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Molecular and phenotypic divergence in the bluethroat (*Luscinia svecica*) subspecies complex

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Abstract

Subspecies complexes may provide valuable insights into the early stages of the speciation process. The bluethroat (*Luscinia svecica*) consists of many morphologically distinct subspecies that differ most strikingly in the ornamental colour pattern of the male throat. We investigated the genetic and phenotypic differentiation in this subspecies complex, using (i) microsatellite genotyping (11 loci) of a sample of 364 individuals from bluethroat populations in Europe and Asia, and (ii) spectrometric and morphological measurements of a sample of 131 museum skin specimens. Population genetic analyses, based on microsatellite allele frequency variation, revealed a slight but significant overall population differentiation (F_{ST} = 0.042). There was a well-differentiated southern group of subspecies with white or no throat spots and a less-differentiated northern group of chestnut-spotted populations. Phylogenetic analyses indicated that the southern all-blue and white-spotted forms are ancestral to the chestnut-spotted subspecies. In addition to the qualitative variation in throat plumage pattern already described in the literature, we found significant quantitative variation among subspecies in hue, chroma and brightness of the ultraviolet (UV)/blue throat coloration, and this variation seemed to be unrelated to the phylogenetic distance between subspecies.

Keywords: microsatellites, phylogeography, plumage colour variation, population genetics, reflectance spectrometry, sexual selection

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Introduction

Studies of speciation often focus on closely related species or groups of species that are already in an advanced stage of the speciation process, with strong reproductive isolation mechanisms and low frequencies of hybridization (Coyne & Orr 2004). In contrast, species with highly differentiated populations (hereafter referred to as subspecies) represent the early stages of the speciation process and should thus be a valuable complement to the more traditional studies of higher taxonomic levels. Subspecies complexes are by definition characterized by poorly developed isolation mechanisms and higher frequencies of hybridization and introgression, yet subspecies are often both morphologically and geographically well-defined. In such isolated populations, adaptation or drift may induce changes in traits used in mate choice, and thus affect the evolution of reproductive isolation mechanisms between allopatric populations (Mayr 1963). Furthermore, upon secondary contact between distinct subspecies, the speciation process may continue even in sympatry or parapatry, e.g. through the process of assortative mating and character displacement

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resulting in reinforcement of reproductive barriers (Dobzhansky 1937; Sætre *et al.* 1997), or via sexual conflict leading to rapid evolution of such barriers (Gavrilets 2000).

The great diversity among species in secondary sexual traits and the degree to which these are sexually dimorphic is largely attributed to the forces of sexual selection (Andersson 1994; Panhuis *et al.* 2001; Badyaev & Hill 2003; Hill & McGraw 2006). Phenotypic variation at the intraspecific level is less well-understood. In some avian species, there is extensive subspecific variation in the expression of secondary sexual traits (Irwin *et al.* 2001; Ödeen & Björklund 2003; Cheviron *et al.* 2006), which, like at higher taxonomic levels, may reflect divergent actions of sexual selection. Alternatively, adaptations to different ecological signal conditions, e.g. light environments for visual communication, may account for subspecies differences (signal optimization; Endler 1990). Finally, subspecies diversification may be due to nonadaptive changes resulting from genetic drift, founder effects and other random processes accumulating over time.

The bluethroat (*Luscinia svecica*) comprises a complex of about 10 recognized subspecies distributed in Europe, Asia and Alaska (Cramp 1988). The subspecies are distinguished partly by body size, but the most striking variation is in the ornamental plumage pattern and coloration of males. The central spot of the male throat patch shows a qualitative colour variation (chestnut, white or absent). In addition, variation in the colour (to human eyes) of the violet/blue throat feathers has been described in the literature (Gladkov 1968; Cramp 1988), although not with objective colour quantification. Most importantly, in a Norwegian population of the nominate subspecies *L. s. svecica*, the throat patch of bluethroat males, in particular its strong ultraviolet (UV) colour component (Andersson & Amundsen 1997), is subjected to sexual selection (Johnsen *et al.* 1998a, b; Johnsen *et al.* 2001).

The phylogenetic relationships among bluethroat subspecies are not well resolved. Questiau *et al.* (1998) used mitochondrial DNA (mtDNA) variation (control region and cytochrome *b* gene) to investigate the relationship between the subspecies *L. s. svecica* and *L. s. namnetum*. They found that although the subspecies were genetically distinct, the magnitude of the differentiation was small, indicating a recent split and rapid diversification in size and plumage coloration. In a larger sample of populations, Zink *et al.* (2003) found a relatively high degree of population differentiation, but little support for the currently recognized subspecies, based on the same mitochondrial markers.

Here, we use nuclear microsatellite DNA variation to investigate population differentiation among bluethroat populations. Microsatellites are increasingly used in phylogeographical studies of closely related species or subspecies (Petren *et al.* 1999; Gibbs *et al.* 2000; Lee *et al.* 2001; Chan & Arcese 2002; Koskinen *et al.* 2002). Microsatellite markers are particularly suited to differentiate between populations that have recently diversified, because they show a high degree of polymorphism caused by high mutation rates (Jarne & Lagoda 1996; Ellegren 2004).

The aims of the present study are to: (i) investigate the degree of population differentiation and construct a phylogeographical scenario for the current distribution of 11 bluethroat populations, representing seven subspecies, based on microsatellite allele frequency variation, (ii) objectively describe colour variation in the same seven subspecies using reflectance spectrometry of museum specimens, and (iii) investigate whether colour variation is related to the phylogeographical history of the species.

Materials and methods

Study species

The bluethroat is a small (18 g) migratory passerine bird. It is highly sexually dichromatic, with males displaying a colourful throat patch (Fig. 1). Females generally lack the throat patch or show a rudimentary version of it. The throat and breast are dominated by a large patch of ultraviolet (UV)/blue plumage (structural coloration), with or without a central spot of varying coloration (see below), and a band of chestnut-red plumage below (melanin-based; Staffan Andersson, unpublished data). Males display the throat patch both in inter- and intrasexual communication (Peiponen 1960). In a series of experiments involving manipulation of the male ornament, we have shown that the throat colour, in particular its UV component, is a sexual signal (Andersson & Amundsen 1997) subject to sexual selection in the wild (Johnsen *et al.* 1998a, b, 2001). There is high within-population variation between males both in the structural and the melanin-based parts of the ornament, and some of this variation is related to male age (Johnsen *et al.* 2001). In addition to these within-population patterns of variation, there is extensive variation in throat coloration between subspecies (Fig. 1). The central spot of the ornament shows qualitative variation, e.g. being chestnut-colored in the nominate *L. s. svecica*, white in *L. s. cyanecula* (Central Europe) and *L. s. namnetum* (Atlantic coast, France), and mostly absent in *L. s. magna* (Caucasus) and *L. s. azuricollis* (Spain). There is also quantitative variation in blue coloration between subspecies, which humans perceive as variation in the lightness of blue. However, spectrometry shows that the most pronounced variation is in the relative amount of UV reflectance (this study).

