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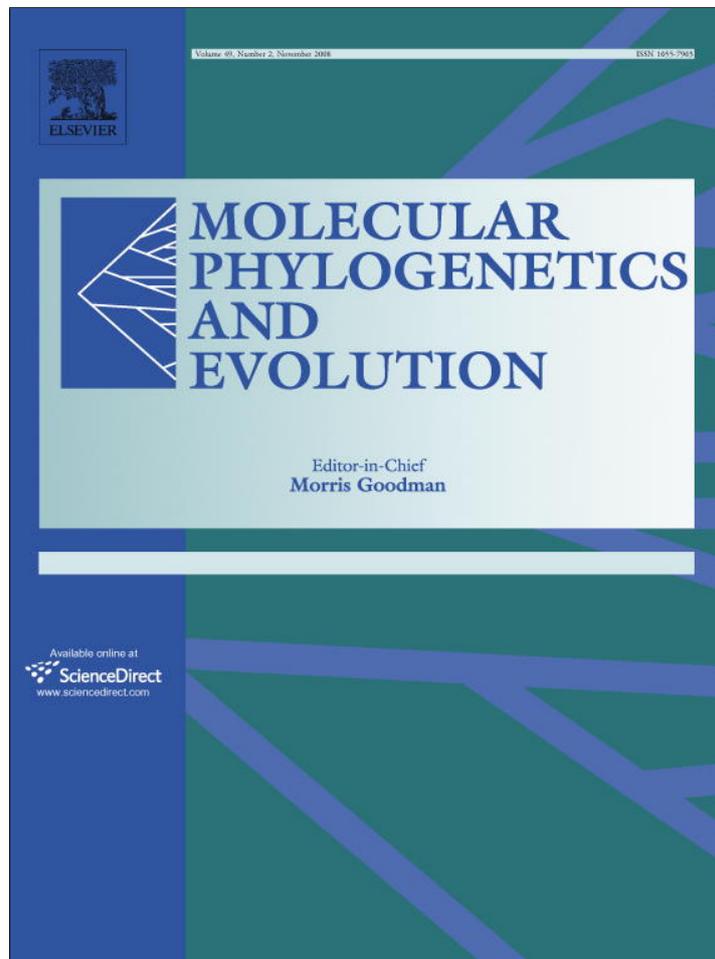


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Evidence of a highly complex phylogeographic structure on a specialist river bird species, the dipper (*Cinclus cinclus*)

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ABSTRACT

This study details the phylogeographic pattern of the white-throated dipper (*Cinclus cinclus*), a Palearctic, temperate, passerine bird that is exclusively associated with flowing water. Our results reveal a complex phylogeographic structure with at least five distinct lineages for the Western Palearctic region. As for many species of the Western Palearctic fauna and flora, this genetic structure is probably linked to the isolation of populations in different southern refuges during glacial periods. Furthermore, the isolation of populations in Scandinavia and/or Eastern regions, but also in Morocco and probably in Corsica, was accentuated by ecological and biogeographic barriers during Quaternary interglacial periods. During glacial periods, Italy, Sicily and the Balkano-Carpathian region acted as major refuge zones for the dipper. At the end of the last ice age, Western Europe was repopulated by dippers from an Italian refuge, while Eastern Europe was recolonised by Balkano-Carpathian birds. A large contact zone between these two lineages was evidenced and extends from Luxembourg to Hungary. Finally, our results indicate the need to clarify the taxonomic status of the dipper, especially concerning the European subspecies whose validity appears uncertain.

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1. Introduction

The last 700,000 years of the Quaternary were characterised by a succession of glacial and interglacial periods, with a tempo of $\pm 100,000$ years (Webb and Bartlein, 1992). Major changes occurred in the composition and location of European biotas as a response to these climatic fluctuations (Comes and Kadereit, 1998).

During glacial periods, temperate species could only survive in suitable habitats, mainly located in southern areas. Evidence from fossil and pollen data suggests that three main glacial refuges for temperate species were located in the southern peninsulas of Iberia, Italy, and the Balkans (Bennett et al., 1991; Hewitt, 1996; Blondel, 1997). More recently, other areas located near the Caucasus, the borders of the Black Sea and in the Carpathian region were also identified as potential glacial refuges (Hewitt, 2004; Defontaine et al., 2005).

As the ice cover retreated during interglacial periods, refuge populations expanded northwards, colonizing new ice-free regions (Taberlet et al., 1998). These contractions and expansions in the

ranges of temperate species have left signatures in both the geographical distribution and the genetic diversity of extant populations (Bennett et al., 1991; Hewitt, 1996; Avise, 2000).

When compared to other taxa, birds present an enigma regarding postulated levels of geographical structure and magnitude of gene flow (Avise, 2000). On one hand, their generally high dispersal capacities should prevent the appearance of strong genetic structures, but on the other hand, many species show a strong philopatry (Newton, 1998). This generates obvious geographical differences between conspecific populations. Phylogeographic studies conducted on a growing number of bird species in the world have uncovered a great variety of mtDNA patterns (Zink, 1997; Avise, 2000). However, in Europe, with the exception of a few large studies (Griswold and Baker, 2002; Brito, 2005), most phylogeographic studies concerning birds have been restricted to subsets of the three main putative refuges, due to restricted sampling, or to constraints in species range (Brito, 2005). Results of these studies are contradictory. (i) Some species did not show any clear phylogeographic structure (Seutin et al., 1995; Questiau et al., 1999; Zink et al., 2002a,b; Koopman et al., 2005). Several reasons might be evoked to account for this: restricted sample and/or the use of molecular markers with insufficient sensitivity (Crochet

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et al., 2000), recent recolonisation events (Gay et al., 2004) and erratic behaviour (Questiau et al., 1999; Koopman et al., 2005). (ii) In contrast, other birds displayed phylogeographic patterns congruent with the Mediterranean refuges hypothesis (Griswold and Baker, 2002; Liukkonen-Anttila et al., 2002; Kvist et al., 2004; Brito, 2005). (iii) Finally, Nordic and/or marine species displayed distinct patterns with refuges located more northward (Tiedemann et al., 2004; Ruokonen et al., 2005) and/or close to the Atlantic Ocean, the Mediterranean and Black Sea (Kidd and Friesen, 1998; Liebers et al., 2001; Moum and Arnason, 2001).

