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Climate-driven range shifts and demographic events over the history of Kruper's Nuthatch *Sitta krueperi*

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Capsule This study is the first ever documented evidence of an interglacial refugium during the Last Interglacial for birds in Anatolia and suggests the need of a re-examination of the effects of the Last Interglacial on the geographic distribution and genetic structure of species.

Aims We tested whether, in accordance with the 'refugia within refugia' model, multiple refugia existed for Kruper's Nuthatch *Sitta krueperi* during the Last Glacial Maximum or the species survived along the coastal belt of Anatolia through the Late Quaternary glacial–interglacial cycles.

Methods An ecological niche model was developed to predict the geographic distribution of Kruper's Nuthatch under reconstructed past (the Last Interglacial and the Last Glacial Maximum), present, and projected future bioclimatic conditions. Also, robust coalescent-based analyses were used to assess demographic events over the history of Kruper's Nuthatch.

Results Kruper's Nuthatch survived the Last Glacial Maximum almost along the coastal belt of Anatolia, but not in multiple refugia, and surprisingly, contrary to expectations, it survived the Last Interglacial in southern Anatolia, but not along the coastal belt of Anatolia.

Conclusion A kind of the 'refugia within refugia' model (i.e. the 'refugium within refugium' model) was supported because range shifts took place within Anatolia (itself also a refugium) for Kruper's Nuthatch.

Global climate changes through the Quaternary glacial–interglacial cycles have had significant impacts on the geographic distribution and genetic structure of numerous taxa (Hewitt 2000, 2004, Swenson & Howard 2005, Provan & Bennett 2008). The effects of these climatic oscillations have been well documented especially for temperate European species, which shifted their range into and out of three well-known southern refugia located in the Iberian, Italian, and Balkan peninsulas during glacial and interglacial periods, respectively (Hewitt 2000, 2004, Taberlet & Cheddadi 2002). Apart from these classical refugia, Anatolia is also important both as a refugium and as a source of re-colonization for European biota (Cooper *et al.* 1995, Ibrahim *et al.* 2002, Çıplak 2008, Ansell *et al.* 2011, Bilgin 2011, Hewitt 2011). The number of phylogeographic studies concentrating on or including samples from Anatolia has increased steadily, so that

we now have information from a wide variety of taxa, such as bats (Bilgin *et al.* 2009), rodents (Dubey *et al.* 2006, Gündüz *et al.* 2007, Gür 2013), birds (Perktaş *et al.* 2011, Albayrak *et al.* 2012, Perktaş & Quintero 2013), turtles (Fritz *et al.* 2009), amphibians (Akın *et al.* 2010), insects (Çıplak *et al.* 2010, Mutun 2010, İpekdal 2012, Kaya *et al.* 2012, 2013), and plants (Koch *et al.* 2006, Jakob *et al.* 2007, Ansell *et al.* 2011).

As the number of studies accumulates, we are gaining insight into the general patterns of inter- and intraspecific diversity within Anatolia (Çıplak *et al.* 2008, Bilgin 2011). One pattern that has been recently put forth is the occurrence of potential suture zones (where lineages from the different refugia meet) within Anatolia (Bilgin 2011), indicating that Anatolia did not constitute a single homogenous refugium, but rather harboured populations isolated in multiple refugia primarily during glacial (Bilgin 2011 and references therein) or during interglacial periods (Gür 2013). This is similar to the 'refugia within refugia'

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model, indicating that range shifts into and out of refugia (i.e. latitudinal and/or longitudinal shifts) take place at regional scales (i.e. within the refugia themselves) for endemic and/or local species (Harris & Sá-Sousa 2001, 2002, Gündüz *et al.* 2007, Stefani *et al.* 2012, Gür 2013). There is a growing amount of literature concentrating on the 'refugia within refugia' model mostly from the Iberian Peninsula, which has been shown to harbour multiple refugia for a wide variety of taxa, such as mammals, birds, reptiles, amphibians, insects, and plants (Gómez & Lunt 2007, Ferrero *et al.* 2011).

One example of the 'refugia within refugia' model for Anatolia has recently been described by Albayrak *et al.* (2012) for Kruper's Nuthatch *Sitta krueperi*. They showed that Kruper's Nuthatch exhibits phylogeographic structuring, with subdivision into three mitochondrial (mt)DNA haplogroups, and interpreted these haplogroups as indicating that the species survived the Last Glacial Maximum in three glacial refugia.

Although it is feasible that Anatolia did not constitute a single homogeneous refugium due to its topographic and climatic complexity (Şekercioğlu *et al.* 2011), species-specific habitat requirements and the distribution of suitable habitats for species of interest through the Quaternary glacial–interglacial cycles should be taken into account before broad generalizations are made. Thus, it is logical to ask whether multiple glacial refugia could have existed in Anatolia for free-flying, forest-dwelling bird species, such as Kruper's Nuthatch that inhabits temperate coniferous forests (BirdLife International 2014), because it is known that the coastal belt of Anatolia retained up to about 90% of its forest cover during the Last Glacial Maximum (Fig. 6 in Şenkul & Doğan 2013).

It is also useful to clearly define what is meant by the term 'refugium' because the term's broad usage has led to confusion (Bennett & Provan 2008). In this study, we used the definition put forth by Stewart *et al.* (2010): 'the geographical region or regions that a species inhabits during the period of a glacial/interglacial cycle that represents the species' maximum contraction in geographical range'. This approach also forces one to explicitly test for changes in the geographic distribution and population size of species through time and determine the timing of these events before drawing conclusions about glacial or interglacial refugia because there are numerous ways in which species can respond to climatic and environmental changes

(Bennett & Provan 2008, Stewart 2008). While genetic diversity within species (e.g. lineages and populations) can be used by robust coalescent-based analyses to infer past demographic events (population fluctuations) (Drummond *et al.* 2005, Cornuet *et al.* 2008), ecological niche modelling allows us to understand past climate-driven range shifts (expansions or contractions) and therefore the Quaternary refugial distributions of species (Carstens & Richards 2007, Knowles *et al.* 2007, Knowles & Alvarado-Serrano 2010, Hung *et al.* 2012, Peterson & Lieberman 2012).

In this study, we aimed to further advance the understanding of the phylogeography of Kruper's Nuthatch (see Albayrak *et al.* 2012) by adding new samples from Caucasus and Midilli into samples from the other regions (Kerr *et al.* 2009, Schindel *et al.* 2011, Albayrak *et al.* 2012) so as to represent almost all of the known geographic distribution of the species, which is vital for obtaining robust phylogeographic results (Pavlova *et al.* 2006, Perkaş *et al.* 2011, Perkaş & Quintero 2013). We developed an ecological niche model and used robust coalescent-based analyses to conduct a complete phylogeographic study of Kruper's Nuthatch, including both previously published (Kerr *et al.* 2009, Schindel *et al.* 2011, Albayrak *et al.* 2012) and new mtDNA cytochrome c oxidase subunit 1 (COI) sequences. By assessing both climate-driven range shifts (expansions or contractions) and demographic events (population fluctuations) over the history of Kruper's Nuthatch, we tested whether, in accordance with the 'refugia within refugia' model, multiple refugia existed for the species during the Last Glacial Maximum, as suggested by Albayrak *et al.* (2012), or the species survived along the coastal belt of Anatolia, which retained up to about 90% of its forest cover during the Last Glacial Maximum (Şenkul & Doğan 2013), through the Late Quaternary glacial–interglacial cycles.

