



Song divergence between subspecies of reed bunting is more pronounced in singing styles under sexual selection



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Song divergence between populations of a species can lead to reproductive isolation and speciation. However, birds may have different singing styles used in distinct social contexts, and songs of each style may change at different rates over time and space. Here, we tested whether song divergence between subspecies of reed bunting, *Emberiza schoeniclus*, differs with singing style, by comparing song traits of its three singing styles among three subspecies breeding in northern and western Europe. We show that the two singing styles under sexual selection (dawn and fast songs, related to obtaining extrapair and social mates, respectively) diverged significantly more than the slow songs (used as an all-clear signal to nest-attending social females). Multiple song traits differed significantly between the subspecies in all singing styles, with *E. s. lusitanica* generally being intermediate between *E. s. schoeniclus* and *E. s. witherbyi*, and the pattern of song complexity opposing the expected latitudinal gradient (of increasing complexity with increasing latitude). Cluster analyses of populations indicate that sexually selected singing styles are better for discriminating subspecies, describing a scenario of a major split in song features between the migratory, northern *E. s. schoeniclus* and the two resident, southern subspecies, rather than a clinal variation. The greater song divergence in fast and dawn singing styles suggests that sexual selection may be playing an important role in the incipient speciation of reed buntings.

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Song is an important premating isolation barrier between passerine species (Catchpole & Slater, 2008; Marler & Slabbekoorn, 2004), and song divergence between populations of a species can lead to reproductive isolation and speciation (Martens, 1996; Price, 2008). Song divergence often follows morphological divergence (e.g. in bill or body size) resulting from a shift in ecology (e.g. in diet or feeding actions; Christensen, Kleindorfer, & Robertson, 2006; Grenier & Greenberg, 2005; Huber & Podos, 2006; Ratcliffe & Grant, 1985), or adaptation to the local acoustic environment (Cardoso & Price, 2010; Kirschel et al., 2009). However, it could also result from random cultural evolution and/or drift, especially in small, isolated populations (Lynch, 1996; Williams, Levin, Norris, Newman, & Wheelwright, 2013; Xing, Alström, Yang, & Lei, 2013).

In many birds and some marine mammals, there can be differences in the patterns of within-species spatial variation from one type of vocalization to another (Baker, 2011). Among passerines, some species have several singing styles: functionally

nonequivalent song types used in specific contexts (Bradbury & Vehrencamp, 1998; Brunner & Pasinelli, 2010; Ewin, 1976; Hasselquist & Bensch, 1991; Nemeth, 1996). The songs used in each singing style may show distinct geographical patterns (Byers, 1996; Kroodsmma, 1981) and change over time at different rates (Byers, Belinsky, & Bentley, 2010), suggesting that divergence between populations may be more pronounced in certain singing styles. Thus, as some social contexts are more relevant to reproductive isolation, certain singing styles could be of greater importance for speciation.

The reed bunting, *Emberiza schoeniclus*, is a Palearctic passerine with several subspecies described on the basis of morphology (Byers, Olsson, & Curson, 1995; Cramp & Perrins, 1994). It has three singing styles, two of which are used for female attraction: fast song, the only style used by unpaired males, has been related to the attraction of a social mate (Ewin, 1976; Nemeth, 1996), and dawn song, which is sung by paired males and is associated with obtaining extrapair paternity (Suter, Ermacora, Rielle, & Meyer, 2009). In contrast, slow songs are used by paired males apparently as an all-clear signal to their nest-attending females (Wingelmaier, Winkler, & Nemeth, 2007) and do not influence extrapair paternity or the number of fertilizations (Bouwman, Dijk, Wijmenga, & Komdeur, 2007). In

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addition, all three singing styles are probably important in the context of territory defence (Brunner & Pasinelli, 2010; Ghot, 1976). Individual males have repertoires of 10–30 different syllables that are used to build the songs of the three singing styles (Cramp & Perrins, 1994; Ewin, 1978; Suter et al., 2009). Dawn and fast singing are stereotyped styles because of the stricter rules of syllable and song arrangement (Brunner & Pasinelli, 2010; Suter et al., 2009). Concomitantly, compared to the slow singing style, the fast style has greater syllable sequence predictability and greater syllable repetition (Ewin, 1978). Song sharing, matched countersinging and local dialects have been recorded at the syllable level, but not at the song level (Ehrenguber, Pasinelli, & Egli, 2006; Ewin, 1976, 1978).