The white-throated dipper has a very large and fragmented distribution across Europe, Asia and North Africa (Fig. 1) (Cramp, 1988; Hagemeyer and Blair, 1997). The representatives of this family (Cinclidae) are exclusively riverine, being entirely restricted to fast flowing rivers and streams, and their diet is almost exclusively comprised of benthic macro-invertebrates (Tyler and Ormerod, 1994; D'Amico and Hémerly, 2007). An important clinal variation

concerning body size and plumage colors has been described for the white-throated dipper. This has led to the description of many subspecies whose validity is often questionable, as such subspecies are not always geographically isolated, nor morphologically well defined (Tyler and Ormerod, 1994). Ten out of the 13 recognized subspecies (Cramp, 1988) are present within the Western Palearctic region, and other three occur in the eastern Palearctic. White-throated dippers are generally sedentary monogamous birds. Limited dispersion movements observed for this species (Tyler and Ormerod, 1994; O'Halloran et al., 2000) probably strongly limit gene flow between populations. All these characteristics make this bird a particularly interesting model for a phylogeographic study. A first study conducted by Lauga et al. (2005) on this species concluded to a lack of a phylogeographic pattern, highlighting the need to reconsider the intraspecific taxonomy of such a species.

The present study aims to clarify the phylogeographic patterns of white-throated dippers in the Western Palearctic, using a larger

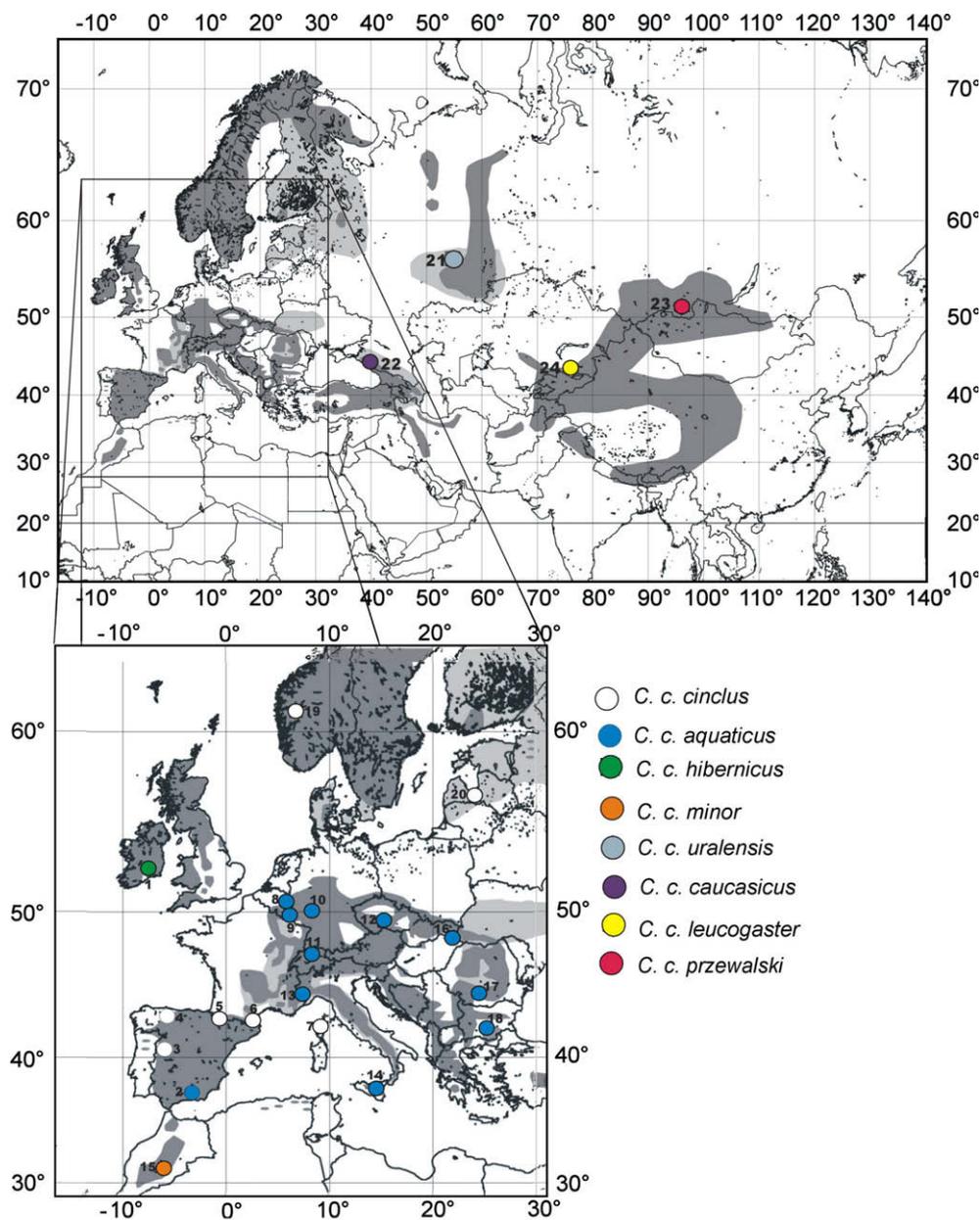


Fig. 1. Geographical distribution of *Cinclus cinclus* samples, symbols colors refer to the different subspecies as described by Cramp (1988) (see Appendix 1 for samples characterisation). Dark grey zones correspond to the breeding range distribution of the dipper; light grey zones correspond to wintering zones.

