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ORIGINAL PAPER

Rapid sperm evolution in the bluethroat (*Luscinia svecica*) subspecies complex

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Abstract Spermatozoa are among the most variable animal cell types, and much research is currently directed towards explaining inter- and intraspecific variation in sperm form and function. Recent comparative studies in passerine birds have found associations between the level of sperm competition and both sperm length and sperm velocity. In species with sperm competition, postcopulatory sexual selection may shape the morphology of sperm as adaptations to the female environment. The speed of evolutionary change in sperm morphology at the species level is largely unknown. In this study, we analysed variation in sperm morphology among morphologically distinct and geographically isolated bluethroat subspecies in Europe. Consistent with previous studies, our analyses of mtDNA and nuclear introns suggest recent divergence and lack of lineage sorting among the subspecies. We found significant divergence in total sperm length and in the length of some sperm components (i.e. head and midpiece). There was a significantly positive relationship between pairwise divergences in sperm morphology and mitochondrial DNA, suggesting a role for genetic drift in sperm divergence. The magnitude of sperm length divergence was considerably higher than that in other geographically structured passerines, and even higher than that observed between several pairs of sister species. We hypothesize that the rapid sperm evolution in bluethroats is driven by sperm competition, and that strong postcopulatory sexual selection on sperm traits can lead to rapid speciation through reproductive incompatibilities.

Keywords Sperm competition · Sperm morphology · Sperm size variation · Reproductive isolation

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Introduction

Sexual selection is a potent evolutionary force that may lead to rapid changes in traits related to success in competition for mates, like ornaments and armaments (Andersson 1994). Divergence in such secondary sexual traits and associated mate preferences may lead to precopulatory prezygotic isolation between populations and hence catalyse the early stages of speciation (Panhuis et al. 2001; Grether 2010;

Maan and Seehausen 2011). Similarly, postcopulatory sexual selection (i.e. sperm competition and cryptic female choice) exerts strong selection on primary sexual traits (e.g. gametes and the tissues producing them), and divergence in such traits may also lead to prezygotic reproductive isolation, and ultimately speciation (Coyne and Orr 2004).

Spermatozoa are among the most variable animal cell types, exhibiting considerable variation in morphology and behaviour at all levels of organisation (e.g. Cohen 1977; Pitnick et al. 2009). Recent comparative studies have suggested that sperm competition influences the evolution of a range of male reproductive traits (Birkhead and Møller 1998). For example, species experiencing stronger sperm competition have larger testes (relative to their body size) (Møller 1991; Harcourt et al. 1995; Hosken 1997; Stockley et al. 1997; Byrne et al. 2002) and produce more sperm (Rowe and Pruett-Jones 2011). Furthermore, total sperm length is associated with the level of sperm competition in a range of taxa including, insects (Gage 1994; Morrow and Gage 2000), fish (Stockley et al. 1997; Balshine et al. 2001), frogs (Byrne et al. 2003), mammals (Gomendio and Roldan 1991; Breed and Taylor 2000; but see Hosken 1997; Gage and Freckleton 2003) and birds (Lüpold et al. 2009b; Kleven et al. 2009; but see Immler and Birkhead 2007).

Variation in sperm size between males in a population is also associated with sperm competition. Specifically, the coefficient of between-male variation in total sperm length is negatively related to the level of sperm competition faced by males in passerine birds (Calhim et al. 2007; Kleven et al. 2008; Lifjeld et al. 2010), suggesting that sperm competition exerts strong stabilizing selection on sperm morphology. Sperm traits may also respond to directional selection from sperm competition (e.g. Kleven et al. 2009; Lüpold et al. 2009a), in addition to being influenced by drift (Laskemoen et al. 2013). Moreover, sperm morphology appears to be shaped via interaction with the female reproductive environment (Woolley 1970; Briskie et al. 1997; Morrow and Gage 2001; Higginson et al. 2012). Therefore, a complex range of selection pressures and drift are likely to drive the evolution of sperm morphology and, consequently, sperm traits may move towards different optima in different populations, especially if the spatially isolated populations differ in strength or form of selection.

Passerine birds exhibit considerable variation in total sperm length, ranging from 42.7 μm in the red-backed shrike (*Lanius collurio*) (Briskie et al. 1997) to 291 μm in reed bunting (*Emberiza schoeniclus*) (Dixon and Birkhead 1997). Sperm length generally shows high phylogenetic dependence in comparative studies (e.g. Kleven et al. 2009). However, closely related species may also exhibit large divergence in total sperm length, e.g. common chiffchaff (*Phylloscopus collybita*) 116.2±1.6 μm (Laskemoen & Lifjeld unpublished) and willow warbler (*Phylloscopus trochilus*) 93.5±1.9 μm (Lifjeld et al. 2010). Within the family Muscicapidae, total sperm length varies from 101.2 μm (collared flycatcher *Ficedula albicollis*) to 279.9 μm (nightingale *Luscinia megarhynchos*) among 12 investigated species (own unpublished data). To date, relatively few studies have investigated intraspecific variation in sperm morphology, despite the fact that analyses at these levels (between-individuals within a population and between populations) should provide considerable insight into both the selection pressure shaping the evolution of sperm morphology and the speed of evolutionary change in sperm traits. Additionally, intraspecific studies may reveal contrasting patterns to studies at the interspecific level (e.g. Lüpold et al. 2009b), thus a comprehensive understanding of the evolution of sperm traits requires examination at both levels of organisation.

