

Recent divergence between two morphologically differentiated subspecies of bluethroat (Aves: Muscicapidae: *Luscinia svecica*) inferred from mitochondrial DNA sequence variation

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Abstract

We assessed the mitochondrial DNA sequence divergence of a 718 bp fragment of the control region and 1007 bp of the cytochrome *b* gene between two allopatric morphologically different subspecies of bluethroat (*Luscinia svecica*). None of the 17 total haplotypes was shared between *L. s. namnetum* and *L. s. svecica*. However, the mean distances between subspecies were very low for both fragments (0.00168 ± 0.00001 (mean \pm SE) for the control region; 0.00306 ± 0.00016 for the cytochrome *b* gene). Only one substitution made the two subspecies genetically differentiated, highlighting their recent divergence. Interestingly, the control region was not more variable than the cytochrome *b* gene.

Keywords: control region, cytochrome *b*, intraspecific phylogeny, *Luscinia svecica*, mitochondrial DNA, subspecies differentiation

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Introduction

Many bird species are subdivided into several subspecies based on morphological data. In intraspecific phylogeography studies using mitochondrial DNA (mtDNA), these polytypic species can be classified into several categories corresponding to the models defined by *Avise et al.* (1987). The two main categories can be described as follows: (i) strong phylogeographic structure of mitochondrial haplotypes in accordance with the distribution of the previous described morphs that are biogeographically isolated, such as the rufous-sided towhee (*Pipilo erythrophthalmus*, Ball & Avise 1992), the common yellowthroat (*Geothlypis trichas*, Ball & Avise 1992), the bananaquit (*Coereba flaveola*, Seutin *et al.* 1994), the fox sparrow (*Passerella iliaca*, Zink 1994), the stonechat (*Saxicola torquata*, Wittmann *et al.* 1995), and the dunlin (*Calidris alpina*, Wenink *et al.* 1996); and (ii) genetic homogeneity without any biogeographic structure even if the species is morphologically and

geographically polytypic such as the song sparrow (*Melospiza melodia*, Zink & Dittmann 1993) and the redpoll finch (*Carduelis flammea*, Seutin *et al.* 1995).

The bluethroat (*Luscinia svecica*, Muscicapidae, Sibley & Ahlquist 1990) is a polytypic species that breeds across a large range from western Palearctic to eastern Eurasia, from arctic upper limits to temperate and steppe middle latitudes. Ten subspecies have been described. They have been clustered into two phyla according to the red or white colour of the spot on the blue throat.

In our study we survey mtDNA differentiation in two allopatric subspecies of bluethroat that exhibit the most divergent morphological traits. *L. s. namnetum* is the smallest race and has a white spot on the throat. It breeds in western France along the Atlantic coast (Mayaud 1939; Eybert *et al.* 1989). *L. s. svecica* has a red spot on the throat and is larger in several morphometric measurements. Its breeding area ranges from Scandinavia across northern Russia.

By sequencing segments of the cytochrome *b* gene and the control region, we expected to reveal either (i) high genetic distances between morphs that are always

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geographically separated in relation to an ancient isolation, or, alternatively, (ii) low genetic distances between morphs reflecting a very recent separation.

Materials and methods

Samples

Growing feathers or blood were sampled for 10 individuals in each of the two subspecies *Luscinia svecica namnetum* (four localities) and *L. s. svecica* (three localities). Sampling locations are described in Table 1.

DNA methods

DNA from a 3 mm piece of the base of the growing feathers or 400 µL of blood plus lysis buffer (Seutin *et al.* 1991) was extracted as described by Taberlet & Bouvet (1991), except that DNA was precipitated in ethanol. The precipitate was diluted in 200 µL of TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) (Sambrook *et al.* 1989).