number of populations spread throughout the entire region and based on the analysis of three molecular markers. This study will help to determine whether a bird species that is strongly associated with fast flowing waters, and more adapted to cold conditions, reacted to the Quaternary climate changes in the same way as other temperate bird species. More specifically, this study aims to answer the following questions: (i) Is the white-throated dipper genetically heterogeneous? If so, is this heterogeneity geographically structured? (ii) How did the Quaternary climatic oscillations affect the distribution of this species? (iii) In which regions did the dipper survive during the Quaternary ice age? (iv) How did the dipper recolonise Europe after the last pleniglacial?

Answers to these questions will provide a new insight into the intraspecific taxonomy of dippers, allowing a reconsideration of the validity of some of the numerous current subspecies.

2. Materials and methods

2.1. Sample collection

Hundred and six tissue samples from white-throated dippers were obtained from 24 populations located mostly in the Western Palearctic and representing 8 of the 13 formerly described subspecies (Cramp, 1988) (Fig. 1, Appendix 1). Tissue samples from eastward *uralensis*, *caucasicus*, *leucogaster* and *przewalski* subspecies were provided by the Burke Museum (Washington, USA) (Museum numbers: UWBM 46430, 61537, 64752, 64772, 70974 and 71403). *Cinclus mexicanus* and *Cinclus pallasii* were used as outgroups for our study (Museum numbers: UWBM 46768 and 51144) according to previous phylogenetic studies performed on this genus (Voelker, 2002).

With the exception of the samples provided by the Burke Museum, tissue samples consisted of 20–100 μ l of blood, collected under licence by puncturing the brachial or tarsal vein. Blood was immediately placed in an APS Buffer (Arctander, 1988) and frozen at -20°C for long-term conservation. Individuals were quickly released in the wild after blood samplings.

2.2. DNA extraction and amplification

Total DNA was extracted using the Qiagen DNeasy Tissue Kit (cat. no. 69506) following the manufacturer instructions.

We amplified 3 fragments of the mitochondrial genome, including the cytochrome *b* gene, the ND2 gene and domains II and III of the control region (CR). Total length of the amplified fragments is equal to 3096 bp. Each 50 μ l polymerase chain reaction (PCR) contained ± 50 ng of total DNA, 350 mM of each dNTP, 0.5 pmol of each primer, 2 units DNA polymerase and a final concentration of 1.5 mM MgCl_2 .

The primers used for amplification and sequencing are listed in Table 1. Amplification protocol for the cytochrome *b* gene (length = 1079 bp) consisted in 40 cycles of 30 s at 94°C (denaturation step), 30 s at 49.5°C (annealing step) and 75 s at 72°C (elongation step). Protocols were identical for the ND2 gene (length = 1085 bp) and the control region (length = 932 bp), except for the annealing temperature that was respectively equal to 55°C (ND2) and 59°C (CR). Both strands of these mitochondrial regions were sequenced using automated sequencers from the Macrogen society (Seoul, Korea).

2.3. Sequence analyses

The sequences were aligned using the ED editor of the MUST package (Philippe, 1993). A combined matrix for the control region (926 bp), ND2 (1075 bp) and cytochrome *b* (1079 bp) sequences was used to determine the phylogeographic structure of the dipper.

Networks were constructed using the minimum spanning network method (MINSPNET in ARLEQUIN 2.0, Schneider et al., 2000), statistical parsimony (TCS, Clement et al., 2000) and median-joining network (Bandelt et al., 1999; Network 4.000 available at www.fluxus-engineering.com). Phylogenetic reconstructions were also performed using neighbour joining (NJ; Saitou and Nei, 1987) and maximum parsimony (MP; Fitch, 1971) algorithms implemented in PAUP 4.0b8 (Swofford, 2000), as well as the maximum likelihood algorithm (ML; Felsenstein, 1981) implemented in the PHYML program (Guindon and Gascuel, 2003). Phylogenetic trees were rooted by sequences from *C. mexicanus* and *C. pallasii*. NJ and ML analyses were performed using the HKY + I + G model (Hasegawa et al., 1985; Yang, 1993; Gu et al., 1995) suggested for the data by MODELTEST 3.0 (Posada and Crandall, 1998) with a gamma distribution shape parameter of $\alpha = 0.9684$ (Yang, 1996) and a proportion of invariable sites of $I = 0.5224$. The robustness of the nodes on the trees was assessed by bootstrap re-sampling (BP; Felsenstein, 1985) with 1000 random repetitions for MP, NJ and ML analysis.

2.3.1. Genetic structure and demographic trends

An analysis of molecular variance (AMOVA) was performed using the ARLEQUIN 2.0 program to examine hierarchical population genetic structure (Schneider et al., 2000). Analyses were performed on 20 populations within six groups, these groups reflecting the geographic distribution of the studied animals.

Haplotype (*h*) and nucleotide (π) (Nei, 1987) diversities and their standard deviations (Tajima, 1993) were estimated using the DNASP 4.0. software (Rozas et al., 2003).