Contemporary populations and DNA analyses

In total, 364 putatively unrelated individuals (227 males, 137 females) were captured by mist- and clap netting at 11 bluethroat breeding localities (representing seven subspecies) in Europe and Asia (Fig. 2, Table 1), during the years 1996–

Fig. 1 Representatives of seven bluethroat *Luscinia svecica* subspecies, showing the whole range of variation in throat coloration. From left to right: *L. s. magna*, *L. s. azuricollis*, *L. s. cyanecula*, *L. s. namnetum*, *L. s. volgae*, *L. s. pallidogularis*, *L. s. svecica*. Copyright: the Natural History Museum, UK.

Fig. 2 Map of Eurasia, showing the geographical location (black dots) of the 11 contemporary bluethroat populations that were part of the genetic analyses. The pictures show the predominant throat spot colour morph for each respective population.

2003. We took a blood sample (25–50 µl) by brachial venipuncture, and recorded the colour of the throat spot of adult males.

DNA was extracted using QIAamp blood extraction kits (Qiagen). Individuals were genotyped with up to 11 heterologous microsatellite markers (mean ± SD: 10.79 ± 0.50, range: 7–11), using standard PCR conditions. Details about the microsatellite loci are given in Appendix I. The PCR products were run on manual polyacrylamid gels with radioactive labelling (see Johnsen *et al.* 1998b for details) or on automated sequencers (CEQ™ 8800 Genetic Analysis System, Beckman Coulter, and ABI 310/3100, Applied Biosystems).

Population genetics and phylogeny

We tested for Hardy–Weinberg equilibrium within each microsatellite locus (across populations) and within each population (across loci), using GENEPOP 3.4 (http://wbiomed.curtin.edu.au/genepop/). After controlling for multiple tests using sequential Bonferroni correction (Rice 1989) (critical $P = 0.0045$), three loci showed significant or

Table 1 Details of the 11 sampled contemporary bluethroat *Luscinia svecica* populations

Locality	Subspecies	Latitude	Longitude	Altitude (m)	*n* males/ females	Colour of male throat spot		
						Red	White	Blue
Sandfjorden, Norway	*L. s. svecica*	70°30′N	30°32′E	1	15/3	15	0	0
Stugudal, Norway	*L. s. svecica*	62°55′N	11°52′E	785	11/10	11	0	0
Heimdalen, Norway	*L. s. svecica*	61°25′N	8°52′E	1100	65/53	65	0	0
Kostanay, Kazakhstan	*L. s. pallidogularis*	51°38′N	64°13′E	175	11/6	11	0	0
Krkonoše Mts., Czech R.	*L. s. svecica*	50°45′N	15°35′E	1380	22/11	21	1	0
Thüringen, Germany	*L. s. cyanecula*	50°21′N	10°44′E	275	13/7	0	13	0
Třeboň, Czech R.	*L. s. cyanecula*	49°03′N	14°43′E	420	21/13*	0	20	0
Guérande, France	*L. s. namnetum*	47°20′N	2°25′W	0	11/10	0	11	0
Valduerna, Spain	*L. s. azuricollis*	42°20′N	5°58′W	810	27/9†	0	4	15‡
Suusamyr, Kyrgyzstan	*L. s. tianshanica*	42°10′N	73°45′E	2200	16/6	16	0	0
Mount Aragats, Armenia	*L. s. magna*	40°30′N	44°15′E	2250	15/9	0	4	11§

*One partial albino male with unknown throat spot colour. †Includes nine presumably unrelated juvenile birds (8 males, 1 female), caught in the breeding area after the breeding season (between 30 July and 20 September). ‡Three specimens with some white feathers underneath the blue feathers. §Two specimens with some white feathers underneath the blue feathers. Altitude values are in metres above sea level.

close to significant departure from Hardy–Weinberg (*Hru7*, *Ppi2*, and *Cuμ10*; all $P < 0.005$), whereas the remaining eight loci conformed to Hardy–Weinberg expectations (all $P > 0.05$). The departure at *Hru7* may be partly due to null alleles, because six populations showed tendencies for heterozygotes deficiency (all $P < 0.05$) at this locus, and previous studies also found low-frequencies of null alleles (0.6–2%) (Johnsen *et al.* 1998b, 2000). The other two loci showed either heterozygote deficiency or excess, depending on the population. Excluding these three loci from the analyses did not affect the main conclusions of this study. We therefore report the analyses including these loci to maximize the power of the tests, unless otherwise stated. Population details for the microsatellite data are given in Appendix II. The Øvre Heimdalen population showed significant deviation from Hardy–Weinberg overall ($P < 0.001$), while the remaining populations did not deviate significantly after Bonferroni correction (all $P > 0.016$).

Using STRUCTURE 5.0 (Pritchard *et al.* 2000), we estimated the number of genetic clusters (K) present in our sample without using information about the breeding populations from which the samples were taken. We assumed that individuals can have mixed ancestry in these analyses (admixture model). We performed 10 iterations at each level of K with a burn-in length of 50 000 and 1 million repetitions. Samples were then assigned to the estimated K populations, using the Bayesian Markov chain Monte Carlo (MCMC) estimation procedure implemented in the program.

Population differentiation was further examined with analyses of molecular variance (AMOVA). Overall genetic differentiation was estimated by the F_{ST} fixation index (Weir & Cockerham 1984), as calculated from the computer program FSTAT (Goudet 1995). Significance of differentiation (both overall and between each pair of populations) was determined by the permutation test (5000 iterations) implemented in the program. F_{ST} estimates may be biased when sample sizes are low relative to the number of alleles at the microsatellite loci used (S. Bensch, personal communication). Thus, to test whether our set of 11 loci was robust with respect to this potential problem, we also performed F_{ST} analyses using four different subsets of the 11 loci: (i) the eight loci that conformed to Hardy–Weinberg expectations (see above), (ii) a random half ($n = 6$) of all the loci, (iii) the remaining half ($n = 5$) of all the loci, and (iv) the three loci with the smallest number of alleles (6, 10 and 12 alleles, respectively). Pairwise F_{ST} estimates based on all these four reduced data sets were highly correlated with estimates based on the full set of loci (11 vs. subset 1: $r = 0.99$, $P < 0.001$, 11 vs. subset 2: $r = 0.99$, $P < 0.001$, 11 vs. subset 3: $r = 0.99$, $P < 0.001$, 11 vs. subset 4: $r = 0.87$, $P < 0.001$; all $n = 55$ pairwise comparisons). Moreover, the F_{ST} estimates based on subset 2 was highly correlated with those based on subset 3 ($r = 0.97$, $P < 0.001$). The overall estimate of F_{ST} was also very similar between the five different combinations of the 11 loci (average F_{ST}: 0.045, range: 0.040–0.053). Thus, our F_{ST} estimates do not depend on the specific microsatellite markers used. Using ARLEQUIN 2.0 (Schneider *et al.* 2000), we also performed an AMOVA based on the genetic clusters revealed by the Bayesian analysis.

