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# Red-Backed Shrike *Lanius collurio* Whole-Genome Sequencing Reveals Population Genetic Admixture

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**Abstract:** The Red-backed Shrike (*Lanius collurio*) is a medium-sized, carnivorous passerine, occurring throughout the western Palearctic. As with numerous other bird species, its numbers are declining, mainly due to anthropogenic factors. Therefore, revealing the population structure and genetic diversity is paramount in ensuring the survival of the species. However, until present, only mitochondrial DNA has been targeted to reveal the genetic structure of the species. These studies suggested a panmictic population structure. In this study, we employed next-generation sequencing of 88 Red-backed Shrikes from 11 countries and used single nucleotide polymorphisms (SNPs)) to investigate the population structure. Even with such high-resolution DNA data, we found considerable genetic variability, but our results indicate no genetic structure in the Red-backed Shrike, suggesting a panmictic population. Migrant birds from Israel and Kuwait could not be attributed to breeding populations. Panmixia is the genetic legacy of the widespread and continuous distribution of the species, high locomotion capacities, and, most importantly, the numerous ice ages from the past few million years, which forced various populations to retract to refugia and expand their ranges several times, and to interbreed both in the glacial refugia and during warm periods in Eurasia.

**Keywords:** whole-genome sequencing; SNPs; Z chromosome; Red-backed Shrike; population genomics; panmixia; Western Palearctic; migration



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## 1. Introduction

DNA sequencing began a new era in population genetics in the late 1970s with the arrival of the Sanger sequencing technique [1]. This technique endowed researchers with the ability to assess population differentiation at the molecular level. At its core, the study of population genetics was a combination of Darwin's natural selection theory and Mendel's genetic principles, as emphasised by the early researchers in the field [2–4]. This indicates that, previously, population genetic research was performed while considering morphological and behavioural aspects coupled with theoretical features [5]. Current DNA high-throughput sequencers [6] generate an outstanding amount of genomic data, allowing for unprecedented views on variation patterns among individuals and species alike [7]. Once the technological capabilities of sequencing became so efficient, their running costs subsequently decreased, making them accessible to a wider range of research groups. Following this, it became possible to expand focus beyond model organisms, e.g., *Drosophila melanogaster* and *Mus musculus*, and to study species whose genetic backgrounds were unknown. In the past decade, the number of genomes from non-model organisms has grown at an astonishing rate [8], which broadened our understanding of population history in terms of genetic drift, rate of mutation occurrence, gene flow, and even natural selection [9].

Avian genomics has played a crucial role in the development of non-model organism genomics, mainly through the efforts of the Birds 10K Avian Phylogenomics Consortium [10]. This international initiative aims to obtain genomes from all extant bird species

(around 11,000), with circa 450 genomes already assembled [11]. The history of avian genomics unfolded relatively slowly in the beginning of the omics era [12–14], with most sequencing aimed at resolving avian phylogenies [15–17] or targeting species with high economic importance such as the chicken (*Gallus gallus*) [18] and the turkey (*Meleagris gallopavo*) [19]. However, in the past few years, increasing numbers of studies have employed high-throughput sequencing to decode the population genetic background, evolutionary history, and speciation of various other bird species [20–24].

The shrike family (Aves, Laniidae) currently comprises a total of 34 species, divided into four genera: *Corvinella*, *Urosteles*, *Eurocephalus*, and *Lanius* [25], the last one being considered true shrikes. Until present, apart from sequencing the genome of the Loggerhead Shrike (*Lanius ludovicianus*) by the B10K consortium, no next-generation sequencing (NGS) research has targeted the shrike family. Nonetheless, a number of genetic studies have focused on shrike species, notably to elucidate phylogeographical [26–31] and speciation patterns [26,32–34]. In the western Palearctic, the most abundant shrike species is the Red-backed Shrike *Lanius collurio* (RBS), with more than 6 million breeding pairs [35,36]. Previous mitochondrial DNA research on the RBS has revealed population genetic variability, but admixture across the birds from western Palearctic, although two haploclades without geographic separation, are obvious [37].

Mitochondrial DNA can reveal the phylogeographic details of recent evolutionary history, but nuclear DNA should be more informative regarding the distant past and offer data on separation events going back many thousands of years. In this study, we used next-generation DNA sequencing and SNPs to elucidate the population genetic structure of the breeding RBS in the western Palearctic from nuclear DNA. We also explored if SNPs could help identify the breeding site of migrant birds captured in Israel and Kuwait.

