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A wide geographical survey of mitochondrial DNA variation in the great spotted woodpecker complex, *Dendrocopos major* (Aves: Picidae)

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One of the fundamental goals of phylogeographical studies should be to achieve a comprehensive geographical sampling of any investigated group. In this study, we conducted the most comprehensive geographical investigation to date for the great spotted woodpecker complex (*Dendrocopos major*), including populations from North Africa and Eurasia [including specimens from China, Japan and southern Caucasia (Anatolia, Azerbaijan and Iran)], in order to evaluate its genetic structure and population history. At the same time, we tested species limits within the *D. major* complex, which currently includes 14 recognized subspecies based on morphology and coloration. We based our study on haplotypes for the mitochondrial gene NADH dehydrogenase subunit 2 (ND2). Most haplotypes were obtained from museum toe pads, although we also used some previously published data. We also tested gene flow through MDIV, and estimated divergence dates among lineages using BEAST. The analysis of 352 base pairs of the ND2 gene from 155 individuals sampled from 33 populations showed significant phylogeographical structure across the breeding range. Our results found four distinct and reciprocally monophyletic clades: China, Japan, Iran–Azerbaijan and Eurasia–North Africa, with no phylogeographical structure within them. Coalescent-based gene flow analysis showed restricted gene flow between China and Japan and between Japan and Eurasia. On the basis of the gene flow and phylogenetic analysis results, we propose the recognition of at least four different species within the complex. We also propose that, within the Eurasia–North Africa clade, a rapid population expansion through 'leading edge expansion' from refugia in Iberia, Kursk and North Africa, as well as irruptive and loop migration, can explain the lack of phylogeographical structure. *Biological Journal of the Linnean Society*, 2013, **108**, 173–188.

ADDITIONAL KEYWORDS: BEAST – Eurasia – gene flow – MDIV – North Africa – phylogeography – population genetics – southern Caucasia – species limits – ND2.

INTRODUCTION

The late Quaternary glacial–interglacial cycles are known to have played a dominant role in shaping the genetic diversity within species. Most phylogeographical studies of Eurasian birds have concentrated on the effects of these glacial cycles. Studies have shown that Eurasian bird species expanded their ranges from southern refugia after the Last Glacial Maximum (LGM: Hewitt, 1996, 2000, 2004; Griswold & Baker, 2002; Brito, 2005; Zink *et al*., 2009; Perktas, Barrowclough & Groth, 2011). It has been proposed that during the LGM, both the geographical split of populations and the ecological factors stimulating species divergence should have been critical in the process of avian diversification (Weir & Schluter, 2004; Zink *et al*., 2006; Milá *et al*., 2007).

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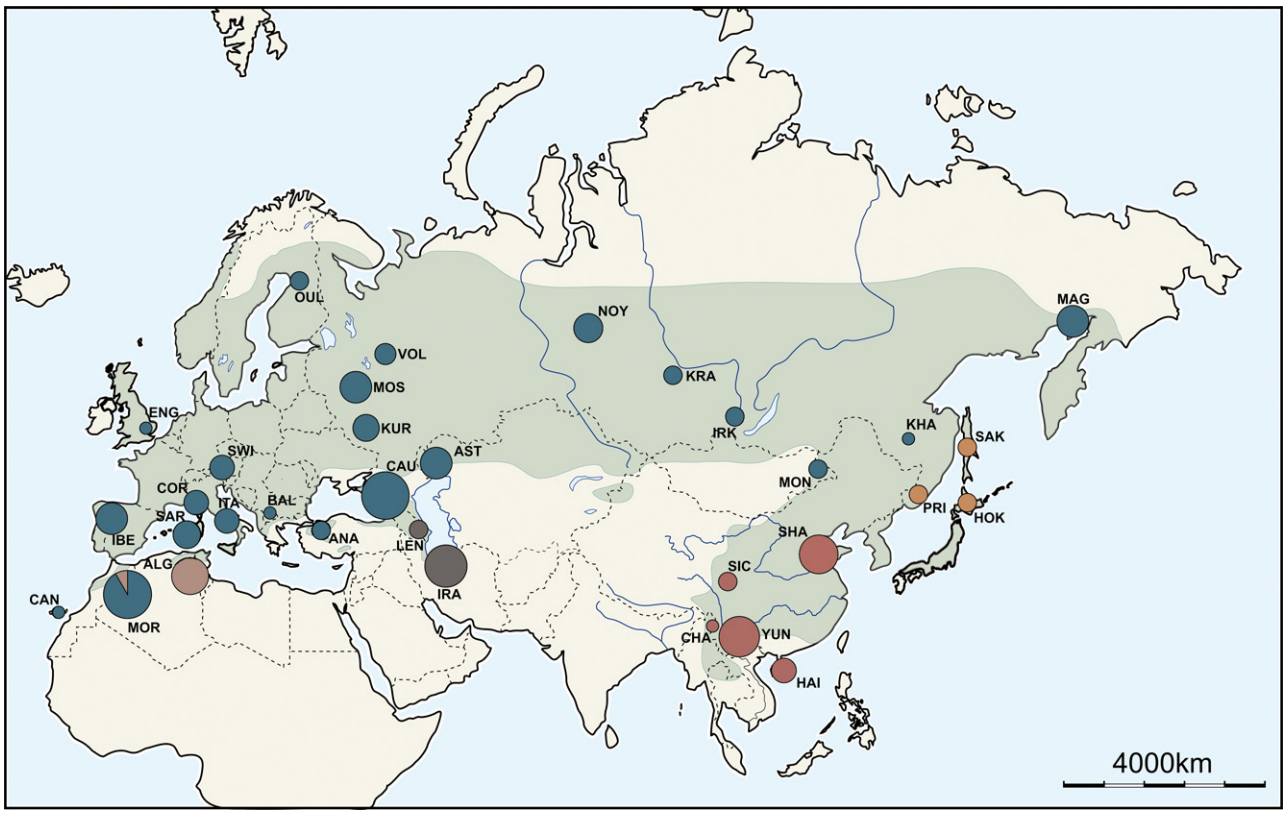

Figure 1. Distribution and genetic structure of *Dendrocopos major* in North Africa, Eurasia, China and Japan. The range of the species complex is shown in green. Sample locations are indicated as pie diagrams with abbreviations of the population names. Abbreviations are given in Table 1. Each pie diagram is proportional to the sample size. The colours of the pie diagrams indicate the haplotype groups in Figures 2 and 3.

Recent advances in phylogeographical and coalescent methods have made it possible to perform detailed assessment of the role played by the LGM in species diversification (Brito, 2005; Peters, Gretes & Omland, 2005).

Comprehensive sampling of the populations within the entire distribution of any given species should be the fundamental goal of any phylogeographical study, as phylogeographical inferences and the establishment of species limits cannot be achieved without filling sampling gaps. Unfortunately, relatively few phylogeographical studies of birds have covered most of the geographical distribution of widespread species in Eurasia (e.g. Pavlova *et al*., 2006; Zink *et al*., 2009). Moreover, even fewer of these studies have included specimens from Anatolia and southern Caucasia, particularly from Iran and Azerbaijan (e.g. Perktas *et al*., 2011). Therefore, phylogeographical studies of Eurasian birds usually lack specimens from the aforementioned areas. Blondel & Mourer-Chauvire (1998) have proposed that few potential refugia were available during the LGM for forest-related bird species in the Western Palaearctic region. In order to test this idea, it is important to explore the phylogeography of forest bird species throughout southern Eurasia. In addition, southern Eurasia, including Anatolia, Iran and North Africa, has been suggested as an important spot for molecular diversity in birds (Griswold & Baker, 2002; Zink, Drovetski & Rohwer, 2002a; Albayrak *et al*., 2011; Perktas *et al*., 2011). Therefore, in this study, we focus on the entire distribution range of the great spotted woodpecker complex (*Dendrocopos major*) to investigate its genetic structure and population history based on phylogeographical and coalescent methods.