Two segments of the mtDNA were amplified (718 and 1007 bp for the control region (CR) and the cytochrome *b* (Cb), respectively) using primers H1248 and L437 defined in oscine birds for the control region (Tarr 1995), and H15915 (Irwin *et al.* 1991) and L14841 (Kocher *et al.* 1989) for the cytochrome *b*. Amplifications were performed in a final volume of 25 µL (750 mM Tris-HCl pH 9.0, 200 mM (NH₄)₂SO₄, 50 µM of each dNTP, 2 mM MgCl₂, 1 U of *Taq* Polymerase (Eurogentec), 25 pmol of each primer and 2 µL of the DNA. Cycling conditions were 93 °C for 40 s, 50/55 °C for 40 s and 72 °C for 90/180 s for 25/30 cycles

depending on the amplified mtDNA segment in a 9600 thermocycler (Perkin Elmer Cetus). PCR products were purified on QiaQuick PCR columns (Qiagen). Sequencing was performed using the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer) in a 20 µL volume containing 15 ng of purified DNA, 3.2 pmol of primer, according to the manufacturer's instructions. Sequencing reactions were electrophoresed for 7 h on a ABI PRISM™ 377 DNA sequencer (Perkin Elmer) in a 5% Long Ranger™ gel (FMC).

Data analysis

Multiple sequence alignments were obtained using the Clustal program implemented in the Sequence Navigator 1.0 software (Perkin Elmer) (Higgins *et al.* 1992). The distances were calculated with the program DNADIST implemented in the computer package PHYLIP version 3.57c (Felsenstein 1995). They have been calculated under the same model as in the maximum likelihood program DNAML with a ratio TS/TV = 2 (TV, transversion; TS, transition) and using the empirical frequencies of nucleotides. The insertion-deletion (indel) events were not taken into account for the calculation. A maximum likelihood tree was computed using the program DNAML. The haplotypic and nucleotide diversities were calculated using the program ARLEQUIN version 1.0 (Schneider *et al.* 1996). We also generated 100 000 trees at random in PAUP version 3.1.1 to test whether our data set had a phylogenetic signal (Hillis & Huelsenbeck 1992).

Sample	CR/Cb haplotype	Subspecies	Locality
nam01	CR/Cb01	<i>L. s. namnetum</i>	Brière, F, 47° 23' N 2° 10' W
nam02	CR/Cb02	<i>L. s. namnetum</i>	Brière, F, 47° 23' N 2° 10' W
nam03	CR/Cb03	<i>L. s. namnetum</i>	Guérande, F, 47° 20' N 2° 25' W
nam04	CR/Cb04	<i>L. s. namnetum</i>	Guérande, F, 47° 20' N 2° 25' W
nam05	CR/Cb04	<i>L. s. namnetum</i>	Guérande, F, 47° 20' N 2° 25' W
nam06	CR/Cb05	<i>L. s. namnetum</i>	Guérande, F, 47° 20' N 2° 25' W
nam07	CR/Cb06	<i>L. s. namnetum</i>	Gannedel, F, 47° 39' N 2° 05' W
nam08	CR/Cb04	<i>L. s. namnetum</i>	Tagus estuary, P, 38° 47' N 8° 53' W
nam09	CR/Cb07	<i>L. s. namnetum</i>	Tagus estuary, P, 38° 47' N 8° 53' W
nam10	CR/Cb08	<i>L. s. namnetum</i>	Tagus estuary, P, 38° 47' N 8° 53' W
sve01	CR/Cb09	<i>L. s. svecica</i>	Oural, R, 66° 35' N 66° 12' E
sve02	CR/Cb10	<i>L. s. svecica</i>	Oural, R, 66° 35' N 66° 12' E
sve03	CR/Cb11	<i>L. s. svecica</i>	Oural, R, 66° 35' N 66° 12' E
sve04	CR/Cb12	<i>L. s. svecica</i>	Oural, R, 66° 35' N 66° 12' E
sve05	CR/Cb13	<i>L. s. svecica</i>	Gumbaritsy, R, 60° 41' N 32° 56' E
sve06	CR/Cb14	<i>L. s. svecica</i>	Gumbaritsy, R, 60° 41' N 32° 56' E
sve07	CR/Cb15	<i>L. s. svecica</i>	Gumbaritsy, R, 60° 41' N 32° 56' E
sve08	CR/Cb16	<i>L. s. svecica</i>	Kola peninsula, R, 67° 55' N 35° 50' E
sve09	CR/Cb10	<i>L. s. svecica</i>	Kola peninsula, R, 67° 55' N 35° 50' E
sve10	CR/Cb17	<i>L. s. svecica</i>	Kola peninsula, R, 67° 55' N 35° 50' E

Table 1 Bluethroat samples used in the mtDNA analysis and their collection location. F, France; P, Portugal; R, Russia. Their corresponding mtDNA haplotypes (CR/Cb) are given

