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Deep genome-wide phylogeographic structure indicates cryptic diversity in the Middle Spotted Woodpecker (*Dendrocoptes medius*)

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Deep phylogeographic structure in mitochondrial DNA not reflected in morphological variation has been uncovered in a number of species over the past few decades. However, inferred phylogeographic structure based solely on mitochondrial DNA can be misleading and might not reflect the true history of evolutionary lineages. Consequently, such cases should be further investigated based on genome-wide data. One of these examples is provided by the Middle Spotted Woodpecker *Dendrocoptes medius*, a non-migratory habitat specialist associated with old deciduous forests of the Western Palaearctic. It displays strong genetic divergence in mitochondrial DNA between Asian and European populations despite there being only slight variation in morphology between them. Here, we found a clear genomic divergence between Asian and European populations that is consistent with mitochondrial divergence patterns. As revealed by isolation by distance analyses, this differentiation in two lineages was not merely an effect of geography. Genomic population structure indicates that both the Asian and European lineages might each have been separated in more than one refugium during the last glacial maximum. The Middle Spotted Woodpecker might represent a case of cryptic diversity throughout its distribution range, as has been previously found for other taxa across the tree of life. However, we also found footprints of gene flow from the Asian into the European populations, suggesting at least limited introgression upon secondary contact. The processes and mechanisms that might prevent lineage fusion between the morphologically cryptic but genetically divergent lineages of the Middle Spotted Woodpecker need to be further investigated especially in the area of potential secondary contact.

Keywords: introgression, phylogeography, Picidae, population genomics.

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The last decade has revealed many species with surprisingly deep phylogeographic structure in mitochondrial DNA (mtDNA) that is not reflected in morphological variation (e.g. Lohman *et al.* 2010,

Webb *et al.* 2011, Milá *et al.* 2012, Saitoh *et al.* 2015, Alaei Kakhki *et al.* 2018, Fuchs *et al.* 2018, Schweizer *et al.* 2018, Zhang *et al.* 2019). The higher mutation rate of mtDNA compared with that of the nuclear genome, facilitated by reduced effective population size due to its haploidy and chiefly maternal inheritance, makes it a valuable tool to study phylogeographic patterns of recently diverged lineages (Avise 2009). However, phylogeographic structure inferred solely based on mtDNA can be misleading for various reasons. In general, as gene trees can vary substantially across the genome (e.g. Nater *et al.* 2015), phylogeographic reconstruction based on single recombination units, such as mtDNA, can contradict the true history of lineage diversification as a consequence of incomplete lineage sorting, introgression or selection (Edwards 2009, Toews & Brelsford 2012). Divergence patterns in mtDNA might then reflect the biogeographic rather than evolutionary history of lineages (Drovetski *et al.* 2018a). Moreover, deep mtDNA divergence can also be prevalent in the absence of any nuclear genomic differentiation as a result of processes such as introgression from an extinct species ('ghost introgression'; Hogner *et al.* 2012, Zhang *et al.* 2019), male-biased dispersal (Dai *et al.* 2013), speciation reversal (Webb *et al.* 2011) or non-neutral evolution of mtDNA (Pavlova *et al.* 2013, Fossoy *et al.* 2016). Additionally, mtDNA does not usually enable informative evaluation of putative gene flow among different evolutionary lineages (e.g. Bastos-Silveira *et al.* 2012, Zemanova *et al.* 2017). Nonetheless, there are cases where patterns of mtDNA variation contradicting morphological disparity were shown to reflect evolutionary histories that were also recovered by genome-wide data (e.g. Harris *et al.* 2018, Schweizer *et al.* 2019a, 2019b).

Consequently, cases where phylogeographic or phylogenetic patterns inferred from mtDNA contradict morphological variation should be further investigated through analyses based on genome-wide data. Such an example is the Middle Spotted Woodpecker *Dendrocoptes medius*. The species is a non-migratory, low-dispersal habitat specialist (Pasinelli 2003) that is morphologically rather uniform over its wide distribution area, which ranges from northern Spain over large parts of central and eastern Europe, the Balkans and Anatolia to the Caucasus, and an isolated area in the Zagros Mountains of Iran and Iraq (Robles & Pasinelli 2020, Winkler *et al.* 2020). Four subspecies are usually recognized (Winkler *et al.* 2020, Gill *et al.* 2022): *D. m. medius* from continental Europe; *D. m. caucasicus* occurring in northern Turkey, the Caucasus, Transcaucasia and probably northwestern Iran; *D. m. anatoliae* from western and southern Anatolia; and *D. m. sanctijohannis*, which breeds in the Zagros Mountains in southwestern Iran and northeastern Iraq. Differences in size and plumage among subspecies are slight and partly clinal (Vaurie 1959, Cramp 1985). A recent study, however, found a strong phylogeographic break between Asian (including the Caucasus) and European populations (Kamp *et al.* 2019). Although slight differences were also found in a single nuclear marker, this pattern was mainly driven by mtDNA divergence, with the two lineages being reciprocally monophyletic in all three mtDNA markers analysed (ATP6, Control Region, Cytochrome b). Coalescence of the Asian and European clades was dated to 1.5 million years ago, a level of divergence found between taxa sometimes treated as distinct species, for instance in the Great Spotted Woodpecker *Dendrocopos major* and Green Woodpecker *Picus viridis* complexes (Pons *et al.* 2011, 2019, Perktas & Quintero 2013, del Hoyo & Collar 2014, Perktas *et al.* 2015, Gill *et al.* 2022).

The strong mitochondrial divergence between Middle Spotted Woodpecker lineages is surprising given the apparent lack of pronounced morphological differences between the Asian and European lineages. Moreover, the Marmara Sea, with the Dardanelles and Bosporus Straits separating the two lineages, should not represent a strong physical barrier for a bird. Indeed, such a strong phylogeographic break across this area has not been documented in birds before (cf. Kamp *et al.* 2019). In addition, the lack of phylogeographic mtDNA structure within the two lineages (Kamp *et al.* 2019) precluded any conclusion on their recent population history. Here, we use genome-wide data to: (1) assess the history of population divergence in the Middle Spotted Woodpecker and to test for potential mito-nuclear discordance, and (2) infer the recent population history of the different populations of the Middle Spotted Woodpecker.