METHODS

Study species

Kruper's Nuthatch is mostly distributed along the coastal belt of Anatolia (Fig. 1) and inhabits temperate coniferous forests, from sea level up to about 2400 m. In Anatolia, Kruper's Nuthatch occurs mainly in forests of black pine *Pinus nigra*, fir *Abies cilicica*, cedar *Cedrus libani*, red pine *Pinus brutia*, and juniper *Juniperus* spp., while, in the Caucasus, the species occurs mainly in forests of spruce *Picea* and Caucasian

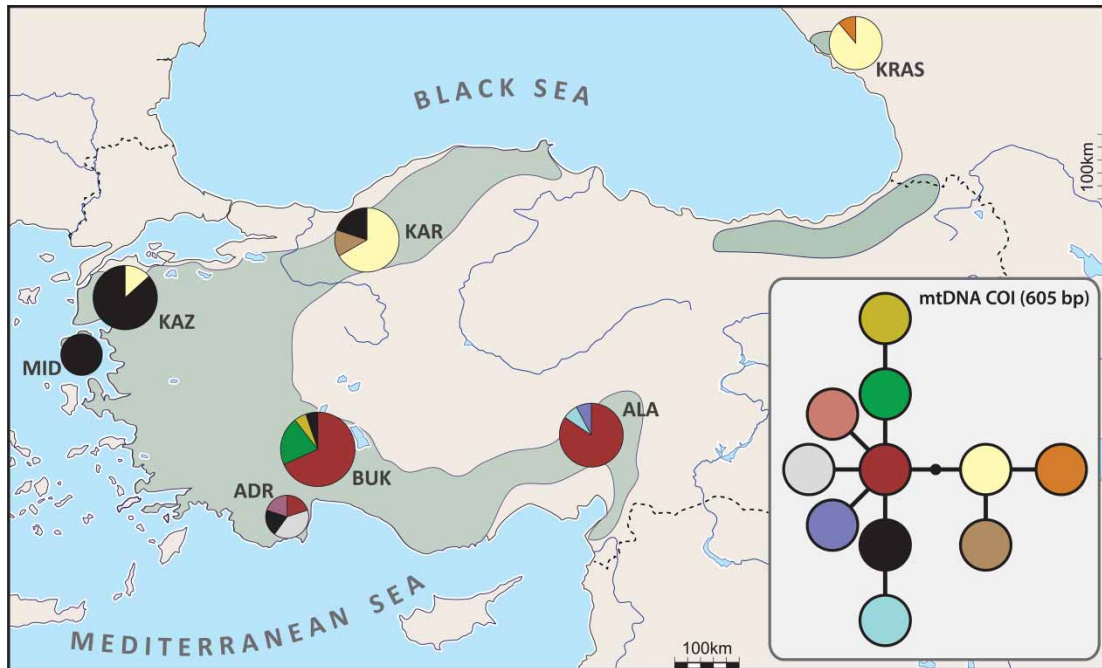


Figure 1. The geographic distribution (shown in green) and genetic structure of Kruper's Nuthatch. Sample locations are indicated as pie diagrams with abbreviations of populations. Each pie diagram is proportional to sample size. The colours in pie diagrams indicate different mtDNA COI haplotypes in the haplotype network.

fir *Abies nordmanniana*, but also in forests of pine *Pinus* (Albayrak & Erdoğan 2006). The breeding population size of Kruper's Nuthatch was estimated to be 80 000–170 000 breeding pairs, equating to 240 000–510 000 individuals (BirdLife International 2014).

Samples and laboratory studies

In this study, 9 new mtDNA COI sequences, which were obtained from tissue samples in the ornithology collections of the Yale Peabody Museum, were combined with 72 previously published ones ($n = 3$ from Kerr *et al.* 2009; $n = 1$ from Schindel *et al.* 2011,

$n = 68$ from Albayrak *et al.* 2012). Thus, 81 mtDNA COI sequences were obtained (Table 1, Fig. 1). Voucher information can be found, together with sequence information, in GenBank (KM260745-753).

Extractions of total genomic DNA from tissue samples were performed with the DNeasy Kit (Qiagen), with incubation overnight. We amplified a 605-bp fragment of mtDNA COI gene by polymerase chain reaction (PCR) using a combination of two primers (Passer F1 and Passer R1; see Albayrak *et al.* 2012 for further details). DNA extractions and amplifications were conducted in a separate laboratory using fresh PCR reagents and laboratory equipment to avoid

Table 1. Genetic diversity of Kruper's Nuthatch, based on mtDNA COI sequences.

Region/population	n	Haplotype number	% Private haplotypes	Haplotype diversity (H_d)	Nucleotide diversity (π)
<i>Southern Anatolia</i>		8	64		
Aladağlar (ALA)	13	3		0.538	0.0028
Adrasan (ADR)	5	4		0.295	0.00076
Antalya (BUK)	19	4		0.900	0.00231
<i>Northwestern Anatolia</i>		2	0		
Kaz Mountains (KAZ)	15	2		0.189	0.0014
Midilli (MID)	5	1		0.248	0.00116
<i>Northern Anatolia and Caucasus</i>		4	18		
Kartalkaya (KAR)	15	3		na	na
Krasnodar (KRAS)	9	2		0.431	0.0022
				0.533	0.00211
				0.222	0.00037

contamination. We followed PCR procedure described by Albayrak *et al.* (2012). Negative controls were always used to detect contamination. Amplification products were visualized by electrophoresis and purified using ExoSAP. Purified PCR products were sequenced with the same primers as used for the amplifications on a 3730 Automated DNA Sequencer (Perkin-Elmer, ABI) following the standard protocol.

Molecular phylogeography

mtDNA COI sequences were aligned and edited in Sequencher version 5.2.3 (Gene Codes, Ann Arbor, MI, USA). Standard measures of genetic variation, such as number of haplotypes, haplotype diversity (H_d) and nucleotide diversity (π) were estimated for Kruper's Nuthatch by DnaSP version 5.10 (Librado & Rozas 2009). We constructed a statistical parsimony network to investigate phylogenetic relationships between mtDNA COI haplotypes using the algorithm of Templeton *et al.* (1992) by TCS version 1.21 (Clement *et al.* 2000). Haplotype networks are usually better than bifurcating phylogenetic trees at representing the relationships of intraspecific populations (Posada & Crandall 2001, Freeland *et al.* 2011, Forister *et al.* 2008; Ferreri *et al.* 2011). Based on the results of the haplotype network (i.e. that the overall amount of genetic variation was low; see Results for further details), we combined all mtDNA COI sequences from all populations while assessing demographic events (population fluctuations) over the history of Kruper's Nuthatch. This also made the results of ecological niche modelling and molecular phylogeography (i.e. demographic analyses) more comparable (Gür 2013).

