
2. Hansen, A.K.; Justesen, M.J.; Olsen, M.T.; Solodovnikov, A. Genomic population structure and conservation of the red listed *Carabus arcensis* (Coleoptera: Carabidae) in island-mainland habitats of Northern Europe. *Insect Conserv. Divers.* **2018**, *11*, 255–266. [CrossRef]
3. Schmidt, I.K.; Riis-Nielsen, T. Lynghederne. In *Naturen i Danmark: Det Åbne Land*; Vestergaard, P., Ed.; Gyldendal: Copenhagen, Denmark, 2007; pp. 213–246.
4. Brunbjerg, A.K.; Høye, T.T.; Eskildsen, A.; Nygaard, B.; Damgaard, C.F.; Ejrnæs, R. The collapse of marsh fritillary (*Euphydryas aurinia*) populations associated with declining host plant abundance. *Biol. Conserv.* **2017**, *211*, 117–124. [CrossRef]
5. Sigaard, P.; Pertoldi, C.; Madsen, A.B.; Søgaard, B.; Loeschcke, V. Patterns of genetic variation in isolated Danish populations of the endangered butterfly *Euphydryas aurinia*. *Biol. J. Linn. Soc.* **2008**, *95*, 677–687. [CrossRef]
6. Nielsen, O.F. *De Danske Græshopper*; Apollo Books: Stenstrup, Denmark, 2000; p. 192.

7. Odgaard, B.V. Fra Bondestenalder til nutid. In *Naturen i Danmark: Geologien*; Sand-Jensen, K., Ed.; Gyldendal: Copenhagen, Denmark, 2006; pp. 333–360.
8. Wandeler, P.; Hoeck, P.E.; Keller, L.F. Back to the future: Museum specimens in population genetics. *Trends Ecol. Evol.* **2007**, *22*, 634–642. [CrossRef] [PubMed]
9. Holmes, M.W.; Hammond, T.T.; Wogan, G.O.U.; Walsh, R.E.; LaBarbera, K.; Wommack, E.A.; Martins, F.M.; Crawford, J.C.; Mack, K.L.; Bloch, L.M.; et al. Natural history collections as windows on evolutionary processes. *Mol. Ecol.* **2016**, *25*, 864–881. [CrossRef] [PubMed]
10. Eskildsen, A.; Carvalheiro, L.G.; Kissling, W.D.; Biesmeijer, J.C.; Schweiger, O.; Høye, T.T. Ecological specialization matters: Long-term trends in butterfly species richness and assemblage composition depend on multiple functional traits. *Divers. Distrib.* **2015**, *21*, 792–802. [CrossRef]
11. Stoltze, M. *Danske Dagsommerfugle*; Gyldendal: Copenhagen, Denmark, 1996; p. 383.
12. Helsing, F. Dagsommerfugle. In *Den Danske Rødliste 2019*; Moeslund, J.E., Nygaard, B., Ejrnæs, R., Bell, N., Bruun, L.D., Bygebjerg, R., Carl, H., Damgaard, J., Dylmer, E., Elmeros, M., et al., Eds.; DCE—Nationalt Center for Miljø og Energi: Aarhus, Denmark, 2019.
13. Fric, Z.; Wahlberg, N.; Pech, P.; Zrzavý, J. Phylogeny and classification of the *Phengaris-Maculinea* clade (Lepidoptera: Lycaenidae): Total evidence and phylogenetic species concepts. *Syst. Entomol.* **2007**, *32*, 558–567. [CrossRef]
14. ICZN. Opinion 2399 (Case 3508)—*Maculinea* Van Eecke, 1915 (Lepidoptera: Lycaenidae): Precedence over *Phengaris* Doherty, 1891 not granted. *Bull. Zool. Nomencl.* **2017**, *74*, 117–119. [CrossRef]
15. Maes, D.; Van Dyck, H. Habitat quality and biodiversity indicator performances of a threatened butterfly versus a multispecies group for wet heathlands in Belgium. *Biol. Conserv.* **2005**, *123*, 177–187. [CrossRef]
16. Gilmer, M. Die Futterpflanze, das Ei und die junge Raupe von *Lycaena alcon*, F. *Soc. Entomol.* **1902**, *17*, 92–93.