A mismatch distribution analysis was also performed to investigate indications of demographic expansions using DNASP 4.0. We examined the 'raggedness' value (R_g) and the sum of squared

Table 1
List of primers used for amplification and sequencing

Primer name	Sequence	Amplification	Sequencing	Source
<i>Control region</i>				
H450rev	5'-ATG-CCA-CTG-GIT-CCT-ATT-TC-3'	X	X	Present study
CINDW	5'-TGT-ATG-ACC-GCG-GTG-GCT-GG-3'	X	X	Present study
<i>ND2</i>				
L5215	5'-TAT-CGG-GCC-CAT-ACC-CCG-AAA-AT-3'	X	X	Hackett (1996)
H6313	5'-CTC-TTA-TTT-AAG-GCT-TTG-AAG-GC-3'	X	X	Johnson and Sorenson (1998)
L5578	5'-TCT-GAT-TCC-CAG-AAG-TAC-TAC-AAG-G-3'		X	Voelker (2002)
L5758	5'-GGG-TGA-ACG-GGC-CTA-AAC-CAA-AC-3'		X	Voelker (2002)
<i>Cyt. b</i>				
L14841	5'-AAA-AAG-CTT-CCA-TCC-AAC-ATC-TCA-GCA-TGA-TGA-AA-3'	X	X	Kocher et al. (1989)
H16065	5'-GGA-GTC-TTC-AGT-CTC-TGG-TTT-ACA-AGA-C-3'	X	X	Helm-Bychowski and Cracraft (1993)
L15383	5'-GGA-CAA-ACA-CTA-GTA-GAA-TG-3'		X	Pasquet et al. (1999)

deviation (SSD) to assess the statistical significance of the distribution within ARLEQUIN 2.000 (Schneider et al., 2000) under the rapid expansion model.

Expansion times (t_e) were estimated using the relationship between t_e and Tau (τ) (Rogers and Harpending, 1992). We also calculated Fu's F_s value (Fu, 1997) as an indicator of demographic expansion. All these values were calculated using DNASP 4.0.

2.3.2. Divergence time analyses

The genetic distance (%-Kimura 2 parameters, K_2P) between lineages was obtained using the MEGA 2.1 program (Kumar et al., 2001). Where necessary, K_2P distances were corrected for ancestral mtDNA polymorphism using Edwards and Beerli formula (2000). Since we found no listed fossils of white-throated dipper (Mourer-Chauviré, 1995; Bochenski, 1997), we referred to Voelker's (2002) paper on the phylogeny of *Cinclidae* to have a calibration point in order to estimate divergence times between the different lineages of White-throated dipper. According to Voelker, the separation event between *Cinclus cinclus* and *C. pallasii* should have occurred about 2.54 Myr ago. This approach can give only a rough estimation of the separation times between the different lineages, especially when such times are rather recent; indeed, Ho et al. (2005) clearly showed the danger of overestimation for the recent dates of separation when calibration points are too ancient. Nevertheless, we performed the calculation as it was the only possibility to have an estimation of the chronology of appearance of the different *C. cinclus* lineages.

3. Results

3.1. Sequence variation

The three studied genes were weakly variable (28, 30 and 63 variable sites and 16, 24 and 22 parsimony informative sites for the control region, ND2 and cytochrome *b* genes, respectively). Therefore, the phylogenetic reconstructions performed on these genetic markers taken independently, gave important polytomies with weak genetic structures among dipper populations. According to these results, we preferred to concatenate our markers to improve our topologies.

Using this combined data matrix, a total of 51 haplotypes were identified among the 106 white-throated dippers. Out of the 3096 bp sequenced, 205 sites were variable and 158 were parsimony informative. The average values of transitions to transversions ratio is equal to 4.6110, and base composition is: T 23.7%, C 32%, A 31% and G 13.3%.

3.2. Phylogenetic analyses

The minimum spanning network (Fig. 2) shows the existence of several lineages that are well differentiated from a genetical and a geographical point of view:

- A large West European lineage, including specimens from Italy, Spain, France (except Corsica), Switzerland, Belgium, Germany, Czech Republic, Hungary and Ireland;
- an East European lineage, including Bulgarian and Romanian populations as well as some animals from Hungary, Czech republic, Germany and Luxembourg;
- a North-East European lineage (birds from Norway, Latvia, Ural);
- a lineage including Moroccan dippers;
- another lineage including Caucasian birds;
- finally a 'miscellaneous' group including an isolated animal from Ireland, another isolated animal from Corsica, and the 2 asiatic birds.

A minimum of four mutational steps separate these lineages from each other, with the Asiatic birds being the most distant and separated from the rest of the sample by a minimum of 26 mutational steps.

Within the West European lineage, almost all of the haplotypes are connected in a large star-like topology. The central haplotype of such a topology corresponds to a large number of animals (30) coming from various geographical origins. Nonetheless, some geographical structuration, with North Italian, Andalusian, Sicilian and Irish subgroups, seems to appear within the lineage.

Other network methods (statistical parsimony and median-joining network) give the same topology. Phylogenetic reconstructions performed using maximum parsimony algorithms, neighbour-joining and maximum likelihood methods (not shown here) confirm these results: the composition of major lineages is identical to those formed by the minimum spanning network (with a high bootstrap support ranging from 73% to 100%), with the only exception of the isolated animal from Ireland which is included in the North–East European lineage.

3.3. Population structure and genetic diversities

3.3.1. Partitioning of genetic variation and gene flow

The AMOVA analysis (Table 2) shows that the mtDNA variation observed in our samples is almost equally distributed among groups (43.04%) and among populations within groups (40.86%). This indicates a substantial genetic structure among the six groups we have defined, but also among the populations that constitute these groups. In contrast, mtDNA variation within populations was much lower (16.10%).

3.3.2. Genetic structure and demographic trends

To test the Mediterranean refugia hypothesis and to locate potential glacial refuges for the dipper, nucleotide and haplotype diversities were calculated for the different lineages and populations from these lineages (see Table 3 for results and grouping characterisation). In spite of the important size of sequences obtained, dippers display low levels of nucleotide diversity, ranging from 0.00018 (Ireland) to 0.00132 (Sicily).