The components of genetic variation distributed among populations were estimated using hierarchical Fst analysis and statistical significance was estimated using a bootstrap procedure with 1000 replicates in Arlequin version 3.5.1.3 (Excoffier & Lischer 2010). F_{st} statistic was also used for investigating a genetic isolation-by-distance pattern. A matrix of genetic distances between all pairs of populations was estimated from $F_{st}/(1 - F_{st})$ values. A matrix of ecological distances (km) between all pairs of populations was estimated from Google Earth version 7.1.2.2041. Because straight-line geographical distances contained possible physical barriers to dispersal, ecological distances were considered (Acevedo *et al.* 2012). A Mantel test with 10000 random permutations was performed between matrices of

genetic [$F_{st}/(1 - F_{st})$] and ecological (log) distances (Slatkin 1993, Rousset 1997).

In the mismatch distribution, population expansion was evaluated using pairwise differences between mtDNA COI sequences under the sudden expansion model of Rogers and Harpending (1992) by DnaSP version 5.10 (Librado & Rozas 2009). Significance of the sudden expansion model was evaluated by calculating the raggedness index (r) (Harpending 1994) and Ramos-Onsins and Rozas' test statistic (R_2) (Ramos-Onsins & Rozas 2002). Associated P -values of these test statistics were calculated by coalescent simulations. When the mismatch distribution is not significantly different from that simulated under the sudden expansion model and the observed and simulated frequencies of pairwise differences are similar, the parameter tau (τ), estimated from the mismatch distribution, can be used to estimate the time since population expansion (TSE). τ equals $2ut$, where u is the mutation rate per gene per generation and t the time in years since population expansion (Rogers 1995). While estimating the time since population expansion, mutation rates (see below for details) and a generation time of one year were used in the tool (<http://www.uni-graz.at/zoowww/mismatchcalc/mmc1.php>) developed by Schenekar and Weiss (2011).

A Bayesian skyline plot was used to determine how the effective population size (N_{ef}) changed through time as implemented in BEAST version 1.7.1 (Drummond *et al.* 2012). The Bayesian skyline plot estimates the posterior distribution for the effective population size at intervals along a phylogeny, thus allowing inferences of population fluctuations over time. The Markov chain Monte Carlo analysis was run for 100 million generations (sampling every 10000 generations) using the Hasegawa–Kishino–Yano substitution model, strict molecular clock model (see below for mutation rates) and piecewise-linear skyline model. The first 10% of generations were discarded as burn-in. Results were checked using Tracer version 1.5 (Rambaut & Drummond 2007) and all effective sample size values were over 1000.

We used the MDIV (Nielsen & Wakeley 2001) to discriminate between historical isolation and continuous gene flow between pairs of regions. The MDIV is based on a Bayesian approach, uses the Hasegawa–Kishino–Yano substitution model, and calculates the divergence time and gene flow between pairs of populations. There are three parameters in the MDIV: population parameter theta ($\theta = 2N_e\mu$) and the migration rate ($M = 2N_{em}$) and the divergence time

($T = tdiv/2Ne$) between pairs of populations. The MDIV was first run using default search settings and default priors (for the parameters, θ and T), then run for 2 million generations following a burn-in period of 500 000 generations, and repeated three times to ensure convergence upon the same posterior distributions for each of the parameter estimates (see below for mutation rates).

In the mismatch distribution, the Bayesian skyline plot, and the MDIV, we used both the potential range of mutation rates for mtDNA of birds (Brito 2005, Pereira & Baker 2006, Weir & Schluter 2008) and a mutation rate for mtDNA COI gene of Kruper's Nuthatch estimated by coalescent simulations (see below for further details). Those mutations rates corresponded to 2%, 2.62% (the estimated one), 3%, and 4% sequence divergence per million year (Myr).

We used the approximate Bayesian computation implemented in DIYABC version 2.0 beta (Cornuet *et al.* 2008) to examine coalescent simulations of demographic events over the history of Kruper's Nuthatch. DIYABC calculates the posterior probabilities of alternative possible biogeographic scenarios by generating simulated data sets and comparing them to the observed dataset. We designed a simple one-population model, together with two alternative possible biogeographic scenarios. The scenario 1 envisages a population expansion beginning after the Last Interglacial (130 000 to 116 000 years ago), whereas the scenario 2 accounts for a population contraction during the last glacial period until the Last Glacial Maximum (21 000 years ago) and a population expansion afterwards based on the hypothesis by Albayrak *et al.* (2012). In both scenarios, the prior distributions for all historical parameters (i.e. effective population sizes and times of demographic events) were set as uniform (see Fig. 6 for further details about the prior distributions). The prior distribution for mutation rate was also set as uniform between $5.00E-09$ and $2.00E-08$ (corresponded to 1% and 4% sequence divergence per Myr). The substitution model was Hasegawa–Kishino–Yano. Summary statistics were number of haplotypes, number of segregating sites, mean of pairwise differences, and variance of pairwise differences. We generated 2 million simulated datasets (1 million for each scenario) and estimated the posterior probability of each scenario using logistic regression on the 20 000 simulated data sets closest to the observed data set. Confidence on the best scenario choice between both alternate ones (confidence in scenario choice) was evaluated by determining type-I

error in order to test how many times the most strongly supported model did not have the highest posterior probability when it was the true model (see Barker *et al.* 2012).

Ecological niche modelling

Species occurrence data were compiled from the following sources: KuşBank (<http://www.kusbank.org>) and Global Biodiversity Information Facility (GBIF; <http://www.gbif.org>). These sources provided georeferenced occurrence records. Only the occurrence records from the breeding season (1 March to 30 June; Harrap & Quinn 1996) over the period 1992–2012 were considered because, even though Kruper's Nuthatch is resident, some post-breeding dispersal and seasonal altitudinal movements have been noted (Harrap & Quinn 1996). To correct for biased sampling effort and to reduce spatial dependence, the occurrence records were spaced at least 20 km apart by removing intervening ones randomly (Pearson *et al.* 2007). Thus, a total of 78 occurrence records, covering almost all of the known geographic distribution of Kruper's Nuthatch, were used in ecological niche modelling (see Fig. 7).