## 2. Materials and Methods

### *2.1. Sampling, DNA Extraction, and Sequencing*

We obtained blood and tissue samples from 88 Red-backed Shrikes in 11 countries: Bulgaria, Czech Republic, Germany, Israel, Kuwait, Latvia, Norway, Poland, Romania, Russia, and Sweden (see Figure 1). Birds were captured and sampled by experienced bird ringers, following national regulations. All samples were from breeding birds, except the birds from Israel and Kuwait, which are migratory individuals. Complete details of the samples used are found in Table S1. We extracted genomic DNA following a classic phenol-chloroform protocol [38]. After isolation, we quantified DNA quality with a WPA Biowave II spectrophotometer (Biochrom Ltd, Cambridge, UK).

Whole-genome sequencing was performed by Berry Genomics (Beijing, China) at 15× coverage, with paired ends, on an Illumina Novaseq 6000 machine. Sequencing was completed in two batches: the first in January 2018 (n = 56) and the second in December 2018 (n = 32).

### *2.2. NGS Data Analysis*

We assembled the raw sequences (fastq files) and mapped them using the Burrows–Wheeler Aligner [39]. We employed the complete genome of the Loggerhead Shrike (*Lanius ludovicianus*) as a reference. Currently, this is the only complete genome available for the shrike family. It split from the RBS circa 8 Mya [26]. In the new alignments, we removed the clonal reads via SAMtools [40], with the rmdup command. Using the same software, we created a multiple VCF file containing the genomic variation from all 88 alignments. We then filtered the VCF file by (i) removing the indels and (ii) setting a minor allele frequency (MAF) of 0.1. We added this filtering step as some software requires the indels to be removed, while the second procedure helped to exclude errors in sequencing. Until this stage, our methodology closely resembles the technique employed by the Genome Analysis Toolkit (GATK) pipeline as best practice for variant discovery [41].