The *D. major* complex has a broad distribution (Vaurie, 1965), including the Canary Islands, northwestern Africa, Anatolia and the Caucasus region to northern Iran, all Europe, Russia, northern Mongolia to Amurland, Sakhalin, Japan, and from China to Sikang, Burma, northern Indochina and Hainan (Fig. 1). Taxonomy below the species category is not clear, as is the case for many other bird species. Different authors have proposed different taxonomies within the complex. For instance, Vaurie (1965) proposed two different groups, *major* and *cabanisi*, with

various numbers of subspecies in each. Currently, the complex includes 14 accepted subspecies based on morphology and coloration differences (following Winkler, Christie & Nurney, 1995), although, at times, as many as 27 subspecies have been proposed (Peters, 1948). Because the ranges of these subspecies are arbitrarily described, species limits within the complex are not accurately known.

So far, few phylogeographical studies have been conducted on this species complex and none has covered its entire distribution. In the most comprehensive study to date based on the mitochondrial NADH dehydrogenase subunit 2 (ND2) and cytochrome-*b* genes, Zink *et al*. (2002a) did not find phylogeographical structure in populations from central and northern Europe or in those from Russia. These authors suggested that, during the Pleistocene, a refugium or refugia might have existed further south from the Caucasus region. Based on the same gene regions as Zink *et al*. (2002a), Garcia-del-Rey *et al*. (2007) found a significant difference between populations from the Canary Islands and those from the mainland, but not among the Islands themselves. Finally, using mitochondrial control region (CR) sequences from central and northern Europe, Britain and Ireland, McDevitt *et al*. (2011) designed a phylogeographical study to investigate the population history of the newly established populations of *D. major* in Ireland. Mitochondrial CR sequences showed a small, but significant, genetic structure in that study. Based on these results, the authors suggested that Ireland was colonized from Britain.

There are no detailed phylogeographical surveys of the populations of *D. major* from the southeastern region of Eurasia and China. Therefore, in this article, we conduct a comprehensive geographical survey of the phylogeographical structure of the *D. major* complex, building in part on the studies of Zink *et al*. (2002a) and Garcia-del-Rey *et al*. (2007), in order to evaluate the genetic structure and population history of this species complex throughout its entire distribution. Because the use of basal taxonomic units (phylogenetic species *sensu* Cracraft, 1997) is essential to understand the temporal and spatial patterns of diversification within any group, we also tested the species' limits within the complex. In order to achieve our objectives, we used haplotypes obtained from the mitochondrial ND2 gene region throughout the study.

MATERIAL AND METHODS

Sample collection

One hundred and fifty-five specimens, representing 33 populations of the *D. major* complex in Eurasia and North Africa, were obtained for this study (Fig. 1). DNA was extracted from toe pads from the ornithology collections of both the American Museum of Natural History ($N = 81$) and the Field Museum of Natural History ($N = 10$). Voucher information can be found, together with sequence information, in GenBank (JX276551–JX276641). In addition, 64 DNA sequences based on previous studies ($N = 63$ from Zink *et al*., 2002a; $N = 1$ from Garcia-del-Rey *et al*., 2007) were included to obtain broad sampling, and were re-evaluated.

DNA extraction, gene amplification and sequencing

Extractions of total genomic DNA from toe pads were performed with the DNeasy Kit (Qiagen), with incubation overnight after the addition of dithiothreitol (DTT) to the incubation buffer. We amplified a 352-bp fragment of the mitochondrial ND2 gene region via polymerase chain reaction (PCR) using a combination of six primers (PV 1–6) that were designed for the green woodpecker (Perktas *et al*., 2011). Moreover, two additional primers (forward PV3x, GCAACCACT GGGCAATAGCCTGAAC; reverse PV4x, CCTAGTTT TATAGCAATTGCAGCTGT) were designed for the Chinese specimens, as PV3 and PV4 (Perktas *et al*., 2011) did not work for them. For all specimens, the ND2 target fragment was amplified as three smaller fragments of approximately 220 bp, with 90–100-bp overlap between primers. DNA extractions and amplifications were conducted in a separate laboratory using fresh PCR reagents and laboratory equipment to avoid contamination. GoTaq Hot Start Polymerase (Promega) was used in genomic amplifications, and various annealing temperatures during PCR procedures were used to obtain the best results, depending on the different primer sets, ranging between 52 and 58 °C. The following PCR protocols were followed: denaturation at 95 °C for 5 min, followed by 42 cycles of 96 °C for 20 s, annealing for 15 s and 72 °C for 20 s. These cycles were followed by a final extension at 72 °C for 1 min. 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.

Statistical analyses

DNA sequences were aligned and edited in Sequencher v.4.7 (Gene Codes, Ann Arbor, MI, USA). The number of haplotypes, haplotype diversity (h) and nucleotide diversity (π) for the amplified region were calculated using DnaSP 5.10 (Rozas *et al*., 2003).

The neutrality of the data was tested using Tajima's *D* (Tajima, 1989) as implemented in Arlequin v.3.5 (Excoffier & Lischer, 2010). In addition, pairwise differences and Fu's F_s were also calculated in DnaSP 5.10 (Rozas *et al*., 2003), whereas raggedness indices were calculated in Arlequin v.3.5 (Excoffier & Lischer, 2010). Fu's F_s is the most suitable statistic for the evaluation of demographic expansion with moderate sample sizes such as that in this study (Ramos-Onsins & Rozas, 2002; Barrowclough *et al*., 2004). A negative F_s is usually attributed to recent population expansion. Demographic expansion was only calculated for the locations that had adequate sample sizes: southern Europe (Iberia), Russia (Kursk and Moscow), Caucasus and North Africa (Morocco and Algeria). Finally, to better understand the patterns of geographical variation within the *D. major* complex, an analysis of molecular variance (AMOVA; Excoffier, Smouse & Quattro, 1992) was performed, and pairwise population F_{st} values were also calculated in Arlequin v.3.5 (Excoffier & Lischer, 2010). For both the descriptive statistics and AMOVA, we combined some of the samples with low sample sizes as follows: England, Oulu and Switzerland were renamed Northern Europe; Irkutsk and Krasnoyarsk were renamed Irkutsk; Mongolia and Krasnoyarsk were renamed Mongolia; and Changyinhku (eastern Myanmar) and Yunnan were renamed Yunnan. As we only included one sequence from the Canary Islands, we did not include that sample in AMOVA. Thus, in total, we used 27 different populations throughout the analyses.