Bioclimatic data were obtained for past (the Last Interglacial, 130 000 to 116 000 years ago and the Last Glacial Maximum, 21 000 years ago), present (1950–2000), and future (2080) conditions. Reconstructed Last Interglacial bioclimatic data, obtained from the WorldClim – Global Climate Data (<http://www.worldclim.org/past>) at a spatial resolution of 30 s, are based only on the Community Climate System Model (CCSM; Otto-Bliesner *et al.* 2007). These bioclimatic data were subsequently resampled to a spatial resolution of 2.5 min. Reconstructed Last Glacial Maximum bioclimatic data, obtained from the WorldClim – Global Climate Data (<http://www.worldclim.org/past>) at a spatial resolution of 2.5 min, are based on both the CCSM3 (Collins *et al.* 2006) and the Model for Interdisciplinary Research on Climate (MIROC3.2; K-1 Model Developers 2004). Reconstructed Last Interglacial and Last Glacial Maximum bioclimatic data were previously used successfully in a similar study of a mammal species in the same region (Gür 2013). Present bioclimatic data, obtained from the WorldClim – Global Climate Data (<http://www.worldclim.org/current>) at a spatial resolution of 2.5 min, are based on interpolation using a thin-plate smoothing spline of observed climate at weather stations from a large number of global,

regional, national, and local sources, mostly for the period of 1950–2000, with latitude, longitude, and altitude as independent variables (Hijmans *et al.* 2005a). Projected future bioclimatic data, obtained from the CGIAR Research Program on Climate Change, Agriculture and Food Security (CCAFS) (<http://www.ccafs-climate.org>) at a spatial resolution of 2.5 min, are based on the Canadian Centre for Climate Modelling and Analysis Coupled Global Climate Model (CCCMA–CGCM2), the Commonwealth Scientific and Industrial Research Organization Mark Global Climate Model (CSIRO–Mk2), and the Hadley Centre for Climate Prediction and Research Hadley Climate Model (HCCPR–HadCM3) under one of the Intergovernmental Panel on Climate Change (IPCC) Special Report Emissions Scenarios (SRES; A2a).

Ecological niche modelling was based on all 19 bioclimatic variables in the above-mentioned bioclimatic data (see below). All these variables were masked to include only 24° to 46°E and 33° to 46°N.

Using the maximum entropy machine learning algorithm in MAXENT version 3.3.3 k (Phillips *et al.* 2004, 2006, Elith *et al.* 2011), which is among the most effective methods of ecological niche modelling (Elith *et al.* 2006), an ecological niche model was developed to predict the geographic distribution of Kruper's Nuthatch under reconstructed past (the Last Interglacial, 130 000 to 116 000 years ago and the Last Glacial Maximum, 21 000 years ago), present (1950–2000), and projected future (2080) bioclimatic conditions. To test for model over-fitting as a result of all 19 bioclimatic variables, an ecological niche model based on the three most significant bioclimatic variables in predicting the present distribution of Kruper's Nuthatch was also developed (Galbreath *et al.* 2011, Gür 2013). Both the ecological niche models gave qualitatively similar predictions for reconstructed past, present, and projected future bioclimatic conditions, suggesting that model over-fitting as a result of all 19 bioclimatic variables was not a critical issue (online only supplementary Fig. S1).

MAXENT was run with the default settings (see Gür 2013 for details). To test for model complexity as a result of the default settings, the regularization multiplier was set at 0.05, 0.25, 0.50, 0.75, 1, 2, 5, and 10 (Richmond *et al.* 2010, Gür 2013, see Warren & Seifert 2011 for further details about model complexity). A regularization multiplier of 1 (the default) provided the appropriate model complexity because the ecological niche model performance did

not differ when setting the regularization multiplier at the above-mentioned values. Fade by clamping (reducing the effect of projecting onto bioclimatic conditions not encountered during training) was performed because novel bioclimatic conditions were encountered during projecting (Elith *et al.* 2010). A five-fold cross-validation was also performed in which a different 80% of the occurrence records were used to train the ecological niche model and 20% were used to test it for each of five runs. Thus, each of the occurrence records was used to test the ecological niche model once. The importance of each bioclimatic variable in the ecological niche model was assessed using the percentage contribution and permutation importance. MAXENT computes the area under the receiver operating characteristic curve (AUC) to evaluate the ecological niche model performance. An AUC > 0.5 indicates that the ecological niche model performs better than a random prediction. Given test data, MAXENT also computes binomial probabilities (one-sided) for all 11 thresholds to test the null hypothesis that the test occurrence records are predicted no better by the ecological niche model than by a random prediction.

MAXENT generates logistic prediction values for bioclimatic suitability of specific geographic areas and reports these values ranging from 0 (least suitable) to 1 (most suitable; Phillips *et al.* 2004, 2006, Elith *et al.* 2011). To define bioclimatically suitable geographic areas in the past (the Last Interglacial and the Last Glacial Maximum), in the present, and in the future, 1 out of 11 thresholds applied by MAXENT was chosen: the '10 percentile training presence' threshold. The logistic prediction values of this threshold were averaged across the five-fold cross-validation runs (mean \pm sd = 0.310 \pm 0.015, range = 0.290–0.327). Application of this threshold resulted in the prediction for present bioclimatic conditions largely in agreement with the known geographic distribution of Kruper's nuthatch (see Results).

Given two reconstructed Last Glacial Maximum (the CCSM3 and the MIROC3.2) and three-projected future (the CCCMA–CGCM2, the CSIRO–Mk2, and the HCCPR–HadCM3) bioclimatic data, two and three predictions for Last Glacial Maximum and future bioclimatic conditions, respectively, were obtained. These two and three predictions (i.e. logistic prediction values) were averaged to generate a final summary prediction for Last Glacial Maximum and future bioclimatic conditions, respectively (Waltari & Guralnick 2009).

All GIS operations were conducted using DIVA-GIS version 7.5.0.0 (Hijmans *et al.* 2005b).

RESULTS

Molecular phylogeography

We obtained a 605-bp fragment of mtDNA COI gene from 81 individuals representing 7 populations of Kruper's Nuthatch (Table 1, Fig. 1). The Caucasus population (Krasnodar (KRAS)) was not included in Albayrak *et al.* (2012). In total, 11 haplotypes were found, one of which is unique for this study (from the Caucasus population, KRAS). Eleven variable sites were detected and no transversions were found. Six of the transitions were A/G and five were C/T. Summary statistics for mtDNA COI sequences were given in Table 1.