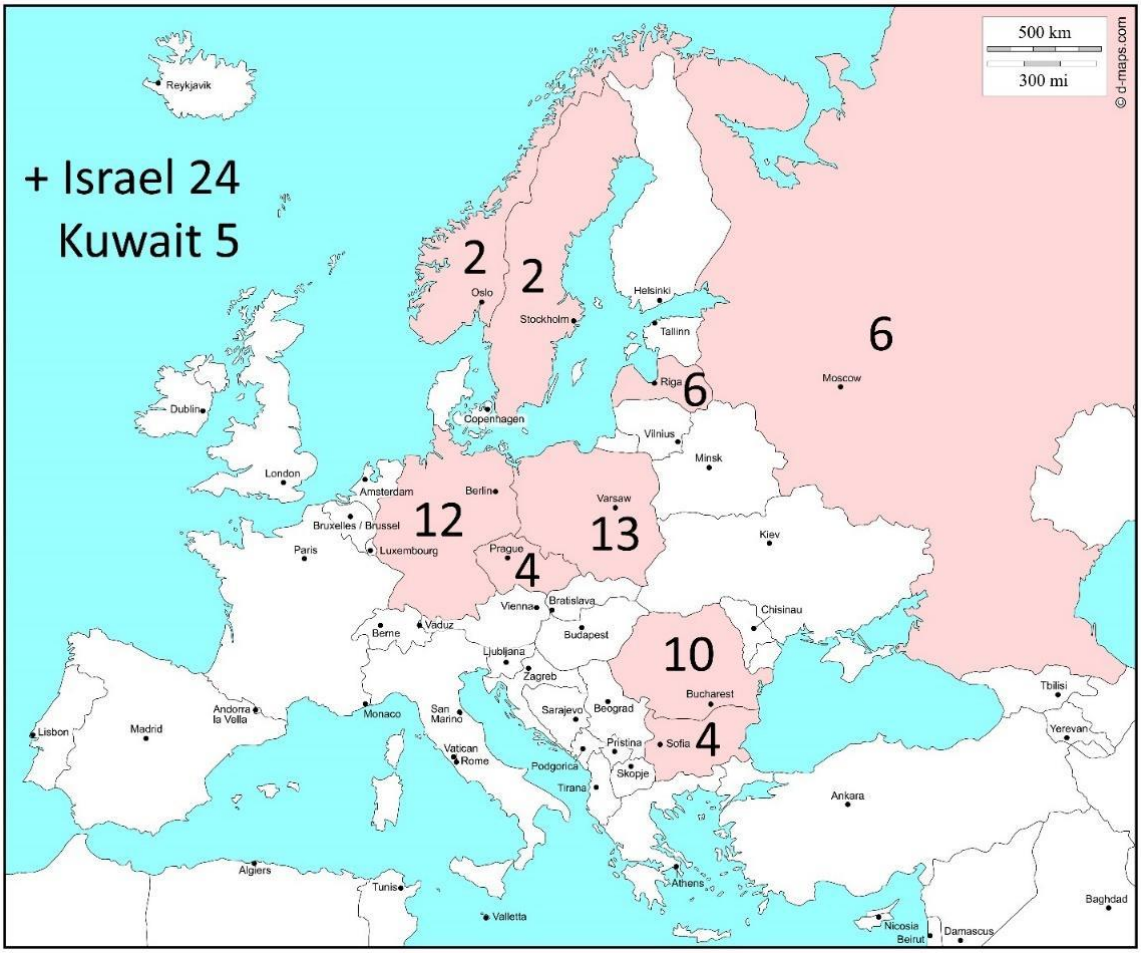


**Figure 1.** Map with countries from which we obtained samples, as well as the total number of samples per country.

For the first analysis step, we performed a principal component analysis (PCA) via PLINK [42] and used R [43] to visualise the results. We further investigated population membership of our individuals using Admixture software [44], and for visualising the runs, we uploaded the output files in the online POPHELPER Structure Web App v1.0.10 (https://roymf.shinyapps.io/structure/, accessed on 14 January 2020). Third, we constructed a tree based on single nucleotide polymorphisms (SNPs) from the sex chromosome Z. To do so, we initially downloaded the chromosome Z from a Collared Flycatcher (*Ficedula albicollis*) (NC_021700) from GenBank and then compared it to our Loggerhead Shrike genome reference, from which we then extracted the same scaffolds. Finally, with the list of scaffolds belonging to the chromosome Z, we were able to extract the respective scaffolds from our multiple VCF file. Via the vcf2phylo pipeline [45], we extracted the chromosome SNPs and built an IQ-TREE [46]. We visualised the results in FigTree 1.4.4 [47].

A schematic representation of our NGS data analysis workflow is available in Figure 2.