Phylogenetic analyses

Phylogenetic relationships between unique haplotypes were reconstructed using maximum parsimony (MP) in PAUP 4.0b10 (Swofford, 2001), maximum likelihood (ML) in GARLI (Zwickl, 2006) and a Bayesian inference analysis in MrBayes v.3.1.1 (Huelsenbeck & Ronquist, 2001) via the CIPRES Science Gateway 3.1. MP analyses were performed under the heuristic search option, using the tree bisection–reconnection (TBR) branch swapping algorithm and 1000 replicates. A bootstrap procedure with 1000 replicates was used to investigate the stability of the phylogenetic relationships. The ML analysis was performed employing HKY as the nucleotide substitution model, as selected through JModeltest 0.1.1 (Posada, 2008) using the Akaike Information Criterion (AIC), and estimating the base frequencies in GARLI. Two parallel analyses were run, which were automatically terminated when no significant improvements in topology were found after two million generations. One hundred bootstrap replicates were run in GARLI to assess support. The best model for the Bayesian analysis (HKY + G) was selected through MrModelTest 2.2 (Nylander, 2004) using AIC. Four simultaneous Markov chains were run in two independent analyses for 20 million generations each, sampling trees every 1000 generations for a total of 20 000 trees, with 9000 kept in each analysis. The resulting 36 000 sampled trees were used to compute the posterior probabilities of each node.

Following previous studies and lists (Zink *et al*., 2002a; Moyle, 2005; Benz, Robbins & Peterson, 2006; Fuchs *et al*., 2006, 2007), we used the following outgroups throughout all the phylogenetic analyses: *Dendrocopos leucotos* (GenBank accession number: DQ188168), *D. medius* (DQ361294), *D. mahrattensis* (DQ361280), *D. macei* (DQ361296), *D. minor* (DQ188180), *D. moluccensis* (AY625192), *D. hyperythrus* (DQ479169), *D. canicapillus* (DQ361303), *Picoides tridactylus* (DQ188164) and *Jynx torquilla* (DQ479151). Finally, to better visualize the relationship between haplotypes, we created a statistical parsimony network via TCS v 1.21 (Clement, Posada & Crandall, 2000) and the method described by Bandelt, Forster & Röhl (1999), where each branch represents a single nucleotide substitution. The only haplotype from the Canary Islands included in the study (taken from Garcia-del-Rey *et al*., 2007) has 41 base pairs missing. Therefore, this haplotype was not included in the network.

Molecular dating

In order to obtain an approximate date of divergence among the different lineages within the *D. major* complex, we employed a strict-clock approach in BEAST v.1.7.2 (Drummond & Rambaut, 2007) using all the individuals sampled, but with only *D. leucotos* as an outgroup. The substitution rate used for the strict-clock was 0.0137 substitutions per site per million years (Pereira & Baker, 2006). Model parameters for this analysis were selected in BEAUti with the following settings: HKY + G as substitution model, a Yule process as a tree prior and the initial root height estimated from the data (0.9 Mya); this, in turn, was used as a normal distribution prior for the root, with a standard deviation of 0.2. The analysis was run for ten million generations, sampling every 1000 generations. The results from the analysis were analysed in Tracer v.1.5 for convergence and summarized in TreeAnnotator v.1.7.2 using 10 000 'burn-in' trees. Finally, the resulting tree was visualized and edited using FigTree v.1.3.1 (Rambaut, 2009).

Gene flow

In addition to estimating the divergence times among the clades of the *D. major* complex, we estimated the

gene flow between the clades under the HKY model using the program MDIV (Nielsen & Wakeley, 2001). MDIV uses a Bayesian approach to simultaneously estimate divergence times and migration rates between two populations by calculating integrated likelihood surfaces for three parameters: θ ($\theta = 2N_e\mu$), the migration rate (twice the migration rate, $M = 2N_em$) and the scaled divergence time ($T = t_{div}/2N_e$).

MDIV was first run using default search settings and default priors (for the parameters θ and T). We then set our prior value of T to equal 50 and M to equal 2 to obtain well-behaved posterior distributions. MDIV analyses were run three times to ensure convergence of the posterior distributions for each of the parameter estimates. Each run ran for two million generations following a burn-in period of 500 000 generations. The highest posterior density (HPD) credible interval was computed for gene flow (M) according to the smallest possible interval within 95% of the posterior probability. As we found only one haplotype from Iran and Azerbaijan (details are given in the results section), it was not possible to estimate the effective population size for this clade. Therefore, for the MDIV analysis, we combined the samples from Iran and Eurasia. Moreover, the only individual from the Canary Islands (Garcia-del-Rey *et al*., 2007) was excluded from this analysis because of the small sample size.

RESULTS

Sequences and descriptive statistics

We obtained 352 bp of the mitochondrial ND2 gene region representing a total of 33 populations. Sequences were aligned without indels. Information for all haplotypes, including location and frequencies, is presented in Table 1. Twenty-nine unique haplotypes were identified among 155 sequences, 17 of which were unique to this study. Haplotype seven (Hap-7) was the most common haplotype within the study area, and was detected in 89 individuals from 21 populations. Within populations, haplotype diversity (h) ranged from 0 to 0.833, whereas nucleotide diversity (π) ranged from 0 to 0.00284 (Table 2). None of the populations sampled differed from neutral expectations according to Tajima's D (Table 2).

Phylogenetic analyses

MP analyses of the *D. major* complex, including the 10 outgroups, returned 36 most parsimonious trees of 276 steps (consistency index, 0.5978; retention index, 0.6959). Forty-four variable characters were parsimony uninformative, whereas 94 were parsimony informative. The two independent ML analyses returned trees with an identical structure with those recovered by MP and Bayesian inference analyses. We present the MP tree together with the haplotype network for each clade (Fig. 2), as well as the ML tree with both bootstrap values and posterior probabilities (Fig. 3). Results from the phylogenetic analyses, as well as from the haplotype network, indicate the presence of four distinct clades: China, Japan, Iran–Azerbaijan and Eurasia–North Africa. Along the different trees, the different clades are separated from each other by long branches and are reciprocally monophyletic with good bootstrap values ($\geq$ 70%; Hillis & Bull, 1993; Baldauf, 2003) and high posterior probabilities (Fig. 3). Within the Eurasia–North Africa clade, the populations from Morocco, Algeria and Tunisia are paraphyletic with respect to the remainder of Eurasia.

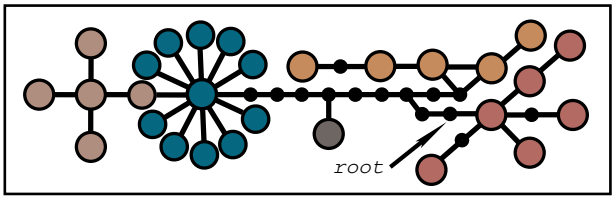

Figure 2. Parsimony network based on the mitochondrial NADH dehydrogenase subunit 2 (ND2) sequences for the *Dendrocopos major* complex. Haplotype groups are indicated by different colours, and dots indicate missing haplotypes.

Geographical variation and demography

Based on the AMOVA results, we found substantial genetic variation among the four clades (Table 3). It is plausible to say that this variation can be attributed to the fact that each one of these clades corresponds to a different phylogenetic species (see the discussion section).

To further understand the genetic variation among populations within the Eurasia–North Africa clade, we performed a separate AMOVA (Table 3). The results showed that much of the genetic variation within the Eurasia–North Africa clade is a result of the unique haplotypes from Morocco, Algeria and Tunisia. Phylogeographical structure as well as the geographical variation within this clade suggested that, although the populations from North Africa might have been historically isolated from those of Europe, there should have been recent gene flow between European and North African populations.

