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ORIGINAL ARTICLE

Are Ring Ouzel (*Turdus torquatus*) populations of the low mountain ranges remnants of a broader distribution in the past?

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Abstract The Ring Ouzel (*Turdus torquatus*) has an arctic–alpine distribution, with small populations also occurring at the higher altitudes of the low mountain ranges north of the Alps. The populations of the Alps and mountain ranges are classified as the subspecies *T. torquatus alpestris*, and those of the northern populations as *T. torquatus torquatus*. Since birds are mobile when, e.g., compared to mammals of the same size, it is likely that the populations in the low mountain ranges originated from dispersal events after the last ice age. We analyzed an mtDNA fragment of Ring Ouzel museum specimens from across Europe. We found a shallow gene tree with little differentiation between the subspecies and therefore incomplete lineage sorting. However, both, subspecies as well as low mountain range populations were characterized by private alleles respectively. Furthermore, when we grouped sequenced specimens according to their origin in an alpine group, a group from the low mountain ranges, and two northern groups, we found significant differentiation between the alpine and the low mountain range group, similar to the difference between the alpine and the two northern groups. This suggests an origin of populations on the low mountain ranges similar to that of the arctic–alpine disjunction, and that these populations are remnants of a broader distribution in the past.

Keywords Arctic–alpine distribution · Biogeography · Climate warming · mtDNA · Low mountain ranges · *Turdidae*

Zusammenfassung

Populationen der Ringdrossel (*Turdus torquatus*) in europäischen Mittelgebirgen: Reste einer einst weiteren Verbreitung?

Die Ringdrossel (*Turdus torquatus*) zeigt eine arktisch-alpine Verbreitung mit kleinen Populationen in höheren Lagen europäischer Mittelgebirge. Die Populationen der Alpen und der Mittelgebirge werden als Alpenringdrossel (*Turdus torquatus alpestris*), die nordischen Populationen dagegen als Nordische Ringdrossel (*Turdus torquatus torquatus*) bezeichnet. Da Vögel im Vergleich zu Säugetieren verhältnismäßig mobil sind, stellt sich die Frage, ob die Populationen in den Mittelgebirgen durch Ausbreitungsereignisse nach der letzten Eiszeit entstanden sind. Zur Klärung dieser Frage haben wir Balgmaterial aus europäischen Naturkundemuseen genutzt, um ein mtDNA Fragment zu analysieren. Wir fanden eine geringe genetische Differenzierung zwischen den Unterarten, wobei mehrere Haplotypen in beiden Taxa vorkamen. Trotzdem

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waren die Unterarten durch private Allele charakterisiert. Private Allele traten aber auch in den Proben aus den Mittelgebirgen auf. Des Weiteren fanden wir, wenn die sequenzierten Individuen in Gruppen gemäß ihrer Zugehörigkeit zu Alpen, Mittelgebirgen bzw. nordische Verbreitungsgebiete eingeteilt wurden, eine signifikante Differenzierung zwischen Alpen und Mittelgebirgspopulationen, ähnlich dem Unterschied zwischen Alpen und nordischen Populationen. Offensichtlich sind die Vorkommen der Ringdrossel in den Mittelgebirgen Reste einer einst weiteren Verbreitung.

Introduction

The Pleistocene glaciations were among the most important drivers of present distributional patterns of plants and animals across the Palaearctic realm (Holdhaus 1954; de Lattin 1967). Molecular work has shown that these events also produced imprints on the genetic structure within populations and/or between closely related species (Hewitt 1996; Schmitt 2007; Shafer et al. 2010): (1) Mediterranean species that survived the glaciations in southern refugia, (2) continental species that colonized Europe from refugia in the eastern Palaearctic, and (3) arctic–alpine species whose range expanded during cold periods and became restricted to northern and alpine refugia during warmer periods. Within the latter scenario, the populations of the low mountain ranges are regarded as remnants of a periglacial distribution. However, events of long-distance dispersal may also be responsible for populations of alpine species on low mountain ranges.

Of the species of the three main patterns, those of the Mediterranean refugia have been the focus of most genetic studies, and only recently have several molecular studies on arctic/alpine species appeared in the literature (reviewed in Schmitt 2007). Unexpectedly, these studies suggest that the biogeography of arctic–alpine species is not that straightforward (Schmitt 2007). The arctic and alpine populations often show little genetic differentiation, and Fennoscandian populations may have originated from dispersal events (Despres et al. 2002; Skrede et al. 2006; Reisch 2008). Furthermore, some species living in the low mountain ranges north of the Alps (e.g., in the Bohemian Forest) are genetically differentiated from alpine populations (Muster and Berendonk 2006).