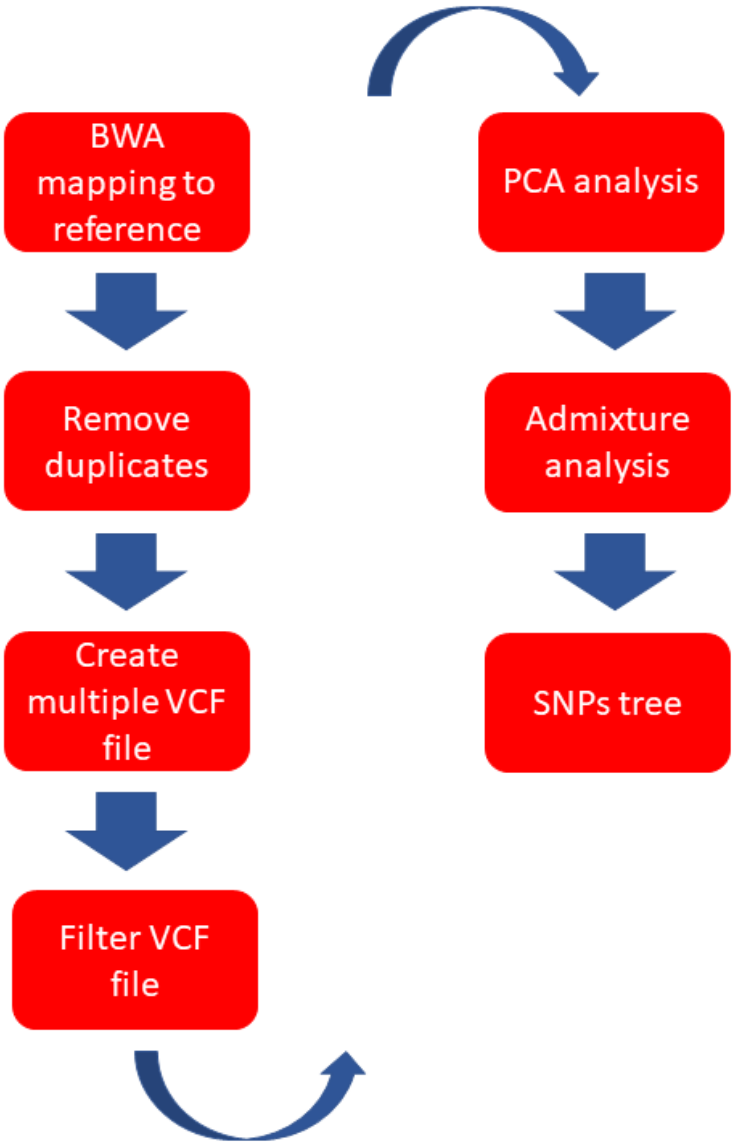


**Figure 2.** Schematic representation of our complete NGS data analysis workflow.

## 3. Results

We obtained short-read (i.e., 150 base pairs) whole-genome sequences for 88 individuals. On average, each sequence had 63,883,753 clean reads and 19,165,126,023 clean bases (Table 1, and full details in Table S1). Our PCA did not reveal any group structure among the 11 populations in our dataset (Figure 3). Although with minimal variation, it appears that several of the German birds are different, but this aspect was not confirmed by our other analyses.

**Table 1.** Average quality values of the 88 whole-genome sequences.

| | Raw Reads | Raw Bases | Clean Reads | Clean Bases | Read Length | Q30 Percent |
|---|---|---|---|---|---|---|
| **Average** | 64,015,481 | 19,204,644,286 | 63,883,753 | 19,165,126,023 | 150;150 | 94.00;91.22 |

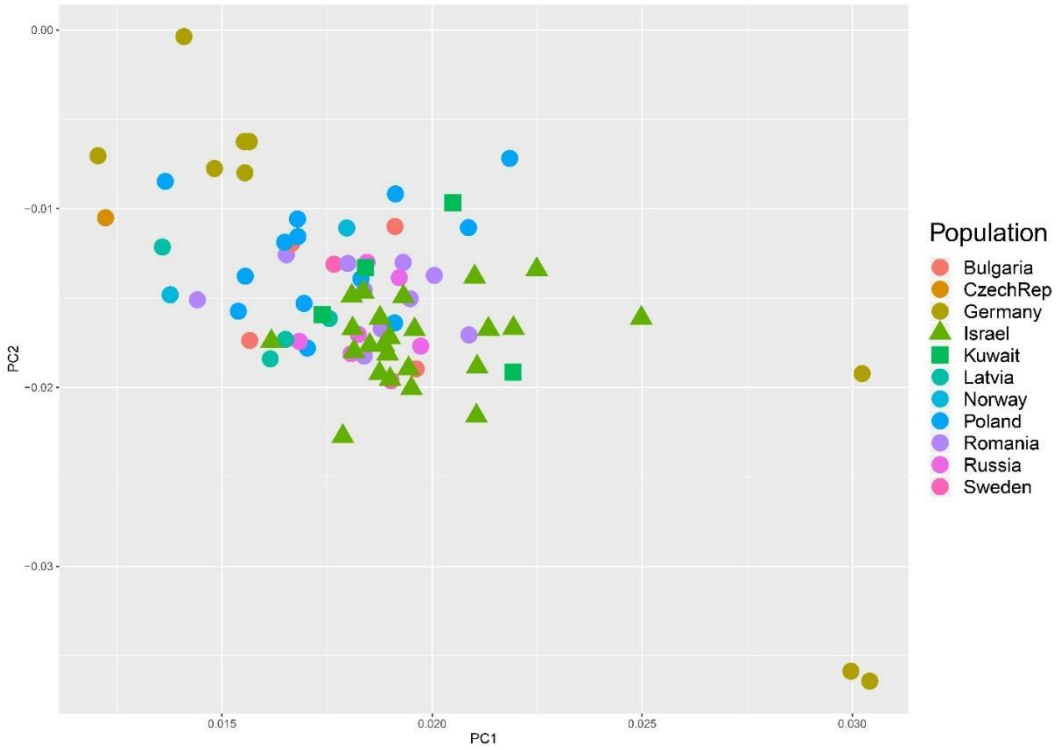


**Figure 3.** PCA containing both breeding and migratory individuals included in our dataset. Breeding birds are represented by circles, while migratory individuals are distinguished by triangles (Israel) and squares (Kuwait).