In order to find probable refuge areas throughout Eurasia and North Africa, we used both Fu's F_s and pairwise differences to explore the deviation from constant population size within populations. As

Table 1. Frequencies of 28 mitochondrial NADH dehydrogenase subunit 2 (ND2) haplotypes within 33 population samples of the *Dendrocopos major* complex

	111111222222222233333333 113355678814459001113445680122235 789781903170203684030289254073456950 1	SHA	YUN	SIC	CHA	HAI	BAL	SWI	ITA	COR	SAR	IBE	ENG	OUL	CAU	ANA	MAG
Hap-1	CTTGCCGCCTGATCCCTTTCCAACCTTCAAATAATAG	9	7		1	1											
Hap-2	.C.....C.....			1													
Hap-3	.....T.....			1													
Hap-4	.....T....C.....		2														
Hap-5	.....A					2											
Hap-6	.....T.....A					1											
Hap-7	.....T.....TT.CC....TTC.....G...						3	4	6	4	6	4	1	2	13	2	6
Hap-8	.....T.....C.TT.CC....TTC.....G...																
Hap-9	.....TA.....TT.CC....TTC.....G...																
Hap-10	.....T.....CTTT.CC....TTC.....G...																
Hap-11	.....T.....CTTT.CC..T.TTC.....G...																
Hap-12	.....T...G..CTTT.CC....TTC.....G...																
Hap-13	.....TA.....CTTT.CC....TTC.....G...																
Hap-14	.....T.....TT.CC...GTTC.....G...											1					
Hap-15	.....T.....TT.CC....TTCC.....G...											1					
Hap-16	.....T....A...TT.CC....TTC.....G...											1					
Hap-17	.....T.....TT.CC....TTC.....GC..														1		
Hap-18	...A.T.....G...T.CC.....C.....G...																
Hap-19	G.....G...T...T.....C..G.GN.G...																
Hap-20	G.....G...T...T.....C..G.GN.G.--																
Hap-21	.....G...T...T.....C..G.G..G...																
Hap-22	.....G...T...T.....C..G.G.....																
Hap-23	.....G...T...T.....C..G.GC.....																
Hap-24	.....T.....TT.CC....TTC.....NNG...																
Hap-25	....YT.....TT.CC....TTC.....G...																
Hap-26	.....T.....TT.CC....TTC...G...G...														1		
Hap-27	.....T..T.....TT.CC....TTC.....G...																
Hap-28	..C..T.....TT.CC....TTC.....G...																

Table 1. *Continued*

	111111222222222233333333 113355678814459001113445680122235 789781903170203684030289254073456950 1	IRK	AST	MOS	MON	KRA	KUR	NOY	VOL	KHA	MOR	ALG	IRA	LEN	HOK	SAK	PRI
Hap-1	CTTGCCGCCTGATCCCTTTCCAACCTTCAAATAATAG																
Hap-2	.C.....C.....																
Hap-3	.....T.....																
Hap-4	.....T....C.....																
Hap-5	.....A																
Hap-6	.....T.....A																
Hap-7	.....T.....TT.CC....TTC.....G...	2	5	6	2	1	3	5	3	2	9						
Hap-8	.....T.....C.TT.CC....TTC.....G...										1						
Hap-9	.....TA.....TT.CC....TTC.....G...										2						
Hap-10	.....T.....CTTT.CC....TTC.....G...											5					
Hap-11	.....T.....CTTT.CC..T.TTC.....G...											1					
Hap-12	.....T...G..CTTT.CC....TTC.....G...											1					
Hap-13	.....TA.....CTTT.CC....TTC.....G...											1					
Hap-14	.....T.....TT.CC...GTTC.....G...																
Hap-15	.....T.....TT.CC....TTCC.....G...																
Hap-16	.....T....A...TT.CC....TTC.....G...																
Hap-17	.....T.....TT.CC....TTC.....GC..																
Hap-18	...A.T.....G...T.CC.....C.....G...												11	3			
Hap-19	G.....G...T...T.....C..G.GN.G...														1		
Hap-20	G.....G...T...T.....C..G.GN.G.--														1		
Hap-21	.....G...T...T.....C..G.G..G...															1	1
Hap-22	.....G...T...T.....C..G.G.....															1	
Hap-23	.....G...T...T.....C..G.GC.....																1
Hap-24	.....T.....TT.CC....TTC.....NNG...		1														
Hap-25	....YT.....TT.CC....TTC.....G...		1														
Hap-26	.....T.....TT.CC....TTC...G...G...																
Hap-27	.....T..T.....TT.CC....TTC.....G...			1													
Hap-28	..C..T.....TT.CC....TTC.....G...						1										

Haplotypes shown in bold are unique for this study. One haplotype (Hap-29) from the Canary Islands (CAN) is not given in the table because the first 41 bases were missing. SHA, Shandong; YUN, Yunnan; SIC, Sichuan; HAI, Hainan; CHA, Changyinhku; BAL, Balkan; SWI, Switzerland; ITA, Italy; COR, Corsica; SAR, Sardinia; IBE, Iberia; ENG, England; OUL, Oulu; CAU, Caucasus; ANA, Anatolia; MAG, Magadan; IRK, Irkutsk; AST, Astrakhan; MOS, Moscow; MON, Mongolia; KRA, Krasnoyarsk; KUR, Kursk; NOY, Noyabrsk; VOL, Vologda; KHA, Khabarovsk; MOR, Morocco; ALG, Algeria; IRA, Iran; LEN, Lenkoran; HOK,Hokkaido; SAK, Sakhalin; PRI, Primor'e.

Table 2. Genetic characteristics and demographic structure of the *Dendrocopos major* complex populations included in this study [Number of individuals (N); number of haplotypes (H); haplotype (h) and nucleotide (π) diversity]

Populations	N	H	h	π	Fu's F_s	Tajima's D
Shantung	9	1	NC	NC	NC	NC
Sichuan	2	2	NC	NC	1.09861	NC
Yunnan	10	2	0.356	0.00202	1.52347	0.01889
Hainan	4	3	0.833	0.00284	–0.88730	–0.70990
Balkans	3	1	NC	NC	NC	NC
North Europe	7	1	NC	NC	NC	NC
Italy	6	1	NC	NC	NC	NC
Corsica	4	1	NC	NC	NC	NC
Sardinia	6	1	NC	NC	NC	NC
Iberia	7	4	0.714	0.00244	–1.79788*	–1.35841
Morocco	12	3	0.439	0.00133	–0.72455	–0.84971
Algeria	8	4	0.464	0.00142	–1.83232*	–1.31009
Caucasus	15	3	0.257	0.00076	–1.54636*	–1.49051
Anatolia	2	1	NC	NC	NC	NC
Lenkoran	3	1	NC	NC	NC	NC
Iran	11	1	NC	NC	NC	NC
Hokkaido	2	2	NC	NC	NC	NC
Sakahlin	2	2	NC	NC	NC	NC
Primor'e	2	2	NC	NC	NC	NC
Magadan	6	1	NC	NC	NC	NC
Irkutsk	3	1	NC	NC	NC	NC
Astrakhan	7	3	NC	NC	NC	0
Moscow	7	2	0.286	0.00081	–0.09474	–1.00623
Mongolia	4	1	NC	NC	NC	NC
Kursk	4	2	0.500	0.00142	0.17185	–0.61237
Noyabrsk	5	1	NC	NC	NC	NC
Vologda	3	1	NC	NC	NC	NC
Canary	1	1	NC	NC	NC	NC

NC, not computed because of sample size and/or nonpolymorphism in the data.
*$P < 0.05$.