Previous work has shown that the fast songs of nonmigratory, thick-billed central and western Mediterranean birds (*E. s. intermedia* and *E. s. witherbyi*) differ from those of the largely migratory, thin-billed central European *E. s. schoeniclus* (Matessi, Dabelsteen et al., 2000, 2001). However, the divergence in other singing styles and differential divergence across styles have never been studied. The differences in fast song seem to be recognized by the birds, as males react differently to the playback of their own and foreign fast songs (Gordinho, Hasselquist, & Neto, n.d.; Matessi, Pilastro et al., 2000, 2001).

In this study, we analysed quantitative properties of songs of the three singing styles in three subspecies of reed bunting breeding in northern and western Europe: *E. s. schoeniclus* (hereafter *schoeniclus*), widespread from France and the U.K. north and eastwards to beyond the Western Palearctic; *E. s. lusitanica* (hereafter *lusitanica*), endemic to northwestern Iberia, for which there was no previous quantitative study of song (for qualitative notes see Martínez & Romay, 2012); and *E. s. witherbyi* (hereafter *witherbyi*), from eastern Iberia, Balearics, southern France and, at least formerly, North Africa and Sardinia (Atienza, 2006; Cramp & Perrins, 1994; Fig. 1). These three subspecies probably constitute the closest link between thin- and thick-billed groups of subspecies, as *lusitanica* is intermediate in bill traits and bill thickness increases towards the east among the thick-billed subspecies; it is thus particularly interesting to study speciation in this system (Neto et al., 2013).

Our aims were (1) to compare quantitative properties of songs from each of the three singing styles between the three subspecies, (2) to quantify the extent to which males can be assigned to the correct subspecies on the basis of song properties in each singing style and (3) to evaluate whether the geographical pattern of song differentiation conforms to the subspecies distributions. Considering that (1) vocal signals with different functions can exhibit different geographical patterns (Baker, 2011), (2) singing styles used for female attraction can act as a behavioural isolating mechanism (Kroodsma, 1981) and (3) stronger sexual selection results in faster evolution/divergence (Kraaijeveld, Kraaijeveld-Smit, & Maan, 2011; Seddon et al., 2013), we hypothesized that fast and dawn singing styles (those used for female attraction) diverged faster and more extensively between reed bunting subspecies than the slow style.

METHODS

Ethical Note

Field procedures used to obtain the sound recordings in which this study was based comply with the current laws of the countries where they were obtained (Portugal, Spain, France, Switzerland and Sweden).

Fieldwork

Most of the recordings were obtained using a 570 mm Telinga parabola with a Twin Science microphone and a Marantz PMD660

solid-state recorder (settings: 48 kHz sampling frequency, WAV 16-bit format) by L.G. (Fig. 1, Table 1). Additional recordings were obtained by E.M. using a similar Telinga parabola with a Stereo mic and a DA-P1 Tascam recorder (in 2004 and earlier), by the ‘Sound Approach to Birding’ team, and by Jean Roché (Fig. 1, Table 1). Further recordings were obtained at three Swiss lakes in 2006 by Patrick Brunner (part of which were published in Brunner & Pasinelli, 2010) and in 2009–2010 by Gilberto Pasinelli (Fig. 1, Table 1).

Given the rate of cultural evolution in birdsong (Byers et al., 2010), large temporal gaps in data may be a potential source of bias. However, in our data set, the overall distribution of recording years (Mann–Whitney *U* test: $P > 0.05$) and the median year of recording (median test: $P > 0.05$) did not differ between subspecies.