The phylogenetic reconstruction is based on the genetic distance, D_A (Nei *et al.* 1983), following the recommendations of Takezaki & Nei (1996). D_A, which assumes that mutations follow the infinite-allele model of evolution (Kimura & Crow 1964), was calculated using DISPAN (Ota 1993), and an UPGMA dendrogram (Sneath & Sokal 1973) was constructed with DISPAN and viewed in TREEVIEW 1.6.6 (Page 1996). The UPGMA method assumes a nearly equal rate of molecular change in all subgroups (molecular clock); this

Table 2 Details of specimens from the seven bluethroat *Luscinia svecica* subspecies from skin collections

Breeding area	Subspecies	Latitude	Longitude	*n* males/females	Colour of male throat spot		
					Red	White	Blue
Russia	*L. s. svecica*	52–66°N	40–115°E	22/9	22	0	0
Turkestan	*L. s. pallidogularis*	37–51°N	55–66°E	11/5	11	0	0
Central Europe	*L. s. cyanecula*	48–54°N	5–25°E	28/8	0	23	5†
Bretagne, France	*L. s. namnetum*	46–47°N	2°W	18/5	0	18	0
Tian Shan	*L. s. tianshanica*	41–43°N	80–82°E	6/1	6	0	0
Caucasus*	*L. s. magna*			10/1	0	2	8‡
Gredos, Spain	*L. s. azuricollis*	39–40°N	4°W	7/0	0	2	5§

*All specimens collected on spring migration. †All five specimens with some white feathers underneath the blue feathers. ‡Two specimens with some white feathers underneath the blue feathers. §Three specimens with some white feathers underneath the blue feathers.

assumption is likely to hold in closely related and recently diverged taxa (Felsenstein 2004).

Museum specimens

We examined bluethroat specimens in the skin collections at the Natural History Museum of London (Tring), the Muséum National d'Histoire Naturelle in Paris, the Natural History Museum of Oslo and the Swedish Museum of Natural History in Stockholm, and took measurements from all available skins in full breeding plumage. The data set consists of 131 skins (102 males, 29 females), with collection year ranging from 1856 to 1969 (median: 1917) and collection date from 27 March to 29 July (median: 9 May). Most skins ($n = 114$) had been collected on the presumed breeding grounds, whereas the remaining 17 skins (all 11 specimens from the subspecies *L. s. magna* and 6 of 16 specimens from *L. s. pallidogularis*) had been collected during spring migration (Table 2). The measured specimens belonged to the same seven subspecies (according to the specimen labels) as the sampled contemporary populations (Tables 1 and 2). For both sexes, we obtained measurements of tarsus (method 2 in Svensson 1992), bill (from distal end of nostril to tip) and wing length (flattened and straightened) and used the mean in the analyses. In addition, we measured spectral reflectance of male throat plumage (see below) and scored the colourfulness of females on a 10-point scale, where 1 is least and 10 is most colourful (Amundsen *et al.* 1997). Age (second year or older) was determined according to Svensson (1992).

Colour measurements

We used a PS1000 diode-array spectrometer system (Ocean Optics), a DH2000 deuterium-halogen light source and a fibre-optic probe with 'probe pointer' to obtain coincident normal reflectance (Andersson & Prager 2006) from a 4-mm wide plumage spot, relative to a WS-2 white standard (Avantes). Five scans, removing the probe between each, were taken from the same spot of the male UV/blue chin plumage. More details on bluethroat reflectance measurements can be found in Johnsen *et al.* (2001). From the collected reflectance spectra, we subsequently calculated three objective colourimetric measurements (Andersson & Prager 2006): brightness (spectral intensity; $R_{320–700}$), hue [spectral location; $\lambda(R_{max})$] and chroma [spectral purity; $(R_{max} - R_{min})/R_{average}$].

We obtained colour measurements from 102 male specimens, but measurements from one individual were excluded because of damaged throat plumage. There was a significant negative correlation between hue and chroma ($n = 101$, $r_s = –0.22$, $P = 0.026$), a positive albeit nonsignificant correlation between hue and brightness ($n = 101$, $r_s = 0.17$, $P = 0.09$), and no significant correlation between chroma and brightness ($n = 101$, $r = –0.09$, $P = 0.39$). In other words, a UV skew was associated with relatively high chroma and low brightness.

Statistical procedure

We tested for variation in coloration and size between groups using ANCOVAS, including group identity and age of the specimen (second year or older) as fixed factors, and year and date of collection as covariates. To get an estimate of overall size, we performed a principal component analysis (PCA) on the three morphological measurements (wing, tarsus and bill length). The first principal component had an eigen value of 2.1, explained 71.3% of the variance and was highly positively correlated with all three variables (all $r > 0.82$, all $P < 0.001$, $n = 90$). It should thus be a good representation of overall size. We performed standard statistical tests using SPSS 11.0.

We tested for isolation by distance using the Mantel test (Mantel 1967), using MANTEL 2.0 (http://www.sci.qut.edu.au/nrs/mantel.htm), with 10 000 random iterations. Geographical distances between contemporary populations

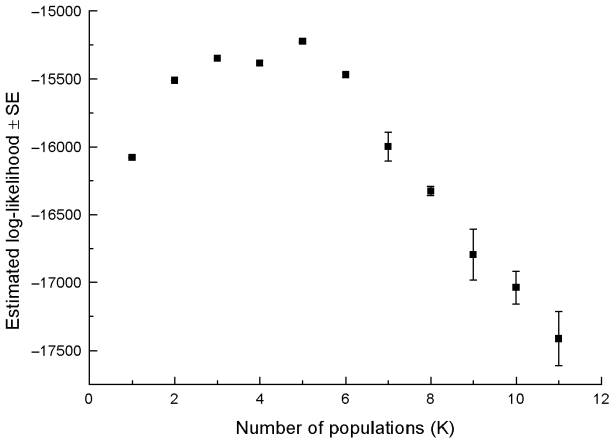

Fig. 3 Scatter plot showing the estimated log-likelihood (± SE) of each number of populations, $K = 1–11$, with 10 iterations per K. Based on calculations from STRUCTURE 5.0. See Methods for additional details.

were calculated based on latitude and longitude using the online distance calculator at http://sniptools.com/latitudeLongitude.php. To test for relationships between size and colour variables and subspecies differentiation, we constructed contrasts between all pairs of subspecies on the mean of each variable and tested for associations using Mantel tests.

Results

Population genetics and phylogeny

Population structure was first investigated using STRUCTURE 5.0. Although the highest average log-likelihood was found for $K = 5$, with all 10 iterations at this level of K giving a higher posterior probability than all iterations at all other levels of K (Fig. 3), the estimated probability was rather similar from $K = 2$ through $K = 6$. Thus, we investigated the pattern of assignment of individuals from the 11 different *Luscinia svecica* breeding populations across the above range of K (Table 3). The main results were the following: first, a high proportion of individuals from the Spanish *L. s. azuricollis* population was assigned to one genetic cluster at all values of K. Second, at $K = 3$, the majority of individuals from the German *L. s. cyanecula*, the Czech *L. s. cyanecula* and the French *L. s. namnetum* were assigned to a second cluster, while at $K = 5$, this cluster was split in two, with individuals from the French *L. s. namnetum* forming one cluster and those from the *L. s. cyanecula* populations forming the other. Third, individuals from the remaining seven populations were assigned mixed membership with roughly equal proportions, at $K \geq 4$. At the level of K that best describes our data ($K = 5$; Fig. 3), all *L. s. namnetum* samples and all but one *L. s. azuricollis* sample were assigned to their own, specific populations with a very high probability (Fig. 4). The picture was a bit less clear with respect to the two *L. s. cyanecula* populations, but the majority of the samples belonged to one genetic cluster (Fig. 4). For the remaining populations, there seemed to be no structure. Rather, these populations appeared to consist of individuals of mixed origin. These populations included all the chestnut-spotted populations, plus the all-blue *L. s. magna*. Confining the analyses to the eight loci that conformed to Hardy–Weinberg expectations produced virtually identical results (data not shown).