Results and Discussion

Combining the results for the control region and the cytochrome *b* gene, we revealed 17 haplotypes for all sequences obtained for 10 individuals from each of the two morphologically differentiated subspecies of bluethroat *Luscinia svecica namnetum* and *L. s. svecica* (eight for *L. s. namnetum* and nine for *L. s. svecica*) (Table 2). There was no common haplotype shared between the two subspecies. There was a total of five and 16 variable positions with substitutions in *L. s. namnetum* and *L. s. svecica*, respectively. The maximum likelihood tree suggested that the individuals of each subspecies clustered in separated groups (Fig. 1). This result is mainly due to one site (position 3 in the cytochrome *b* sequence) that separates the subspecies. The calculated skewness $g_1 = -0.502469$ after generating 100 000 random trees indicated a significant phylogenetic signal at $P < 0.01$.

For the control region, comparisons of all 20 sequences revealed a total of 13 variable nucleotide positions that define 12 different sequence types (Table 2). Of these 13 positions, seven were indels. The six remaining variable positions were substitutions (five TS and one TV). The calculated distances between the CR sequence types ranged from 0.00000 (indels were not taken into account for the distance calculation; however, different indel positions defined several sequence types, which explains the zero distance obtained between some control region sequence types) to 0.00560 with an average of 0.00191 ± 0.00018 (mean \pm SE). The calculated distances between the individuals of the two subspecies ranged from 0.00000 to 0.00560 with an average of 0.00168 ± 0.00001 . The mean intrasubspecific distance was 0.00049 ± 0.00009 for *L. s. namnetum* and 0.00186 ± 0.00019 for *L. s. svecica*. One sequence type included individuals from both subspecies (nam03, nam04, nam05, nam08, sve03, sve06, sve10).

For the cytochrome *b* gene, comparison of all sequences revealed a total of 15 variable nucleotide positions (all substitutions: 14 TS and one TV) corresponding to 12 different sequence types (Table 2). No Cb sequence type was shared between the two subspecies. The distances between the Cb sequence types ranged from 0.00100 to 0.00700 and averaged 0.00369 ± 0.00020 . Between the individuals of the two subspecies the distances ranged from 0.00100 to 0.00700 with an average of 0.00306 ± 0.00016 . The intrasubspecific distance was 0.00095 ± 0.00012 for *L. s. namnetum* and 0.00284 ± 0.00026 for *L. s. svecica*. The mean distance between subspecies of bluethroat was surprisingly less for the control region than for the cytochrome *b* gene. In a comparison of haplotypic and nucleotide diversities (Nei 1987), *L. s. namnetum* had a lower haplotypic diversity ($h \pm SD = 0.8667 \pm 0.0850$

for the control region, 0.6667 ± 0.1633 for the cytochrome *b*) than *L. s. svecica* (0.9111 ± 0.0773 and 0.8667 ± 0.1072 for the control region and cytochrome *b*, respectively). Both subspecies had very low nucleotide diversity (*L. s. namnetum*: $\pi \pm SD = 0.002901 \pm 0.001998$ for the control region, 0.000949 ± 0.000797 for the cytochrome *b*; *L. s. svecica*: 0.002500 ± 0.001777 for the control region and 0.002825 ± 0.001835 for the cytochrome *b*).

To summarize, our results of the sequence analysis are as follows. (i) We revealed a large number of mtDNA haplotypes, but the genetic distances between them were very low for the cytochrome *b* gene as well as for the control region. An important fact is that intra- and intersubspecific distances were of the same magnitude. (ii) Furthermore, we did not detect a faster rate of divergence in the control region compared to the cytochrome *b* gene as would be expected (Greenberg *et al.* 1983). (iii) There was phylogeographic structuration of the haplotypes between the two morphological lineages.