Sound Processing

We screened an initial pool of 239 recordings (116 *witherbyi*, 76 *lusitanica*, 47 *schoeniclus*) and visually attributed each of them to one of the three singing styles (114 fast, 80 slow, 45 dawn). Dawn song is highly distinctive because it is sung ‘continuously’, with intervals between songs of similar magnitude to intervals between syllables within songs (Brunner & Pasinelli, 2010; Suter et al., 2009). Fast and slow songs were distinguished based on the length of the intervals between the first two syllables (>0.3 s suggesting slow song) and the length of the interval between songs (if shorter than the length of songs, being suggestive of slow song; Nemeth, 1996; Brunner & Pasinelli, 2010). We only analysed songs from individual males that could be unambiguously identified based on the location of song posts and, especially, on the introductory syllables used by each bird, which are individual specific (Nemeth, 1996). We therefore excluded recordings that could possibly be of the same individuals, resulting in a sample size of 143 different males (Table 1). From each male, we analysed songs of just one singing style, digitized 100 consecutive syllables and classified them into syllable types, using syllable type catalogues for each area created for this work (following Suter et al., 2009). For dawn songs, the frequency of occurrence of syllables and short pauses between song bouts were used to identify introductory syllables (Brunner & Pasinelli, 2010). Those introductory syllables were then used to define songs within the continuous song bouts. We digitized up to 20 songs per individual male (following Brunner & Pasinelli, 2010), but in some cases this was not possible due to recording length. Overall, on average 18 ± 4 SD (range 6–20) songs per male were used.

Songs were screened and digitized in Raven Pro 1.3 (www.birds.cornell.edu/brp/raven) using default spectrogram settings, resulting in a time resolution of 2.67 ms and a frequency resolution of 187.5 Hz. In each song, seven traits were measured from the spectrogram (following Suter et al., 2009): maximum frequency (MaxF); minimum frequency (MinF); song length (SL), the duration of each song; first interval (FI), the duration of the interval between the first two syllables; song interval (SI), the interval between two consecutive songs; number of syllables (NS); and number of different syllables (NDS). Although extracting frequency measures from spectrograms can be problematic (Zollinger, Podos, Nemeth, Goller, & Brumm, 2012), it nevertheless produces good results (Cardoso & Atwell, 2012) and, importantly, does not introduce biases to the subspecies and style comparisons.

Statistical Analysis

The quantitative properties of song were compared between the three subspecies with general linear mixed models (GLMMs), in which the male identity was included as a random (subject) effect and subspecies and singing style were fixed factors (Grafen & Hails, 2002). The song variables were approximately normally distributed