Three haplotypes (red, black, and yellow; see Fig. 1) were found in higher frequencies than the rest. Red haplotype, which is central in the haplotype network and therefore the ancestral one (see Discussion), was common to, and only found in, southern Anatolia (i.e. Adrasan (ADR), Aladağlar (ALA), and Antalya (BUK)). Black haplotype was common to northwestern Anatolia (i.e. Kaz Mountains (KAZ) and Midilli (MID)), whereas yellow haplotype was common to northern Anatolia and Caucasus (i.e. Kartalkaya (KAR) and KRAS). Black, yellow, and low-frequency haplotypes differed from red haplotype by one, two, or three base pairs, giving rise to a star-like haplotype network (Fig. 1). Southern Anatolia had a high frequency of private haplotypes (7 out of 11 haplotypes, 64%). However, northern Anatolia and Caucasus had a low frequency of private haplotypes (2 out of 11 haplotypes, 18%), whereas northwestern Anatolia had no private haplotypes (Fig. 1, Table 1). Also, there tended to be more nucleotide and haplotype diversity in southern Anatolia than in other regions (Table 1). All of these results were consistent with those of ecological niche modelling in that southern Anatolia was an interglacial refugium (see below).

Hierarchical F_{st} analysis of mtDNA COI sequences provided a quantitative perspective on the patterns of geographic variation. Sixty per cent of genetic variation was distributed among seven populations (Table 2). We investigated major sources of this genetic variation. All of genetic variation among seven populations existed at the level of variation among three regions: southern Anatolia (ADR, ALA, and

Table 2. The components of genetic variation distributed among populations estimated using hierarchical F_{st} analysis for Kruper's Nuthatch.

Hierarchy	Percentage of variation
Among seven populations $F_{st} = 0.60$ ($P < 0.001$)	60
Two regions (Northwestern Anatolia and northern Anatolia and Caucasus: MID, KAZ, KAR, KRAS/southern Anatolia: BUK, ADR, ALA)	
Among regions	23
Among populations within regions $F_{st} = 0.63$ ($P < 0.001$)	40
Three regions (Northern Anatolia and Caucasus: KAR, KRAS/northwestern Anatolia: MID, KAZ/southern Anatolia: BUK, ADR, ALA)	
Among regions	62
Among populations within regions $F_{st} = 0.65$ ($P < 0.001$)	3

BUK), northwestern Anatolia (KAZ and MID), and northern Anatolia and Caucasus (KAR and KRAS). That is, most of genetic variation was attributable to these three regions (Table 2). However, genetic distances were positively and significantly correlated with ecological distances, indicating a genetic isolation-by-distance pattern (Fig. 2). Also, the overall amount of genetic variation was low (0.77% maximum divergence; Fig. 1). All of these results justified that we combined all mtDNA COI sequences from all populations while assessing demographic events

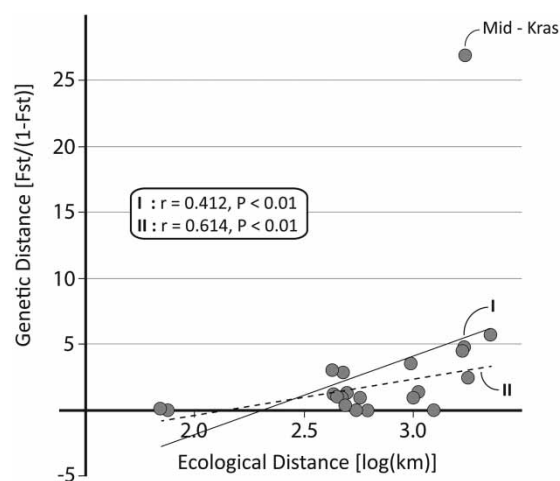


Figure 2. Isolation by distance of populations of Kruper's Nuthatch. The genetic distance between MID and KRAS was found to be quite high [$F_{st}/(1 - F_{st}) = 27$], so a genetic isolation-by-distance pattern is illustrated with (I) and without (II) that genetic distance.

(population fluctuations) over the history of Kruper's Nuthatch.

The mismatch distribution for Kruper's Nuthatch was unimodal (Fig. 3) and indicated a low average number of differences between mtDNA COI sequences ($\tau = 1.58$). Furthermore, both the raggedness index ($r = 0.17$; $P > 0.05$) and Ramos-Onsins and Rozas' test statistic ($R_2 = 0.098$; $P > 0.05$) failed to reject the null hypothesis of population expansion. τ (1.58) estimated from the mismatch distribution suggested that population expansion began after the Last Interglacial and before the Last Glacial Maximum (see Fig. 3 for the time since population expansion). Despite a lack of demographic signal at deeper time scales, the results of the Bayesian skyline plot did not contradict population expansion beginning before the Last Glacial Maximum (Fig. 4). The MDIV (based on comparisons between southern Anatolia (ADR, ALA, and BUK) and northwestern Anatolia (KAZ

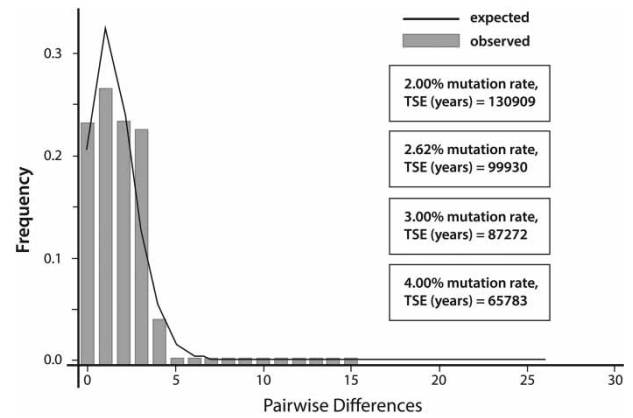


Figure 3. The mismatch distribution for Kruper's Nuthatch.

and MID), and southern Anatolia and northern Anatolia and Caucasus (KAR and KRAS)) suggested that the divergence time between pairs of regions was consistent with pre-Last Glacial Maximum