Considering the results obtained for the control region, it is surprising that this noncoding region, known to evolve quickly, revealed so little variability. In humans, the control region is known to evolve 10-times faster than the rest of the molecule (Greenberg *et al.* 1983). Unfortunately, data on the evolution of the control region in birds are still scarce (Ramirez *et al.* 1993; Quinn & Wilson 1993; for a review see Baker & Marshall 1997) and studies using sequencing data on this region give opposing results. In the grey-crowned babbler (Edwards 1993; Edwards & Kot 1995) and in some shorebirds (Wenink *et al.* 1994; Wenink *et al.* 1996), the control region is a useful and powerful tool to resolve intraspecific phylogeography. However, low variability is also found in some polytypic bird species such as the knot *Calidris canutus* (Baker *et al.* 1994) and the loggerhead shrike *Lanius ludovicianus* (Mundy *et al.* 1997). The lowest intraspecific divergences inferred from control region sequences or restriction sites have been reported in endangered species: the plain pigeon (*Columba inornata*, Miyamoto *et al.* 1994) and the whooping crane (*Grus americana*, Snowbank & Krajewski 1995).

We feel that our results are unlikely to be due to inadvertent amplification of a nuclear copy of the control region (Sorenson & Fleicher 1996; Zhang & Hewitt 1996). Only 25 (and even 20) cycles were sufficient for obtaining a strong PCR product using diluted DNA as template (40–100 ng). It seems unlikely that a nuclear copy (1000-fold less in number than mitochondrial copies) was amplified through such a small number of cycles. Moreover, we never observed any background in the sequences due to the possible presence of several different copies of the investigated fragment for the same individual.

Despite the very low level of genetic differentiation, we revealed phylogeographic differentiation between the

Table 2 Variable positions for the CR(Control region)/Cb(Cytochrome *b*) haplotypes. Full points indicate identical nucleotides to haplotype CR/Cb01. A dash indicates a single-base deletion. The different (CR) and (Cb) sequence types have been entered in the EMBL Nucleotide Sequence Database under the accession numbers Y08038, Y10061, Y08039, Y10062, Y08040, Y10063, Y08037, Y10066, Y08036, Y10068, Y08043, Y08051, Y08052, Y08053, Y08055, Y08056, Y08044, Y08045, Y10071, Y08046, Y08047, Y08048, Y08049, Y10073

nam:	Control region																Cytochrome <i>b</i>															
	24	94	123	150	390	519	618	627	657	678	679	705	3	65	209	231	232	317	395	429	584	596	611	683	816	974	1002					
CR/Cb01	-	-	-	C	T	C	T	T	A	-	-	-	G	C	C	A	T	C	C	C	T	C	C	G	T	G	T					
CR/Cb02	-	-	-	.	C	.	.	C	.	C	-	-	.	.	.	G	C					
CR/Cb03	-	-	-	C	-	C	-	-	.	.	.	G	C	C					
CR/Cb04	-	-	-	C	-	C	-	-	.	.	.	G	C					
CR/Cb05	A	A	G	-	-	-	C					
CR/Cb06	-	-	-	-	-	G	.	.	.	G	C					
CR/Cb07	-	-	-	.	C	.	.	.	C	-	-	-	.	.	.	G	C					
CR/Cb08	-	-	G	C	-	-	-	.	.	.	G	C					
sve:	-	-	-	T	.	A	.	.	C	-	-	-	A	.	.	G	C					
CR/Cb10	-	-	-	T	.	.	.	C	C	-	-	-	A	.	.	G	C					
CR/Cb11	-	-	-	C	-	C	-	-	A	.	.	G	C					
CR/Cb12	-	-	-	C	C	-	-	-	A	.	.	G	T	.	.	C	.	C					
CR/Cb13	-	-	-	T	.	.	.	C	-	C	-	-	A	T	.	G	.	T	A	C					
CR/Cb14	-	-	-	C	-	C	-	-	A	T	.	G	C					
CR/Cb15	-	-	-	T	.	.	.	C	C	-	-	-	A	.	.	G	.	.	A	A	.	.	C					
CR/Cb16	-	-	-	T	.	.	C	C	-	C	-	-	A	T	T	G	C	C					
CR/Cb17	-	-	-	C	-	C	-	-	A	T	.	G	C					