One example of a bird with an arctic–alpine distribution is the Ring Ouzel (*Turdus torquatus*). In contrast to other classic examples of this disjunction (e.g., *Lagopus mutus*, *Picoides tridactylus*), the Nordic populations are limited to Scotland and Fennoscandia. Three subspecies of the Ring Ouzel have been described for Europe and western Asia: (1) the Nordic Ring Ouzel (*Turdus torquatus torquatus*), which inhabits Great Britain and Fennoscandia; (2) the Alpine Ring Ouzel (*Turdus torquatus alpestris*), which lives in the Alps, Pyrenees, Balkan, Greece, and Asia Minor, and which also occurs in small populations in the low mountain ranges north of the Alps; and (3) the Eastern Ring Ouzel (*Turdus torquatus amicorum*), which is mainly distributed in the Caucasus (Glutz von Blotzheim 1988; del Hoyo et al. 2005). The Ring Ouzel is philopatric and returns to specific breeding and wintering areas each year, and the northern subspecies (*Turdus torquatus torquatus*) winters south of the alpine populations in the Atlas Mountains (Glutz von Blotzheim 1988; Greenwood 1980; Berthold 1988; Ogonowski and Conway 2009).

Given the distribution of the subspecies, several plausible scenarios could explain the origin of the populations of the low mountain ranges, and these can be tested using molecular data. One possibility is that populations of the low mountain ranges originated from dispersal events from the Alps. In this case, these populations would not be genetically differentiated from alpine populations. Although populations of the low mountain ranges are small, the inflow of genes from the Alps may stabilize genetic variability. Another possibility is that, although populations from the low mountain ranges belong to the alpine subspecies, they may have originated from the biogeographic reorganizations during climate warming after the last glaciations, and they are isolated from the alpine populations. In this case, one would expect that unique and/or old genotypes occur within populations occurring on low mountain ranges. Furthermore, the small size of these populations may have led to a loss of genetic variation. A third possibility considers that the Nordic Ring Ouzel winters south of the alpine subspecies, and therefore that individuals from the Nordic subspecies migrate over Europe across the populations of the alpine subspecies. This possibly leads to introgression of genes from the Nordic subspecies, and the small populations north of the Alps may therefore be melting pots of the genetic material from two subspecies. This predicts that Nordic genotypes occur in the low mountain ranges and that genetic variation is high. We tested these scenarios by analyzing a mitochondrial DNA fragment of museum specimens of Ring Ouzels from across Europe.

Materials and methods

We used material from ornithological collections to estimate genetic differentiation between groups of the Ring Ouzel. The use of museum specimens allowed us to cover Europe and to include samples from areas where populations seem to be extinct (Table S1). For our purpose, we required that specimens sampled on low mountain ranges

were sampled from resident populations. For this, we evaluated all available information on the museum labels (e.g., subspecies, place, date of capture, state of the bird). However, this information was often not complete, and therefore we decided to group specimens according to the following pragmatic criteria. First, all specimens of the subspecies *alpestris* collected in the Alps formed the groups "Alps". Second, all specimens of the subspecies *alpestris* collected north of the Alps on the low mountain ranges (e.g., Bavarian Forest, Ore Mountains) were classified as "low mountain range". Third, all specimens of the subspecies *torquatus* collected in northern Great Britain were grouped under the name "Scotland". Finally, all specimens of the subspecies *torquatus* collected in northern Europe or the Faroe Islands were treated as one group ("Scandinavia"). Altogether, 46 specimens (overall, we extracted DNA from 53 individuals) were grouped in this way (see Table S1). We cannot be entirely sure that all specimens in our material from low mountain ranges were residents. Therefore, we use the term group instead of population to make this point clear. Note also that, in principle, it is possible that individuals of the subspecies *torquatus* that migrate across the low mountain ranges of Europe breed there (see also "Introduction"). However, with our procedure for grouping specimens, we ignored such cases.

To minimize the destruction of the valuable material, we sampled only feathers and/or small amounts of toe pad tissue. In some instances, blood samples were provided by the museums. Tissue material was stored in 70 % ethanol. We additionally included muscle tissue from two specimens from ongoing field work (Table S1). DNA was extracted in an isolated work area to separate samples and the extracted DNA from PCR amplifications. During the whole process, negative control extractions and amplifications were performed to screen for contaminants entering the process at any stage.