A limited number of recent studies of passerine birds demonstrate significant variation in sperm morphology among different populations (Lüpold et al. 2011; Schmoll and Kleven 2011; Laskemoen et al. 2013). Moreover, in addition to variation in sperm morphology among redwing-blackbird (*Agelaius phoeniceus*) populations, Lüpold et al. (2011) found a gradual increase in sperm size from southwest to northeast of the breeding range, and a negative relationship between sperm length and body size across population. However, in this study and the study of Schmoll and Kleven (2011) on coal tits (*Periparus ater*), the degree of genetic differentiation between populations was unknown and thus no inference could be drawn regarding the speed of sperm diversification. Laskemoen et al. (2013) found significant variation in sperm morphology among barn swallow populations (*Hirundo rustica*). Furthermore, the subspecies with the highest genetic distance also showed the largest difference in sperm morphology, leading the authors to hypothesise that variation in sperm morphology between subspecies might reflect the genetic distance between taxa (Laskemoen et al. 2013). Studies incorporating information on both variation in sperm traits along with data concerning genetic divergence are necessary to examine the speed and direction of evolution on sperm traits.

Here, we examined both sperm morphology and genetic divergence in the bluethroat (*Luscinia svecica*) subspecies complex. The bluethroat is a small (~18 g) passerine bird, which ranges from upper arctic limits to temperate and steppe middle latitudes, and breeds from the western Palearctic to eastern Eurasia (Cramp 1988). Currently, around ten subspecies are recognized based primarily on differences in male plumage characteristics and to some extent size (Cramp 1988). All subspecies are sexually dimorphic: males are larger and exhibit striking sexual ornamentation, whereas female generally exhibit drab plumage (Johnsen et al. 2006). Specifically, males have a colourful throat patch with blue and chestnut surrounding a conspicuous central spot (white,

red or absent depending on the subspecies), which is displayed during courtship (Peiponen 1960; Johnsen and Lifjeld 1995). There is also evidence that subspecies vary in song characteristics (Turcokova et al. 2010). Currently, the phylogenetic relationships among the bluethroat subspecies are not well resolved and subspecific status is somewhat contentious. Using mtDNA markers (control region, cytochrome b), Questiau et al. (1998) found support for two distinct subspecies clusters (*svecica* and *namnetum*), whereas Zink et al. (2003), despite reporting a relatively high degree of population differentiation (F_{ST}=0.29), found little support for subspecies recognition in a study of seven morphs previously identified as subspecies based on morphological differences. Using 11 microsatellite loci, Johnsen et al. (2006) found evidence for differentiation (F_{ST}=0.042) across bluethroat populations in Europe and Asia. Moreover, that study found support for genetic differentiation between some morphologically distinct subspecies, most notably *svecica*, *namnetum*, *azuricollis* and *cyanecula* (Johnsen et al. 2006).

Laskemoen et al. (2007) demonstrated considerable between-male and within-male variation in sperm morphology in the nominate subspecies *svecica*. In the present study, we focus on the four most distinct subspecies identified by Johnsen et al. (2006) (i.e. ssp. *svecica*, *namnetum*, *azuricollis*, *cyanecula* and in addition ssp. *volgae*), and investigate between-population variation in sperm morphology in relation to variation in two mtDNA regions and two nuclear introns. Our study had two main aims: (1) to test if there is a relationship between genetic divergence and sperm divergence among these five study populations and (2) to compare differences in sperm evolution between bluethroat and other species with known sperm divergence.

Material and methods

Field work

We collected samples from each of the five bluethroat subspecies (*azuricollis* from Spain, *cyanecula* from central Europe, *namnetum* from France, *svecica* from Norway and *volgae* from Russia; see Table S1 for details of localities and Table S2 for information about individual samples) during the peak of the breeding season in 1996–2011. Five individuals of *L. megarhynchos*, collected by Marc Naguib in Germany in 2000, were used as outgroup. Birds were caught on their home territories using song playback and mist nets or clap nets using mealworm as bait. Sperm samples were collected via cloacal massage (Wolfson 1952). The ejaculate was collected with a micro capillary tube and fixed in 5 % formalin (PBS) solution. At the same time, 25 μl of blood was collected by brachial venipuncture and stored in 96 % ethanol. DNA was later extracted following the protocol for the E.Z.N.A blood kit (Omega Bio-Tek, Inc, Norcross, GA, USA) and the QIAmp blood kit (QIAGEN, Inc. Valencia, CA, USA).

Sperm morphology

For each individual, approximately 15 μl of diluted sperm was applied to a glass microscope slide and allowed to air-dry. Next, the slide was gently rinsed with distilled water and allowed to air-dry once again. We captured digital images (×160 magnification) of sperm cells using a Leica DFC420 camera mounted on a Leica DM6000 B digital light microscope (Leica Microsystems, Switzerland) and measured sperm length using specialised image analysis software (Leica Application suite v. 2.6.0 R1). For each male, the following measurements were obtained (±0.1 μm): head length, midpiece length, tail length and total length (i.e. head + midpiece + tail). Following the recommendation of Laskemoen et al. (2007), we measured 10 morphologically normal sperm from a minimum of 10 males per subspecies (with the exception of *volgae*, for which samples were only available from nine males). Additionally, we calculated the within male (CV_{wm}) and between male coefficient of variation in total sperm length (CV_{bm}), the latter being an index for sperm competition across passerines (Lifjeld et al. 2010). We then adjusted CV_{bm} according to the sample size using the formula $CV_{bm} + (1/(4n))$ (Sokal & Rohlf, 1995), because CV_{bm} has been documented to be underestimated in small population samples (Laskemoen et al., 2007). Hereafter, CV_{bm} will refer to the adjusted value. To avoid observer effects, all measurements were conducted by one person (TL).