Table 3. Hierarchical estimates of F_{st} between population samples of the *Dendrocopos major* complex based on mitochondrial NADH dehydrogenase subunit 2 (ND2) sequences of mitochondrial DNA

	Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation
Among four clades	Among clades	3	281.908	3.91618	92.73
	Among populations within clades	23	22.531	0.14878	3.52
	Within populations	126	19.954	0.15836	3.75
	Total	152	324.392	4.22332	
	F_{st}	0.96250 ($P < 0.001$)			
Between two regions (Eurasia/North Africa)	Among populations	1	6.383	0.18940	48.97
	Within populations	107	21.121	0.19740	51.03
	Total	108	27.505	0.38679	
	F_{st}	0.48966 ($P < 0.001$)			

we only found polymorphisms in Iberia, Caucasus, Moscow, Kursk, Morocco and Algeria, we only calculated pairwise differences for these six populations (Fig. 4). None differed significantly from the expectation of sudden population expansion (Iberia: raggedness index, 0.286; $P = 0.23$; sudden expansion sum of squared deviation, 0.048; $P = 0.35$; Caucasus: raggedness index, 0.265; $P = 0.57$; sudden expansion sum of

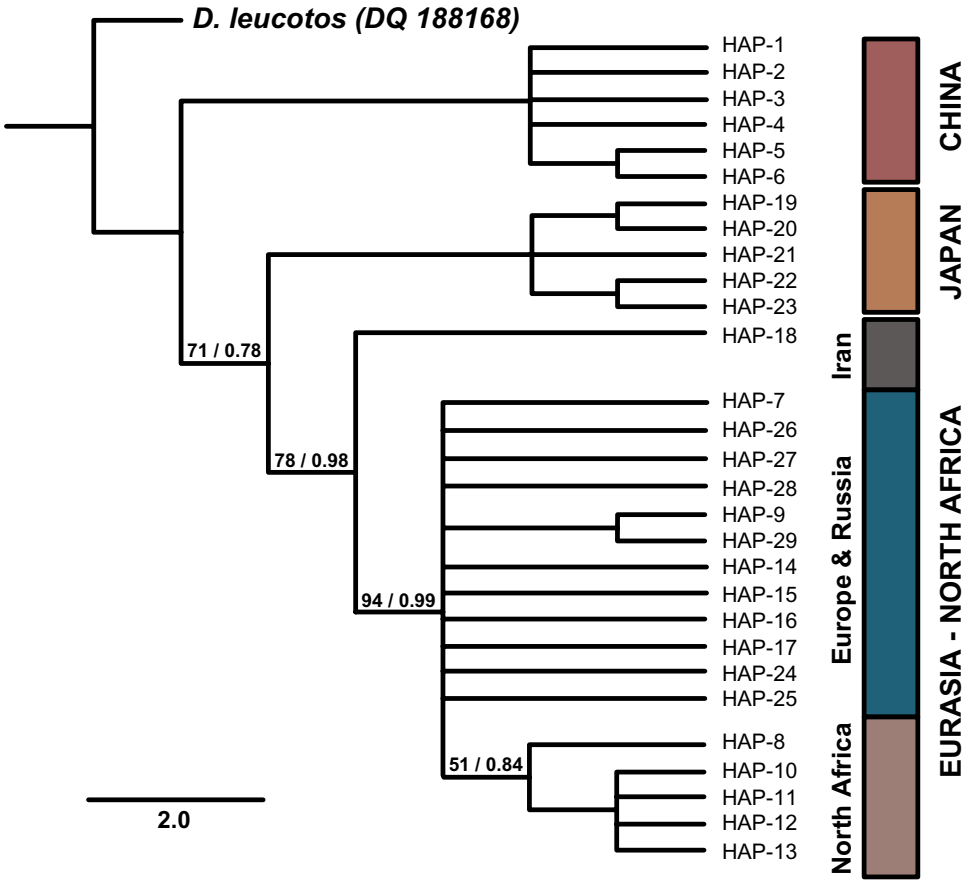

Figure 3. Maximum likelihood tree with bootstrap values and posterior probabilities, showing the existence of four major groups within the *Dendrocopos major* complex.

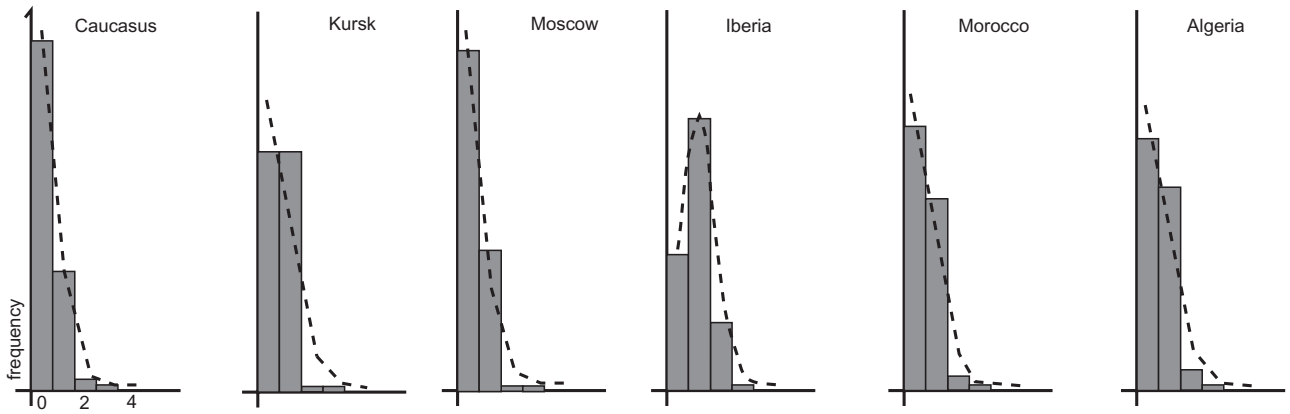

Figure 4. Pairwise distributions of several populations within *Dendrocopos major* based on the mitochondrial NADH dehydrogenase subunit 2 (ND2) sequences. Broken line represents the expected distribution in each plot.

squared deviation, 0.004; $P = 0.41$; Moscow: raggedness index, 0.302; $P = 0.68$; sudden expansion sum of squared deviation, 0.248; $P = 0.08$; Kursk: raggedness index, 0.250; $P = 0.96$; sudden expansion sum of squared deviation, 0.021; $P = 0.71$; Morocco: raggedness index, 0.167; $P = 0.41$; sudden expansion sum of squared deviation, 0.011; $P = 0.36$; Algeria: raggedness index, 0.227; $P = 0.39$; sudden expansion sum of squared deviation, 0.033; $P = 0.23$; Rogers & Harpending, 1992). These results were concordant

with Fu's F_s statistic (Table 2). It is noteworthy that the most common haplotype (Hap-7) was found in Morocco, but not in the other African localities. We further explored the inference of demographic structure for the polymorphic populations (Iberia, Caucasus, Kursk, Moscow, Morocco and Algeria) using haplotype (h) and nucleotide (π) diversity. Within Eurasia, Iberia is the oldest growing population (Fig. 4) and has the highest values for both h and π. In addition, Kursk, in the northern Caucasus region, also has high values of h and π, similar to those found for Morocco and Algeria. These results might support the idea of the presence of refugia in Iberia, the northern Caucasus region and North Africa. Conversely, the Caucasus and Moscow have the lowest values for both h and π.

Molecular dating and gene flow

Results from the dating analysis (Fig. 5) suggest that the *D. major* complex and *D. leucotus* split around 0.9 Mya [95% confidence interval (CI), 0.5–1.10 Mya]. Within the complex, the split between the Chinese lineage and the remainder of the group occurred around 0.8 Mya (95% CI, 0.4–1.0 Mya). The Japan lineage split from the Iran–Azerbaijan and Eurasia–North Africa clades around 0.7 Mya (95% CI, 0.3–0.8 Mya), whereas the Iran–Azerbaijan and Eurasia–North Africa clades split at *c.* 0.5 Mya (95% CI, 0.2–0.6 Mya) and the North African lineage diverged from the European at around 0.3 Mya.