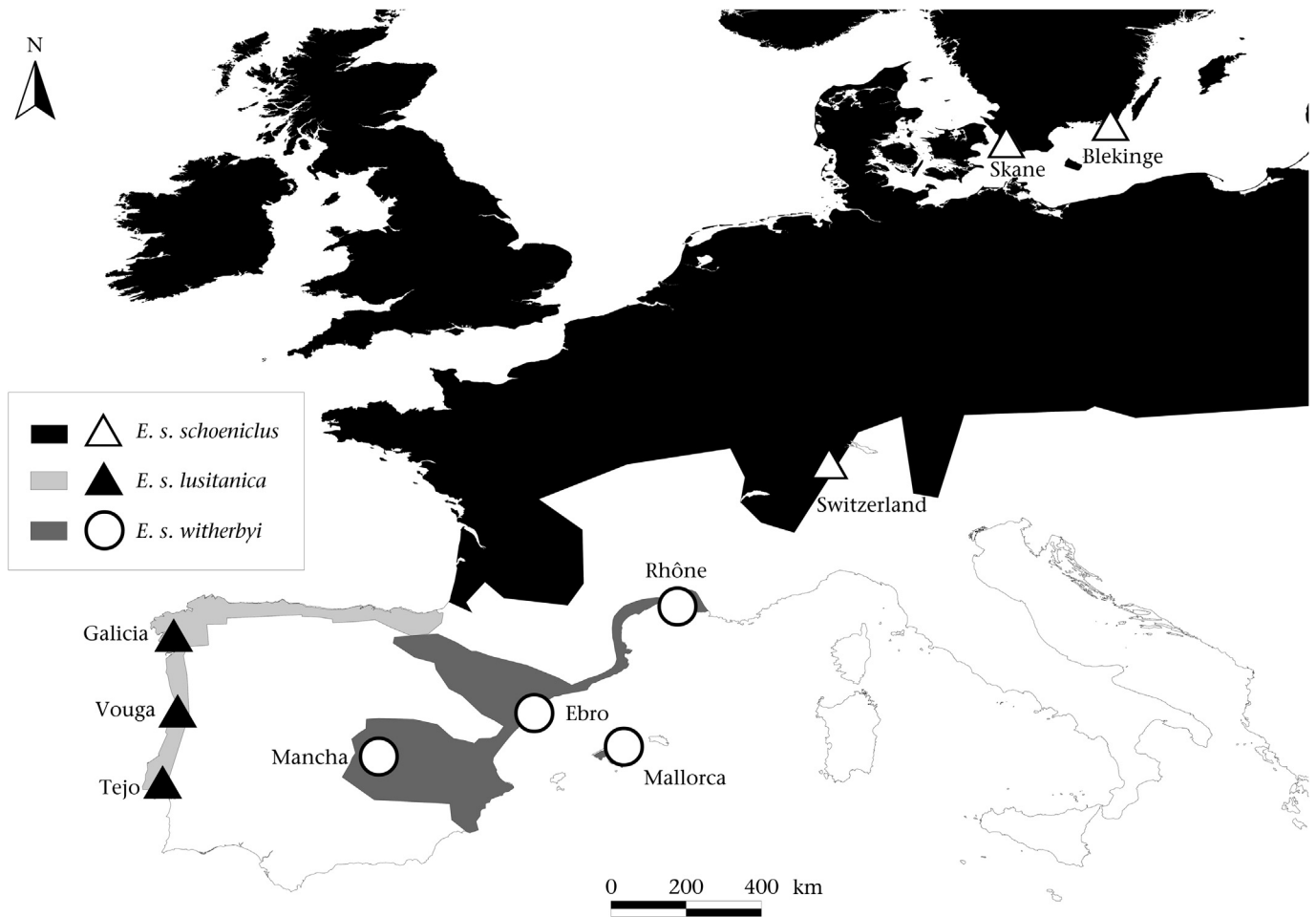


Figure 1. European breeding range of the studied subspecies of reed bunting (*Emberiza schoeniclus schoeniclus*, *E. s. witherbyi* and *E. s. lusitanica*) and positions of recording sites. Iberian range according to [Atenza \(2006\)](#), distribution in France from [Byers et al. \(1995\)](#).

within each subspecies and singing style, with the exception of SI, which was log-transformed for this analysis. In addition to the seven individual variables, we reduced the dimensionality of the data with a principal component analysis (PCA) from which two principal components (PC) with eigenvalues greater than one were extracted (explaining 67% of the variance; KMO = 0.63, Bartlett's

test of sphericity: $\chi^2_{21} = 8034$, $P < 0.001$; [Appendix Table A1](#)). The two PCs are easily interpretable ([Appendix Table A1](#)): PC1 (which explains 37% of the variance) mostly concerns differences in frequency and syllable number whereas PC2 (30% of the variance) mainly represents the differences in components of cadence (intervals between syllables and intervals between songs). Parameters

Table 1
Location, year and sample size of recordings of reed bunting song analysed in this study