PCR and sequencing

We sequenced two mitochondrial regions (COI-region and control region, n=84 each) and two Z-linked introns (BRM-15 and VLDLR-7, n=53 each; see Table S3 for primer combinations and PCR conditions). All markers were amplified in PCR reaction volumes of 10 μL, containing dH_2O, 1X PCR buffer II (Applied Biosystems, Foster City, CA, USA), 1.5 mM magnesium, 0.2 mM dNTP (ABgene, Epsom, UK), 0.5 mM forward and reverse primer, 3 % dimethyl sulfoxide, 0.25 U AmpliTaq DNA polymerase (Applied Biosystems) and approximately 50 ng DNA template. Amplifications were run on a DNA Engine Tetrad 2 (MJ Research, Waterton, MA, USA) using the following profile: 95 °C for 1 min, 94 °C for 30 s, annealing temperature 52–56 °C (depending on primer combination; see Table S3) for 30 s and 72 °C for 1 min. This profile was then repeated for a further 34 cycles before a final elongation step of 72 °C for 10 min. In order to confirm amplification success and to exclude any contamination, 3 μl of PCR product was electrophoresed in 1 % agarose TBE.

The remaining PCR product was purified by digesting unincorporated nucleotides and primers using diluted (1:9) ExoSap-It (United States Biochemical, Cleveland, OH, USA) run at 37 °C for 45 min, followed by 15 min at 80 °C to inactivate the enzyme. The PCR products were then sequenced using BigDye Terminator v 3.1 Cycle Sequencing kit (Applied Biosystems). Next, sequences were aligned (using ClustalW) and manually edited in the program Mega v 5.05 (Tamura et al. 2011). For each locus, all sequences were truncated to the length of the shortest sequence for comparison. Altogether, we included samples from five different subspecies and eight different localities/populations (Table S2). Sperm samples and mitochondrial regions were analysed from individuals sampled at the same locality during a single collection episode, with the exception of individuals sampled in Wroclaw (Poland) for which blood and sperm were collected from the same locality but from different birds during different years (2009 and 2010, respectively). For the Z-introns, samples were obtained from the same localities, but in different years, as both sperm and mtDNA samples, with the exception of *cyanecula*, for which samples were obtained from Thüringen (Germany) and Trebon (Czech Republic). Importantly, introns were sequenced from females only which allowed us to obtain exact haplotypes, since female birds are hemizygous on the Z-chromosome. We did not sequence any introns from the *volgae* population.

Statistical methods

We tested for differences in sperm morphology (means per male) among subspecies using ANOVAs and post hoc Tukey HSD tests using Statistica v 7.1 (StatSoft Inc). DNAsp v 5 (Librado and Rozas 2009) was used to calculate the nucleotide diversity (π) (Hudson et al. 1987) and Tajima's D (Tajima 1989) for both mitochondrial and nuclear regions. The Tajima's D tests showed no significant deviation from neutrality for either of the subspecies, in either of the regions (Table S4).

We implemented a model test in Mega v 5.05, using BIC (Bayesian Information Criterion) scores to find the best fitting substitution model for the two mitochondrial markers combined (all further analyses were conducted for the two mtDNA regions combined) and each intron separately. For mitochondrial regions, a gene tree based on maximum likelihood was made using Mega v 5.05 with the substitution model HKY + G, with *L. megarhynchos* as outgroup. For nuclear regions, maximum likelihood were constructed using the K2P (VLDLR-7) and Tamura-3-parameter (BRM-15) model using the same outgroup. Bootstrap values were calculated in Mega v 5.05 using 10,000 iterations. Translation from nucleotide to amino acid sequences of the analysed regions revealed no stop codons, frameshifts or systematic double peaks in the COI region, indicating an absence of pseudogenes.

In order to examine the genetic structure of the populations, analysis of molecular variance (AMOVA) was run using Arlequin v 3.5 (Excoffier et al. 2005). The variance was partitioned into variation between populations and variation within populations. Pairwise species differentiation was estimated using F_{ST} (Weir and Cockerham 1984), with default settings in the population comparison. These F_{ST} values can be used as short-term genetic distances between populations. The null hypothesis is no differences between the populations, and the P value is given as the proportions of simulations giving a F_{ST} value larger or equal to the observed one. Similarly, we calculated a measure of phenotypic divergence, P_{ST} that expresses the proportion of total variance in sperm length that can be attributed to the variation among populations. P_{ST} was calculated using the following formula, $P_{ST} = \frac{\sigma_B^2}{\sigma_B^2 + 2h^2\sigma_W^2}$, where σ_B^2 is the phenotypic variance between populations, σ_W^2 is the phenotypic variance within populations and h^2 denotes the heritability (Leinonen et al. 2006). We calculated σ_B^2 and σ_W^2 from one-factor ANOVA following Sokal and Rohlf (1995; p. 216, Box 9.2). The heritability was conservatively set to 0.62, based on an estimate of heritability of flagellum length (midpiece plus tail) reported for the zebra finch (*Taeniopygia guttata*) by Birkhead et al. (2005). Next, in order to test for a correlation between pairwise estimates of genetic divergence (F_{ST}) and sperm length divergence (P_{ST}), we performed a Mantel test (Mantel 1967) to correct for the multiple uses of the same populations. The Mantel test was conducted in R v 2.11.1 (R Development Core Team 2009) using the package ade4 (Dray and Dufour 2007) and the P value obtained through Monte Carlo simulations with 9,999 replicates.