METHODS

Sampling

We obtained tissue or blood samples of Middle Spotted Woodpeckers from six geographical areas

encompassing the eastern and western mtDNA lineages: Armenia (Lesser Caucasus one individual, Armenian Highlands in Transcaucasia four individuals), Greater Caucasus in Russia (five individuals from four sampling sites), and North Aegean Island of Lesvos in Greece (four close sampling sites with one, two, two and four individuals, respectively) from the Asian populations; Balkans with mainland Greece (two sampling sites with one and two individuals) and Serbia (one individual), Switzerland (three sampling sites with seven individuals each) and Spain (nine individuals from six close sampling sites) for the European populations (Fig. 1, Table 1). With the exception of *D. m. sanctijohannis* of the Zagros Mountains of Iraq and Iran, our sampling locations represented all currently recognized subspecies as well as three formerly recognized subspecies: *D. m. lilianae* of Iberia, *D. m. splendidior* of the Balkans and *D. m. laubmanni* of Transcaucasia (Winkler *et al.* 2020).

Samples from 57 individuals were used for analyses after quality checks of the data (see below), some of which have already been used in Kamp *et al.* (2019) (cf. Table 1). DNA was extracted with a modified salt extraction protocol (Aljanabi & Martinez 1997), a standard phenol–chloroform protocol (Sambrook *et al.* 1989) or by ecogenics GmBH (Balgach, Switzerland) using the DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany).

Data preparation

Genotyping by sequencing (Elshire *et al.* 2011) was conducted by ecogenics GmbH (Balgach, Switzerland). Individually MID-tagged reduced-representation libraries were generated using the standard enzyme combination of *Eco*RI/*Mse*I and sequenced on an Illumina NextSeq sequencing system (single-read 75 base pairs). The quality of raw reads was then checked with FASTQC 0.10.1 (Andrews 2010). Individuals with fewer than one million reads were excluded. Leading and trailing low-quality bases of reads were removed using TRIMMOMATIC 0.39 (Bolger *et al.* 2014). A genome assembly of a female Downy Woodpecker *Dryobates pubescens* (GCA_000699005.1) (Zhang *et al.* 2014) was then used as reference to align trimmed reads using BWA MEM 0.7.17 (Li 2013).

Single nucleotide polymorphisms (SNPs) were called and genotyped in analysis of next-generation sequencing data (ANGSD) (Korneliussen *et al.* 2014). Only SNPs were retained that had a maximum P value of 10^{-3} for being variable, a minimum mapping quality of 20, a minimum base quality score of 20, a minimum total read depth of 120, a minimum individual read depth of 5 and a minimum minor allele frequency of 0.01 (-SNP_pval 1e-3 -minQ 20 -minMapQ 20 -setMinDepth 120 -geno_minDepth 5 -minMaf 0.01). Only uniquely mapped reads and biallelic SNPs with less than 10% missing data across sampled individuals were retained (-uniqueOnly 1 -skipTriallelic 1 -minInd 52).

Population genomic structure

We first computed a principal component analysis with PCAngsd (Meisner & Albrechtsen 2018) to obtain an overview of the genomic structure within and among the different populations of the Middle Spotted Woodpecker. We accounted for the uncertainty of called genotypes using individual genotype likelihoods. Eigenvectors from the covariance matrix were computed with the function 'eigen' in R 3.6.2 (RCoreTeam 2019). We then additionally performed an admixture analysis in NGSadmix (Skotte *et al.* 2013) to check for individual assignments to different numbers of ancestral populations. We performed 10 independent runs each for different numbers of K ranging from 1 to 14. The optimum number of populations was evaluated with CLUMPAK (Kopelman *et al.* 2015).

Nucleotide diversity (π) of each population was computed in ANGSD. The unfolded site frequency spectrum was estimated using a reference genome assembly from Downy Woodpecker (GCA_000699005.1) to characterize ancestral states and to provide a prior to compute allele frequency probabilities in ANGSD (-dosaf 1). Missing data were allowed in one individual per SNP. Pairwise nucleotide diversity for each site was estimated using thetaStat based on the site frequency spectrum. The average was then used as nucleotide diversity for each population. As a result of the small sample sizes, individuals from the different sampling sites in the Russian Greater Caucasus were pooled, as were individuals from the two sampling sites in Armenia (Lesser Caucasus and Transcaucasia) and from Greece and Serbia (Balkans). The different sampling sites from Switzerland were treated separately in the analyses.