The estimates of gene flow ($M = 2N_em$) between the clades obtained from the MDIV analyses indicate that the coalescent time for the most recent common ancestor was sufficient to achieve reciprocal monophyly. We also found a low level of migration between clades, with the exception of Eurasia and North Africa (Table 4, Fig. 6), as a result of the shared haplotypes between Eurasia and Morocco. However, because Algeria and Tunisia have a small isolated distribution within North Africa with unique haplotypes, which are not shared elsewhere in Eurasia or North Africa, MDIV analyses indicated that gene flow is restricted between Morocco and the Algerian and Tunisian populations, with less than one migrating female per generation ($M = 0.084$, 95% CI of HPD, 0.004–0.884).

Table 4. MDIV estimates of θ and gene flow (M) with 95% confidence interval (CI) of highest posterior densities (HPDs)

Clades	θ (95% CI of HPDs)	Gene flow (95% CI of HPDs)
China/Japan	1.530 (0.631–3.800)	0.052 (0.004–0.588)
Japan/Eurasia	2.261 (1.076–4.103)	0.044 (0.004–0.456)
Eurasia/North Africa	1.989 (1.053–3.593)	0.192 (0.008–1.996)

DISCUSSION

Species limits and taxonomic implications

According to the results of the phylogenetic analyses (Figs 2, 3), there are four distinct clades within the traditionally recognized *D. major* complex: Eurasia–North Africa, Iran–Azerbaijan, Japan and China. As mentioned previously, these clades are separated from one another by long branch lengths. Moreover, each of these clades, with moderate bootstrap support values and good posterior probabilities, except for that from China, is reciprocally monophyletic, has disjunct distributions and is diagnosably distinct (Vaurie, 1965). Together, and despite relying only on a short fragment of the mitochondrial ND2 gene region, in our opinion, these results warrant the recognition of at least four different phylogenetic species within the complex (Figs 2, 3): *Dendrocopos major* (Linnaeus, 1758) from Eurasia–North Africa, *Dendrocopos poelzami* (Bogdanov, 1879) from Iran–Azerbaijan, *Dendrocopos japonicus* (Seebohm, 1883) from Japan and *Dendrocopos cabanisi* (Malherbe, 1854) from China. Moreover, the geographical pattern of coloration within the species complex is mostly concordant with these four phylogenetic species, a fact that further advances the idea that each constitutes a phylogenetic species.

A number of different subspecies within *D. cabanisi* and *D. major* have been described throughout the years (i.e. 20 subspecies in Vaurie, 1965; 14 subspecies in Winkler *et al.*, 1995; 24 subspecies in Dickinson, 2003). However, morphological character transitions are only significant among the four clades described in the phylogenetic tree (Fig. 3). The *cabanisi* group from China is darker than *japonicus*, whereas *japonicus* is intermediate between *cabanisi* and the nominate *major* group in terms of coloration as well as in other characters (Vaurie, 1965). A difference in coloration and size can also be appreciated between *poelzami* and the different populations within the nominate *major* group, as *poelzami* is darker in the underparts. Among the different populations within Europe and Russia, coloration differences are subjective and size differences are mostly substantial only between the northern and southern populations (authors' own observations based on morphological measurements, results not shown). Moreover, Vaurie (1965) reported that wing measurements show mostly clinal variation among European subspecies. During the analysis, we did not find any substantial genetic differentiation between

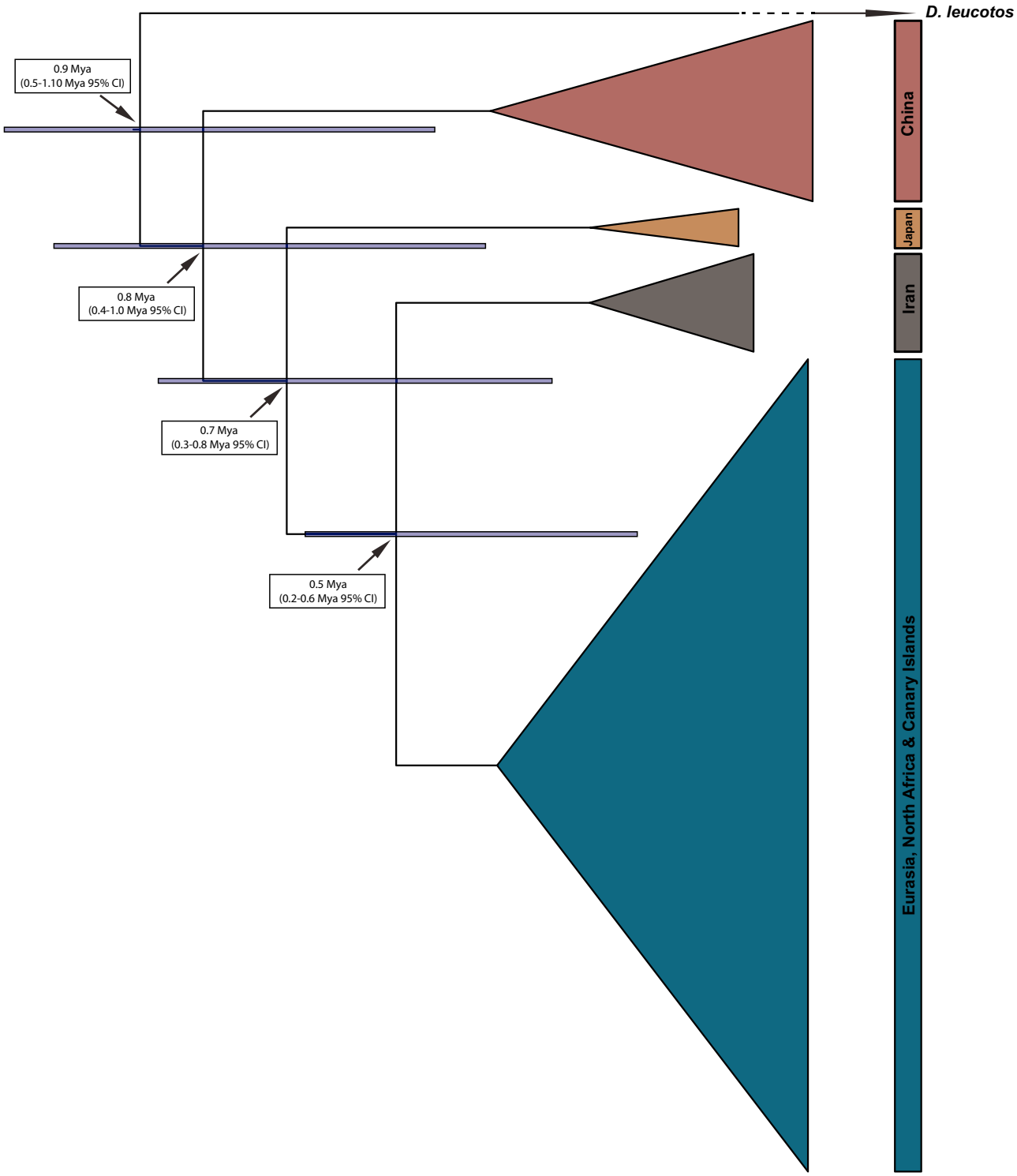

Figure 5. Results from the molecular dating analysis. All data are given in Mya. Only data relevant to the discussion are shown. Blue horizontal bars correspond to the 95% confidence interval of each of the divergence dates.

currently recognized subspecies within *D. cabanisi* and *D. major* (with the exception of the subspecies described for North Africa, see discussion below). Thus, it is our opinion that subspecies status within China, Europe and Russia is arbitrary. A further, more detailed study within the clades described in this article might result in the recognition of more phylogenetic species.