We further examined population differentiation using AMOVA based on all sampled populations. The 11 populations showed low, but significant levels of overall genetic differentiation ($F_{ST} = 0.042$, $P = 0.0002$). Of the 55 pairwise F_{ST} values, 46 were significant after sequential Bonferroni adjustment and another six had a P value lower than 0.05 (Table 4). Six populations (German *L. s. cyanecula*, Czech

Table 3 Proportion of membership of individuals in the predefined populations to each genetic cluster from $K = 2$ through $K = 6$. Population acronyms consist of the first three letters in the locality name followed by the first three letters in the subspecies name (see Table 1 for details)

Genetic cluster	$K = 2$	$K = 3$	$K = 4$	$K = 5$	$K = 6$
1	Val-azu: 0.96	Val-azu: 0.96	Val-azu: 0.95	Val-azu: 0.94	Val-azu: 0.93
2	Remaining 10 pops: 0.97–0.99	Thü-cya: 0.72 Tře-cya: 0.82 Gué-nam: 0.96	Thü-cya: 0.57 Tře-cya: 0.74 Gué-nam: 0.94	Gué-nam: 0.96	Gué-nam: 0.94
3		Remaining 7 pops: 0.73–0.90	Remaining 7 pops: 0.41–0.52	Thü-cya: 0.67 Tře-cya: 0.82	Thü-cya: 0.61 Tře-cya: 0.77
4			Remaining 7 pops: 0.40–0.51	Remaining 7 pops: 0.38–0.42	Remaining 7 pops: 0.27–0.32
5				Remaining 7 pops: 0.35–0.50	Remaining 7 pops: 0.27–0.33
6					Remaining 7 pops: 0.25–0.34

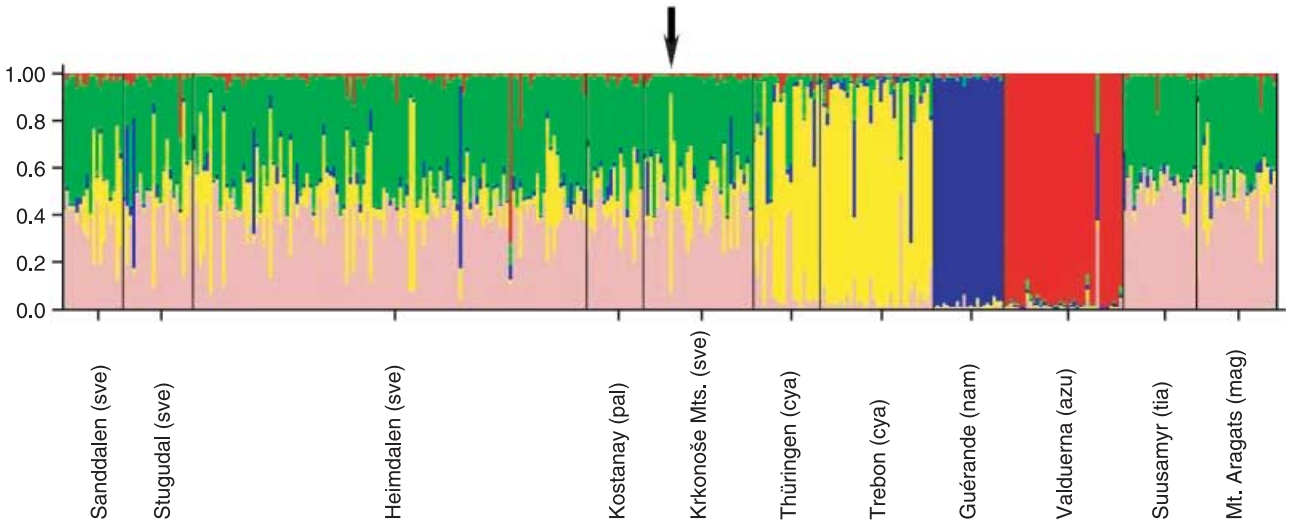

Fig. 4 Bayesian assignment of individuals to each of $K = 5$ genetic clusters, without using prior population information. Each bar represents the estimated membership coefficient (Q) for each individual bird in each cluster, and birds are separated into sampling populations by vertical lines, for illustrative purposes. The arrow indicates a single white-spotted *L. s. cyanecula* male appearing in the Czech *L. s. svecica* population.

Table 4 Pairwise F_{ST} values (below diagonal) and their respective P values (above diagonal). Numbers in bold are significant after sequential Bonferroni correction. Population acronyms consist of the first three letters in the locality name followed by the first three letters in the subspecies name (see Table 1 for details)

	San-sve	Stu-sve	Hei-sve	Kos-pal	Krk-sve	Thü-cya	Tře-cya	Gué-nam	Val-azu	Suu-tia	Ara-mag
San-sve		0.01636	0.53818	0.04909	0.23000	**0.00091**	**0.00091**	**0.00091**	**0.00091**	**0.00091**	**0.00091**
Stu-sve	0.0074		0.05545	0.04909	0.13000	**0.00091**	**0.00091**	**0.00091**	**0.00091**	**0.00455**	**0.00091**
Hei-sve	−0.0004	0.0022		**0.00364**	0.03000	**0.00091**	**0.00091**	**0.00091**	**0.00091**	**0.00091**	**0.00091**
Kos-pal	0.0039	0.0041	**0.0064**		0.02182	**0.00091**	**0.00091**	**0.00091**	**0.00091**	**0.00273**	**0.00091**
Krk-sve	−0.0020	0.0048	0.0029	0.0018		**0.00091**	**0.00091**	**0.00091**	**0.00091**	**0.00091**	**0.00091**
Thü-cya	**0.0126**	**0.0254**	**0.0173**	**0.0176**	**0.0188**		**0.00091**	**0.00091**	**0.00091**	**0.00091**	**0.00091**
Tře-cya	**0.0291**	**0.0293**	**0.0220**	**0.0291**	**0.0259**	**0.0165**		**0.00091**	**0.00091**	**0.00091**	**0.00091**
Gué-nam	**0.1034**	**0.0826**	**0.0796**	**0.0864**	**0.0998**	**0.0916**	**0.0749**		**0.00091**	**0.00091**	**0.00091**
Val-azu	**0.094**	**0.0879**	**0.0968**	**0.0966**	**0.0995**	**0.0979**	**0.1168**	**0. 1744**		**0.00091**	**0.00091**
Suu-tia	**0.0143**	**0.0074**	**0.0115**	**0.0037**	**0.0119**	**0.0275**	**0.0448**	**0.0960**	**0.1059**		**0.00091**
Ara-mag	**0.0147**	**0.0118**	**0.0152**	**0.0196**	**0.0172**	**0.0293**	**0.0373**	**0.0942**	**0.1064**	**0.0186**	