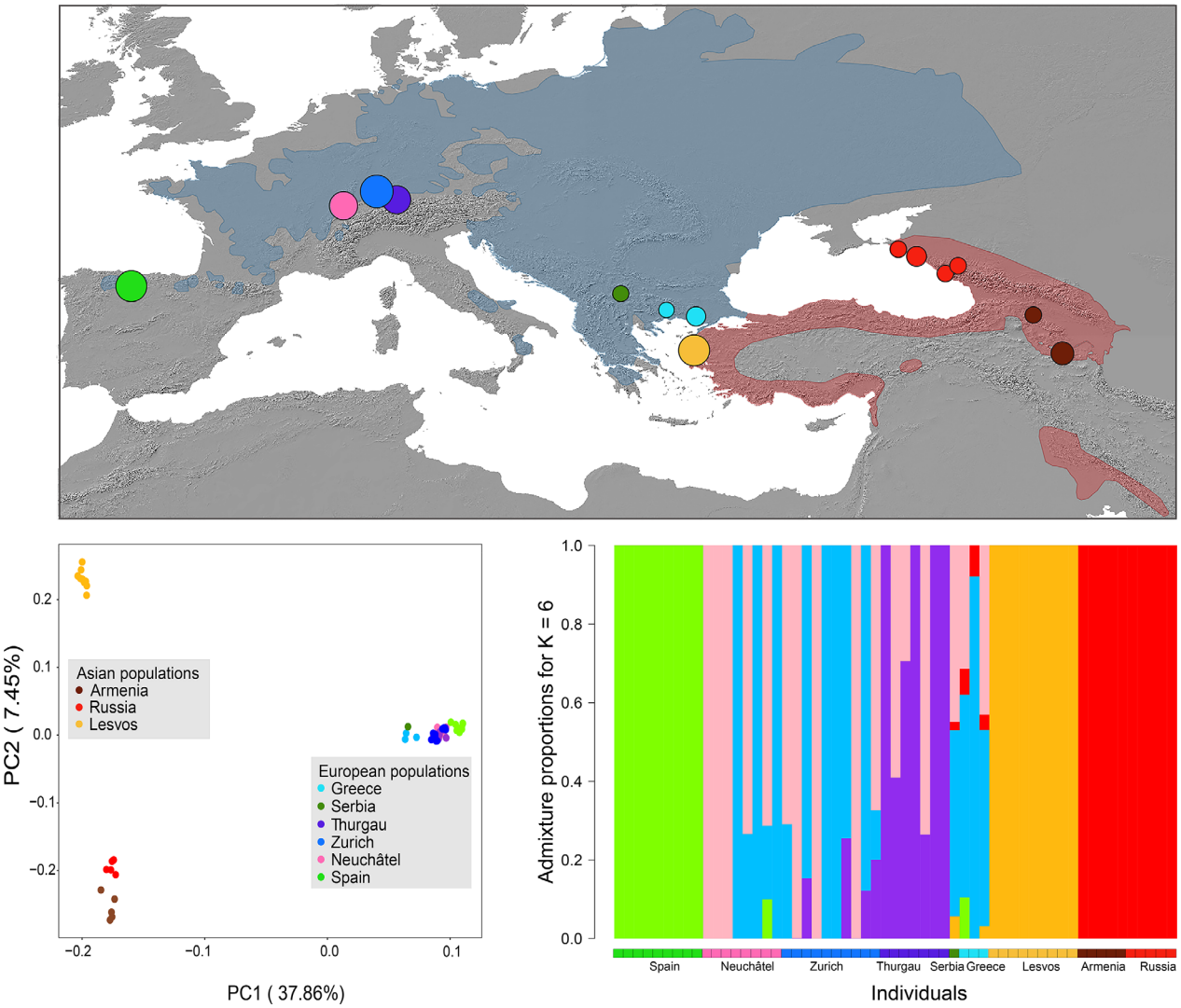

Figure 1. Top: distribution of the European (blue) and the Asian (red) lineages of the Middle Spotted Woodpecker. Circles indicate sample localities with colours corresponding to populations as shown in the principal components analysis (PCA) (bottom left) and sizes of the circles proportional to sample size. Bottom left: PCA using 2743 single nucleotide polymorphisms of the different populations of the Middle Spotted Woodpecker. Bottom right: bar plots showing individual ancestry assignments based on admixture analyses with the best-fitting model for $K > 2$ of six genetic clusters ($K = 6$). Assignment of individuals to the different populations as in the PCA is shown on the bottom of the figure. Different sampling sites were pooled for Greater Caucasus (Russia), for the Lesser Caucasus and Transcaucasia (Armenia), and for mainland Greece (Greece).

The populations of the Middle Spotted Woodpecker sampled for this study were spread across the entire longitudinal range of the European continent. To explore the influence of geographical distance on genomic structure, we tested for isolation by distance between different populations. To this end, Mantel tests (Mantel 1967) were applied to matrices of genetic ($F_{ST}/(1 - F_{ST})$) and logarithmic (ln) Euclidean geographical distances between populations using the R package ade4 (Dray & Dufour 2007) with 999 Monte Carlo permutations. F_{ST} was computed in Arlequin 3.5.2.2 (Excoffier & Lischer 2010) based on SNPs as called with ANGSD (see above) allowing a level of missing data of 0.05. Three populations were considered each for Asia (Greater Caucasus, Lesser Caucasus/Transcaucasia, Lesvos) and Europe (Balkans, Switzerland, Spain).

Table 1. Samples used for this study. Samples included in Kamp *et al.* (2019) are marked in bold.

Sample ID	Location	Lat.	Long.	Geographical area	Deposition	Type of sample	Voucher specimen
Asian lineage							
KS82246	Armenia, Nerkin Hand	39.06	46.52	Armenia, Transcaucasia	Yerevan State University	Blood	
EAK444	Armenia, Nerkin Hand	39.06	46.52	Armenia, Transcaucasia	Yerevan State University	Blood	
IVF1221	Armenia, Nerkin Hand	39.06	46.52	Armenia, Transcaucasia	Yerevan State University	Blood	
KS82243	Armenia, Nerkin Hand	39.06	46.52	Armenia, Transcaucasia	Yerevan State University	Blood	
KS82275	Armenia, Tavush Marz, Zikatar	41.13	44.91	Armenia, Lesser Caucasus	Yerevan State University	Blood	
YPM 140467	Greece, Lesvos, Mount Olympus	39.07	26.38	Lesvos	YPM	Tissue	USNM 637430
YPM 140468	Greece, Lesvos, Mount Olympus	39.07	26.38	Lesvos	YPM	Tissue	USNM637431
YPM 145145	Greece, Lesvos, Petra	39.35	26.17	Lesvos	YPM	Tissue	NHMBEO
YPM 140405	Greece, Lesvos, Polichnitos	39.13	26.22	Lesvos	YPM	Tissue	USNM 637370
YPM 140494	Greece, Lesvos, Polichnitos	39.13	26.22	Lesvos	YPM	Tissue	USNM 637457
YPM 141384	Greece, Lesvos, Polichnitos	39.13	26.22	Lesvos	YPM	Tissue	USNM 640757
YPM 140425	Greece, Lesvos, Polichnitos	39.13	26.22	Lesvos	YPM	Tissue	USNM 637390
YPM 144969	Greece, Lesvos, Vrisa	39.06	26.19	Lesvos	YPM	Tissue	YPM
YPM 145110	Greece, Lesvos, Vrisa	39.06	26.19	Lesvos	YPM	Tissue	YPM
UWBM 64847	Russia, Krasnodarskiy Kray	43.9	40.79	Russia, Greater Caucasus	UWBM	Tissue	UWBM
UWBM 71227	Russia, Krasnodarskiy Kray	44.44	38.4	Russia, Greater Caucasus	UWBM	Tissue	–
UWBM 64715	Russia, Krasnodarskiy Kray	43.5	40.19	Russia, Greater Caucasus	UWBM	Tissue	UWBM
UWBM 64673	Russia, Krasnodarskiy Kray	44.44	38.4	Russia, Greater Caucasus	UWBM	Tissue	UWBM
UWBM 64628	Russia, Krasnodarskiy Kray	44.72	37.45	Russia, Greater Caucasus	UWBM	Tissue	UWBM
European lineage							
YPM 142773	Greece, East Macedonia, Rhodope Mountains	41.11	26.03	Balkans	YPM	Tissue	NHMBEO
YPM 140530	Greece, East Macedonia, Rhodope Mountains	41.34	24.56	Balkans	YPM	Tissue	YPM
YPM 142709	Greece, East Macedonia, Rhodope Mountains	41.11	26.03	Balkans	YPM	Tissue	YPM
YPM 84373	Serbia, Pcinja District	42.33	21.9	Balkans	YPM	Tissue	YPM
8540	Spain, Cantabrian Mountains	42.65	–5.07	Spain	University of Oviedo	Blood	–
3949	Spain, Cantabrian Mountains	42.66	–5.08	Spain	University of Oviedo	Blood	–
3931	Spain, Cantabrian Mountains	42.65	–5.09	Spain	University of Oviedo	Blood	–