Results

Sperm morphology

We found significant variation in total sperm length among the five subspecies (Table 1; $F_{4,77}$=47.0, P<0.001; Fig. 1). This was more or less explained by variation in midpiece length ($F_{4,77}$=26.8, P<0.001) which showed the same pattern as total length. In addition, we found significant variation in head length ($F_{4,77}$=7.47, P<0.001), although this component showed a different pattern than total and midpiece length. In contrast, tail length did not vary between subspecies ($F_{4,77}$=0.80, P=0.53). Post hoc tests revealed that total length and midpiece length was significantly longer in *azuricollis* than in all other subspecies (Table 2). In addition, *svecica* had significantly longer

Table 1 Summary of the different sperm trait measures in the respective subspecies

	Head length (μm)		Midpiece length (μm)		Tail length (μm)		Total length (μm)		CV_{bm} [a]	CV_{wm} [b]
	Mean ± SD	Range	Mean ± SD	Range	Mean ± SD	Range	Mean ± SD	Range		
Azuricollis (*N*=16)	19.28±0.43	18.61–20.18	190.05±7.88	171.62–205.18	15.88±3.98	10.71–26.76	225.21±7.40	213.76–239.79	3.30	1.31
Cyanecula (*N*=14)	18.80±0.59	18.06–19.86	167.79±4.22	162.85–173.27	14.06±2.34	10.24–18.37	200.64±4.05	192.51–205.90	2.04	1.92
Namnetum (*N*=14)	18.63±0.70	17.45–19.72	173.81±5.91	167.91–181.48	14.07±2.42	10.69–18.56	206.51±5.92	198.05–214.62	2.89	1.52
Svecica (*N*=29)	19.69±0.75	18.85–21.07	175.96±6.14	157.78–181.96	15.15±3.59	13.06–19.86	210.79±4.00	202.00–221.04	1.91	1.30
Volgae (*N*=9)	19.46±0.95	17.91–21.29	171.67±7.32	161.39–179-38	14.87 ±4.21	7.45–22.69	205.99±5.00	197.70–213.55	2.45	1.52

[a] Mean CV between males

[b] Mean CV within males

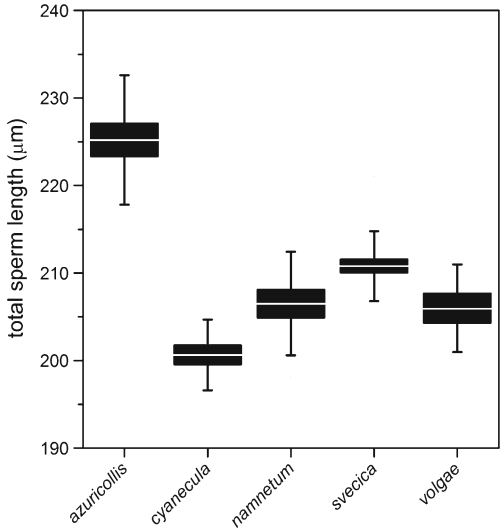

Fig. 1 Box plot illustrating differences in total sperm length for the five subspecies of the bluethroat. *White line* represents mean values, *boxes* indicate ±SE and *whiskers* indicate ±SD. See Results for test statistics

sperm than *cyanecula*, and *cyanecula* had a significantly longer midpiece than *namnetum* (Table 2). Finally, *svecica* had significantly longer sperm head than both *cyanecula* and *namnetum* (Table 2). The overall phenotypic divergence in total sperm length P_{ST} was 0.70 ($F_{4,77}$=47.0, P<0.001), that is, 70 % of the overall between-male variation in sperm length was explained by population.

We found a nearly twofold variation in population-wise CV_{bm} of total sperm length, ranging from 1.91 in *svecica* to 3.30 in *azuricollis* (Table 1). A post hoc test of homogeneity of variances showed significant heterogeneity of sperm variation among populations (Bartletts test χ^2=9.59, P=0.048). Moreover, the mean CV_{wm} varied significantly between populations ($F_{4,77}$=8.44, P<0.001) and ranged from 1.30 to 1.92 (Table 1), with *cyanecula* differing significantly from both *azuricollis* and *svecica* (Table 2). There was no significant correlations between CV_{bm} and mean CV_{wm} (Spearman's correlation: N=5, r_s=0.05, P=0.93).

Variation in mitochondrial DNA

Of the 1,229 bp sequenced for mtDNA in all individuals, we found 27 variable sites and 16 parsimony informative sites (outgroup excluded). The nucleotide diversity (π) for all subspecies combined was 0.0023, and ranged from 0.0006 (*azuricollis*) to 0.0022 (*volgae*) across the five subspecies (Table S4).

The mitochondrial gene tree did not show a clear structure of the subspecies in monophyletic clades, but it did show moderate support (with bootstrap support just above 50 %) for two groups, one consisting of *namnetum*, *cyanecula* and *azuricollis*, and the other consisting of *svecica* (Fig. 2). Interestingly, the

Table 2 Tukey HSD tests of between-subspecies differences in components of sperm length; head, midpiece and total length in addition to CV_{wm}

Trait	Subspecies	*Azuricollis*	*Cyanecula*	*Namnetum*	*Svecica*
Head	*Cyanecula*	0.33			
	Namnetum	0.09	1.00		
	Svecica	0.32	**0.002**	**<0.001**	
	Volgae	0.97	0.18	0.05	0.90
Midpiece	*Cyanecula*	**<0.001**			
	Namnetum	**<0.001**	**0.002**		
	Svecica	**<0.001**	0.40	0.61	
	Volgae	**<0.001**	0.83	0.10	0.93
Total	*Cyanecula*	**<0.001**			
	Namnetum	**<0.001**	0.03		
	Svecica	**<0.001**	**<0.001**	0.10	
	Volgae	**<0.001**	0.13	1.00	0.13
CV_{wm}	*Cyanecula*	**<0.001**			
	Namnetum	0.48	0.03		
	Svecica	1.00	**<0.001**	0.31	
	Volgae	0.60	0.07	1.00	0.46

Significant results (after Bonferroni corrections) highlighted in bold

volgae population was present in both groups (Fig. 2). In addition, the *azuricollis* population formed a monophyletic group, with relatively high bootstrap support (>80 %) for a clade consisting of 19 out of the 21 individuals from this population. The mtDNA gene trees did not have enough resolution or support to determine the most ancestral of these five subspecies.