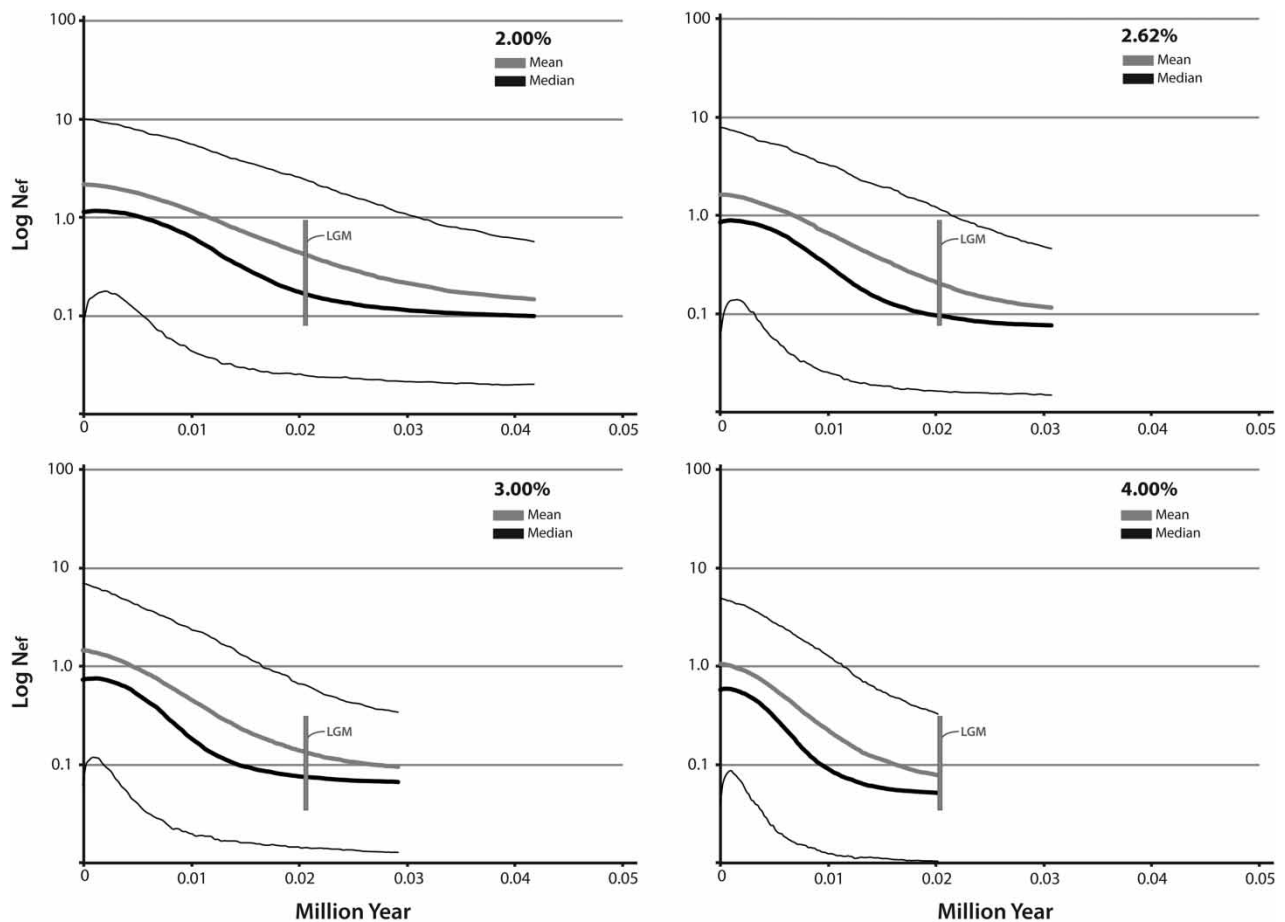


Figure 4. The Bayesian skyline plot for Kruper's Nuthatch.

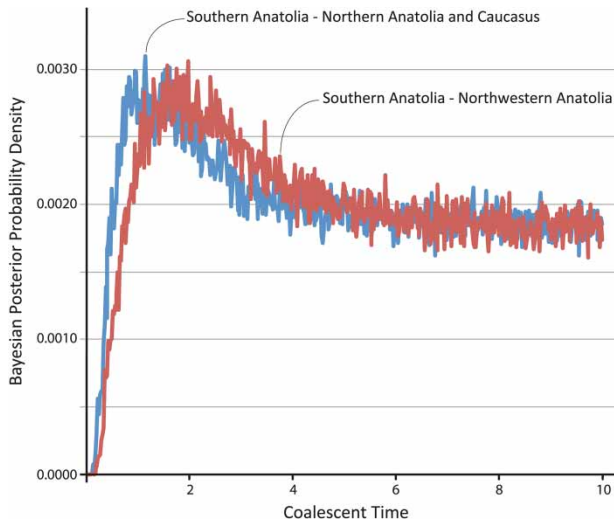


Figure 5. Coalescent-based estimates of probability density for divergence time among regions for Kruper's Nuthatch.

population expansion (Fig. 5, Table 3). Finally, statistical phylogeography (i.e. testing two alternative possible biogeographic scenarios) suggested that the best was scenario 1 (Fig. 6, Table 4). This scenario indicated a population expansion beginning after the Last Interglacial (Fig. 6). The posterior probability based on logistic regression was 0.83 for the scenario 1 (Table 4). Based on properties of the approximate posterior distribution of parameters (i.e. mean values of N_1 , N_{1a} , and t_2) under the scenario 1, population expansion began about 84 000 years ago and the effective population size increased from about 55 000 to 524 000 (Table 5). All of these results were also consistent with those of ecological niche modelling in that Kruper's Nuthatch substantially expanded its range from an interglacial refugium in southern Anatolia to its present distribution (see below).

Ecological niche modelling

For Kruper's Nuthatch, the ecological niche model performed better than a random prediction. The AUC was close to 1 (AUC for training data (mean \pm sd, range) = 0.924 ± 0.004 , 0.919–0.929; AUC for test data (mean \pm sd, range) = 0.879 ± 0.031 , 0.830–0.912, based on the five-fold cross-validation runs). The small standard deviation for the mean AUC for the test data suggested that the ecological niche model performance was robust to variation in the selection of the occurrence records for training and

Table 3. MDIV estimates of divergence time among regions calibrated for different mutation rates. Θ is given with 95% confidence interval (CI) of highest posterior densities (HPDs).

Region 1	Region 2	T_{MRCA}	t_{pop}	Θ (95% CI of HPDs)	Divergence time (T)			
					2% Myr	2.62% Myr	3% Myr	4% Myr
Southern Anatolia	Northern Anatolia and Caucasus	3.08	1.98	1.34 (0.57–2.77)	221,098 (93,273–453,273)	167,384 (71,201–346,010)	146,182 (62,182–302,182)	109,636 (46,636–226,636)
Southern Anatolia	Northwestern Anatolia	2.87	1.16	1.17 (0.44–2.44)	112,165 (42,182–233,917)	146,148 (32,200–178,563)	74,766 (28,121–155,945)	56,082 (21,091–116,959)

T_{MRCA} and t_{pop} are measured in $2N_e \mu$.
Southern Anatolia: BUK, ADR, ALA; Northern Anatolia and Caucasus: KAR, KRAS; Northwestern Anatolia: KAZ, MID.

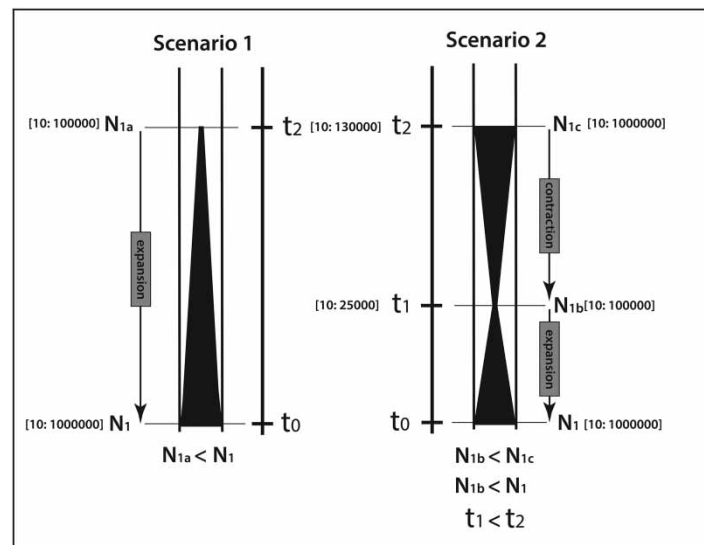


Figure 6. Two alternative possible biogeographic scenarios for Kruper's Nuthatch. t indicates the time scale and N indicates the effective population size. The prior distributions for the time scale and the effective population size are given in the brackets.