(continued)

Table 1. (continued)

Sample ID	Location	Lat.	Long.	Geographical area	Deposition	Type of sample	Voucher specimen
8524	Spain, Cantabrian Mountains	42.69	–5.04	Spain	University of Oviedo	Blood	–
8535	Spain, Cantabrian Mountains	42.79	–5.02	Spain	University of Oviedo	Blood	–
8529	Spain, Cantabrian Mountains	42.62	–5.15	Spain	University of Oviedo	Blood	–
3963	Spain, Cantabrian Mountains	42.73	–5.03	Spain	University of Oviedo	Blood	–
8508	Spain, Cantabrian Mountains	42.62	–5.14	Spain	University of Oviedo	Blood	–
8533	Spain, Cantabrian Mountains	42.79	–5.03	Spain	University of Oviedo	Blood	–
NE101451	Switzerland, Canton of Neuchâtel	47.01	6.93	Switzerland	Swiss Ornithological Institute	Blood	–
NE101455	Switzerland, Canton of Neuchâtel	47.02	6.97	Switzerland	Swiss Ornithological Institute	Blood	–
NE101458	Switzerland, Canton of Neuchâtel	46.1	6.92	Switzerland	Swiss Ornithological Institute	Blood	–
NE101461	Switzerland, Canton of Neuchâtel	47.01	6.92	Switzerland	Swiss Ornithological Institute	Blood	–
NE101463	Switzerland, Canton of Neuchâtel	47	6.93	Switzerland	Swiss Ornithological Institute	Blood	–
NE101464	Switzerland, Canton of Neuchâtel	47.01	6.93	Switzerland	Swiss Ornithological Institute	Blood	–
NE152452	Switzerland, Canton of Neuchâtel	47	6.94	Switzerland	Swiss Ornithological Institute	Blood	–
NE152499	Switzerland, Canton of Neuchâtel	47.01	6.94	Switzerland	Swiss Ornithological Institute	Blood	–
TG152318	Switzerland, Canton of Thurgau	47.65	9.07	Switzerland	Swiss Ornithological Institute	Blood	–
TG152335	Switzerland, Canton of Thurgau	47.64	9.11	Switzerland	Swiss Ornithological Institute	Blood	–
TG152338	Switzerland, Canton of Thurgau	47.64	9.08	Switzerland	Swiss Ornithological Institute	Blood	–
TG152344	Switzerland, Canton of Thurgau	47.65	9.08	Switzerland	Swiss Ornithological Institute	Blood	–
TG152387	Switzerland, Canton of Thurgau	47.64	9.14	Switzerland	Swiss Ornithological Institute	Blood	–
TG152407	Switzerland, Canton of Thurgau	47.63	9.13	Switzerland	Swiss Ornithological Institute	Blood	–

(continued)

Table 1. (continued)

Sample ID	Location	Lat.	Long.	Geographical area	Deposition	Type of sample	Voucher specimen
TG152431	Switzerland, Canton of Thurgau	47.65	9.08	Switzerland	Swiss Ornithological Institute	Blood	–
ZH152306	Switzerland, Canton of Zurich	47.6	8.64	Switzerland	Swiss Ornithological Institute	Blood	–
ZH152321	Switzerland, Canton of Zurich	47.63	8.62	Switzerland	Swiss Ornithological Institute	Blood	–
ZH152323	Switzerland, Canton of Zurich	47.62	8.61	Switzerland	Swiss Ornithological Institute	Blood	–
ZH152325	Switzerland, Canton of Zurich	47.63	8.63	Switzerland	Swiss Ornithological Institute	Blood	–
ZH152327	Switzerland, Canton of Zurich	47.6	8.62	Switzerland	Swiss Ornithological Institute	Blood	–
ZH152329	Switzerland, Canton of Zurich	47.6	8.62	Switzerland	Swiss Ornithological Institute	Blood	–
ZH152351	Switzerland, Canton of Zurich	47.61	8.63	Switzerland	Swiss Ornithological Institute	Blood	–
ZH152353	Switzerland, Canton of Zurich	47.62	8.63	Switzerland	Swiss Ornithological Institute	Blood	–
ZH152360	Switzerland, Canton of Zurich	47.61	8.61	Switzerland	Swiss Ornithological Institute	Blood	–
ZH152361	Switzerland, Canton of Zurich	47.61	8.62	Switzerland	Swiss Ornithological Institute	Blood	–

Abbreviations: NHMBEO, Museum of Natural History, Belgrade; USNM, Smithsonian Institution, National Museum of Natural History; UWBM, University of Washington Burke Museum; YPM, Yale University Peabody Museum of Natural History.