17. Wilcke, J. Nieuwe gegevens over de Biologie van *Lycaena alcon* F. *Tijd Ent* **1947**, *87*, 537–542.
18. Nash, D.R.; Als, T.D.; Maile, R.; Jones, G.R.; Boomsma, J.J. A mosaic of chemical coevolution in a large blue butterfly. *Science* **2008**, *319*, 88–90. [CrossRef] [PubMed]
19. Als, T.D.; Nash, D.R.; Boomsma, J.J. Geographical variation in host-ant specificity of the parasitic butterfly *Maculinea alcon* in Denmark. *Ecol. Entomol.* **2002**, *27*, 403–414. [CrossRef]
20. Nash, D.R.; Andersen, A. *MACULINEA*-sommerfugle og stikmyrer på danske heder-coevolution i tid og rum. *Flora Og Fauna* **2015**, *121*, 133–141.
21. Elmes, G.; Thomas, J.A.; Wardlaw, J. Larvae of Maculinea rebeli, a large-blue butterfly, and their Myrmica host ants: Wild adoption and behaviour in ant-nests. *J. Zool.* **1991**, *223*, 447–460. [CrossRef]
22. Nash, D.R.; Als, T.D.; Boomsma, J.J. Survival and growth of parasitic *Maculinea alcon* caterpillars (Lepidoptera, Lycaenidae) in laboratory nests of three *Myrmica* ant species. *Insectes Sociaux* **2011**, *58*, 391–401. [CrossRef]
23. Kaaber, S. Studies on *Maculinea alcon* (Schiff.) *-rebeli* (Hir.) (Lep. Lycaenidae) with Reference to the Taxonomy, Distribution, and Phylogeny of the Group. *Entomol. Medd.* **1964**, *32*, 277–319.
24. Hirschke, H. Eine neue hochalpine Form der *Lycaena Alcon* F. aus den steirischen Alpen. *Jahresber. Des Wien. Entomol. -Ver.* **1904**, *15*, 109–111.
25. Berger, L. *Maculinea rebeli* Hirschke, bona species. *Lambillionea* **1946**, *46*, 95.
26. Kudrna, O.; Fric, Z.F. On the identity and taxonomic status of *Lycaena alcon rebeli* HIRSCHKE, 1905—a long story of confusion and ignorance resulting in the fabrication of a "ghost species" (Lepidoptera: Lycaenidae). *Nachr. Des Entomol. Ver. Apollo* **2013**, *34*, 117–124.
27. Beuret, H. Contribution a l'ètude du groupe *Maculinea alcon* Schiff.*-rebeli* Hirschke (Lep. Lycaenidae). *Bull. De La Soc. Entomol. De Mulhouse* **1949**, *1949*, 1–6, 10–14, 17–21, 25–28, 33–36, 41–44, 50–51.
28. Habeler, H. Die subalpin-alpinen Lebensräume des Bläulings Maculinea rebeli (HIRSCHKE, 1904) in den Ostalpen (Lepidoptera, Lycaenidae). *Joannea Zool* **2008**, *10*, 143–164.
29. Pecsenye, K.; Bereczki, J.; Tihanyi, B.; Tóth, A.; Peregovits, L.; Varga, Z. Genetic differentiation among the *Maculinea* species (Lepidoptera: Lycaenidae) in eastern Central Europe. *Biol. J. Linn. Soc.* **2007**, *91*, 11–21. [CrossRef]
30. Tartally, A.; Kelager, A.; Fürst, M.; Nash, D. Host plant use drives genetic differentiation in syntopic populations of *Maculinea alcon*. *PeerJ* **2016**, *4*, e1865. [CrossRef] [PubMed]
31. Bereczki, J.; Pecsenye, K.; Peregovits, L.; Varga, Z. Pattern of genetic differentiation in the *Maculinea alcon* species group (Lepidoptera, Lycaenidae) in Central Europe. *J. Zool. Syst. Evol. Res.* **2005**, *43*, 157–165. [CrossRef]
32. Als, T.D.; Vila, R.; Kandul, N.; Nash, D.R.; Yen, S.H.; Hsu, Y.; Mignault, A.; Boomsma, J.J.; Pierce, N.E. The evolution of alternative parasitic life histories in large blue butterflies. *Nature* **2004**, *432*, 386–390. [CrossRef]