For the Admixture software analysis, our assumptions that the birds are subclustered were also proven incorrect (Figure 4). From K = 2 until K = 7, there is no stand-alone population. All individuals from the 11 countries share genetic elements. For example, breeding shrikes from Germany do not show a singular population structure; the corresponding genetic elements also occur in other countries. Furthermore, the $F_{ST}$ values for the admixture analysis (Table 2) offer additional support for almost complete panmixia, with the overwhelming majority of values between 0.051 and 0.097. The only three values that do not fit in this range are from the Czech Republic: the first, compared with the Bulgarian population, at 0.102; the second, compared with Israel, at 0.113; and the third, compared with Latvia, at 0.103. Overall, the population from the Czech Republic has the highest $F_{ST}$ variation (average = 0.094) compared to the other groups. However, in terms of $F_{ST}$ theoretical values, this is still considered almost complete panmixia.

**Table 2.** Fst values for Admixture analysis, considering each of the 11 countries as possible populations.

| | **Bulgaria** | **CzechRep** | **Germany** | **Israel** | **Kuwait** | **Latvia** | **Norway** | **Poland** | **Romania** | **Russia** | **Sweden** |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Bulgaria** | | | | | | | | | | | |
| **CzechRep** | 0.102 | | | | | | | | | | |
| **Germany** | 0.074 | 0.095 | | | | | | | | | |
| **Israel** | 0.093 | 0.113 | 0.085 | | | | | | | | |
| **Kuwait** | 0.072 | 0.093 | 0.064 | 0.083 | | | | | | | |
| **Latvia** | 0.082 | 0.103 | 0.074 | 0.093 | 0.072 | | | | | | |
| **Norway** | 0.064 | 0.085 | 0.056 | 0.075 | 0.054 | 0.064 | | | | | |
| **Poland** | 0.067 | 0.087 | 0.059 | 0.078 | 0.057 | 0.067 | 0.049 | | | | |
| **Romania** | 0.067 | 0.087 | 0.059 | 0.077 | 0.057 | 0.067 | 0.048 | 0.052 | | | |
| **Russia** | 0.077 | 0.097 | 0.069 | 0.087 | 0.067 | 0.077 | 0.059 | 0.062 | 0.061 | | |
| **Sweden** | 0.069 | 0.090 | 0.061 | 0.080 | 0.059 | 0.069 | 0.051 | 0.054 | 0.053 | 0.063 | |


**Figure 4.** Admixture analysis (K = 7), containing the 11 populations in our dataset. In each row, the number of colours indicates the possible number of subgroups in the dataset (as indicated on the y axis).

In several taxa, the DNA from Z sex chromosome was highly informative [48,49]. Our last tool in elucidating the population structure of the RBS, the chromosome Z SNPs tree, also indicates panmixia. Birds from the same country do not cluster together. Instead, there is a general admixture, with individuals occupying random positions on the tree branches, regardless of their natal origin. Migrating birds captured in Israel and Kuwait could not be attributed to a breeding population (Figures 5 and 6).