AMOVA revealed that 61 % of the total variation occurred between subspecies while 39 % occurred within populations, with an overall significant F_{ST} value (F_{ST}=0.61, P<0.001). Additionally, pairwise F_{ST} values between the five subspecies showed relatively high and significant values (see Table 3). The maximum genetic distance between any individual (using K2P substitution model) was estimated to 0.7 % and the mean genetic distance to 0.3 %. The genetic distance between the supported *azuricollis* group (consisting of 19 out of 21 individuals) and the rest of the bluethroats was 0.4 %. Assuming a conventional molecular clock and mutation rate (i.e. 2 % divergence per million years, Bromham and Penny 2003), the maximum time since divergence was 350,000 years, the mean time since divergence150,000 years and the time since divergence between the two groups (*azuricollis* vs the rest) was 200,000 years.

Variation in nuclear DNA

Of the 933 bp sequenced for the nuclear introns in all individuals, we found 39 variable sites and 22 parsimony informative sites (outgroup excluded). The total nucleotide diversity (π) for the introns was 0.0061 for all subspecies combined. The π value ranged from 0.0006 (in *azuricollis*) to 0.0074 (in *cyanecula*) for the two introns combined (Table S4). The two gene trees calculated using the introns BRM-15 and VLDLR-7 (with *L. megarhynchos* as outgroup) showed no structure related to the subspecies (Figs. S1 and S2). AMOVA revealed that 12 % of the total variation occurred between populations, while 88 % occurred within populations, with an overall significant F_{ST} value (F_{ST}=0.12, P value<0.001).

Comparing sperm with genes

There was an overall good concordance between mtDNA divergence (F_{ST}=0.61) and sperm length divergence (P_{ST}= 0.70) for all five populations analysed together. There was also a significant positive correlation between the two divergence measures for all pairwise estimates among populations (Fig. 3; Mantel test: R=0.80, P=0.042). There was, however, no such relationship between intronic F_{ST} estimates and P_{ST} estimates (Mantel test: R=0.24, P=0.34).

Populations with high genetic diversity in mtDNA and Z introns had relatively low variation in sperm length (mtDNA: N=5, r_s=−0.20, P=0.75; intronic DNA: N=5, r_s=−0.90, P= 0.037). This is opposite to expectation from a neutral model of variation in a heritable character, like sperm length.

Discussion

Subspecific divergences in the bluethroat

We found considerable variation in sperm morphology between subspecies, with differences in sperm head and

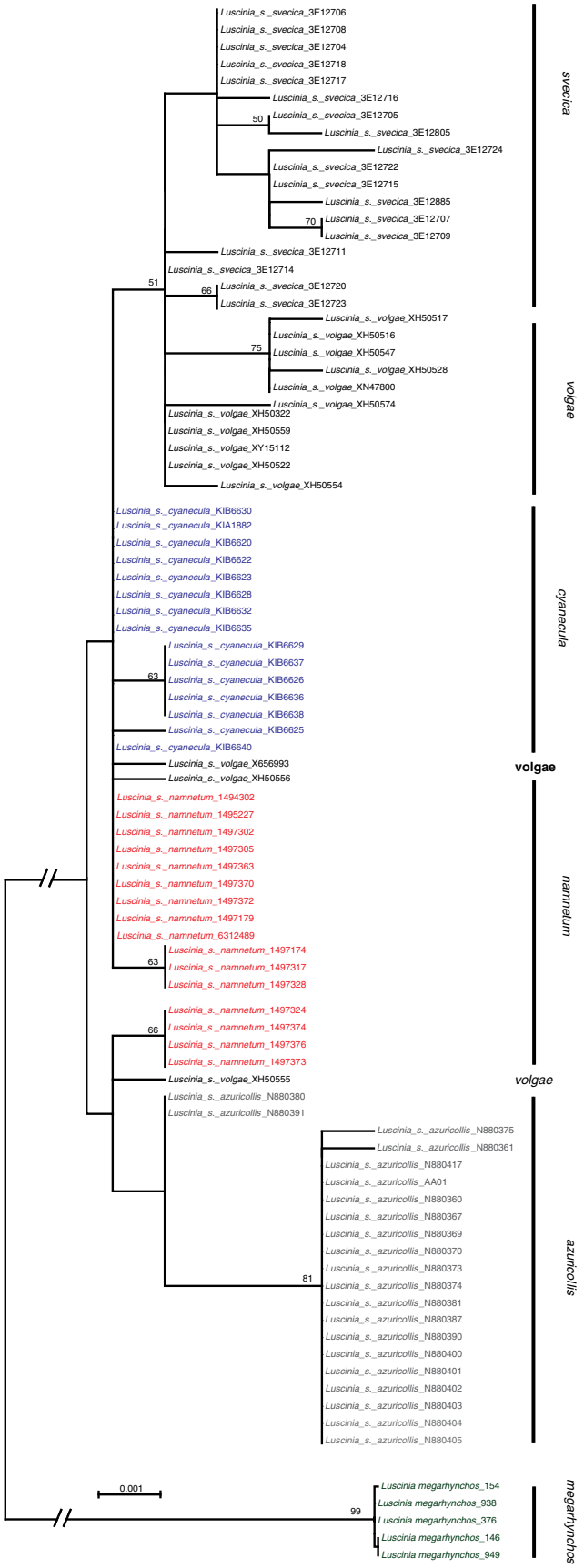

Fig. 2 Maximum likelihood tree (HKY + G model, 10,000 bootstrap replicates) based on the COI and control region combined (1,229 bp) for 84 bluethroats. Only bootstrap values above 50 % are shown. *L. megarhynchos* used as outgroup