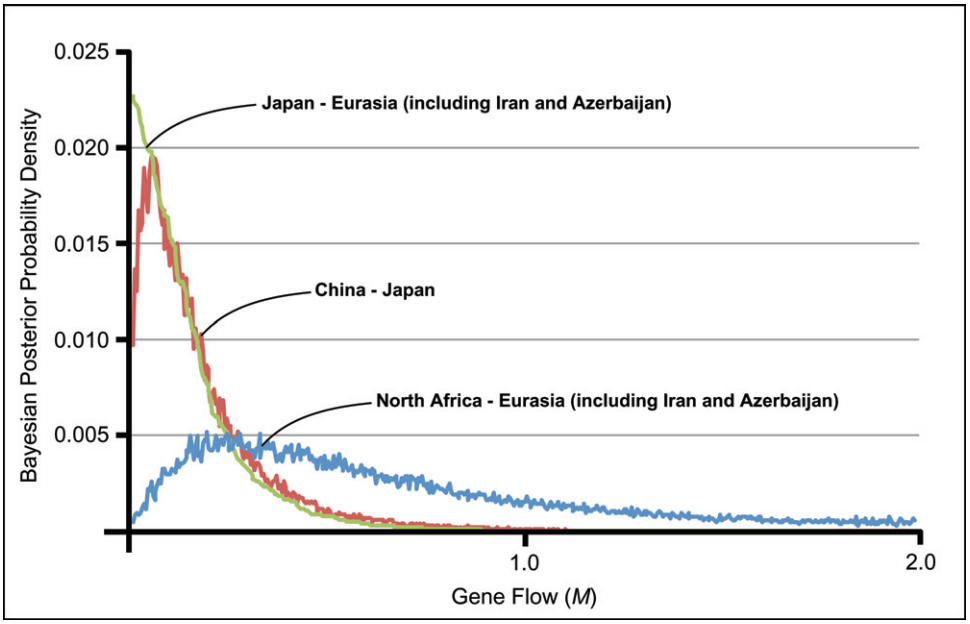

Figure 6. Coalescent-based estimates of the probability density of gene flow (*M*).

The status of the North African clade, which is paraphyletic with respect to Eurasia (Figs 2, 3), requires special attention. The populations from Algeria and Tunisia (*D. major numidus*) are diagnosably distinct from those of Europe (Fig. 7). Moreover, their distribution is allopatric with respect to those of Morocco and southern Europe (Fig. 1), and they have a unique set of haplotypes not shared by any other location (Table 1). For all these reasons, it is very likely that the populations from Algeria and Tunisia (*D. major numidus*) may, in fact, constitute a different phylogenetic species. Although the coalescent-based gene flow analysis indicated a moderate gene flow, there is insufficient evidence with the available data to indicate that this population is different from that of the rest of Europe. The inclusion of a larger set of characters might clarify the status of this currently recognized subspecies. However, the individuals from Morocco have an intermediate morphology between *D. major numidus* and nominate *D. major major* (Fig. 7), which suggests that the Moroccan population could, in fact, be a hybrid. This idea is further supported by the shared haplotype from Europe possessed by the Moroccan individuals (Table 1). Given the amount of data in this study, we cannot make further inferences on the taxonomic status of these two populations, but we recognize the need to further explore this issue.

Interestingly, within *D. major*, Garcia-del-Rey *et al*. (2007) described unique haplotypes from the Canary Islands. The mean sequence divergence between the Canary Islands and Eurasia was reported to be $0.2 \pm 0.1\%$. These findings are at odds with other studies, which found deep divergence between passerines of the Canary Islands and Eurasia (e.g. more than 2% divergence between the Canary Islands and Europe in the common chaffinch; Suarez *et al*., 2009). Based on these results, it is plausible to believe that *D. major* populations from the Canary Islands split from their European counterparts only recently. In our study, we included one haplotype from the Canary Islands from the study by Garcia-del-Rey *et al*. (2007). However, and perhaps because the length of the published sequence does not match exactly the rest of those in our analysis (first 41 base pairs of ND2 are missing), we could not find substantial differences between the haplotype from the Canary Islands and those from the mainland.

Molecular dating and gene flow

Obtaining divergence dates from molecular data is always problematic, as these dates can be under- or overestimated as a result of the data, method or algorithm used. Mitochondrial data are widely used for this purpose. However, the use of mitochondrial data for dating is not without issues, such as saturation because of the higher mutation rate and the correct calibration of the rate of evolution of the gene or genes used (Lovette, 2004). Rather than relying on the 'widely accepted 2% standard avian calibration' (see Lovette's, 2004 comments), we used the rate for ND2 derived by the comprehensive analysis of vertebrate mitogenomic data by Pereira & Baker

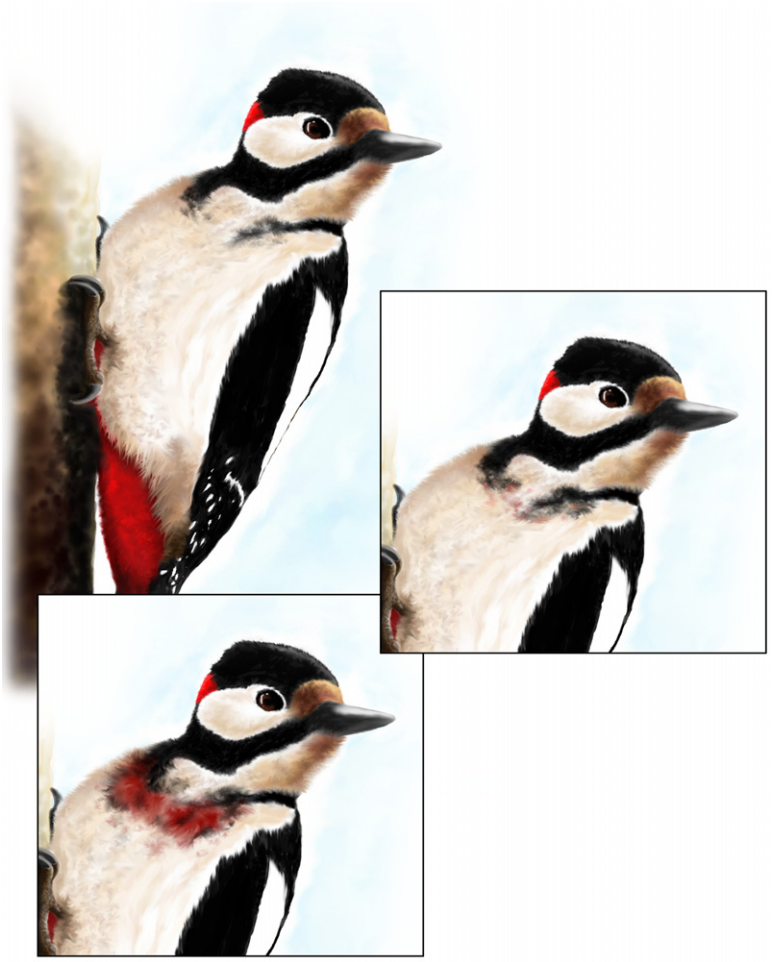

Figure 7. Comparison between nominate *major* and North African supspecies (*Dendrocopos major mauritanus* and *D. major numidus*) according to upper breast coloration pattern. Top left, nominate *major*; middle showing a red crescent – *mauritanus*; bottom with very conspicuous red breast – *numidus*.