L. s. cyanecula, French *L. s. namnetum*, Spanish *L. s. azuricollis*, Armenian *L. s. magna*, Kyrgyz *L. s. tianshanica*) were differentiated from all other populations, with all 10 pairwise comparisons significant (after Bonferroni adjustment). These populations are all from the southern parts of the distribution, and characterized by a white or absent (i.e. blue) central spot, except for the chestnut-spotted *L. s. tianshanica* (Fig. 2). *L. s. magna* and *L. s. tianshanica* were significantly differentiated in these analyses (*magna*: mean $F_{ST} = 0.036$, range: 0.012–0.11; *tianshanica*: mean $F_{ST} = 0.034$, range: 0.007–0.11), although they belonged to the same clusters in the Bayesian analyses. This was not a result of biased F_{ST} values (see Methods section), because analyses with the four subsets of markers (as outlined in the Methods section) confirmed that these populations were distinct (data not shown). For the 10 comparisons involving the remaining five chestnut-spotted populations only, nine were nonsignificant after Bonferroni adjustment. The mean F_{ST} value for these comparisons was 0.003 (range: –0.002–0.007). We also performed an AMOVA based on the genetic clusters identified by Bayesian assignment, pooling all populations with mixed ancestry (see above, $K = 5$; Table 3). Most of the molecular variation resided among individuals within populations (93.4%), while the remaining variation was due to differences among the four groups (5.9%) and to differences among populations within groups (0.8%). The overall F_{ST} value based on this analysis was 0.066 ($P < 0.001$).

There was no evidence that genetic differentiation increased with increasing geographical distance between populations, whether based on the pairwise F_{ST} values

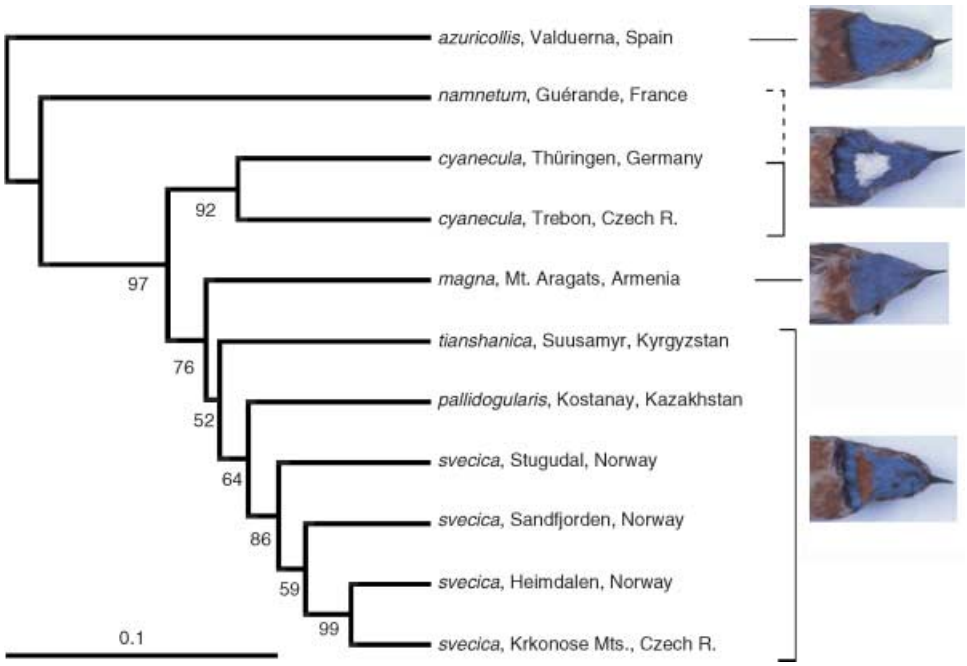

Fig. 5 UPGMA dendrogram with mid-point rooting, based on D_A genetic distances (Nei *et al*. 1983). Numbers below branches indicate bootstrap values; only values above 50% are shown.

(Mantel test: $r = 0.12$, $P = 0.27$) or Nei's D_A genetic distance ($r = 0.26$, $P = 0.13$). The Czech *L. s. svecica* population breeding in the Krkonoše mountains was first recorded breeding there in 1978 (Miles & Formánek 1989), and could conceivably have distorted a pattern of isolation by distance. However, excluding this population did not change the above conclusion (Mantel tests; F_{ST}: $r = 0.12$, $P = 0.27$, D_A: $r = 0.27$, $P = 0.11$).

The UPGMA dendrogram is shown in Fig. 5. Nonspotted *L. s. azuricollis* and white-spotted *L. s. namnetum* have separate branches from the root, while the two white-spotted *L. s. cyanecula* populations form a separate sister clade to that of the nonspotted *L. s. magna* from which all the six chestnut-spotted populations appear to derive. Bootstrap support was generally higher for these major topological features compared to the finer-scaled details within the clade of chestnut-spotted populations (Fig. 5). A virtually identical tree was obtained when restricting the analysis to the eight loci that conformed to Hardy–Weinberg expectations (data not shown).

Subspecies colour and size variation

Males. The colour of the central throat spot was distributed as shown in Table 1 (contemporary breeding populations) and Table 2 (museum specimens). In most populations/subspecies, all males had the same type of throat spot, with the following exceptions. There was a mixture of white-spotted and blue-spotted males in *L. s. cyanecula*, *L. s. azuricollis* and *L. s. magna*, with white-spotted males dominating in the former and blue-spotted males dominating in the latter two subspecies. The sample from the recently established *L. s. svecica* population in the Krkonoše mountains also included one white-spotted male (see arrow in Fig. 4).

For each of the three UV/blue colour variables, ANCOVA revealed significant variation between subspecies (Table 5, Figs 6 and 7a–c). Post-hoc tests revealed that *L. s. namnetum* specimens had a significant UV shift in hue, was more chromatic, and had a lower brightness than all other subspecies. In addition, *L. s. pallidogularis* showed tendencies for a lower chroma than all other subspecies except *L. s. magna* and a higher brightness than *L. s. svecica*, *L. s. cyanecula* and *L. s. namnetum*. When we repeated the above tests using the four genetic groups revealed by the Bayesian analyses as units (Table 3), we found similar patterns: *L. s. namnetum* was more UV reflective, more chromatic and less bright than all other groups and the chestnut-spotted birds were less UV-reflective than *L. s. namnetum*, and *L. s. cyanecula* (data not shown). The proportion of variance residing between groups in these models (hue, 35.8%; chroma, 25%; brightness, 22.0%) was somewhat higher than that between groups in models using subspecies as unit (Table 5).

Variation in the three UV/blue colour variables was nonrandom with respect to spot colour (ANCOVAS, all $P < 0.03$), mainly because chestnut-spotted birds were less UV-reflective, less chromatic and had higher brightness than white-spotted birds (data not shown).