midpiece length, as well as total sperm length between several of the sampled taxa. Seventy percent of the phenotypic variation in total sperm length was confined to the between-population level, while 60 % of the mitochondrial DNA and 12 % of the nuclear variation resided between populations. The time since divergence among individuals in our sample was estimated to be in the range of 150,000–350,000 years, assuming a 2 % sequence divergence per million years (Bromham and Penny 2003; Päckert et al. 2007; Weir and Schluter 2008; but see Pulquério and Nichols 2007). While we acknowledge that these estimates of time since divergence are rather crude and possibly overestimated (Ho et al. 2011), they are consistent with earlier suggestions that bluethroat subspecies diverged during the late Pleistocene glaciation periods (Questiau et al., 1998; Zink et al., 2003). Both mtDNA and nuclear DNA showed a lack of lineage sorting with respect to subspecies. Importantly, these findings suggest that bluethroat subspecies have diverged relatively recently and, as such, constitute 'young' taxa.

Rapid sperm evolution in the bluethroats

The high divergence in sperm morphology together with low genetic divergence suggests rapid evolution of sperm morphology in the bluethroat. Other studies have also reported inter-population variation in sperm morphology (Lüpold et al. 2011; Laskemoen et al. 2013; Schmoll and Kleven 2011). However, relative divergence in total sperm length was considerably higher in bluethroats (11.6 %) than between populations/subspecies in five other species (mean divergence, 2.26 %; range 0.3–3.7 %; see Table 4), as well as between four pairs of sister species (mean divergence, 3.48 %; range 0.3–9.9 %; see Table 5).

Taken together, our findings demonstrate that the bluethroat represents a suite of young taxa exhibiting high divergence in sperm morphology. Few studies including both genetic and sperm divergence are available for comparisons. However, studies of the barn swallow suggest that European and North American populations diverged approximately 840,000 years ago (1.68 % divergence in CO1; Johnsen et al. 2010), but that sperm show just 3.7 % relative divergence in total sperm length (Laskemoen et al. 2013, Table 4). Similar comparisons can be made if we consider divergence between sister species. The relative sperm divergence between sister species ranged from 0.3 % between the house sparrow (*Passer domesticus*) and the Spanish sparrow (*Passer hispaniolensis*, with COI

divergence=3.0 %) to 9.9 % between the common redstart (*Phoenicurus phoenicurus*) and the black redstart (*Phoenicurus ochruros*, with COI divergence=7.5 %). Thus, it appears that the large divergences in sperm morphology within the bluethroat have arisen over a very short time period which suggests that evolutionary change in sperm morphology has been relatively rapid in this subspecies complex. Support for the hypothesis that sperm morphology can undergo rapid evolution, has also been found in other taxa. For example, Landry et al. (2003) showed that sperm morphology evolved rapidly within two clades of the sea urchin (*Echinometra oblonga*). A genetic distance based on COI suggested a split between these two clades about 250,000 years ago, which is comparable in time to the genetic divergence found within the bluethroats (~150,000–350,000 years).

Rapid sperm evolution: drift versus selection?

Laskemoen et al. (2013) hypothesised that variation in sperm morphology at the subspecies level might reflect genetic distances between populations, suggesting that drift is a main driving force behind this relationship. In agreement with this, we found a significant relationship between genetic divergence and divergence in total sperm length among bluethroat subspecies (Fig. 3). This suggests that some of the variation in total sperm length can be explained by genetic drift among isolated populations. However, such a correlation would also be expected if there has been selection for different sperm traits in randomly varying environments. The lack of a positive relationship between neutral genetic variation and sperm variation within populations, suggests that sperm variation is not a simple function of overall genetic variation in the population. Instead, sperm variation in a population may be shaped by postcopulatory sexual selection, mediated by the risk of sperm competition (Kleven et al. 2008, Lifjeld et al. 2010, Laskemoen et al. 2013).

Postcopulatory sexual selection is thought to shape the evolution of sperm traits (reviewed in Pizzari and Parker 2009). For example, more sperm competition may lead to stronger stabilizing selection on sperm length for an optimization to the female reproductive tract, such as the size of female sperm storage tubules (Briskie et al. 1997). This idea is supported by a strong negative relationship between sperm competition and variation in sperm length (Calhim et al. 2007; Kleven et al. 2009; Lifjeld et al. 2010) and a correlation between mean sperm length and the length of sperm storage tubules across species (Briskie et al. 1997; Kleven et al. 2009). Consequently, stabilising selection is thought to result in a reduction in sperm size variation, though such a reduction in variation does not necessarily involve a simultaneous change in mean trait values. However, there is a possibility of long-term directional evolution under a scenario of stabilizing selection given that the optimal phenotype is consistently larger or smaller than the population mean, even if the difference is minute. There will also be effects of genetic drift. Hence, directional evolution does not necessarily require directional selection in the sense that phenotypes at the extreme end of a distribution are favoured.