33. Ugelvig, L.V.; Vila, R.; Pierce, N.E.; Nash, D.R. A phylogenetic revision of the *Glaucopsyche* section (Lepidoptera: Lycaenidae), with special focus on the *Phengaris-Maculinea* clade. *Mol. Phylogenetics Evol.* **2011**, *61*, 237–243. [CrossRef]
34. Sielezniew, M.; Rutkowski, R.; Ponikwicka-Tyszko, D.; Ratkiewicz, M.; Dziekańska, I.; Švitra, G. Differences in genetic variability between two ecotypes of the endangered myrmecophilous butterfly *Phengaris* (= *Maculinea*) *alcon*—the setting of conservation priorities. *Insect Conserv. Divers.* **2012**, *5*, 223–236. [CrossRef]
35. Tartally, A.; Koschuh, A.; Varga, Z. The re-discovered *Maculinea rebeli* (Hirschke, 1904): Host ant usage, parasitoid and initial food plant around the type locality with taxonomical aspects (Lepidoptera, Lycaenidae). *Zookeys* **2014**, *406*, 25–40. [CrossRef]

36. Tartally, A.; Thomas, J.; Anton, C.; Balletto, E.; Barbero, F.; Bonelli, S.; Bräu, M.; Casacci, L.; Csősz, S.; Czekes, Z.; et al. Patterns of host use by brood parasitic *Maculinea* butterflies across Europe. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **2019**, *374*, 20180202. [CrossRef]
37. Bereczki, J.; Pecsenye, K.; Varga, Z.; Tartally, A.; TÓTH, J.P. *Maculinea rebeli* (Hirschke)—a phantom or reality? Novel contribution to a long-standing debate over the taxonomic status of an enigmatic Lycaenidae butterfly. *Syst. Entomol.* **2018**, *43*, 166–182. [CrossRef]
38. Fraser, D.J.; Bernatchez, L. Adaptive evolutionary conservation: Towards a unified concept for defining conservation units. *Mol. Ecol.* **2001**, *10*, 2741–2752. [CrossRef] [PubMed]
39. Ryder, O.A. Species conservation and systematics: The dilemma of subspecies. *Trends Ecol. Evol.* **1986**, *1*, 9–10. [CrossRef]
40. Kelager, A. Biotic Interactions in Space and Time—The Social Parasitic Butterfly *Maculinea alcon* and its Hosts as Model System. PhD Thesis, University of Copenhagen, Copenhagen, Denmark, 2015.
41. Colding-Jørgensen, C.T. Current Genetic Structure of *M. alcon* in Denmark. Bachelor Project Thesis, University of Copenhagen, Copenhagen, Denmark, 2020.
42. Gadeberg, R.M.E.; Boomsma, J.J. Genetic population structure of the large blue butterfly *Maculinea alcon* in Denmark. *J. Insect Conserv.* **1997**, *1*, 99–111. [CrossRef]
43. Vanden Broeck, A.; Maes, D.; Kelager, A.; Wynhoff, I.; WallisDeVries, M.F.; Nash, D.R.; Oostermeijer, J.G.B.; Van Dyck, H.; Mergeay, J. Gene flow and effective population sizes of the butterfly *Maculinea alcon* in a highly fragmented, anthropogenic landscape. *Biol. Conserv.* **2017**, *209*, 89–97. [CrossRef]
44. Dziekańska, I.; Nowicki, P.; Pirożnikow, E.; Sielezniew, M. A Unique Population in a Unique Area: The Alcon Blue Butterfly and Its Specific Parasitoid in the Białowieża Forest. *Insects* **2020**, *11*, 687. [CrossRef]
45. Hauge, M. Change in Genetic Variation of *Maculinea alcon* Populations Over Time. MSc Thesis, University of Copenhagen, Copenhagen, Denmark, 2016.
46. Lomborg, A.E. Population Genetics of *Maculinea* Species in Southern Scandinavia. MSc Thesis, University of Copenhagen, Copenhagen, Denmark, 2006.