Within the West European lineage, the Sicilian population displays significantly higher nucleotide diversity than the North Italian population. These 2 populations display significantly higher nucleotide diversities than all the other populations, while the Irish population displays significantly the lowest nucleotide diversity values ($\pi = 0.00018 \pm 0.00004$) as compared to the other populations. The same schema was observed for haplotype diversity values. However, although the Sicilian population has the highest haplotype diversity value ($h = 0.900 \pm 0.161$), no significant difference is observed between this population and the North Italian, Spanish and Central West European populations ($p > 0.05$).

Within the East European lineage, the Balkano-Carpathian population (Romanian and Bulgarian birds) displays significantly higher nucleotide diversity value (but not haplotype diversity value) as compared to the Central East European population (comprising animals from Hungary, Czech Republic, Germany and Luxembourg).

Although the sampling for some of the populations could appear too low to make these statements, we performed mismatch distribution analyses on populations living in the geographic areas considered as potential refuges in Europe (see Michaux et al. 1998; Carlsson, 2003; Randi et al. 2003; Hewitt 2004) (Fig. 3). These analyses indicate that the Spanish and Northern Italian populations are characterised by a signal of population in expansion, whereas the Sicilian and Balkano-Carpathian populations show a more heterogeneous mismatch distribution that could suggest long term stabil-

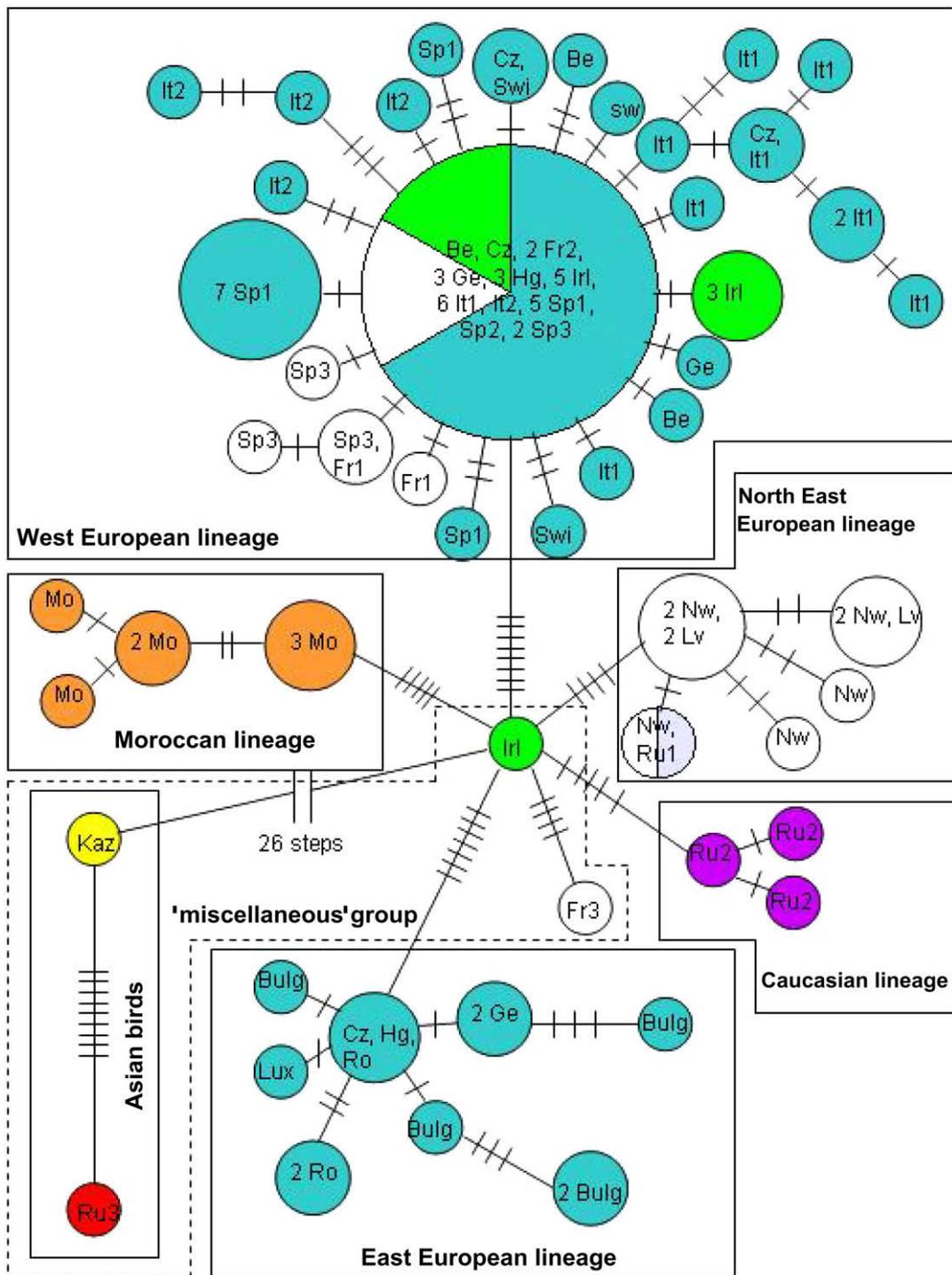


Fig. 2. Minimum spanning tree of the White-throated dipper. Area of circles is proportional to the number of individuals sharing that haplotype. Text inside circles includes number of individuals (if >1) and sampling location—geographical abbreviations correspond to countries listed in the Appendix 1. Subspecies occurrence is shown by different kinds of colors (see Fig. 1).