(2006). Even so, the results from our molecular dating analysis should be taken as rough approximations, as they are derived from only a small fragment of the ND2 gene, and thus are subject to a much wider error range. Nevertheless, we feel that including them yields an extra layer of evidence to the history of the *D. major* complex.

According to the coalescent-based gene flow analysis between the four different clades in the phylogenetic tree, gene flow was close to zero between both China and Japan, and Japan and Eurasia (Fig. 6). However, we found relatively moderate gene flow between Eurasia and North Africa (Table 4). Thus, based on the gene flow analysis and the reciprocal monophyly found among the four clades in the phylogenetic tree, we suggest that gene flow is severely restricted between China and Japan, and among Japan, Iran, and Eurasia.

In conclusion, we found restricted gene flow and a moderate divergence time with reciprocal monophyly among the four different clades. These results concur with the diagnosable distinct morphological characters for each of the four clades (Winkler *et al*., 1995). Hence, each phylogenetic species [*Dendrocopos major* (Linnaeus, 1758), *Dendrocopos poelzami* (Bogdanov, 1879), *Dendrocopos japonicus* (Seebohm, 1883) and *Dendrocopos cabanisi* (Malherbe, 1854)] can also be considered as a separate biological species.

Phylogeograhical structure

Although this study is based on a short piece of the ND2 gene, the results support a substantial genetic difference among the four different clades (China, Japan, Iran–Azerbaijan and Eurasia–North Africa) obtained during the phylogenetic analyses of the *D. major* species complex. Branch lengths (Figs 2, 3) suggest that these four groups have had an independent history for a long time. Moreover, the percentages of molecular variance among species further support

these results (Table 3). As a result of the sedentary behaviour of woodpeckers in the Palaearctic region, as well as the results of our gene flow analysis, we would expect to find phylogeographical structure within the individual species (e.g. Perktas *et al*., 2011; Pons *et al*., 2011). However, and in accordance with the results found by Zink *et al*. (2002a), we did not find any significant phylogeographical structure within each of the four clades (Fig. 2).

Throughout this discussion, we have mostly concentrated on the Eurasia–North Africa clade to draw some demographic inferences for *D. major*. Although some of our individual sample sizes are small, results from the pairwise distributions and Fu's F_s values of some populations within the Eurasia–North Africa clade are consistent with a recent population expansion from different refugia (Table 2, Fig. 4). Genetic structure for *D. major* is concordant with that of other groups of birds throughout Eurasia, such as the three-toed woodpecker (*Picoides tridactylus*, Zink *et al*., 2002b) and some common passerines (*Chloris chloris*, Merilä, Björklund & Baker, 1997; *Fringilla coelebs*, Griswold & Baker, 2002; several *Paridae* species, Pavlova *et al*., 2006). In the case of the three-toed woodpecker, Zink *et al*. (2002b) found one most common haplotype throughout Eurasia, with several other haplotypes just one or two steps apart from this common haplotype. These results suggest that the three-toed woodpecker colonized its current distribution range recently. This colonization should have occurred from a refuge or refugia, because much of the current distribution range of the species was affected by the last glacial cycles (Hewitt, 1999, 2000). Nevertheless, this study could not find any refugia. However, Merilä *et al*. (1997) suggested 'leading edge expansion' as an explanation for the population structure of the greenfinch (*Chloris chloris*). This passerine is widespread throughout Eurasia, and does not show phylogeographical structure in its distribution range. However, southern populations have higher nucleotide diversity than northern populations. According to the authors, these results suggest a recent and fast colonization history from southern latitudes – most probably from multiple refugia – leading to an unstructured tree. Furthermore, Griswold & Baker (2002), and U. Perktas, G. F. Barrowclough and J. G. Groth (unpubl. data), found this same phylogeographical structure for another common passerine bird species in Eurasia, the common chaffinch (*Fringilla coelebs*). In addition, another refuge from the western part of North Africa was described for the common chaffinch (Griswold & Baker, 2002). The presence of the most common haplotype (Hap-7), as well as the results from our gene flow analysis in North Africa, suggest that gene flow between Eurasia and North Africa must have occurred during the last glacial period, as in the case of the common chaffinch. These findings may suggest the occurrence of a refuge or refugia in North Africa. Zink *et al*. (2002a) suggested that a possible refuge for *D. major* might be located in the southern Caucasus region. However, our results are at odds with those of Zink *et al*. (2002a), as they show that the Caucasus Mountains act as a barrier for the clades north (Eurasia, *D. major*) and south (Iran–Azerbaijan, *D. poelzami*) of them. Furthermore, our results suggest that the Iran–Azerbaijan (*D. poelzami*) clade split from that of Eurasia–North Africa (*D. major*) around 0.5 Mya (95% CI, 0.2–0.6 Mya). These inferences might also apply to the populations from the northeast part of Anatolia, because of the continuous distribution of the species between Iran and Anatolia.

Taken together, our results suggest that the distribution of *D. major* experienced a rapid population expansion throughout Eurasia after the departure of extensive glaciers at the end of the LGM. Voous (1947) suggested multiple refugia and a different colonization history for *D. major*. Our data do not support Voous' (1947) suggestion of multiple refugia throughout Europe or Zink *et al*.'s (2002a) suggestion of a refuge or refugia located in the southern Caucasus region. However, as Iberia, Kursk and North African locations (e.g. Morocco) have relatively higher haplotype (h) and nucleotide (π) diversity than all the other locations within the Eurasia–North Africa clade, these locations might have served as refugia for this clade, as suggested by the 'leading edge expansion' theory (Hewitt, 1996).

We found relatively low variation of the ND2 gene within continental Europe and Russia. Winkler *et al*. (1995) suggested that northern populations of *D. major* are irruptive migrants. Moreover, up to 3000 km of loop migration between Europe and Russia has also been suggested (Cramp, 1985). We assume that some invading birds remain in their wintering areas to breed, and this fact could explain both the widespread distribution of the most common haplotype (Hap-7) within Europe and Russia and the general absence of phylogeographical structure within the Eurasia–North Africa clade, a case similar to the pattern found for the great tit (Pavlova *et al*., 2006).

However, the mitochondrial ND2 gene might possess too little variation to recognize possible refugia of *D. major* within Eurasia. In birds, mitochondrial genes other than the noncoding CR show relatively low variation (Saunders & Edwards, 2000). McDevitt *et al*. (2011) found relatively high CR variation for *D. major* within such a small geography. We believe that the ND2 gene is sufficient to distinguish major splits within the *D. major* complex. However, further phylogeographical research on this species complex, which might include CR sequences, is vital to recognize possible refuges within continental Europe and Russia.

As Zink *et al*. (2002a) and Garcia-del-Rey *et al*. (2007) did not include any samples from China, Iran, Azerbaijan, southern Europe and North Africa, this study is the first comprehensive geographical survey of mitochondrial DNA variation in the *D. major* species complex. This comprehensive study shows how the filling of sampling gaps in phylogeographical studies has an effect on the final inferences drawn. Moreover, this study showcases the importance of museum collections as data sources for rare specimens in this type of broad phylogeographical survey. Our results show that there is congruence between the mitochondrial DNA tree and geographical variation, constituting another example of the utility of mitochondrial DNA as a molecular marker for phylogeographical inferences (e.g. Zink & Barrowclough, 2008).

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