Phylogenetic relationships of populations

We used TreeMix (Pickrell & Pritchard 2012) to infer the topology of relationships among different populations and to test for potential gene flow and its direction between different branches in the phylogeny. Based on the results of the admixture analyses (see below), samples from the Greater and Lesser Caucasus and Transcaucasia (Caucasus/Transcaucasia) were pooled. Moreover, we retained three populations for Europe, i.e. Balkans, Switzerland and Spain. Only SNPs with no missing data, and those that were at least 5 kilobase pairs apart to avoid physical linkage were kept for these analyses. Linkage disequilibrium reaches background levels well before this distance in outbred bird populations (Ellegren *et al.* 2012). A maximum-likelihood tree rooted with the population of the Caucasus and Transcaucasia was built first, then an increasing number of migration edges were successively added. The fit of the different models to the data was evaluated by inspecting the residuals of the population matrix. Positive residuals indicate pairs of populations where covariance is underestimated and model fit might be improved by additional events of migration, whereas negative residuals indicate an overestimation of covariance in the particular model (Pickrell & Pritchard 2012). An increasing number of

migration edges were added until residuals were zero. This analysis was performed 20 times to check for convergence among different runs.

RESULTS

Strong genetic differentiation between the Asian and European populations

We obtained 2743 SNPs in total. In an individual-based principal components analysis, there was a strong separation between the Asian and the European populations along the first principal axis, which explained 37.86% of the variance (Fig. 1). The second principal component axis explained 7.45% of the variance and separated the individuals from the Greater Caucasus and Armenia from those of Lesvos in the Asian group. There was only limited genetic separation among individuals sampled from the European group.

Nucleotide diversity was similar across the populations of the Asian group (Fig. 2). Among the European populations, those from Switzerland showed intermediate nucleotide diversity values, whereas those from the Balkans and Spain were distinctly higher and lower, respectively, compared with the populations sampled in Switzerland.

We tested for the overall influence of isolation by distance on patterns of genomic variation using 2423 SNPs; a Mantel test revealed a highly significant correlation between geographical and genetic distances (Fig. 3). However, this was mainly driven by higher values in pairwise comparisons between individuals from the Asian and European populations relative to genetic distance within the respective geographical groups.

Admixture analyses and population histories indicate limited introgression from the Asian into the European populations

In the admixture analyses, $K = 2$ was revealed as the optimal number of clusters (see Supporting information, Fig. S1); one cluster contained all individuals from the Asian group and the other individuals from the European group. However, all individuals from the Balkans (Greece and Serbia) showed some degree of admixture with the Asian cluster (see Supporting information, Fig. S2). To assess if additional structure occurs within the Asian and the European group, we checked the

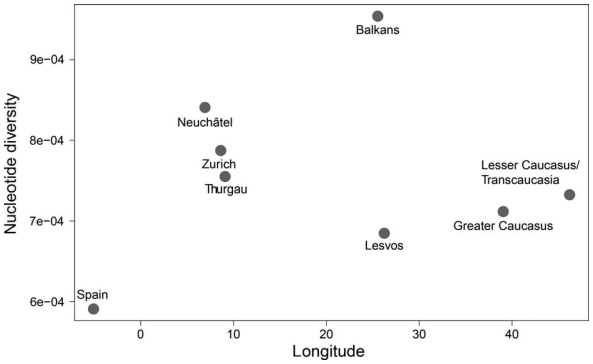

Figure 2. Nucleotide diversity (π) of different populations of the Middle Spotted Woodpecker plotted against longitude in degrees.

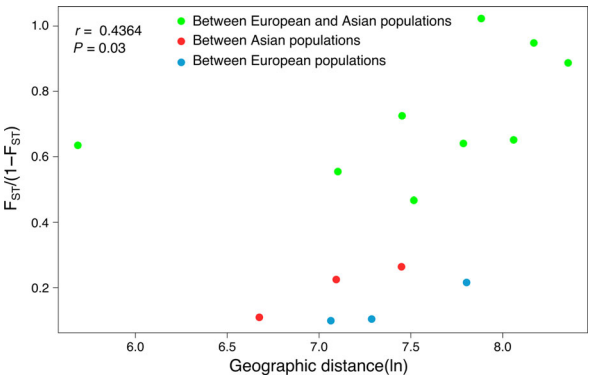

Figure 3. Population-level genetic differentiation between Asian and European lineages of the Middle Spotted Woodpecker relative to geographical distances between populations. Genetic differentiation between the lineages was considerably larger than within them. The point on the far left concerns the comparison between Lesvos from the Asian and the Balkans from the European populations.

results for $K = 6$, which was revealed as the optimum number of clusters for $K > 2$ (see Supporting information; Fig. S1). Within the Asian group, the individuals from the Russian Greater Caucasus and Armenia (Caucasus/Transcaucasia) were assigned to one cluster and individuals from Lesvos to another cluster. There was no indication of mixed ancestry in any individuals from these clusters. In the European group, four clusters were resolved. Although all Spanish individuals were recovered together in a single cluster with no mixed ancestry evident, many Swiss individuals had mixed ancestries with the three remaining European genotype clusters. Furthermore, one Swiss individual also had some genetic contribution from the Spanish cluster. Although the

individuals sampled from the Balkans (three individuals from Greece and one from Serbia) were recovered as primarily comprising European ancestry, as recovered for the Swiss birds, all individuals also showed some degree of admixture with the Asian cluster. Two individuals showed mixed ancestries from both the Caucasus/Transcaucasia and the Lesvos clusters, and two individuals from the Caucasus/Transcaucasia cluster only. One individual from Greece also showed genetic contribution from the Spanish cluster. For runs with $K = 2$ to $K = 5$ and $K = 7$ to $K = 8$, mixed ancestries with the Asian cluster were consistently found in the individuals sampled from the Balkans, and only for $K = 8$ did the individuals from the Balkans form a distinct cluster. Moreover, the Spanish individuals were always treated as a separate cluster, or even as two separate clusters for $K = 7$ (see Supporting information, Fig. S2).