47. Bijlsma, R.; Bundgaard, J.; Boerema, A. Does inbreeding affect the extinction risk of small populations? Predictions from *Drosophila*. *J. Evol. Biol.* **2000**, *13*, 502–514. [CrossRef]
48. Saccheri, I.; Kuussaari, M.; Kankare, M.; Vikman, P.; Fortelius, W.; Hanski, I. Inbreeding and extinction in a butterfly metapopulation. *Nature* **1998**, *392*, 491–494. [CrossRef]
49. Ugelvig, L.; Andersen, A.; Boomsma, J.; Nash, D. Dispersal and gene flow in the rare, parasitic Large Blue butterfly *Maculinea arion*. *Mol. Ecol.* **2012**, *21*, 3224–3236. [CrossRef]
50. Ugelvig, L.; Nielsen, P.; Boomsma, J.; Nash, D. Reconstructing eight decades of genetic variation in an isolated Danish population of the large blue butterfly *Maculinea arion*. *BMC Evol. Biol.* **2011**, *11*, 201. [CrossRef]
51. Hamm, C.A.; Aggarwal, D.; Landis, D.A. Evaluating the impact of non-lethal DNA sampling on two butterflies, *Vanessa cardui* and *Satyrodes eurydice*. *J. Insect Conserv.* **2010**, *14*, 11–18. [CrossRef]
52. Meirmans, P. Genodive version 3.0: Easy-to-use software for the analysis of genetic data of diploids and polyploids. *Mol. Ecol. Resour.* **2020**, *20*, 1126–1131. [CrossRef] [PubMed]
53. Pritchard, J.K.; Stephens, M.; Donnelly, P. Inference of population structure using multilocus genotype data. *Genetics* **2000**, *155*, 945–959. [CrossRef] [PubMed]
54. Evanno, G.; Regnaut, S.; Goudet, J. Detecting the number of clusters of individuals using the software STRUCTURE: A simulation study. *Mol. Ecol.* **2005**, *14*, 2611–2620. [CrossRef]
55. Wickham, H. *Ggplot2—Elegant Graphics for Data Analysis*; Springer New York: New York, NY, USA, 2009.
56. Yu, G.; Smith, D.K.; Zhu, H.; Guan, Y.; Lam, T.T.Y. Ggtree: An R package for visualization and annotation of phylogenetic trees with their covariates and other associated data. *Methods Ecol. Evol.* **2017**, *8*, 28–36. [CrossRef]
57. Kassambara, A. *ggpubr: 'ggplot2' Based Publication Ready Plots*, R package version 0.4.0; R Foundation for Statistical Computing: Vienna, Austria, 2020.
58. Excoffier, L.; Smouse, P.E.; Quattro, J.M. Analysis of molecular variance inferred from metric distances among DNA haplotypes: Application to human mitochondrial DNA restriction data. *Genetics* **1992**, *131*, 479–491. [CrossRef]
59. Chen, H.; Boutros, P. VennDiagram: A package for the generation of highly-customizable Venn and Euler diagrams in R. *BMC Bioinform.* **2011**, *12*, 35. [CrossRef]
60. Michalakis, Y.; Excoffier, L. A generic estimation of population subdivision using distances between alleles with special reference for microsatellite loci. *Genetics* **1996**, *142*, 1061–1064. [CrossRef]
61. Mantel, N. The detection of disease clustering and a generalized regression approach. *Cancer Res.* **1967**, *27*, 209–220.
62. Thioulouse, J.; Dray, S.; Dufour, A.-B.; Siberchicot, A.; Jombart, T.; Pavoine, S. *Multivariate Analysis of Ecological Data with Ade4*; Springer New York: New York, NY, USA, 2018.
63. R Core Team. *R: A Language and Environment for Statistical Computing*; Version 4.1.0; R Foundation for Statistical Computing: Vienna, Austria, 2021; Available online: https//www.R-project.org (accessed on 30 October 2022).
64. Waples, R.S. A generalized approach for estimating effective population size from temporal changes in allele frequency. *Genetics* **1989**, *121*, 379–391. [CrossRef]
65. Nei, M.; Tajima, F. Genetic drift and estimation of effective population size. *Genetics* **1981**, *98*, 625–640. [CrossRef] [PubMed]

66. Do, C.; Waples, R.; Peel, D.; Macbeth, G.; Tillett, B.; Ovenden, J. NeEstimator v2: Re-implementation of software for the estimation of contemporary effective population size (N_e) from genetic data. *Mol. Ecol. Resour.* **2014**, *14*, 209–214. [CrossRef] [PubMed]
67. Noe-Nygård, N.; Knudsen, K.-L.; Houmark-Nielsen, M. Fra istid til og med jægerstenalder. In *Naturen i Danmark: Geologien*; Sand-Jensen, K., Ed.; Gyldendal: Copenhagen, Denmark, 2006; pp. 303–332.