Table 2
Hierarchical AMOVA computed with the twenty populations of dippers distributed in six groups (statistical significance: $p < 0.0001$)

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation
Among groups	5	130.423	1.55752	43.04
Among populations within groups	14	118.137	1.47865	40.86
Within populations	86	50.090	0.58245	16.10
Total	105	298.651	3.61862	100

Table 3
Nucleotide diversity (π) and haplotype diversity (h) of the White-throated dipper lineages and populations

Lineages and populations	Sample size	Haplotypes	Nucleotide diversity (π)	Standard deviation	Haplotype diversity (h)	Standard deviation
Total	106	51	0.00269	0.00029	0.901	0.025
Western Europe	68	27	0.00054	0.00007	0.769	0.053
North Italy	15	9	0.00062	0.00012	0.838	0.085
Sicily	5	5	0.00132	0.00034	0.900	0.161
Spain	24	8	0.00043	0.00008	0.757	0.066
Ireland	8	2	0.00018	0.00004	0.536	0.123
Corsica	1	1	—	—	—	—
Central West Europe	16	8	0.00047	0.00012	0.758	0.11
Eastern Europe	13	8	0.00097	0.00018	0.923	0.050
Balkano-Carpathian	8	6	0.00126	0.00021	0.929	0.084
Central East Europe	5	3	0.00033	0.00009	0.800	0.164
North East Europe	12	6	0.00075	0.00015	0.848	0.074
Morocco	7	4	0.00057	0.00011	0.810	0.130
Caucasus	3	3	0.00043	0.00014	1.000	0.272
Asia	2	2	0.00292	0.00146	1.000	0.500

Spain population includes birds from Spain and from the French Pyrenees; Central West Europe population includes birds from Belgium, Germany, Switzerland, Czech Republic and Hungary belonging to the Western Europe lineage; Balkano-Carpathian population includes birds from Romania and Bulgaria; Central East Europe population includes birds from Luxembourg, Germany, Czech Republic and Hungary belonging to the East European lineage.

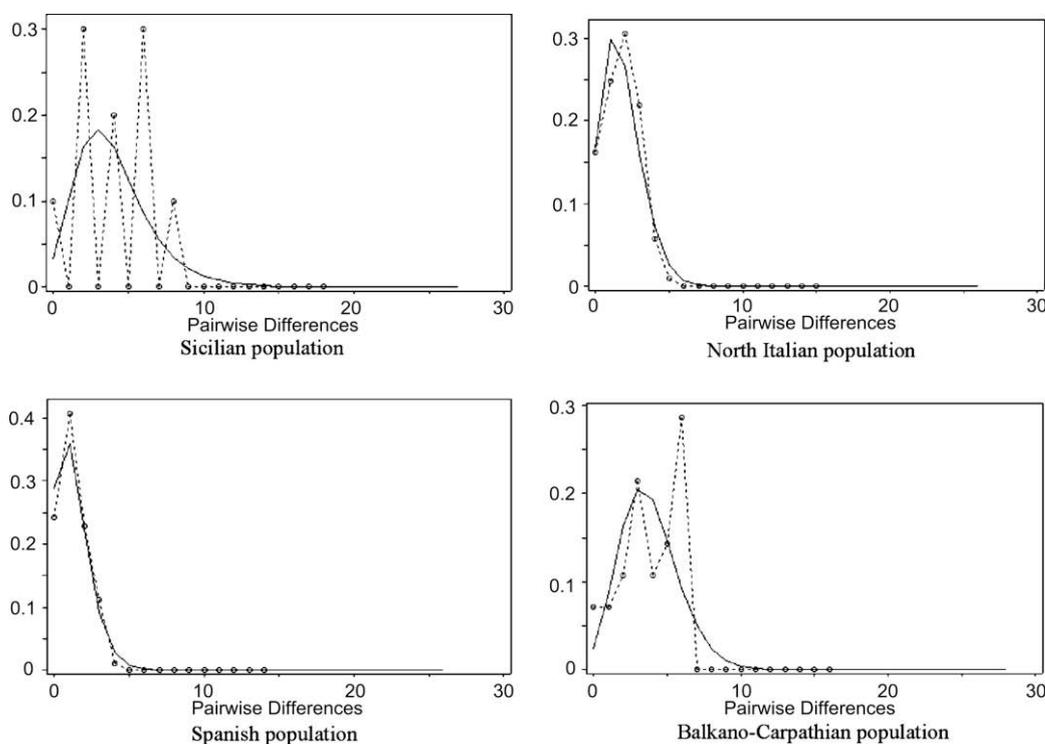


Fig. 3. Mismatch distribution analysis for mtDNA haplotypes from Sicilian, Northern Italian, Spanish and Balkano-Carpathian populations.

ity. The population expansion of Northern Italy and Northern Spain is also confirmed by the Fu's F_s tests (results not shown).

At the lineages level, mismatch distribution analyses conducted on the West European, East European, North East European and Moroccan lineages suggest a signal of recent expansion for these lineages (see Appendix 2). The low number of Caucasian, Asian and Corsican samples did not allow the use of such statistical tests for these groups.

3.4. Divergence time

Table 4 shows mean net distances within and between lineages, and divergence times between these lineages. Due to their isolation and to the genetic distances which separate them from the others lineages (see Fig. 2), the Corsican and the Asiatic individuals

were considered as distinct lineages for the calculation of divergence times. Taking into account the correction for ancestral mtDNA polymorphism, the mean K_2P distance between *C. cinclus* and *C. pallasii* is equal to 8.86%. This value would correspond to a rate of 3.5% K_2P distance per million years for our combined gene dataset. As a consequence, a chronology of separations between the different lineages could be proposed: (i) separation between the Asian lineage and the other lineages, possibly around 240–280,000 years; (ii) separation between the East European and the West European lineage, around 120,000 years; (iii) these last two lineages separated from the other ones (North–East European, Moroccan, Caucasian and Corsican lineages) around 80–95,000 years ago; (iv) finally, the North–East European, Moroccan, Caucasian and Corsican lineages would have been the last ones to separate from each other, around 39–54,000 years.