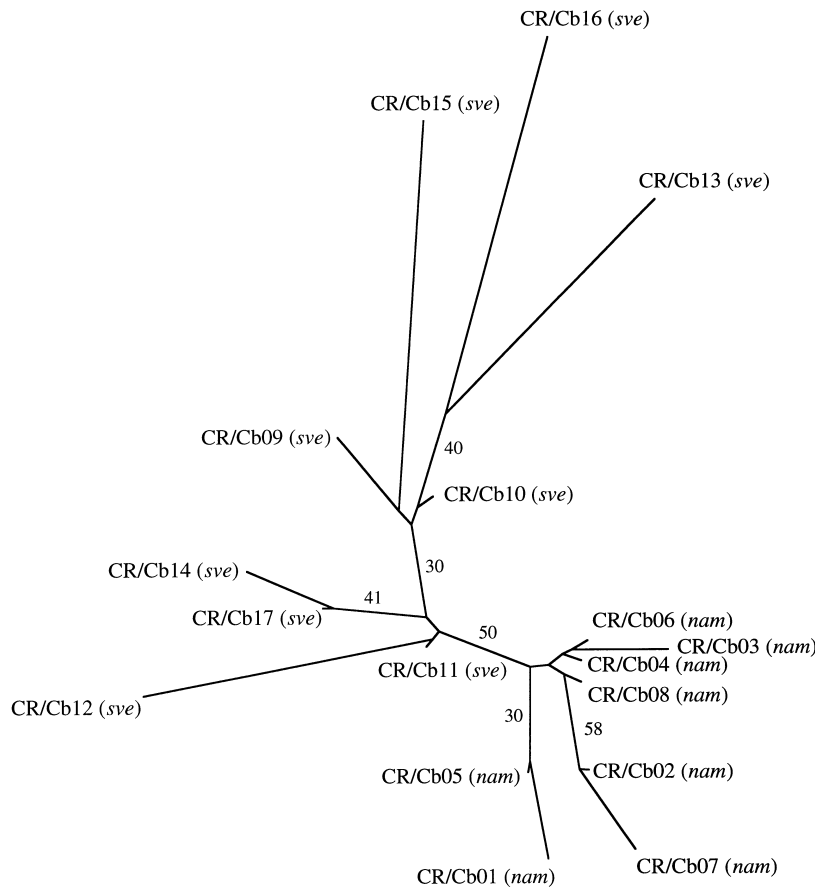


Fig. 1 Maximum likelihood tree obtained for the CR/Cb haplotypes for the two bluethroat subspecies (*nam*) and (*sve*); Bootstrap values (Felsenstein 1985) above 30 are given (100 replicates).

two subspecies. This result is comparable to the one obtained for *Lanius ludovicianus* (Mundy *et al.* 1997) where the genetic structure is in accordance with the described morphs. The difference with other studies demonstrating such clear structure (Ball & Avise 1992; Zink 1994; Wittmann *et al.* 1995) is that the mean distances are among the lowest reported indicating a very recent divergence between the two morphs. We can suspect that bluethroat populations may have suffered a bottleneck probably in the Quaternary cold period greatly reducing the intraspecific diversity in a single population. The high number of haplotypes is a consequence of the long mtDNA fragment that we surveyed (1725 bp) and can probably also be due to their recent emergence by mutations since the rapid population expansion after the bottleneck.

Two populations may have been isolated from each other in allopatry during a subsequent bottleneck event where badge colour could have emerged by chance. Knowing the extreme rapidity of morphological differentiation, the fixation of this character may have been reinforced via genetic drift or sexual selection through female choice (Lande 1981). The spot on the throat is for example used by males in courtship displays and could

consequently be a target of sexual selection. The influence of sexual selection has often been neglected but might be of great importance in intraspecific evolution (Zink 1996). The small number of substitutions and the lower values of haplotypic diversity for *L. s. namnetum* compared to *L. s. svecica* suggested a more severe bottleneck for the population where the white badge emerged or a differential in duration of the bottleneck itself. The two populations may also have experienced a different expansion rate after the bottleneck accounting for the different number of polymorphic sites. The fact that no CR/Cb haplotype was shared between the two subspecies might indicate that no maternal gene flow occurred between them. However, this hypothesis must be taken with caution: we could have missed a common haplotype just because of the small sample size for each population. So we could not reject the possibility of past gene flow between the two populations. Moreover there also could have been gene flow shortly after the bottleneck but those maternal lineages could have been lost by chance. Currently the two subspecies are geographically separated by ≈ 2000 km and their mating and wintering distributions do not overlap; thus further divergence is expected.

In conclusion, we first showed that the divergence between the two morphs has occurred very recently. Second, this result highlights the extreme rapidity of morphological differentiation in this species.

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