Histories of populations based on 1747 SNPs were first explored by estimating a maximum-likelihood tree in TreeMix with no migration edges in the model (Fig. 4, left) and the three populations from the European group were recovered as a distinct clade. When migration was added to the model, model residuals reached values of zero with three migration edges in all 20 runs. In 17 runs, gene flow was inferred from Lesvos into the Balkan populations, as well as from Caucasus/Transcaucasia and the Balkans into Switzerland (Fig. 4, right). In three runs, gene flow was indicated from Lesvos into Spain and the Balkans, as well as from the common ancestor of the European group into Switzerland.

DISCUSSION

An increasing number of morphologically cryptic lineages have been uncovered using molecular data over the past few decades (e.g. Kozak *et al.* 2006, Reeves *et al.* 2008, Lohman *et al.* 2010, Weir *et al.* 2016, Leys *et al.* 2017, Drovetski *et al.* 2018b, Slavenko *et al.* 2020, Tang *et al.* 2021). The Middle Spotted Woodpecker might be an additional case of unexpected diversity in the tree of life. Genome-wide variation in this species revealed a clear divergence between Asian and European populations. This differentiation into two phylogeographic groups was not merely an effect of geographical distance, as isolation by distance analyses revealed a clear separation of comparisons between Asian and European populations from those among populations within these two regions. This unexpected divergence has already been indicated by mitochondrial data (Kamp *et al.* 2019), and we therefore add a case where marked mitochondrial divergence not reflected in morphological disparity reflects the phylogeographic history of a lineage (cf. Stervander *et al.* 2016, Schweizer *et al.* 2019a). Despite the strong divergence, we found signatures of gene flow from the Asian into the European populations, suggesting at least limited introgression upon secondary contact. Which factors might restrict gene flow between the two cryptic evolutionary lineages remains unclear and warrants further research.

Asian and European Middle Spotted Woodpeckers represent distinct evolutionary lineages

Cases of discordance between mtDNA and phenotypic divergence demand thorough investigation with multilocus data. Consistent with patterns of mitochondrial variation (Kamp *et al.* 2019), we corroborate with genome-wide data the existence of two independent evolutionary lineages in the Middle Spotted Woodpecker. The marked genomic differentiation between the Asian and the European populations of this species is surprising, because the Sea of Marmara with the Dardanelles and Bosporus Straits cannot be considered as an effective barrier between them (cf. Kamp *et al.* 2019). Moreover, the species is continuously distributed on both sides of the Sea of Marmara, as well as between European Turkey and Greece (Handrinos & Akriotis 1997, Kirwan *et al.* 2008). Although there are several bird species with different subspecies described on either side of the Sea of Marmara (Roselaar 1995), such a strong phylogeographic break in this region has not been documented. However, the climate in the surroundings of the Sea of Marmara might not have been suitable for Middle Spotted Woodpeckers during the last glacial maximum (Kamp *et al.* 2019). Consequently, the region might have been colonized recently from both the east and the west, potentially leading to a recent secondary contact zone. Indeed, mixed ancestries that included Asian genetic variants were found in all individuals from the Balkans. Additionally, gene flow from Asian into European populations was indicated by the TreeMix analyses. This points to the existence of a secondary contact zone with at least unidirectional

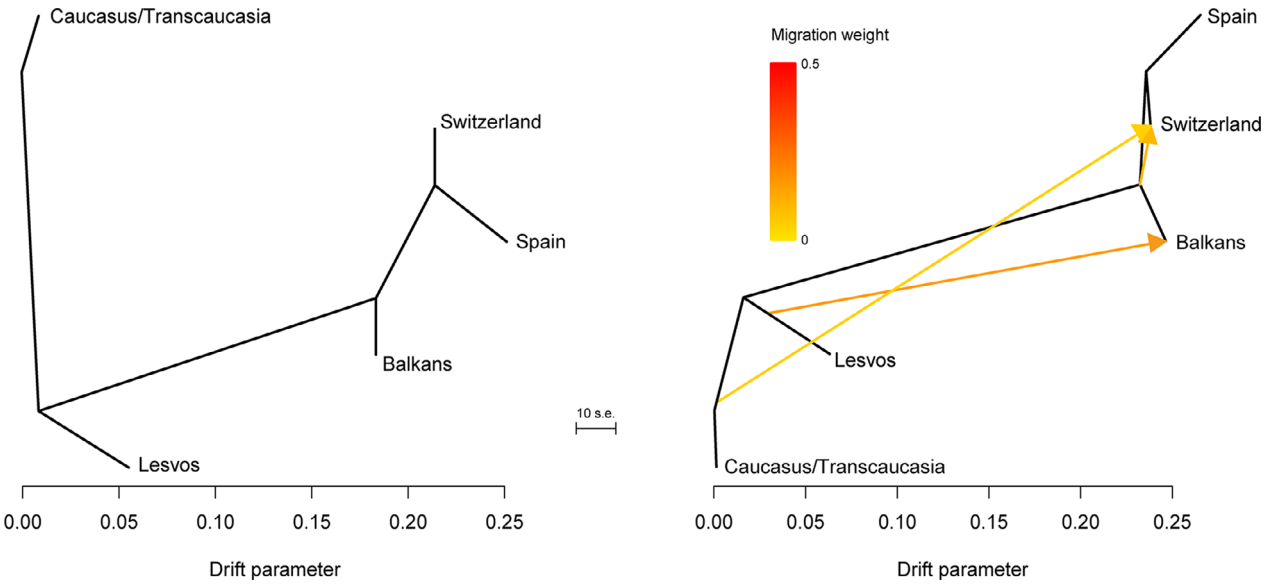

Figure 4. Maximum-likelihood trees as estimated with TreeMix depicting the history of populations on a tree with no migration (left), and with three migration edges shown as coloured arrows indicating gene flow from Lesvos into the Balkan populations, as well as from Caucasus/Transcaucasia and the Balkans into Switzerland. Colours indicate migration weight, and horizontal branch lengths are proportional to the amount of genetic drift that has occurred along the branch.

introgression between the two lineages. However, its location and width remain unclear.