Variable	Mean square	d.f.	*F*	*P*	% of variance
Response variable: hue					
Subspecies	605.8	6	5.5	<0.0001	29.3
Collection date	448.7	1	4.1	0.047	
Residual	110.2	81			70.7
Response variable: chroma					
Subspecies	0.076	6	4.0	0.001	20.8
Collection date	0.010	1	5.2	0.025	
Residual	0.019	81			79.2
Response variable: brightness					
Subspecies	468.0	6	3.9	0.002	17.6
Residual	119.1	94			82.4
Response variable: size (PC 1)					
Subspecies	11.3	6	44.8	<0.0001	77.6
Residual	0.3	83			22.4

Table 5 ANCOVAS testing for subspecies differentiation in male hue, chroma brightness and size. Initial models included subspecies identity and age of specimen (second year or older) as fixed factors, and collection date and year as covariates. Terms with $P > 0.1$ were excluded from the final models

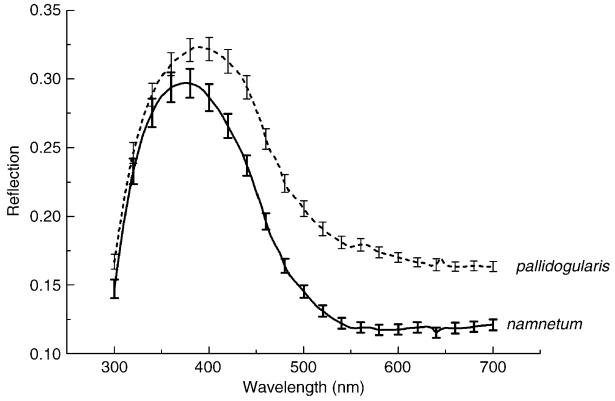

Fig. 6 Mean ± SE reflectance spectra for two *Luscinia svecica* subspecies covering the range of spectral variation in the present sample of bluethroat subspecies: *L. s. namnetum* (solid line; $n = 18$), *L. s. pallidogularis* (short dashes; $n = 10$).

Controlling for subspecies differences, both hue and chroma were significantly associated with collection date, with specimens collected early in spring tending to be more UV-reflective (as found in similar UV/blue plumage in blue tits; Örnborg *et al*. 2002) but less chromatic than specimens collected later in spring. None of the colour variables were related to collection year, indicating that the structural UV/blue plumage can remain fairly constant during long periods of storage in museum collections (but see Pohland & Mullen 2006).

There was significant between-subspecies variation in male size (Table 5, Fig. 7d), with *L. s. magna* being significantly larger and *L.* s. *namnetum* significantly smaller than all other subspecies. When confining the analyses to the four genetic clusters (see above), only *L. s. namnetum* was significantly different from the remaining groups. As a result, the fraction of the variance residing between groups was somewhat smaller (67.5%; Table 5).

Females. The analyses of female coloration and size only included subspecies with more than one female measured (mean number of females: 6.5, range 4–9; *L. s. cyanecula*, *L. s. namnetum*, *L. s. pallidogularis*, *L. s. svecica*). There was no significant between-subspecies variation in degree of female coloration ($F_{3,19} = 0.13$, $P = 0.94$), but old females were more colourful than young ones (mean ± SE score, old females: 3.8 ± 0.2, young females: 1.9 ± 0.5; $F_{1,25} = 11.9$, $P = 0.002$). Females showed significant between-subspecies variance in size ($F_{3,19} = 26.0$, $P < 0.0001$), primarily caused by *L. s. namnetum* females being considerably smaller (mean ± SE PC1: –1.7 ± 0.2) than females from all other subspecies (0.5 ± 0.2).

Genetic distance and variation in UV/blue colour and body size

We tested whether contrasts between subspecies in UV/blue coloration and body size were related to pairwise genetic distances as estimated by Nei's D_A, assuming that the genetic structuring among present-day bluethroats is similar to when the museum skins were collected (median collection year: 1917). There were no significant associations between genetic distance and contrasts (absolute values) in hue (Mantel test: $r = 0.47$, $P = 0.19$), chroma ($r = 0.58$, $P = 0.10$), brightness ($r = 0.27$, $P = 0.26$) or body size ($r = 0.36$, $P = 0.12$).

Discussion

Microsatellite-based population genetic analyses revealed small but significant levels of population differentiation, with southern blue- and white-spotted populations generally being more differentiated than northern chestnut-spotted populations. Reflectance spectrometry showed that bluethroat populations, in addition to the pronounced qualitative