Area	Coordinates	Subspecies	Dawn	Fast	Slow	All styles	Years	Recorders
Galicia	42°22'N, 8°44'W	<i>lusitanica</i>	5	1	4	10	2004	E.M.
Vouga	40°50'N, 8°37'W	<i>lusitanica</i>	10	12	12	34	2010, 2011	L.G.
Tejo	38°51'N, 8°58'W	<i>lusitanica</i>	–	5	3	8	2010, 2011	L.G.
Total		<i>lusitanica</i>	15	18	19	52		
Rhône	43°26'N, 4°31'E	<i>witherbyi</i>	–	2	2	4	1983	J.R.
Ebro	40°39'N, 0°45'E	<i>witherbyi</i>	5	18	6	29	1988, 1994, 2002, 2009, 2012	L.G., E.M., S.A.
Mallorca	39°47'N, 3°06'E	<i>witherbyi</i>	–	3	1	4	2001, 2002, 2003	E.M., S.A.
Mancha	39°31'N, 3°19'W	<i>witherbyi</i>	10	4	3	17	2004, 2011	E.M., L.G.
Total		<i>witherbyi</i>	15	27	12	54		
Switzerland	47°15'N, 8°25'E	<i>schoeniclus</i>	–	13	–	13	2006, 2009, 2010	P.B., G.P.
Skåne	55°39'N, 13°11'E	<i>schoeniclus</i>	5	2	14	21	2011, 2013	L.G., J.N.
Blekinge	56°05'N, 15°51'E	<i>schoeniclus</i>	–	1	2	3	2013	L.G., J.N.
Total		<i>schoeniclus</i>	5	16	16	37		
All range		All ssp.	35	61	47	143		

Samples are given as number of males of each subspecies (ssp.) for each singing style (fast, slow or dawn). Only one singing style was analysed for each male (i.e. there is no repetition of individuals across singing styles). Coordinates are in latitude/longitude, WGS84 datum. Recordings by the authors, Patrick Brunner (PB), Gilberto Pasinelli (GP), Jean Roché (JR) and the Sound Approach to Birding (SA).

were estimated using restricted maximum likelihood (REML; Type III) in SPSS 21.0 (IBM Corporation, Armonk, NY, U.S.A.) using default parameters. The magnitude of subspecies divergence for each singing style was quantified using estimated marginal means (EMM) for the interaction between style and subspecies, and compared with log-likelihood tests.

To quantify the extent to which males could be assigned to the correct subspecies on the basis of song properties in each singing style, we applied the discriminant function analysis (DFA) on the average values of the song variables for each of the 143 males. A tolerance test was performed to determine whether there were any highly correlated variables (Tabachnick & Fidell, 2007). As none of the seven variables were highly correlated (all tolerance values >0.10), we subsequently performed a DFA entering all raw song variables together as independent and subspecies as grouping variable. Post hoc probabilities of correct assignment in DFA were cross-validated by the jackknife method, in which each observation was omitted one at a time, the classification function recalculated with the remaining data, and the omitted observation reclassified (Baker, 2011). In all DFAs, we used *F* tests (Wilk's lambda) to examine whether the overall discriminant models were significant (e.g. Tobias & Seddon, 2009).

Finally, we built hierarchical cluster analysis dendrograms to evaluate the extent to which song traits group the sampling areas into subspecies within each singing style. For that, we used all raw variables in a database with average values for each area, selecting the between-groups linkage cluster method and squared Euclidean distance interval measure.

RESULTS

Acoustic Divergence

According to the GLMMs, all variables were significantly different between subspecies and between singing styles, and significant interactions between subspecies and style were detected for FI, SL and NDS, whereas PC1 (representing variation in frequency and syllable numbers) and NS closely approached the significance threshold (Table 2, Fig. 2). Estimated marginal means and their 95% confidence limits indicate that the divergence between subspecies across singing styles was generally greater for dawn and fast songs than for slow songs (Appendix Table A2, Fig. 2). For instance, the EMM of PC1 was 1.342, 1.175 and 0.520 for dawn, fast and slow songs, respectively. This is further supported by subspecies comparisons within each singing style, which resulted in greater overall significances and subspecies divergence in fast and dawn songs, relative to slow songs (Appendix Table A2, Table 3). Indeed, when we looked at the overall pattern of the nine investigated song parameters (see Table 3), parameters for which at least two of the three subspecies pairwise comparisons differed significantly were much more frequent in the fast (seven of nine) and the dawn (five of nine) singing styles than in the slow (one of nine) singing style. This supports the conclusion that the singing styles under stronger sexual selection (fast and dawn song) have diverged more the singing style used in another context (slow song).