The subspecies *D. m. medius* of the European lineage is usually considered to occur in European Turkey, extending to the western shore of the Sea of Marmara, whereas the range of the subspecies *D. m. caucasicus* of the Asian lineage extends from the Sea of Marmara eastwards (Roselaar 1995). However, given that morphological variation between European and Asian populations is considered at least partly to be clinal, a potential secondary contact zone might not necessarily coincide with the proposed subspecies border and could be located in Europe. Hence, introgression would probably not be detected in the population of Lesvos and the inferred unidirectionality of gene flow might be a sampling artefact. Hybrid zones upon secondary contact might be rather narrow. Nevertheless, recently diverged evolutionary lineages can show rather broad hybrid zones of more than 100 km width (Price 2008). This depends on the strength of selection against hybrids as well as demographic or geographical factors controlling the intensity of migration into the contact zones from the parental populations (e.g. Barton & Hewitt 1985, Beysard & Heckel 2014, McEntee *et al.* 2020). However, given that populations in the Balkans with signs of mixed ancestries lie up to 370 km away from each other, introgression might proceed over a wide geographical distance in the Middle Spotted Woodpecker.

Hybridization upon secondary contact has been documented in a variety of Palaearctic bird species (reviewed in Aliabadian *et al.* 2005). Divergence in the Middle Spotted Woodpecker happened at a similar timescale to the divergence between the Iberian Green Woodpecker *Picus sharpei* and Eurasian Green Woodpecker *P. viridis* (Pons *et al.* 2011, Kamp *et al.* 2019). Their apparently stable contact zone in southwestern France is about 245 km in width with no nuclear introgression detected on both sides of the hybrid zone based on nine Z-linked loci and ten autosomal loci (Pons *et al.* 2019). Although the two Green Woodpeckers differ in coloration of plumage and bare parts as well as in vocalizations (del Hoyo & Collar 2014, Pons *et al.* 2019), evidence for such pronounced differences in morphology between the two lineages of the Middle Spotted Woodpecker is lacking (Cramp 1985) and potential differences in vocalization need to be investigated. However, at least slight differences in habitat preferences might exist. The Middle Spotted Woodpecker is mainly found in deciduous woodland dominated by oaks and avoids any other vegetation type on mainland Greece, but it is common in olive groves on the Island of Lesvos

(Handrinos & Akriotis 1997). Olive groves are also inhabited on mainland Turkey, but oaks seem also to be a more important part of the species' habitat there (Kirwan *et al.* 2008). Consequently, it is unclear which prezygotic isolation factors other than demographic or geographical aspects could limit gene flow between the two lineages of the Middle Spotted Woodpecker. Moreover, given their short time of divergence, it is unlikely that gene flow could be restricted by intrinsic postzygotic isolation (cf. Price & Bouvier 2002, Price 2008). However, genetic incompatibilities might evolve faster than prezygotic isolation, as has been recently shown for relatively young cryptic Amazonian bird species displaying strong intrinsic postzygotic reproductive isolation in the absence of premating isolation (Pulido-Santacruz *et al.* 2018, Cronemberger *et al.* 2020). The amount of gene flow between the two evolutionary lineages of the Middle Spotted Woodpecker, and the potential processes and mechanisms that might restrict it, need to be investigated through more comprehensive geographical sampling especially focused in the area of the potential location of a secondary contact zone.

Our results call for a comprehensive evaluation of phenotypic as well as ecological and behavioural variations in the Middle Spotted Woodpecker. Previous morphological studies were largely qualitative in nature and not comprehensive geographically (Vaurie 1959, Cramp 1985, Kirwan 2005, Kirwan *et al.* 2008). Geographical gradients in body size and plumage colour hypothesized in these studies need to be re-evaluated using quantitative methods, especially in the area separating the European and Asian lineages. Even without strong phenotypic divergence, it can be argued that the two evolutionary lineages of Middle Spotted Woodpecker could be treated at least as incipient species; however, further studies using a more comprehensive geographical sampling should demonstrate that introgression does not extend beyond the contact zone, thereby preventing lineage fusion.

Population structure within Asia and Europe

The genome-wide data used for this study provided much greater resolution with respect to the evolutionary histories of the Asian and European lineages of the Middle Spotted Woodpecker compared with previous analyses of mtDNA variation (cf. Kamp *et al.* 2019). Genomic population structure indicated that both lineages might have been separated in more than one refugium during the last glacial maximum. Overall, a stronger genomic structure was revealed in the Asian compared with the European lineage, with the population of Lesvos being clearly separated from those of Russia and Armenia. Although Russian samples originated from the western Greater Caucasus, Armenian samples were collected either in the Lesser Caucasus or Armenian Highlands in Transcaucasia. Interestingly, no marked difference was found between the different regions, despite them being separated not only by considerable geographical distance, but also by mountain ranges. However, there is suitable habitat for the Middle Spotted Woodpecker along the slopes of the Greater and Lesser Caucasus as well as in the Armenian highlands, and valleys between these ranges might be more likely to act as barriers than the ranges themselves. The slightly lower nucleotide diversity of the Russian population might indicate that the area was colonized from the south. The Caucasus are well known to have acted as a glacial refugium for several forest bird species (e.g. Hung *et al.* 2017), but suitable climate for the Asian lineage of the Middle Spotted Woodpecker during the last glacial maximum existed even further south (Kamp *et al.* 2019). Additionally, the genomic population structure points towards the existence of a second refugium further west. During the last glacial maximum, Anatolia retained much of its forest cover and served as a refugium for two oak species (Bagnoli *et al.* 2016, Ülker *et al.* 2018), and the climate, especially in southern Anatolia, might have been suitable for the Middle Spotted Woodpecker (Kamp *et al.* 2019). Interestingly, nucleotide diversity in the island population of Lesvos was not considerably reduced compared with mainland populations. However, this island is located close to mainland Turkey and was even connected to it during the last glacial maximum (Perissoratis & Conispoliatis 2003).