Table 4. Relative posterior probabilities with 95% credibility intervals for each scenario.

	Scenario 1	Scenario 2
2000	0.8089 [0.7912,0.8266]	0.1911 [0.1734,0.2088]
4000	0.8179 [0.8056,0.8303]	0.1821 [0.1697,0.1944]
6000	0.8188 [0.8087,0.8289]	0.1812 [0.1711,0.1913]
8000	0.8206 [0.8119,0.8294]	0.1794 [0.1706,0.1881]
10000	0.8227 [0.8149,0.8305]	0.1773 [0.1695,0.1851]
12000	0.8251 [0.8181,0.8322]	0.1749 [0.1678,0.1819]
14000	0.8260 [0.8195,0.8325]	0.1740 [0.1675,0.1805]
16000	0.8270 [0.8209,0.8331]	0.1730 [0.1669,0.1791]
18000	0.8280 [0.8223,0.8338]	0.1720 [0.1662,0.1777]
20000	0.8289 [0.8234,0.8343]	0.1711 [0.1657,0.1766]

Table 5. Properties of the approximate posterior distribution of parameters under the scenario 1.

Parameter	Mean	Median	Mode	q(0.025)	q(0.975)
N_1	5.24E+05	4.97E+05	3.81E+05	1.89E+05	9.50E+05
N_{1a}	5.47E+04	5.64E+04	7.83E+04	3.72E+03	9.79E+04
t_2	8.42E+04	8.69E+04	1.15E+05	2.99E+04	1.28E+05

testing. Furthermore, across all 11 thresholds and the five-fold cross-validation runs, the test occurrence records were predicted significantly better by the ecological niche model than by a random prediction ($P < 0.001$).

The prediction for present bioclimatic conditions largely matched the known geographic distribution of

Kruper's Nuthatch, suggesting that the species is very near to equilibrium with climate, but also included areas where the species has not been observed in the breeding season despite extensive observations, including especially the European part of Turkey and Cyprus (Fig. 7). The per cent contribution and/or permutation importance suggested that 'isothermality' (Bio3), 'temperature seasonality' (Bio4), and 'precipitation of coldest quarter' (Bio19) were the three most significant bioclimatic variables in predicting the present distribution of Kruper's Nuthatch. The response curves produced by models created using only one of these bioclimatic variables at a time indicated that Kruper's Nuthatch prefers to inhabit areas where 'isothermality' is ~ 32 – 41 , 'temperature seasonality' is ~ 5.5 – 7.5°C , and 'precipitation of coldest quarter' is $\sim \geq 190$ mm.

The predictions for the reconstructed past (Last Interglacial and Last Glacial Maximum) and present bioclimatic conditions suggested that Kruper's Nuthatch survived the Last Interglacial in an interglacial refugium in southern Anatolia and, afterwards, substantially expanded its range from this interglacial refugium to its present distribution, especially during the last glacial period (including the Last Glacial Maximum). The prediction for the projected future (2080) bioclimatic conditions indicated that Kruper's Nuthatch will slightly contract its range towards the coastal belt of Anatolia (Fig. 7).

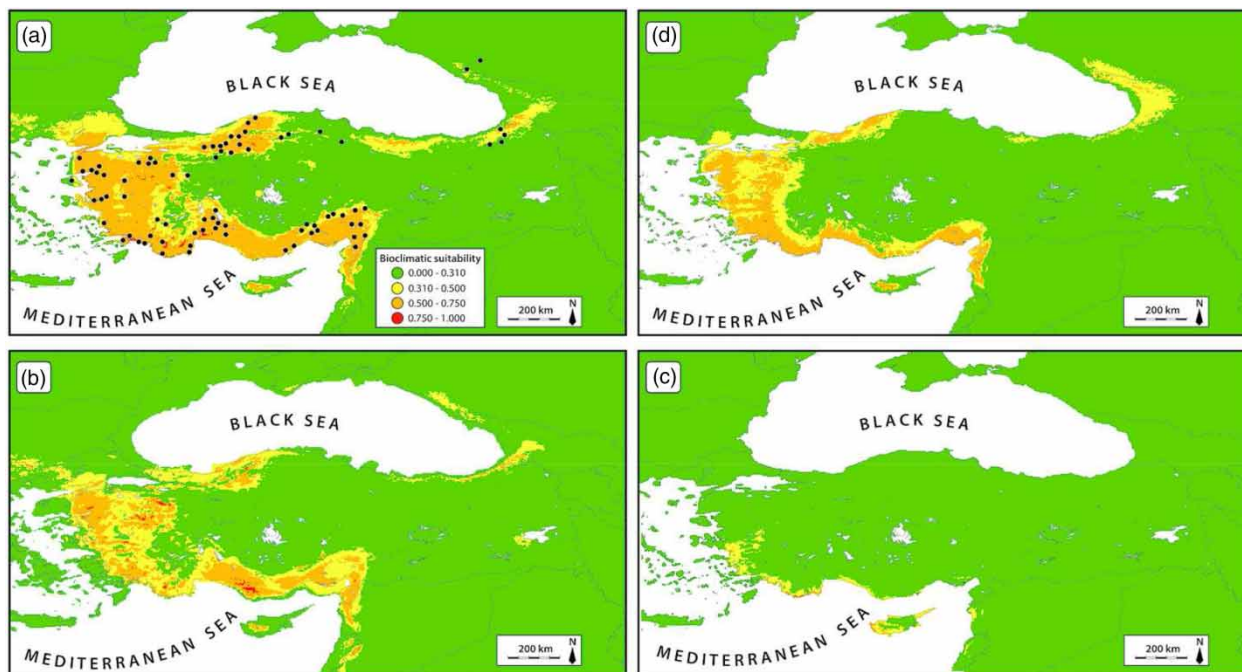


Figure 7. The ecological niche model showing the geographic distribution of Kruper's Nuthatch under (a) present (1950–2000), (b, c) reconstructed past ((b) the Last Glacial Maximum, 21 000 years ago and (c) the Last Interglacial, 130 000–116 000 years ago), and (d) projected future (2080) bioclimatic conditions. The visible area in maps is 24° to 46°E and 33° to 46°N. In (a), filled circles indicate the occurrence records. In (b), note that the Last Glacial Maximum coastline differs from the present and the Last Interglacial coastlines because sea level was lower in the Last Glacial Maximum than both in the present and in the Last Interglacial.