Subspecies Assignment

The DFAs were significant for dawn (Wilk's $\lambda = 0.132$, $\chi^2_{14} = 58.702$, $P < 0.001$), fast (Wilk's $\lambda = 0.319$, $\chi^2_{14} = 62.915$, $P < 0.001$) and slow singing styles (Wilk's $\lambda = 0.433$, $\chi^2_{14} = 34.326$, $P = 0.002$). The three functions showed slightly different abilities to correctly classify cases, with 77.1% for dawn style (80.0% *lusitanica*, 100.0% *schoeniclus*, 66.7% *witherbyi*), 75.4% for fast style (61.1% *lusitanica*, 87.5% *schoeniclus*, 77.8% *witherbyi*) and 74.5% for slow

Table 2

General linear mixed models comparing subspecies and singing styles for each song trait

Dependent variable	Effect	df1	df2	<i>F</i> test	<i>P</i>
PC1	Intercept	1	132.812	2.937	0.089
	Style	2	133.047	22.376	<0.001
	ssp.	2	132.814	24.130	<0.001
	Style* ssp	4	133.086	2.324	0.060
PC2	Intercept	1	132.517	16.616	<0.001
	Style	2	132.738	340.381	<0.001
	ssp.	2	132.519	3.979	0.021
	Style* ssp.	4	132.774	1.939	0.108
Minimum frequency	Intercept	1	132.657	11666.604	<0.001
	Style	2	132.856	3.344	0.038
	ssp.	2	132.637	13.784	<0.001
	Style* ssp	4	132.881	1.001	0.410
Maximum frequency	Intercept	1	134.471	31526.241	<0.001
	Style	2	134.649	7.219	0.001
	ssp.	2	134.452	24.206	<0.001
	Style* ssp	4	134.670	1.942	0.107
Song length	Intercept	1	132.015	1908.479	<0.001
	Style	2	132.374	63.539	<0.001
	ssp.	2	131.980	5.406	0.006
	Style* ssp.	4	132.421	2.750	0.031
First interval	Intercept	1	132.467	1354.372	<0.001
	Style	2	132.729	140.373	<0.001
	ssp.	2	132.440	9.656	<0.001
	Style* ssp.	4	132.762	4.225	0.003
Log ₁₀ (song interval)	Intercept	1	132.456	480.511	<0.001
	Style	2	132.652	348.310	<0.001
	ssp.	2	132.458	2.115	0.125
	Style* ssp.	4	132.683	.351	0.843
Number of syllables	Intercept	1	133.468	1328.939	<0.001
	Style	2	133.610	38.683	<0.001
	ssp.	2	133.454	4.241	0.016
	Style* ssp	4	133.627	2.381	0.055
Number of different syllables	Intercept	1	132.149	1715.307	<0.001
	Style	2	132.324	18.294	<0.001
	ssp.	2	132.131	20.758	<0.001
	Style* ssp.	4	132.345	3.335	0.012

Male was included as a random effect, dependent variables were subspecies (ssp.) singing style (style) and their interaction, and the dependent variables were the two principal components (PC1 and PC2) and the seven variables measured from the songs. *df1* and *df2* refer to the numerator and denominator degrees of freedom, respectively. Significant *P* values are shown in bold.

style (78.9% *lusitanica*, 75.0% *schoeniclus*, 66.7% *witherbyi*). Subspecies diagnosis using song traits is presented in Appendix Table A3.

Geographical Structure

The hierarchical cluster dendrograms for dawn and fast songs clearly segregated the populations of *schoeniclus* from those of the other two subspecies (Fig. 3a, b). In addition, the dendrogram for dawn style agreed with the separation of Iberian populations into two subspecies (Fig. 1a; Atienza, 2006). For fast style, the separation between *lusitanica* and *witherbyi* was less clear, as *witherbyi* populations from Mallorca and Ebro clustered within the *lusitanica* clade (Fig. 3b). As expected from the weaker subspecies discrimination based on slow songs (Table 3 and DFAs results above), the dendrogram for slow songs did not segregate the populations of the three subspecies (Fig. 3c).