Within European populations, admixture analyses revealed no consistent geographical structure among Swiss and Balkan populations of Middle Spotted Woodpecker. The comparatively high nucleotide diversity in the Balkans might indicate the existence of a refugium in this region, where suitable climate existed for the Middle Spotted Woodpecker during the last glacial maximum (Kamp *et al.* 2019). However, it is unclear how this might be influenced by the introgression from alleles from the Asian populations. On the other hand, Spanish individuals were

revealed as a separate cluster, which was not indicated by variation in mtDNA (cf. Kamp *et al.* 2019). Given their very limited amount of admixture with the remaining European populations and their low nucleotide diversity, the Iberian Peninsula was probably a separate glacial refugium for the Middle Spotted Woodpecker that remained isolated and so did not act as a centre of expansion. Colonization of central Europe from a glacial refugium on the Iberian Peninsula played a role in some lineages (Hewitt 2000, 2004), but the Pyrenees might have been too great a barrier for others. Indeed many bird species have isolated lineages in Iberia, often in combination with comparatively low nucleotide diversity, indicating small historical population sizes, which may further restrict their recolonization potential (Ellegren *et al.* 2012, Backström *et al.* 2013, Poelstra *et al.* 2014). Additional sampling of Middle Spotted Woodpeckers on both sides of the Pyrenees may be warranted to evaluate the genetic (and phenotypic) uniqueness of the Iberian population. It is currently negatively affected by habitat fragmentation as well as degradation (Robles *et al.* 2008, Robles & Ciudad 2012, 2017) and is likely to be susceptible to negative effects of climate change (Robles & Pasinelli 2020). The low nucleotide diversity found here may add substantial vulnerability to cope with negative effects of habitat and climate changes. Conservation actions aimed to reverse and mitigate negative effects of habitat fragmentation, habitat degradation and climate change are critical to preserve this unique genetic reservoir of the Middle Spotted Woodpecker.

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CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

ETHICAL NOTE

Samples were collected under the following permits – Armenia: Ministry of Environment RA to Sargis Aghayan, Scientific Centre of Zoology and Hydroecology NAS RA, permission number 5/22.1/51371; Switzerland: permits from the cantonal veterinary offices and the federal office for environment FOEN (NE: permit no. 01/2010; TG: permit no. VoWa 01/09; ZH: permit no. 66/2009) and the federal office for environment FOEN; and Spain: permission of the Junta de Castilla y León (EP/CyL/37/2008).

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Manuel Schweizer: Conceptualization (equal); Data curation (equal); Formal analysis (equal); Funding acquisition (supporting); Investigation (lead); Methodology (equal); Project administration (lead); Resources (supporting); Software (equal); Supervision (lead); Validation (lead); Visualization (lead); Writing – original draft (lead); Writing – review & editing (equal). **Qindong Tang:** Conceptualization (supporting); Data curation (equal); Formal analysis (equal); Funding acquisition (supporting); Investigation (supporting); Methodology (equal); Project administration (supporting); Resources (supporting); Software (supporting); Supervision (supporting); Validation (supporting); Visualization (supporting); Writing – original draft (supporting); Writing – review & editing (equal). **Reto Burri:** Conceptualization (supporting); Data curation (supporting); Formal analysis (equal); Funding acquisition (supporting); Investigation (equal); Methodology (equal); Project administration (supporting); Resources (supporting); Software (supporting); Supervision (supporting); Validation (supporting); Visualization (supporting); Writing – original draft (supporting); Writing – review & editing (equal). **Sergei V. Drovetski:** Conceptualization (supporting); Data curation (supporting); Formal analysis (supporting); Funding acquisition (supporting); Investigation (supporting); Methodology (supporting); Project administration (supporting); Resources (equal); Software (supporting); Supervision (supporting); Validation (supporting); Visualization (equal); Writing – original draft (supporting); Writing – review & editing (equal). **Hugo Robles:** Conceptualization (supporting); Data curation

(supporting); Formal analysis (supporting); Funding acquisition (supporting); Investigation (supporting); Methodology (supporting); Project administration (supporting); Resources (equal); Software (supporting); Supervision (supporting); Validation (supporting); Visualization (supporting); Writing – original draft (supporting); Writing – review & editing (equal). **Kristof Zyskowski:** Conceptualization (supporting); Data curation (supporting); Formal analysis (supporting); Funding acquisition (supporting); Investigation (supporting); Methodology (supporting); Project administration (supporting); Resources (supporting); Software (supporting); Supervision (supporting); Validation (supporting); Visualization (supporting); Writing – original draft (supporting); Writing – review & editing (equal). **Sargis Aghayan:** Conceptualization (supporting); Data curation (supporting); Formal analysis (supporting); Funding acquisition (supporting); Investigation (supporting); Methodology (supporting); Project administration (supporting); Resources (equal); Software (supporting); Supervision (supporting); Validation (supporting); Visualization (supporting); Writing – original draft (supporting); Writing – review & editing (equal). **Marko Rakovic:** Conceptualization (supporting); Data curation (supporting); Formal analysis (supporting); Funding acquisition (supporting); Investigation (supporting); Methodology (supporting); Project administration (supporting); Resources (equal); Software (supporting); Supervision (supporting); Validation (supporting); Visualization (supporting); Writing – original draft (supporting); Writing – review & editing (equal). **Gilberto Pasinelli:** Conceptualization (equal); Data curation (supporting); Formal analysis (equal); Funding acquisition (lead); Investigation (supporting); Methodology (supporting); Project administration (supporting); Resources (equal); Software (supporting); Supervision (supporting); Validation (supporting); Visualization (supporting); Writing – original draft (supporting); Writing – review & editing (equal).

Data Availability Statement

The data supporting this study are openly available on the NCBI sequence read archive (SRR17642604-SRR17642660) under BioProject PRJNA796240 with accession numbers SAMN25040990–SAMN25041046 (raw individual RAD sequences). Filtered single nucleotide polymorphism data are available on Dryad (https://doi.org/10.5061/dryad.m905qfv2z).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. ΔK statistics for admixture analysis from $K = 2$ to $K = 14$.

Figure S2. Individual ancestry assignment for admixture analysis from $K = 2$–5 and $K = 7$–8. Sample origin is indicated below the diagram.