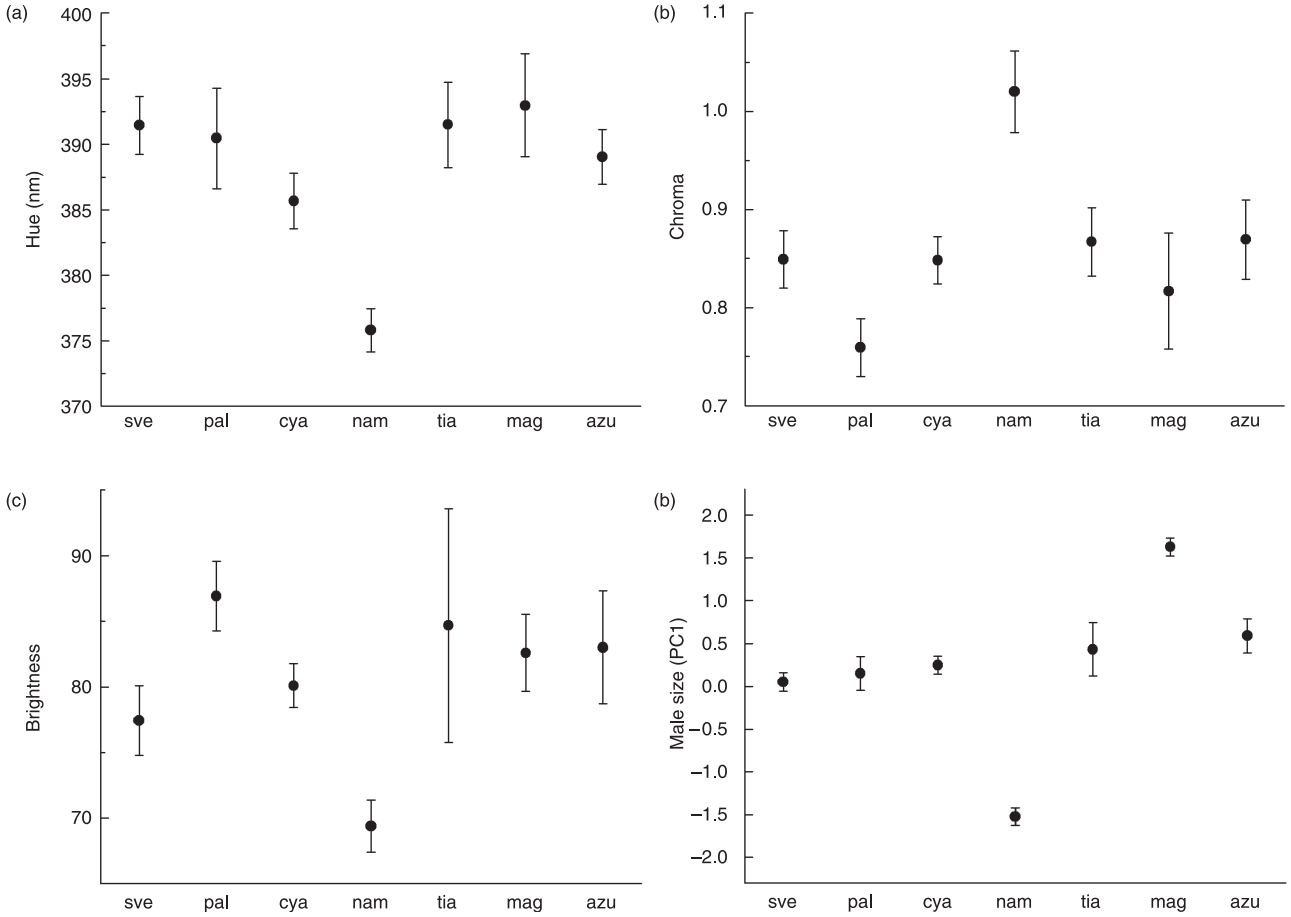

Fig. 7 Subspecies variation in male (a) hue (b) chroma (c) brightness and (d) size. See Table 2 for details on subspecies names.

variation in the colour of the central spot, also differed significantly in aspects of UV/blue coloration.

Bluethroat subspecies differentiation

Our genetic and morphological data show that several of the currently acknowledged *Luscinia svecica* subspecies are well-defined, while others are not. The French *L. s. namnetum* is different from all other subspecies in microsatellite allele frequencies, UV/blue coloration and size. The Spanish *L. s. azuricollis* is well-defined genetically and shows a high frequency of blue-spotted individuals. Central-European *L. s. cyanecula* are significantly differentiated both genetically and morphologically from other populations. Note that the two *L. s. cyanecula* populations were also genetically differentiated from each other according to the F_{ST}-based analyses, while Bayesian assignment revealed a common genetic structure. Caucasian *L. s. magna* are divergent from other subspecies/populations both genetically (based on F_{ST}) and in size. All of these four subspecies are different from all chestnut-spotted populations. However, the chestnut-spotted subspecies (*L. s. svecica*, *L. s. pallidogularis*, *L. s. tianshanica*) are not substantially different from each other morphologically, with the possible exception of *L. s. pallidogularis* (see below), and they show little genetic differentiation, with the exception of *L. s. tianshanica* (based on F_{ST}). The subspecies *L. s. pallidogularis* has been described as having a very pale UV/blue plumage (Gladkov 1968; Cramp 1988), and even though we find few significant differences between this and other subspecies, the paleness perceived by human vision can be traced to a combination of low chroma and relatively high brightness (see Fig. 7b–c). The *L. s. svecica* population in the Czech Krkonoše mountains was first documented breeding there in 1978 (Miles & Formánek 1989), and is one of several recently established *L. s. svecica* populations in the Central European mountain ranges (Meijer & Šťastný 1997). This population was genetically inseparable from the Norwegian *L. s. svecica* populations, and has probably been founded by Scandinavian/Northwest Russian birds on spring migration. The single white-spotted male in the sample from this population was genetically similar to individuals from the *L. s. cyanecula* populations (Fig. 4) and possibly immigrated from one of the Central-European populations of this subspecies.

Our results show both similarities and differences with the results of Zink *et al.* (2003). Both studies find a

well-differentiated southern group and a less-well differentiated northern group. Zink *et al.* (2003) concluded that there is no support for subspecies based on mtDNA markers. In contrast, we find that several of the previously recognized subspecies are well defined when taking both microsatellite allele frequencies and morphology into account. There are several possible explanations for the apparent discrepancy between these results. First, mtDNA markers may not be sufficiently variable to detect differentiation at the subspecies level in a species that seems to have diverged relatively recently, probably after the Pleistocene cold periods (Questiau *et al.* 1998; Zink *et al.* 2003). Several other studies have found discrepancies between morphological divergence and differentiation based on mtDNA (Shaw 2002; Babik *et al.* 2005; but see Omland 1997), indicating that such markers are not always useful in comparisons between closely related species or subspecies. Microsatellites may have a higher power to detect low-level genetic differentiation in rapidly diverging subspecies systems, due to the high mutation rate of such markers. Second, the selection of subspecies differed between the studies; *L. s. magna* was lacking and *L. s. azuricollis* was represented by only one individual in the study of Zink *et al.* (2003), while our genetic analyses did not include the subspecies *L. s. volgae*. This seems likely to have accentuated any differences between the two studies, since *L. s. magna* and *L. s. azuricollis* were among the most diverged subspecies in our study, while *L. s. volgae* is thought to be a hybrid subspecies (Cramp 1988), representing a mix of *L. s. svecica* and *L. s. cyanecula* genomes. As a result, the mtDNA of *L. s. volgae* specimens may stem from either subspecies depending on their maternal origin, and as expected *L. s. volgae* samples were interspersed between *L. s. svecica* and *L. s. cyanecula* samples in the mtDNA haplotype tree (judging from Fig. 2 in Zink *et al.* 2003).

Bluethroat phylogeny

Genetic similarities between populations may be due to common ancestry or gene flow, or more likely, to a combination of the two. It is notoriously difficult to distinguish between these causes of similarity because they are often tightly connected (related populations tend to be located in geographical proximity, making current gene flow more likely). We used genetic distances to construct a tentative bluethroat phylogeny, under the assumption that such distances reflect ancestry, at least to some extent. The UPGMA dendrogram (Fig. 5) showed high bootstrap support (97%) for a separation of the all-blue *L. s. azuricollis* and the white-spotted *L. s. namnetum* relative to the remaining populations. Thus, if we assume that the root of our dendrogram is correctly placed, these are the contemporary populations that are most ancestral in origin. Furthermore, we found high support (92%) for the two white-spotted *L. s. cyanecula* populations forming a separate branch among the remaining more derived populations, while all four *L. s. svecica* populations clustered together with a high level of support (86%). Our dendrogram was rooted according to the midpoint of genetic distances, which is commonly done in microsatellite-based phylogenetic studies (MacHugh *et al.* 1997; Richard & Thorpe 2001). When using the nightingale (*Luscinia megarhyncos*), a species from the same genus, as outgroup in the analysis, an identical topology among the bluethroat populations as the one presented in Fig. 5 was found (A. Johnsen, M. Naguib and V. Amrhein, unpublished data). However, microsatellites may give strongly saturated signals when used between different species, making root placement uncertain. Hence, even if our analysis is consistent with *L. s. azuricollis* and *L. s. namnetum* being the more ancestral forms among our sampled populations, we should consider the alternative that the basal placement of these populations is a result of their relatively low allelic diversity caused by drift (see Appendix II). It is possible that these isolated populations have gone through recent bottlenecks resulting in low genetic diversity, increased frequency of some rare alleles and strong differentiation from all other populations. According to Richard & Thorpe (2001), microsatellite distances and phylogenies are relatively robust to the effect of bottlenecks, even with a small number of loci sampled. Nevertheless, at present we cannot distinguish between these alternatives, and a basal placement of *L. s. azuricollis* and *L. s. namnetum* should therefore be confirmed with other genetic markers.