In comparative studies of passerine birds, there seem to be a general pattern of sperm competition being associated with longer sperm (Briskie et al. 1997; Lüpold et al. 2009a, b, Kleven et al. 2009, Lifjeld et al. 2010) and a gradual increase in sperm length over the evolutionary time scale (Rowe et al. 2013). However, there is still much residual variation in sperm length among species, and also some evidence of a negative association with sperm competition (Immler and Birkhead 2007), which implies that the interpretation of directional change in sperm length as a signature of sperm competition can be rather complex. Nevertheless, the reduced population variation in sperm length induced by sperm competition might be a prerequisite for evolutionary change in the trait. An example of a species pair with a presumed absence of sperm competition, and little or no sperm length divergence, is provided by the Eurasian bullfinch *Pyrrhula pyrrhula* and the Azores bullfinch *Pyrrhula murina*, which both exhibit extremely high between-male variation in sperm length (Lifjeld et al. 2013).

The level of sperm competition faced by males in a population can be estimated from the between-male coefficient of variation in sperm length (CV_{bm}), which is lower in species/populations exhibiting higher rates of extrapair young (EPY) and hence higher levels of sperm competition (Calhim et al. 2007; Kleven et al. 2008; Lifjeld et al. 2010; Laskemoen et al. 2013), presumably because of stabilizing selection on sperm length (Lifjeld et al. 2010). We found marginally significant differences in CV_{bm} between the populations, suggesting that they vary in the level of sperm competition, with *svecica* exhibiting the highest level (CV_{bm}=1.9, translating into a rate of 27 % EPY according to Lifjeld et al. 2010) and *azuricollis* the lowest level (CV_{bm}=3.3, translating into 10 % EPY). Similarly, we found significant variation in the within-male coefficient of variation in sperm length (CV_{wm}), but a lack of correspondence between CV_{wm} and CV_{bm} values. This could be explained by seasonal variation in CV_{wm}, which has been shown for house wrens (*Troglodytes aedon*) (Cramer et al. 2013). Variation in sperm lengths within an ejaculate is presumably not a genetically coded trait, since all sperm originate from the same diploid set of genes, but may be influenced by phenotypic plasticity or production errors during spermatogenesis.

To date, EPP data are only available for two bluethroat populations. In the *svecica* population, Johnsen and Lifjeld (2003) found that 49.5 % of all nests contained at least one EPY and 26.3 % of all young were sired by extrapair males, while the corresponding figures for *namnetum* were 63.8 % of nests and 41.9 % of nestlings (Questiau et al. 1999), both of which are comparatively high rates of sperm competition

Table 3 F_{ST} values (below the diagonal) based on mitochondrial DNA with associated *P* values (above the diagonal)

	Azuricollis	*Cyanecula*	*Namnetum*	*Svecica*	*Volgae*
Azuricollis		**<0.001**	**<0.001**	**<0.001**	**<0.001**
Cyanecula	0.80		**<0.001**	**<0.001**	**<0.001**
Namnetum	0.75	0.20		**<0.001**	**<0.001**
Svecica	0.76	0.56	0.53		**<0.001**
Volgae	0.70	0.36	0.34	0.26	

Significant results (after Bonferroni corrections) highlighted in bold

(mean for all passerines, 27.1 % of nests, 14.9 % of nestlings; Griffith et al. 2002). Given the lack of data on EPY levels and other ecologically relevant variables (e.g. population density, breeding synchrony) for several of the subspecies, it is currently not possible to evaluate whether the different bluethroat subspecies are subjected to different postcopulatory sexual selection pressures. Nonetheless, it seems clear that the different populations have diverged rapidly in sperm length under a general scenario of sperm competition.

Taxonomic implications

Our results, both in terms of mtDNA and sperm morphology variation, are consistent with the findings by Johnsen et al. (2006) showing significant differentiation between the four bluethroat taxa, *svecica*, *namnetum*, *cyanecula* and *azuricollis*, thus supporting their status as independently evolving entities or taxa. This contrasts with the results of Zink et al. (2003), who found no support for subspecies using the mitochondrial control region and cytochrome b. It should be noted that we intentionally selected the most distinct subspecies, both based on microsatellite and phenotypic divergence (Johnsen et al. 2006) and hence the larger degree of population differentiation in mtDNA (F_{ST}= 0.61) compared to Zink et al. (2003) (F_{ST}=0.29) was to be expected. There are also similarities between Zink et al. (2003) and our study: both studies found some support for one northern and one southern group, and mitochondrial nucleotide diversity was equivalent in both studies (i.e. 0.0023). The subspecies *azuricollis*, was described by Mayaud (1958), and was only recently recognised in The Clements Checklist (Clements et al. 2012), whereas the IOC World Bird List (Gill and Donsker 2012) and leading bird handbooks (Cramp 1988; del Hoyo et al. 2006) place the Iberian bluethroats in the subspecies *cyanecula*. Combined with the results of Johnsen et al. (2006), our data strongly support *azuricollis* as a separate subspecies as they are highly divergent in sperm morphology and constitute a monophyletic clade in the mtDNA tree. In contrast, this study shows that the *volgae* subspecies does not fall into one group, but that individuals from this (putative) subspecies are distributed across both mtDNA and nuclear gene trees. These findings, combined with the fact that the sperm measurements of *volgae* males fall between those of *svecica* and *cyanecula* males, and that males in our study population show a mixture of ornamental spot colouration (chestnut, white and a chestnut/white mix; E. Matsyna, unpublished data), render the status of *volgae* as a separate subspecies questionable.