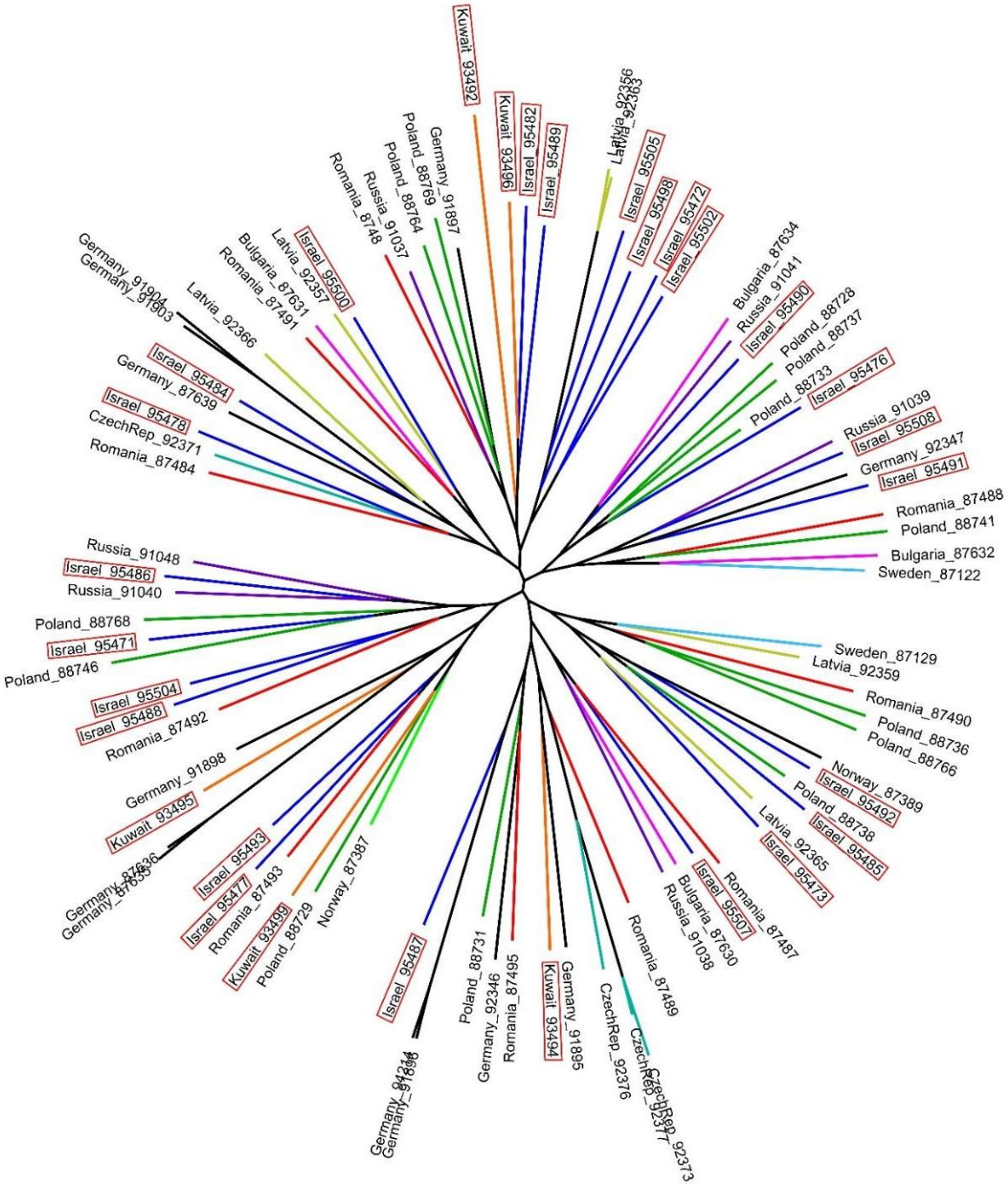


**Figure 5.** Maximum likelihood phylogenetic tree based on the chromosome Z SNPs from both breeding and migrant individuals. Migrant individuals from Israel and Kuwait are highlighted with a red rectangle.