Table 4

Mean net distances expressed as %K₂P within (**diagonal**) and between (lower part of the table) the main lineages of *C. cinclus* and between *C. cinclus*, *C. mexicanus* and *C. pallasii*—Estimated divergence times ($\times 10^3$ years) between these lineages (upper part, in shaded grey) (n/c = not considered)

	Western Europe	Eastern Europe	Northern Europe	Corsica	Caucasus	Morocco	Asia	<i>C. mexicanus</i>	<i>C. pallasii</i>
Western Europe	0.0005	121	88	87	95	82	282		
Eastern Europe	0.0042	0.0009	80	77	88	70	259		
North-East Europe	0.0031	0.0028	0.0008	46	44	41	248		
Corsica	0.0030	0.0027	0.0016	n/c	54	39	240		
Caucasus	0.0033	0.0031	0.0015	0.0019	0.0005	49	251		
Morocco	0.0029	0.0024	0.0014	0.0013	0.0017	0.0005	247		
Asia	0.0098	0.0090	0.0087	0.0084	0.0088	0.0086	0.0034		
<i>C. mexicanus</i>	0.1032	0.1023	0.1028	0.1033	0.1033	0.1035	0.1036	n/c	
<i>C. pallasii</i>	0.0899	0.0891	0.0892	0.0891	0.0891	0.0893	0.0907	0.8567	n/c

Ringed recovery analysis indicates the generation time of the dipper to lie between 1 and 1.5 years. Assuming this and with $u = 0.035$, expansion dates for the four Western Palearctic lineages where a signal of expansion was observed, are all posterior to the LGM, ranging respectively from 10,000 (West European lineage) to 15,700 years BP (East European lineage) for a generation time equal to 1.5 years, and from 6700 to 10,500 years BP if the generation time is equal to one year (Table 5).

4. Discussion

4.1. Phylogeographic patterns

Our results support some of the previous findings on dipper phylogeography (Lauga et al., 2005), i.e. a weak genetic differentiation between West European populations and the existence of geographically widespread haplotypes among these populations.

However, the larger geographic scale and DNA sequence dataset employed in our study clearly shows the existence of geographically well-distinct lineages within the Western Palearctic populations of dippers: (i) a Western European lineage, (ii) an Eastern European lineage, (iii) a North–East European lineage, (iv) a Moroccan lineage and (v) a Caucasian lineage. Furthermore, our dataset highlights the important genetic differentiation between European (and North African) and Asian populations, and the isolation of the Corsican individual within Western Europe.

This is the highest number of lineages ever observed for a bird species in this region. Indeed, most phylogeographic studies performed on European birds resulted in the definition of only one to a maximum of three lineages, or no clear phylogeographic structure for the Western Palearctic (Merilä et al., 1997; Pitra et al., 2000; Liukkonen-Anttila et al., 2002; Pavlova et al., 2003; Randi et al., 2003; Zink et al., 2003; Kvist et al., 2004; Qu et al., 2005). One possible exception could be the tawny owl (Brito, 2005).

Although the nucleotide diversity values observed for the dipper appear very low when compared to other taxa from the same geographical zone, such as micromammals (Michaux et al., 2005), these values are of the same order of magnitude as those observed for several other European bird species. The same is true for haplotype diversity values (see Qu et al., 2005).

Divergence times calculated between the different lineages appear to be rather recent, between the Middle and Late Pleistocene. Avise and Walker (1998) have already underlined the major influ-

ence of Pleistocene conditions in initiating major phylogeographic separations within bird species, and in completing speciations that had been inaugurated earlier. Later studies have also confirmed this concept (Qu et al., 2005).

Although we have to be very careful in estimating the divergence date in our analyses, these estimates help to propose a possible scenario explaining this phylogeographic pattern:

1. The Eastern and Western Palearctic populations were separated at the end of Middle Pleistocene.
2. Later, European dipper populations would have been isolated in at least two distinct refuges during one of the last ice ages, maybe corresponding to the Riss period (around 120,000 years ago). These refuges likely correspond to the Iberian and/or the Italian Peninsulas and Central Europe and/or in the Balkans. This scenario was already observed for many other European species (Santucci et al., 1998; Taberlet et al., 1998; Liukkonen-Anttila et al., 2002; Michaux et al., 2005). The isolation of these populations led to the appearance of the Western and Eastern European dipper genetic lineages. The colonisation of Morocco and Corsica would have happened during the same glacial period, facilitated by a lower sea level (Lambeck et al., 2002).
3. Dippers living in the Western Palearctic probably expanded northward and eastward during a following interglacial, maybe corresponding to the Eemian period (120–72,000 years BP). Later, the large plains existing, as today, in most of northern Europe (i.e. from Northern Belgium and Netherlands to Russia, but also in Central Hungary) would have acted as barriers for this species. Indeed, the lack of a suitable riverine network for dippers through these plains would have, in some way, limited the exchanges between Western European populations and those now isolated in Scandinavia, the Ural Mountains and the Near East-Caucasus regions. These barriers associated with the sedentary behaviour of the dippers and to the isolation by distance with Western European populations, would have led to the appearance of the North East European, as well as the Caucasus lineages.

4.2. Glacial refuges

Haplotype and nucleotide diversity values observed for the different European populations of dipper tend to suggest that the North Italian, Sicilian and Balkano-Carpathian regions could have played the role of major refuge zones for dippers during the LGM.