Small down feathers were frozen in liquid nitrogen and pulverized prior to DNA extraction. Bird toe pads were digested with Proteinase K for 1 h to soften the material before homogenization. Subsequently, homogenized material was digested with Proteinase K for at least 20 h at 55 °C. The DNA was isolated and purified using the DNeasy blood and tissue kit from Qiagen following the manufacturer's instructions. Extracted DNA was resuspended in 50 µl elution buffer and stored at −20 °C. The elution process was repeated to extract all available genetic material. Before the PCR, salts and other PCR inhibitors were eliminated by filtering through MF-Millipore Membrane Filters with 0.025 µm pore size.

We optimized the standard PCR protocol for the gene encoding avian cytochrome *b* (Cibois and Cracraft 2004). The following PCR reactions were used: denaturing for one cycle of 4 min at 95 °C; denaturing for 35 cycles of 30 s at 95 °C; 30 s annealing at 53.6 °C; 30 s extension at 72 °C; and one cycle of 2 min at 72 °C. After the initial PCR reactions, we then applied a nested PCR with the PCR products. Based on primers L14841 and H15149 (Kocher et al. 1989), we designed new nested primers (Table S2). PCR products were sequenced by a commercial company. Sequences were aligned with BioEdit v.7.0.5.3 (Hall 1999) and CodonCode Aligner v.3.5.4 (CodonCode, Dedham, MA, USA).

We identified haplotypes using DnaSP v.5 (Librado and Rozas 2009) and created a haplotype network using TCS v.1.21 (Clement et al. 2000). We estimated the phylogenetic relationship between haplotypes and between overlapping sequences from other *Turdus* species available in GenBank (Fig. S1). For the final analyses, all sequences were trimmed to the length of our fragment. We searched for an appropriate evolutionary substitution model using MEGA 5 (Tamura et al. 2011), and we used the maximum-likelihood criterion and 10,000 bootstrap replications to construct a phylogenetic tree. This tree was only used to check whether our haplotypes are monophyletic. To examine the genetic structure and diversity (haplotype and nucleotide diversities) of Ring Ouzel specimens grouped according to their place of collection or subspecies, we used Arlequin v.3.5 (Excoffier and El Lischer 2010). AMOVA was based on the frequencies of haplotypes. We also calculated classic F_{ST} values between combined samples of the Alps, low mountain ranges, Scotland, and Scandinavia. We decided to use only classic statistics without considering the number of mutations as we analyzed only a small fragment. Increasing the fragment length would increase the number of haplotypes; therefore, our procedure is similar to the pooling of alleles in the analysis of allozymes.

Results

We obtained a 335-bp fragment of the cyt *b* gene from regular PCR, and a 331-bp fragment from nested PCR (excluding the primer region). All sequences were trimmed to 331 bp for alignment and subsequent analysis. We found a total of 12 polymorphic sites with 13 substitutions (11 transitions and 2 transversions; ratio = 5.5), leading to 11 haplotypes (Table 1). Sequence divergence between haplotypes varied between 0.3 and 1.5 % (uncorrected p distance). A phylogenetic analysis of these 11 haplotypes and available sequences of the genus *Turdus* in GenBank showed that these haplotypes form a well-supported clade (for further details and tree, see Fig. S1).

Total haplotype diversity in the whole dataset ($n = 53$ sequences) was 0.69 (±0.06) and total nucleotide diversity was 0.0031 (±0.0023). We sequenced a sufficient number of individuals from the four main areas (Scandinavia,

Table 1 Haplotypes and polymorphic sites of a 331-bp fragment of the cyt *b* gene of the Ring Ouzel *Turdus torquatus* (53 sequences)

Haplotypes	*n*	15010 T	15038 C	15082 T	15100 A	15111 C	15175 G	15193 A	15233 C	15253 A	15266 A	15271 C	15298 A
Position/consensus													
H1	1		T				A		G				
H2	29												
H3	10												G
H4	1			A							G		
H5	1							G					
H6	5			C									
H7	1			T								T	
H8	2									G			
H9	1					T							
H10	1				G								
H11	1	C			G								

The position is labeled according to the mitochondrial reference sequence of chicken (GenBank accession number: AY235571)

n The number of samples for each haplotype

Scotland, Alps, low mountain ranges) and found little differences in haplotype diversity between these groups (Table 2).

The haplotype network (Fig. 1) showed a star-like pattern, with one common haplotype (H2) occurring throughout Europe and with no sign of genetic separation of alpine and Nordic subspecies. However, alpine and Nordic groups (Scandinavia and Scotland) were characterized by private haplotypes (alpine: H3, H10; arctic: H6, H8, H9, H11). Most of these haplotypes differ from the common haplotype by only one mutation. The samples from Scotland showed a composition of haplotypes similar to those of the Scandinavian group. Two haplotypes that differed by two or three mutations compared to H2 were only found in the low mountain ranges north of the Alps: H1 in the Thuringian Forest (population extinct since 1983; Glutz von Blotzheim 1988; Rost and Grimm 2004) and H4 in the Ore Mountains, Saxony (Töpfer 2008). The single individual sampled from the Caucasian Mountains had the same haplotype as that in the Alps and Ore Mountains. The haplotype of the individual sampled in Macedonia was not found elsewhere.