68. Hansen, A. Gentianaceernes, Menyanthaceernes, Asclepiadaceernes og Apocynaceernes udbredelse i Danmark. In *Danmarks Topografisk-Botaniske Undersøgelse*; Dansk Botanisk Forening: Copenhagen, Denmark, 1958; Volume 24, pp. 305–332.
69. Odgaard, B.V. The Holocene vegetation history of northern West Jutland, Denmark. *Opera Bot.* **1994**, *123*, 1–171. [CrossRef]
70. Hansen, J.M.; Aagaard, T.; Stockmarr, J.; Møller, I.; Nielsen, L.; Binderup, M.; Larsen, J.H.; Larsen, B. Continuous record of Holocene sea-level changes and coastal development of the Kattegat island Læsø (4900 years BP to present). *Bull. Geol. Soc. Den.* **2016**, *64*, 1–55. [CrossRef]
71. Hansen, A.K.; Justesen, M.J.; Kepfer-Rojas, S.; Byriel, D.B.; Pedersen, J.; Solodovnikov, A. Ecogeographic patterns in a mainland-island system in Northern Europe as inferred from the rove beetles (Coleoptera: Staphylinidae) on Laeso island. *Eur. J. Entomol.* **2018**, *115*, 256–263. [CrossRef]
72. Rasmussen, S.; Nielsen, J.; Jones, O.; Berg, T.; Pertoldi, C. Genetic structure of the European hedgehog (*Erinaceus europaeus*) in Denmark. *PLoS ONE* **2020**, *15*, e0227205. [CrossRef]
73. Pertoldi, C.; Ruiz-Gonzalez, A.; Bahrndorff, S.; Renee Lauridsen, N.; Nisbeth Henriksen, T.; Eskildsen, A.; Høye, T. Strong isolation by distance among local populations of an endangered butterfly species (*Euphydryas aurinia*). *Ecol. Evol.* **2021**, *11*, 12790–12800. [CrossRef]
74. Meirmans, P.; Hedrick, P. Assessing population structure: F(ST) and related measures. *Mol. Ecol. Resour.* **2011**, *11*, 5–18. [CrossRef]
75. Zhang, D.-X.; Hewitt, G.M. Nuclear DNA analyses in genetic studies of populations: Practice, problems and prospects. *Mol. Ecol.* **2003**, *12*, 563–584. [CrossRef]
76. Bereczki, J.; Tóth, J.P.; Sramkó, G.; Varga, Z. Multilevel studies on the two phenological forms of Large Blue (*Maculinea arion*) (Lepidoptera: Lycaenidae). *J. Zool. Syst. Evol. Res.* **2014**, *52*, 32–43. [CrossRef]
77. Dincă, V.; Dapporto, L.; Somervuo, P.; Vodă, R.; Cuvelier, S.; Gascoigne-Pees, M.; Huemer, P.; Mutanen, M.; Hebert, P.; Vila, R. High resolution DNA barcode library for European butterflies reveals continental patterns of mitochondrial genetic diversity. *Commun. Biol.* **2021**, *4*, 315. [CrossRef] [PubMed]
78. Habel, J.C.; Schmitt, T. The genetic consequences of different dispersal behaviours in Lycaenid butterfly species. *Bull. Entomol. Res.* **2009**, *99*, 513–523. [CrossRef] [PubMed]
79. Krauss, J.; Schmitt, T.; Seitz, A.; Steffan-Dewenter, I.; Tscharntke, T. Effects of habitat fragmentation on the genetic structure of the monophagous butterfly *Polyommatus coridon* along its northern range margin. *Mol. Ecol.* **2004**, *13*, 311–320. [CrossRef]
80. Sielezniew, M.; Patricelli, D.; Rutkowski, R.; Witek, M.; Bonelli, S.; Buś, M.M. Population genetics of the endangered obligatorily myrmecophilous butterflyPhengaris(=Maculinea)arionin two areas of its European range. *Insect Conserv. Divers.* **2015**, *8*, 505–516. [CrossRef]
81. Andersen, A. Population genetics of reintroduced and source populations of the socially parasitic Large Blue Butterfly *Maculinea arion*. MSc Thesis, Department of Biology, University of Copenhagen, Copenhagen, Denmark, 2012.
82. Taberlet, P.; Luikart, G. Non-invasive genetic sampling and individual identification. *Biol. J. Linn. Soc.* **1999**, *68*, 41–55. [CrossRef]