DISCUSSION

This study combined molecular phylogeography and ecological niche modelling in order to understand how Kruper's Nuthatch has responded to global climate changes through the Late Quaternary glacial–interglacial cycles. Molecular phylogeography allowed assessment of climate-driven range shifts (expansions or contractions) under the assumption that range shifts should be accompanied by demographic events (population fluctuations) (Jezkova *et al.* 2011, Gür 2013). Ecological niche modelling also directly allowed assessment of climate-driven range shifts (expansions or contractions) under the assumptions of species–climate equilibrium and stability of ecological niches through time (Nogués-Bravo 2009, Gür 2013). Thus, in general, we tested whether, in accordance with the 'refugia within refugia' model, multiple refugia existed for Kruper's Nuthatch during the Last Glacial Maximum, as suggested by Albayrak *et al.* (2012), or the species survived along the coastal belt of Anatolia, which retained up to about 90% of its forest cover during the Last Glacial Maximum (Şenkul & Doğan 2013), through the Late Quaternary glacial–interglacial cycles.

This complete phylogeographic study of Kruper's Nuthatch suggested that the species survived the Last Interglacial in an interglacial refugium in southern Anatolia and, afterwards, substantially expanded its range from this interglacial refugium to its present distribution, especially during the last glacial period (including the Last Glacial Maximum). That is, Kruper's Nuthatch survived the Last Glacial Maximum almost entirely along the coastal belt of Anatolia, but not in multiple refugia, and surprisingly, contrary to expectations, it survived the Last Interglacial in southern Anatolia, but not along the coastal belt of Anatolia. All of these results supported a kind of the 'refugia within refugia' model (i.e. the 'refugium within refugium' model) because range shifts took place within Anatolia (itself also a refugium) for Kruper's Nuthatch.

The statistical parsimony network indicated the star-like haplotype network, suggesting a population expansion. Usually, the central haplotype in the star-like haplotype network could be interpreted as the ancestral haplotype (Freeland *et al.* 2011). The red haplotype was central in the haplotype network and therefore interpreted as the ancestral one. This haplotype was common to, and only found in, southern

Anatolia, which also had a high frequency of private haplotypes. Moreover, there tends to be more nucleotide and haplotype diversity in southern Anatolia than in other regions. All of these results suggested that southern Anatolia was a refugium. The mismatch distribution, the Bayesian skyline plot, the MDIV, and the statistical phylogeography mostly suggested that this population expansion began after the Last Interglacial and before the Last Glacial Maximum.

A more or less continuous haplotype network, with a few partly localized, high-frequency (i.e. red, black, and yellow) and more highly localized, low-frequency haplotypes (i.e. others) with small mutational changes, might indicate historically intermediate gene flow among populations not subdivided by firm long-term barriers (e.g. the Last Glacial Maximum ones) to dispersal (Avice's phylogeographic category V, Avice 2000). That is, this pattern provided no evidence of longstanding genetic differences of populations isolated by multiple glacial refugia. A positive and significant correlation between genetic and ecological distances, that is, a genetic isolation-by-distance pattern, might be consistent with this explanation and, in the light of the results, especially of ecological niche modelling, seems to be a most reasonable explanation of genetic differentiation within Kruper's Nuthatch. Even though a population expansion occurred from a single refugium, genetic differentiation within species might be largely formed by isolation by distance of populations (Zigouris *et al.* 2013, Manthey *et al.* 2014). However, in this study, the question remains whether or not the results of molecular phylogeography can be verified using information from nuclear genes.

The results of molecular phylogeography were consistent with those of ecological niche modelling in that Kruper's Nuthatch has substantially expanded its range from an interglacial refugium in southern Anatolia to its present distribution. Ecological niche modelling also suggested that this range expansion will not continue and Kruper's Nuthatch will slightly contract its range towards the coastal belt of Anatolia in the future (2080). The predictions for reconstructed past (Last Interglacial and Last Glacial Maximum) and projected future (2080) bioclimatic conditions are more robust if the certain assumptions are met: species-climate equilibrium and stability of ecological niches through time (Nogués-Bravo 2009). The geographical distribution of Kruper's Nuthatch can be predicted successfully from present bioclimatic conditions, suggesting that the species is very near to equilibrium with climate. For example, the prediction for present

bioclimatic conditions successfully included a probably isolated breeding area recently discovered in the Ak Mountains in central Anatolia (Albayrak & Erdoğan 2010). Thus, the assumption of species-climate equilibrium was confirmed. Although the assumption of stability of ecological niches through time could not be assessed, it appears to hold true for Kruper's Nuthatch because the results of molecular phylogeography were consistent with those of ecological niche modelling (Gür 2013).

The effects of the Last Interglacial on the geographic distribution and genetic structure of species have not been sufficiently discussed although there are a limited number of recent studies that have paid attention to this phenomenon (Gür 2013, Wang *et al.* 2013). In general, present distribution of a species should be concordant with its Last Interglacial distribution because several studies have shown that climatic conditions during the Last Interglacial are more or less similar to those during the present (Qu *et al.* 2011). Thus, we expected that the Last Interglacial distribution of Kruper's Nuthatch would have been similar to its present distribution. Interestingly, however, the ecological niche modelling did not support this expectation (see above). It has also been suggested that temperature and temperature seasonality during the Last Interglacial were higher than those during the present (Cowie 2007). During the Last Interglacial, these climatic conditions might have pushed Kruper's Nuthatch to southern Anatolia. However, interpretation of vegetation and climatic conditions of Anatolia during the Last Interglacial is important to the complete understanding of why Kruper's Nuthatch survived the Last Interglacial in an interglacial refugium in southern Anatolia. The geographic distribution of Kruper's Nuthatch during the Last Glacial Maximum was consistent with the observation that the coastal belt of Anatolia retained up to about 90% of its forest cover during the same time period (Şenkul & Doğan 2013). That Kruper's Nuthatch will slightly contract its range towards the coastal belt of Anatolia in the future (2080) was also consistent with the observation that coniferous forests will undergo a contraction in eastern Mediterranean in the same time period (Zeydanlı *et al.* 2011).

All in all, the results of molecular phylogeography and ecological niche modelling suggested a different biogeographic history of Kruper's Nuthatch than the one suggested by Albayrak *et al.* (2012). Consequently, these results are the first ever documented evidence of an

interglacial refugium during the Last Interglacial for birds in Anatolia and suggest the need of a re-examination of the effects of the Last Interglacial on the geographic distribution and genetic structure of species.

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SUPPLEMENTAL MATERIAL

Figure S1, the ecological niche model (based on three most significant bioclimatic variables) showing the current, reconstructed past and predicted future geographic distribution of Kruper's Nuthatch can be accessed at: [10.1080/00063657.2014.977220](https://doi.org/10.1080/00063657.2014.977220).

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