When specimens from Scandinavia, Scotland, low mountain ranges, and the Alps were analyzed (for the number of individuals; see Tables 2, 3), the analysis of molecular variance revealed significant genetic variation among groups (15 %; $p = 0.0029$; AMOVA based on haplotype frequencies). When all specimens from each subspecies were combined, the analysis of molecular variance showed significant differences between subspecies (14 %; $p = 0.0049$ using the same 46 individuals; see also Table S1). The pair-wise F_{ST} between groups showed significant differentiation between the Alps and Scotland, Scandinavia, and the low mountain ranges (Table 3). Surprisingly, the combined samples collected across the low mountain ranges were significantly differentiated from the Alps but not from the two northern groups.

Discussion

When we compared an mtDNA fragment of the two subspecies of the Ring Ouzel occurring in northern Europe (Scandinavia, Scotland) and the Alps, we found only a shallow genetic signal within the mitochondrial genome.

Table 2 Number of sequenced individuals n_S, number of haplotypes n_H, number of polymorphic site n_{PS}, haplotype diversity *h*, and nucleotide diversity π of the specimen sampled across four major regions

Population	n_S	n_H	n_{PS}	h ± SD	π ± SD
Alps	13	3	2	0.56 ± 0.11	0.0020 ± 0.0019
Low mountain ranges	9	4	6	0.58 ± 0.18	0.0040 ± 0.0031
Scandinavia	17	5	4	0.57 ± 0.13	0.0022 ± 0.0019
Scotland	7	4	3	0.81 ± 0.13	0.0032 ± 0.0027
Total	46	11	12	0.71 ± 0.06	0.003 ± 0.002