The phylogenetic patterns suggested by our analysis are consistent with patterns of spot coloration and current geographical distribution of populations, with one notable exception (see below). Studies of mtDNA variation have suggested that plumage divergence in bluethroats is of recent origin, possibly occurring after the last major ice age (Questiau *et al.* 1998; Zink *et al.* 2003). Our results point to the possibility that the isolated populations in Southern Europe represent the ancestral morphs that inhabited glacial refugia, and that the chestnut-spotted morphs evolved during the process of northward population expansion following the retreat of the ice. This scenario of throat spot evolution has the intuitive appeal of starting with a simple all-UV/blue upper throat, which evolved into throat ornaments with increasing complexity. Mechanistically, the reflectance properties of both the all-UV/blue and the white spot feathers are based on homologous structures in the feather barbs (Andersson & Amundsen 1997; S. Andersson, unpublished results), while the chestnut spot derives from brown melanin pigments deposited within the keratin structure (S. Andersson, unpublished results). The fact that there are white-spotted individuals in both all-blue populations included in our study supports the hypothesis that there is a close connection between all-blue and white-spotted forms. This might have been caused by recent gene

flow, although at least in the genetically distinct *L. s. azuricollis*, where all but one individual belonged to a unique genetic cluster (see Fig. 4), this seems unlikely.

The all-blue *L. s. magna* clustered with the chestnut-spotted populations and not with the morphologically similar *L. s. azuricollis*. Assuming that the overall topology of our dendrogram is correct, this implies that either the all-blue state or the white-spotted state must have evolved independently twice. However, the intermediate position of *L. s. magna* should be confirmed with additional genetic markers before a conclusion can be reached. At this stage, we can only conclude that *L. s. azuricollis* and *L. s. magna* are not closely related despite their similar phenotypic appearance.

The high degree of consistency between genetic differentiation and throat spot coloration suggests that this character may serve as a subspecies discrimination cue in areas of secondary contact. Some of the subspecies also differ in song characteristics (B. Chutný, T. Kumstatova and V. Pavel, unpublished data), which may provide additional cues used in mate choice in contact zones. However, the frequent occurrence of hybridization between some subspecies, prominently indicated by the presence of hybrid subspecies (*L. s. volgae* and *L. s. abbotti*; Gladkov 1968; Ali & Ripley 1982), shows that discrimination and assortative mating is not complete. During the last few decades, the establishment of *L. s. svecica* populations in the Central European mountain ranges has created new contact zones between chestnut-spotted and white-spotted (*L. s. cyanecula*) populations, and hybridization is known to occur in at least one such area (B. Chutný and V. Pavel, unpublished data). Furthermore, a very recent expansion of breeding ranges has led to secondary contact between *L. s. cyanecula* and another white-spotted subspecies, *L. s. namnetum* in northwestern France (Eybert *et al.* 1999). It remains to be seen whether these subspecies have built up reproductive isolation mechanisms in allopatry that are strong enough to keep them apart in sympatry, or whether extensive gene flow eventually will lead to merging of the subspecies.

Spectrometry revealed extensive variation between subspecies in all aspects of male UV/blue plumage coloration (hue, chroma and brightness). This variation does not seem to be strongly related to phylogenetic relationships, as there was no significant relationship between genetic distance and contrast in colour. It therefore seems unlikely that the quantitative variation between populations in UV/blue coloration is a result of genetic drift alone. Alternative processes that may account for population variation in UV/blue plumage, including signal optimization and sexual selection, will be explored in future research.

Conclusion

The bluethroat subspecies complex displays significant genetic and morphological differentiation and many of the previously acknowledged subspecies, notably the southern ones, are supported by our data. However, we find little support for treating the chestnut-spotted populations as different subspecies. Given the high level of subspecies divergence and the geographical isolation of many populations, the bluethroat may be at a relatively advanced stage of the speciation process compared to other subspecies complexes. Our study suggests a role for assortative mating based on throat spot coloration in the process of subspecies divergence in the bluethroat. Future studies should investigate the function of this and other secondary sexual characters in subspecies discrimination, e.g. by examining patterns of mating in hybrid zones and by experimental manipulation of male spot coloration.

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This study is part of an ongoing research programme on the ecology and evolution of genetic mating system and sexual signalling in bluethroats, led by Jan T. Lifjeld and Arild Johnsen at the Natural History Museum in Oslo. The team of collaborators consists of scientists working on avian visual signalling and the evolution of reproductive behaviour (Staffan Andersson, Bart Kempenaers), passerine ecology and phylogeography — with a special interest in bluethroat subspecies divergence — (Václav Pavel, Sophie Questiau, Javier Garcia Fernandez, Michael Raess), and insect phylogeny (Eirik Rindal).

Appendix I

Characteristics of the 11 microsatellite markers used

Locus	*n*	*k*	H_E	H_O	Reference
Ase19	354	21	0.83	0.80	Richardson *et al*. 2000
Cuμ4	358	20	0.87	0.82	Gibbs *et al*. (1999)
Cuμ10	359	18	0.90	0.74	Gibbs *et al*. (1999)
Fhu2	363	12	0.47	0.40	Ellegren (1992)
Hru7	356	13	0.56	0.44	Primmer *et al*. (1995)
Lm6	327	9	0.60	0.57	Marc Naguib, personal communication
Mcyμ4	364	26	0.91	0.88	Double *et al*. (1997)
PAT MP 2–43	362	22	0.88	0.87	Otter *et al*. (1998)
Phtr2	363	19	0.86	0.80	Fridolfsson *et al*. (1997)
PmaC25	356	6	0.53	0.48	Saladin *et al*. 2003
Ppi2	364	37	0.96	0.90	Martinez *et al*. (1999)

n, number of individuals; *k*, number of alleles; H_E, expected heterozygosity; H_O, observed heterozygosity.

Appendix II

Microsatellite diversity for the 11 contemporary bluethroat populations

Population	*n*	*k*	H_O	Allelic richness* ± SD	F_{IS}
Sandfjorden, Norway	18	8.82	0.73	8.65 ± 4.03	0.012
Stugudal, Norway	21	10.36	0.74	9.67 ± 4.08	0.047
Heimdalen, Norway	118	15.27	0.72	9.45 ± 4.44	0.032
Kostanay, Kazakhstan	17	9.27	0.73	9.27 ± 4.10	0.050
Krkonoše Mts., Czech R.	33	11.36	0.70	9.19 ± 4.36	0.063
Thüringen, Germany	20	7.64	0.65	7.40 ± 2.75	0.097
Třeboň, Czech R.	34	10.00	0.70	8.12 ± 3.82	0.039
Guérande, France	21	6.00	0.62	5.77 ± 2.59	0.072
Valduerna, Spain	36	6.45	0.60	5.50 ± 2.89	0.084
Suusamyr, Kyrgyzstan	22	9.91	0.72	9.21 ± 4.26	0.047
Mount Aragats, Armenia	24	9.82	0.76	8.87 ± 3.54	0.008

*Adjusted for sample size using FSTAT; *n*, number of individuals; *k*, mean number of alleles per locus; H_O, mean observed heterozygosity; F_{IS}, inbreeding coefficient.