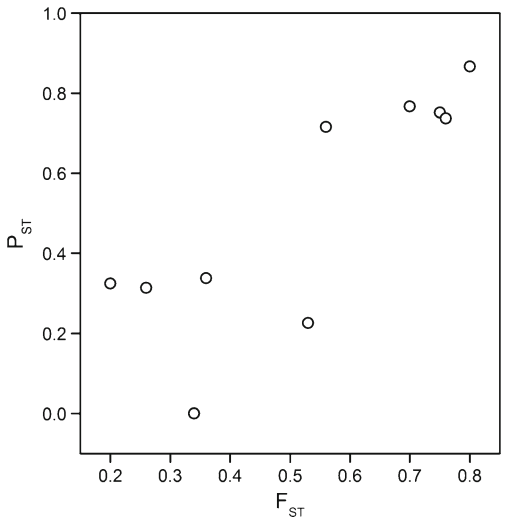

Fig. 3 Plot illustrating pair-wise correlation between the genetic divergence (F_{ST}) in mtDNA and total sperm length divergence (P_{ST}) among bluethroat subspecies. See Results for test statistics

Sperm divergence and speciation

Given the apparently rapid divergence of sperm morphology in bluethroats and some other taxa and the comparative evidence that sperm competition leads to both stabilizing and directional selection on sperm morphology, a plausible hypothesis is that sperm competition increases the likelihood of sperm divergence in allopatric populations, which in turn may result in reproductive isolation upon secondary contact and, ultimately, speciation (Howard et al. 2009; Pitnick et al. 2009). If so, one would predict that species with high sperm competition should show higher levels of sperm divergence between allopatric populations than those with low sperm competition, and that speciation should be more rapid in species with high sperm competition than in

Table 4 Summary of minimum, maximum and relative difference in sperm length between populations/subspecies of passerine birds

Common name	Scientific name	Min (μm)	Max (μm)	Number of populations	Significant differentiation in sperm?	Relative difference (%)[a]	Source
Pied flycatcher	*Ficedula hypoleuca*	102.9	104.2	3	No	1.3	Lifjeld et al. (2012)
Red-winged blackbird	*Agelaius phoeniceus*	140.0	146.0	17	Yes	2.9	Lüpold et al. (2011)
Barn swallow	*Hirundo rustica*	87.9	91.2	7	Yes	3.7	Laskemoen et al. (2013)
Coal tit	*Periparus ater*	91.7	94.6	2	Yes	3.1	Schmoll and Kleven (2011)
Common redstart	*Phoenicurus phoenicurus*	163.6	164.1	4	No	0.3	Hogner et al. (2012)
Bluethroat	*Luscinia svecica*	200.6	225.2	5	Yes	11.6	This study

[a] Standardized difference in sperm length ((max−min)/mean)

those with low sperm competition. While the last prediction is difficult to evaluate at present due to lack of data in the literature, the first prediction is supported by available data on within-species sperm divergence in passerines, although the data points are few (Table 4): the two species with small and non-significant sperm divergence both have low levels of sperm competition (pied flycatcher: Lifjeld et al. 1991, 15 % of nests, 4 % of nestlings; common redstart: 11 % of nests, 2 % of nestlings; Kleven et al. 2007) compared to the four species with significant sperm divergence (% nests, range, 48–72; % nestlings, range, 26–42) (Weatherhead and Boag 1995; Johnsen and Lifjeld 2003; Kleven et al. 2005; Schmoll et al. 2005). The hypothesis that sperm competition promotes speciation through its effects on sperm divergence clearly warrants further investigation.

In conclusion, high divergence in sperm morphology combined with low genetic divergence suggests that sperm morphology has evolved rapidly in the bluethroat subspecies complex. While the relative importance of selection (e.g. via sperm competition) and drift is unknown, we suggest that selection is likely to have played an important role in driving sperm evolution because of the relatively short time span over which change has occurred. Moreover, our findings suggest that sperm divergence may play an important role in the early stages of the speciation process. Divergences in both primary (this study) and secondary sexual characters (song: Turcokova et al. 2010; Turcokova et al. 2011; plumage: Johnsen et al. 2006) among several distinct bluethroats subspecies (Johnsen et al. 2006), suggests a role for both precopulatory and postcopulatory

Table 5 Comparison of differentiation in total sperm length between four species pairs of passerines

Common name	Scientific name	Total length (μm)	SD (μm)	No.	Difference (μm)[a]	Relative difference (%)[b]	Source	COI divergence (%)[c]
Black redstart	*Phoenicurus ochruros*	180.8	3.5	5			Laskemoen, Albrecht, Lifjeld unpublished data	
Common redstart	*Phoenicurus phoenicurus*	163.7	5.8	75	17.1	9.9	Hogner et al. (2012)	7.5
Collared flycatcher	*Ficedula albicollis*	101.2	2.4	14			Laskemoen, Albrecht, Lifjeld unpublished data	
Pied flycatcher	*Ficedula hypoleuca*	103.5	2.8	80	2.3	2.2	Lifjeld et al. (2012)	2.1
Azores bullfinch	*Pyrrhula murina*	45.6	4.3	11			Lifjeld et al. (2013)	
European bullfinch	*Pyrrhula pyrrhula*	46.3	4.3	13	0.7	1.5	Lifjeld et al. (2013)	1.0
House sparrow	*Passer domesticus*	99.7	3.2	27			Laskemoen, Sætre, Johnsen, Lifjeld unpublished data	
Spanish sparrow	*Passer hispaniolensis*	100.0	2.4	16	0.3	0.3	Laskemoen, Sætre, Johnsen, Lifjeld unpublished data	2.8

[a] Absolute difference in mean total sperm length

[b] Standardized difference in sperm length ((max−min)/mean)

[c] Johnsen, Lifjeld and Hogner unpublished data

sexual selection in the diversification of this subspecies complex.

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Ethical standards All authors declare that the present study complies with the current laws and ethical standards of animal research in Czech Republic, France, Germany, Norway, Poland, Russia and Spain.

Conflict of interest The authors declare that they have no conflict of interest.

Genbank accession numbers VLDLR7: KC595671–KC595724
BRM15: KC595725–K C595778
Control region: KC595779–KC595862
COI: KC789558–KC789641

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