SD Standard deviation

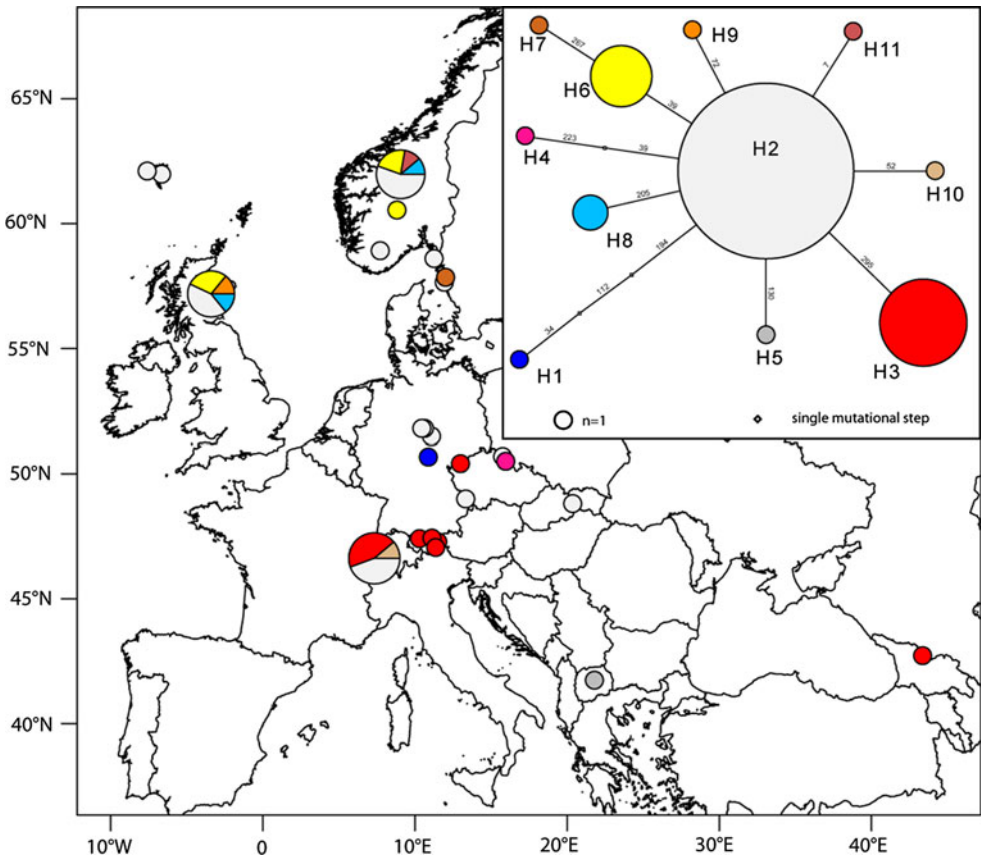

Fig. 1 Haplotype network and distribution of the haplotypes detected across 48 specimens of Ring Ouzel (*Turdus torquatus*) assigned to groups according to sampling location (for details, see text). The area of the *circles* of the haplotype network indicates the number of sequenced individuals with this haplotype. Mutational steps are indicated along the lines connecting haplotypes. The colors of the map and haplotype network correspond. Single individuals are represented by *colored dots*; for areas with more individuals, haplotype composition is indicated by *pie charts* (for further details, see Table S1)

Table 3 Conventional pairwise F_{ST} values between sampled areas

	Alps	Low mountain ranges	Scandinavia
Low mountain ranges	**0.21**		
Scandinavia	**0.30**	−0.017	
Scotland	**0.23**	0.031	−0.028

For sample sizes, see Table 2. Bold values are significant at the 5 % level based on 1,023 permutations

The most common haplotype occurred in both subspecies. In shallow gene trees, common haplotypes are often also the ancestral haplotype; therefore, the two subspecies are likely of recent origin, probably after the last glaciation with an incomplete lineage sorting. The finding that even morphologically different subspecies show low divergences and share the same haplotype has also been observed in other bird species (Ball and Avise 1992; Tarr and Fleischer 1993; Greenberg et al. 1998; Zink 2004). Nevertheless, this pattern is consistent with the traditional concept of arctic–alpine distributions, according to which the present populations are remnants of a broader distribution inbetween the ice sheets during the last glaciation event. Unfortunately, fossil records of Ring Ouzels do not provide independent evidence, because the European *Turdus* species are difficult to distinguish osteologically (Tyrberg 1991).

We also found private haplotypes for the individuals sampled on low mountain ranges which suggested that this group of individuals may be from populations that are at least in part isolated from both the northern and the alpine populations. The occurrence of haplotype H3 throughout the alpine samples but not within the individuals sampled in Scandinavia and Scotland may have two mutually non-independent explanations. First, this could be an old haplotype that was either lost in the northern populations or originated after the separation of the two subspecies. Second, this haplotype could have originated from dispersal events from the Alps to the low mountain ranges. The fact that we did not record any of the private haplotypes from the northern populations in the Alps indicated that there is no or very little introgression of genes from Nordic populations. Therefore, populations of the low mountain ranges are not melting pots of the two subspecies, but instead seem to be fairly isolated.

Although the present populations of the low mountain ranges are small, the genetic diversity of the individuals sampled in these areas was surprisingly high. At present, we cannot offer a convincing explanation for this finding. Perhaps there is still considerable gene flow between populations of the low mountain ranges or the populations were much larger in the recent past, or both. However, the small number of sequenced individuals precludes any firm conclusions on such details. We must make two additional

cautionary notes. First, using museum material did not always allow us to trace the origin and breeding status. The gaps in our sampling of populations could be a problem given the unclear delimitation of subspecies of the Ring Ouzel (Glutz von Blotzheim 1988). Nevertheless, the unique haplotype found in the Macedonian individual indicated that there might be considerable genetic variation across the southern populations of this species. Second, although the use of museum specimens allowed genetic information to be retrieved from areas where the Ring Ouzel is extinct, the low-quality DNA yielded only short PCR products, and degraded DNA could have miscoding lesions. However, our comparison of the polymorphic sites provided no indication of damage hotspots. Furthermore, Gilbert et al. (2007) demonstrated that the majority of miscoding lesions consist of C → T and G → A transitions, and although such transitions occurred in our dataset, our conclusions do not depend on them (see Table 1).

Climate change is at present the most pressing environmental issue (Parmesan 2006; IPCC 2007a, b; Huntley et al. 2008). In the Bavarian Forest, Bässler et al. (2010) modeled the future distribution of the Ring Ouzel along the altitudinal gradient suggesting that this species might disappear by 2100 due to climate change. However, Scherrer and Körner (2011) suggested that the rugged topography of mountain areas provides refuges with very different microclimates that allow plant species to survive. Birds act on a wider spatial scale than plants, and therefore this idea cannot be transferred easily to these animals. Anyhow, if the Ring Ouzel living in the low mountain ranges is a distinct lineage, the extinction of these populations will also lead to an extinction of the private haplotypes and so threaten the genetic diversity of this species.

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