DISCUSSION

Geographical variation in bird vocalizations, such as the large divergence we describe in reed buntings, can arise through several mechanisms, and may have profound consequences for the segregation of populations within a species. In passerines, vocalizations

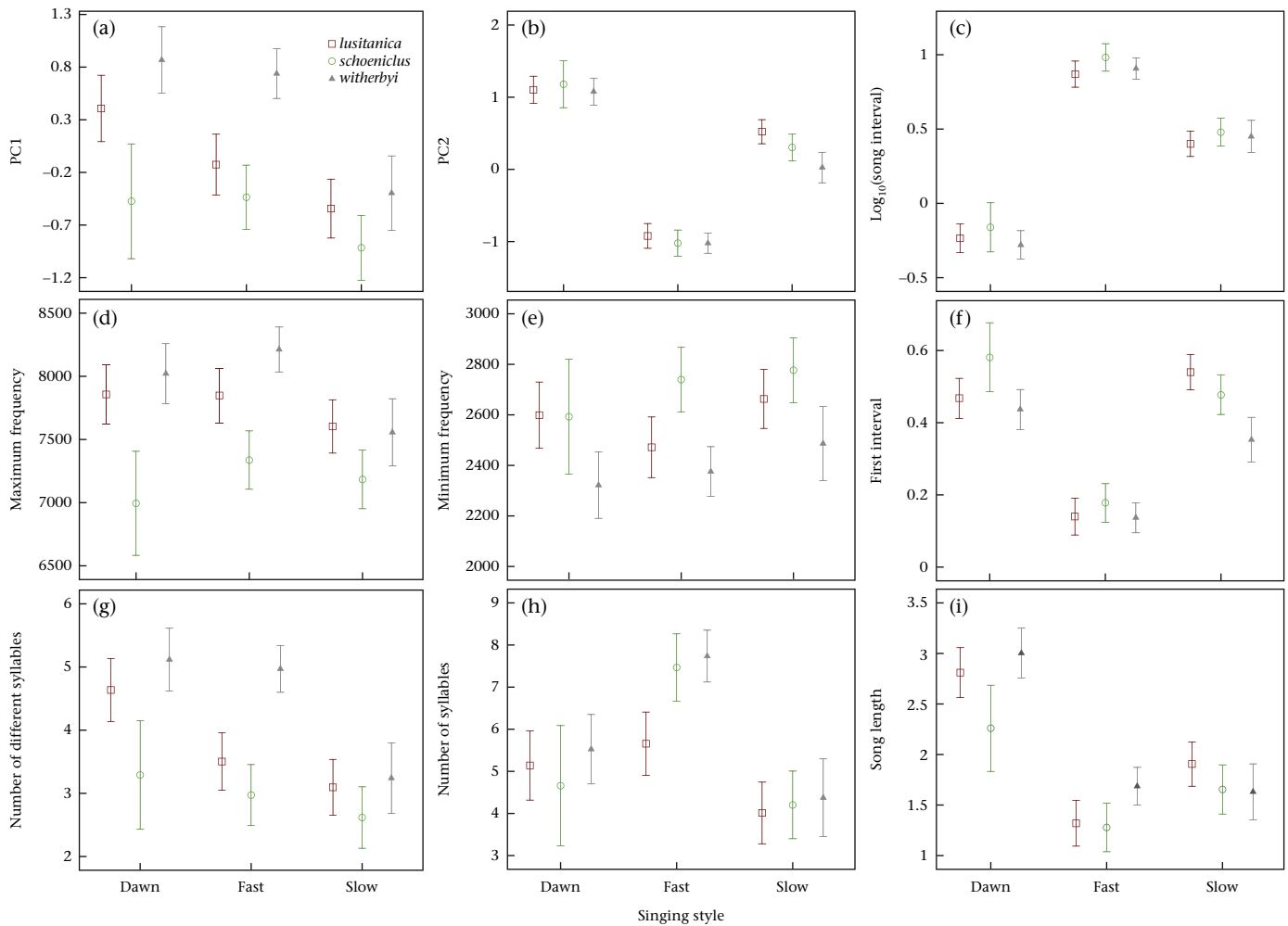


Figure 2. Estimated marginal means and 95% confidence intervals of (a) PC1 (describing differences in frequency and syllable numbers), (b) PC2 (describing differences in intervals between syllables and intervals between songs), (c) $\log_{10}(\text{song interval})$, (d) maximum frequency, (e) minimum frequency, (f) first interval, (g) number of different syllables, (h) number of syllables and (i) song length per subspecies and singing style, derived from general linear mixed models. For statistics see Table 2.