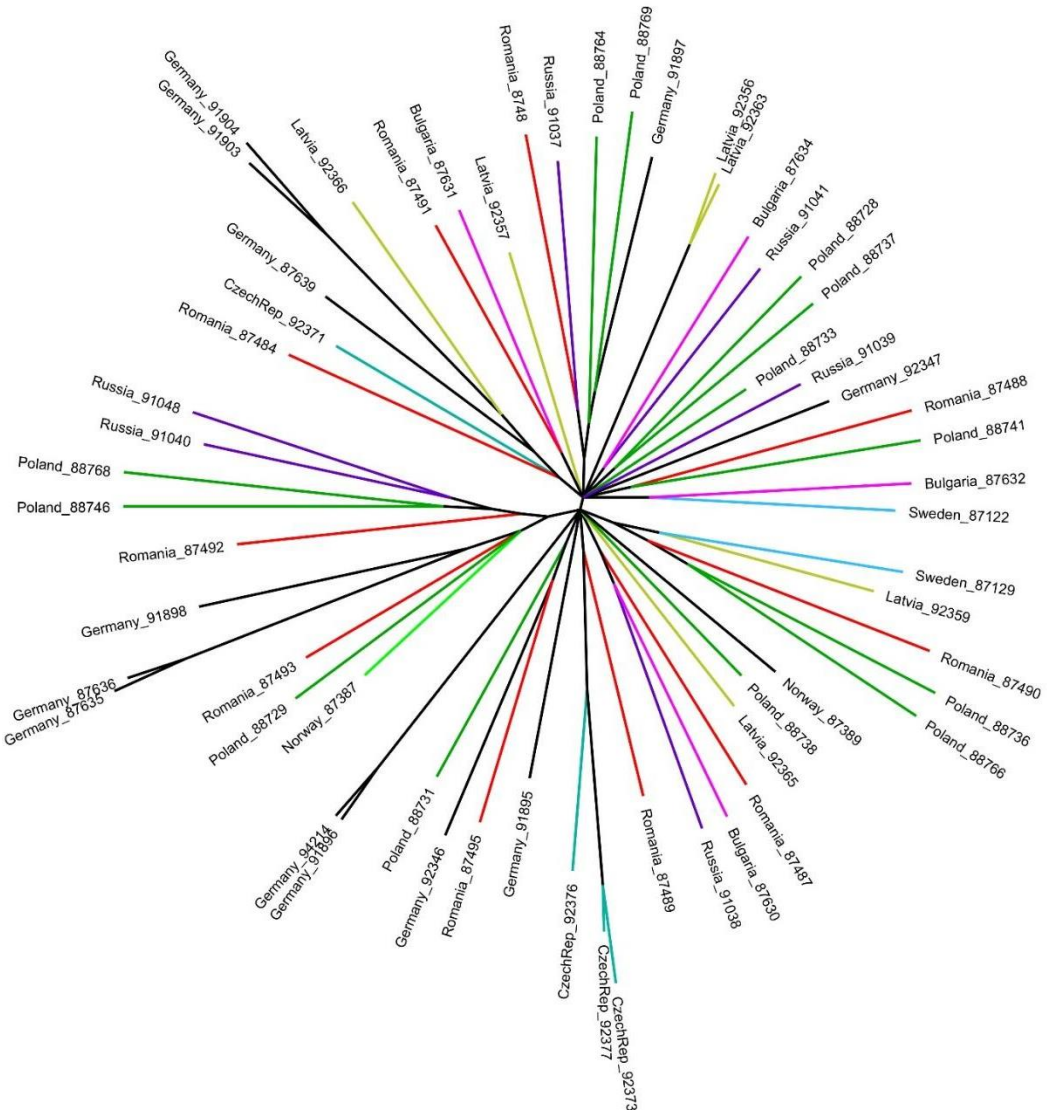


**Figure 6.** Maximum likelihood phylogenetic tree based on the chromosome Z SNPs, only from breeding individuals.

## 4. Discussion

Our insight into the population genetic structure of the Red-backed Shrike, provided by next-generation sequencing of 88 individuals from 11 countries, revealed complete admixture, also known as panmixia. These findings confirm the results from our previous study, based only on mitochondrial DNA [37]. Across the western Palearctic, genomic studies have indicated population differentiation in a number of species. For the Saker Falcon (*Falco cherrug*), SNP sets differentiated between birds of central Europe, eastern Europe, and central Asia, with the westernmost populations showing demographic isolation [20]. For the Great Tit (*Parus major*), all continental European populations share a common genetic background, except the birds from Spain, Corsica, and Sardinia; similarly, birds from the UK also exist within their own cluster [21]. For Barn Swallows (*Hirundo rustica*) breeding in Sweden, Germany, and Switzerland, an assessment based on ddRAD, mtDNA sequencing, and microsatellites did not detect any unique genetic elements between the populations [23]. However, the European Turtle Dove (*Streptopelia turtur*) is characterised by population genetic admixture, according to a study that employed both RAD and mtDNA sequencing [22].