Mismatch distribution analyses conducted on the Sicilian and Balkano-Carpathian populations (Fig. 3) also suggest that these populations would have remained constant during the last ice age, further evoking the existence of some refuge areas for the dipper in these regions. Fu's F_s tests also support this hypothesis (results not shown). Such a hypothesis of a Sicilian and/or a

Table 5

Expansion times (t_e) expressed in years for the different lineages and for a generation time equal to 1 or 1.5 years

Lineage	t_e (generation time = 1.5 years)	t_e (generation time = 1 year)
East Europe	15,725	10,484
West Europe	10,008	6672
North East Europe	12,583	8389
Morocco	11,863	7909

Balkano-Carpathian refuge has already been proposed for several other species including birds (Santucci et al., 1998; Michaux et al., 1998, 2005; Carlsson, 2003; Randi et al., 2003; Brito, 2005; Deffontaine et al., 2005; Nieberding et al., 2005; Kotlik et al., 2006).

In spite of a limited sampling for the Caucasus region, our results strongly support the existence of a particular lineage in this region which is well separated from all the other populations. The existence of a refuge in the Caspian/Caucasian region has already been proposed for different European temperate species (Durand et al., 1999; Deffontaine et al., 2005), but it is difficult to be more precise about such a lineage without better sampling in this region and in the neighbouring areas.

Corsica and North Africa have also been suggested as refuge zones for different European bird species (Marshall and Baker, 1999; Griswold and Baker, 2002; Kvist et al., 2004). Moreover, these regions are also known for their numerous endemic species and subspecies (Pasquet, 1998; Thibault and Bonnacorsi, 1999; Prodon et al., 2002; Thévenot et al., 2003). Our results tend to support such a hypothesis for North Africa. The hypothesis of a distinct Corsican dipper lineage must, however, be treated with caution since we only analysed one individual from this region.

Finally, the existence of a North-East European lineage suggests a northern and/or eastern refuge for this lineage. Northern refuges have already been proposed for different bird species (Tiedemann et al., 2004; Ruokonen et al., 2005), and other taxa (Fedorov et al., 1999; Hewitt, 2004; Nieberding et al., 2004; Ruokonen et al., 2005).

Scandinavia was completely glaciated during the LGM (Hewitt, 1996) and, although dippers are capable of enduring very low temperatures, it is not very likely that they could have survived in Scandinavia during this period, due to a restricted access to food resources when streams were frozen. Another more likely possibility is that dippers from the North East European lineage survived in places located more Eastward, close to the Ural Mountains. Indeed, Northern Russia was not so affected by ice compared to its neighbouring Scandinavian region (Hewitt, 1996), and the Ural region has already been proposed as a refuge zone for other species (Carlsson, 2003; Hewitt, 2004; Deffontaine et al., 2005). However, given the limited sampling we have for these regions, it is possible to discard neither of these two hypotheses.

4.3. Postglacial expansion

As observed for expansion date estimations of many Palearctic and Nearctic birds species (Qu et al., 2005), recolonisation of the Western Palearctic by dippers seems to have started immediately after the last ice age, i.e. between 16,000 and 10,000 years BP.

4.3.1. West European lineage

The star-like topology observed on the network as well as the mismatch distribution analyses following a Poisson model suggest that the West European lineage probably recolonised Europe recently from a small number of animals surviving in a single refuge.

On the basis of our results, a plausible hypothesis is that the recolonization of the Western European region began from an Italian refuge: at the end of the last glaciation, Italian birds expanded westwards to France and the Iberian Peninsula, but also northwards to reach Britain, Ireland, Belgium, Germany, Switzerland, Czech Republic and Hungary, crossing and/or bypassing the Alps.

4.3.2. East European lineage

As for the West European lineage, mismatch distribution for the East European lineage suggests that the East European lineage probably expanded just after the last ice age. When temperatures increased at the beginning of the last interglacial, Balkano-Carpathian populations expanded North-Westwards, following the Carpathian mountains and bypassing the Hungarian plain to

colonize Northern Hungary, Slovakia, the Czech Republic, Germany and Luxembourg. This lineage entered into contact with the Western European one in these last regions, where they form a very large contact zone extending from Luxembourg to Hungary.

4.3.3. Other lineages

It is impossible to propose a pattern of postglacial recolonisation, due to our limited sampling.

5. Conclusions

As already reported for many Palearctic bird species (Seutin et al., 1995; Zink et al., 2002a; Pavlova et al., 2003; Koopman et al., 2005), the lack of concordance we observed between the morphological races as described by Cramp (1988) and the genetic lineages as defined by our results clearly raise the question of the validity for some of these subspecies, a fact that had already been quoted by Tyler and Ormerod (1994). Several recent examples propose that extensive morphological or behavioural differentiation can evolve rapidly in birds with little or no genetic differentiation (Zink and Dittmann, 1993; Ödeen and Björklund, 2003). This results from a rapid local adaptation to fluctuating resources (Questiau et al., 1999) and/or to sexual selection (Ödeen and Björklund, 2003). Therefore, this study highlights the necessity to reconsider the status of the different European subspecies and the relevance of the characters employed to discriminate these subspecies.

Above all, this study gives evidence of the impact of glacial periods on the genetic structure of a temperate species. However, in contrast to many other phylogeographic studies performed on birds and for the first time in Europe, our study also demonstrates the impact of interglacial periods. These gave rise to probably the highest number of different refuges ever observed in Europe for a single vertebrate species, and led to very complex phylogeographic patterns for the dipper within its Western Palearctic breeding range, with the determination of several geographically well distinct genetic lineages.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2008.07.025.

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