Unfortunately, we could not assess the natal origin of the RBSs sampled along the migratory flyways in Israel and Kuwait, which represented a special point of interest for us. For a number of species, previous studies were successful in tracking population membership of migrating birds. Using stable isotopes, Kloskowski et al. [50] were able to connect wintering areas with breeding areas for the Red-necked Grebe (*Podiceps grisegena*). Employing the same technique, Guillemain et al. retraced the natal origin of Greylag Geese (*Anser anser*) wintering in Camargue, France [51]. European breeding Ortolan Buntings (*Emberiza hortulana*) were also assigned to their east African wintering grounds based on stable isotopes extracted from tail feathers [52]. Last, migrating Pied Flycatchers (*Ficedula hypoleuca*) and European Robins (*Erithacus rubecula*) over the Italian Alps were connected to their breeding areas via stable isotopes as well as bird ringing data [53]. The isotope analysis, however, offers only limited information, as it reflects the recent situation, whereas DNA data go back thousands and even millions of years.

Our panmixia results for the RBS are in concordance with the general situation of bird species inhabiting the western Palearctic. Out of 145 species that have been the focus of population genetic studies, the majority of them show genetic admixture, as indicated by a recent literature review [54]. Only species on the Atlantic islands or on separated mountain ranges show a higher degree of genetic isolation and speciation. Extended gene flow among populations on the landmass of the western Palearctic leads to homogenisation of mutations and contributes to general admixture, as shown in the Northern Wheatear (*Oenanthe oenanthe*) [55]. Furthermore, as in the case of the Red-backed Shrike, low philopatry rates [56] also significantly reduce population differentiation. Overall, for avian species, their high locomotion capacity also acts as a catalyst for gene flow, dispersal, and admixture [57].

Although the phylogeography and population history of other highly mobile vertebrates, e.g., Killer Whales [58] and saltwater fishes [59], have been resolved via high-throughput sequencing, we think the flying ability of many bird species represents a strong underlying factor for their general genetic admixture. This is particularly evident in comparison to several tropical passerines, which have reduced flying capabilities and show a thoroughly differentiated genetic structure. Furthermore, the numerous ice ages in the past few million years had a crucial influence on the panmixia of the majority of bird species in the northern hemisphere. These glaciations and the harsh conditions they imposed forced bird populations to retreat into southern refugia, where different lineages mixed. During the interglacial periods, when the ice pack melted and the majority of the hemisphere was once again hospitable, these bird populations expanded and lineages from different refugia came into contact. These consistent and consecutive admixtures of lineages in the past millennia were a decisive cause in the panmixia of modern bird species in the northern hemisphere.

In theory, population membership can be linked to certain genetic variation, but in practice, population differentiation ranges from complete isolation to full panmixia [60]. There are still limitations in understanding population histories from large, whole-genome datasets, especially regarding the number of samples, magnitude of sequencing depth, and complexity of computational analyses, among others [61]. Although an array of performant analysis software [62] has helped elucidate the history and dynamics of many species, the current bioinformatic pipelines still show limitations for selecting population specific genetic elements [63]. Today, we are witnessing the generation of datasets containing thousands of genomes; the main challenge derived from the ever-increasing size of these datasets is building software capable of processing the amount of data. However, as more efficient software and scanning algorithms emerge, coupled with the constantly improving sequencing techniques [6], future research on population genomics will greatly improve and further shed light on evolutionary aspects of the natural world.

## 5. Conclusions

High-throughput sequencing of 88 RBSs from 11 countries and subsequent data analysis could neither detect an unambiguous population genetic substructure nor could it allocate migrating RBS from Israel and Kuwait to their natal populations. Although genetic variation is evident, neither PCA, admixture analysis, nor SNP tree investigation could provide us with differentiating elements between the 88 samples used in the current study. Our results are in concordance with mtDNA studies of RBS and previous research on western Palearctic bird species, of which the majority are characterised by population genetic admixture. This is the legacy of multiple ice ages, which forced a back-and-forth population expansion process, in which different subpopulations mixed. Although our bioinformatic approach was performed with the most up-to-date and recommended software, we think that there are limitations in the amount of data that current software can process. Finally, we emphasise the need for additional population sampling and deeper sequencing to challenge our current results.

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/d14030216/s1, Table S1: Complete details of samples used.

**Author Contributions:** L.G.P. and M.W. conceived the study. L.G.P. and E.W. analysed the data. L.G.P. wrote the manuscript. E.W. and M.W. provided regular intellectual input, reviewed drafts of the paper, and approved the final manuscript. All authors have read and agreed to the published version of the manuscript.

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