are, to a large extent, learnt from parents and neighbours, but the learning process is imperfect and copying errors are common. In such cases, the appearance of geographical variation in vocalizations depends on the timing of the learning period and on the dispersal of the juveniles (Podos & Warren, 2007). However, part of the vocalizations, in particular their general structure (including many of the traits studied here), do not depend on social experience and are subject to evolutionary forces such as selection and drift (Podos, Huber, & Taft, 2004). In addition, most bird species often have several kinds of vocalizations that are used in different contexts, and hence may be subject to different evolutionary forces. Thus, the greater divergence of structural traits in dawn and fast singing styles that we describe in reed buntings strongly suggests that sexual selection was an important cause of acoustic divergence in this species. Vocalizations, in particular songs, are important for sexual selection and species recognition among birds (Price, 2008). Consequently, geographical differences in such vocalizations among populations may allow individuals to distinguish local birds from immigrants, for example to avoid inbreeding or mating with maladapted individuals (Bensch, Hasselquist, & von Schantz, 1994; Edmands, 2007; Gordinho et al., n.d.; Hansson, Bensch, & Hasselquist, 2004; Keller & Waller, 2002; Marr, Keller, & Arcese, 2002), being highly relevant in the context of the incipient speciation occurring in reed buntings.

Song Divergence in Reed Buntings

In this study, we compared quantitative properties of three singing styles with known functions among populations of three subspecies of reed bunting, which differ morphologically in traits that seem to confer local adaptation (e.g. bill size and shape; Byers et al., 1995; Neto et al., 2013, 2015). Although subspecies divergence in relation to the singing style varied depending on the particular trait that was measured, it is clear that, overall, dawn and fast songs diverged more extensively than slow songs, as is reflected by the first principal component (PC1) of variation (see Fig. 2). The number of different syllables contributed most to this pattern, whereas song length and the total number of syllables were particularly divergent in dawn and fast songs, respectively. On the other hand, the first interval, which also showed a significant interaction between subspecies and singing style, seems to have contributed little to the overall pattern, having only a slightly greater divergence in dawn and slow songs, and produced a different relative positioning of the subspecies (see Fig. 2). Slow songs are less differentiated, but significant differences between the subspecies were nevertheless detected in three of the seven original variables (contrasting with six of seven variables with significant differences for both dawn and fast songs; see Table 3). The subspecies *witherbyi* stands out as being particularly divergent from the other two subspecies in most

Table 3
General linear mixed models comparing subspecies within each singing style

	Dawn		Fast		Slow	
	F test	P	F test	P	F test	P
PC1	12.42	<0.001	3.56	0.037	15.92	<0.001
	sch ≠ wit; lus ≠ wit; sch ≠ lus		sch ≠ wit; lus ≠ wit		sch ≠ wit	
PC2	0.65	0.527	3.42	0.042	5.35	0.007
	None		sch ≠ wit; sch ≠ lus		lus ≠ wit	
First interval	6.47	0.004	5.11	0.01	2.01	0.144
	sch ≠ wit; sch ≠ lus		none		sch ≠ wit	
Log ₁₀ (song interval)	1.21	0.313	0.42	0.657	2.86	0.066
	None		sch ≠ lus		None	
Maximum frequency	13.70	<0.001	3.02	0.059	18.73	<0.001
	sch ≠ wit; sch ≠ lus		sch ≠ wit; lus ≠ wit; sch ≠ lus		sch ≠ lus	
Minimum frequency	4.54	0.018	6.34	0.004	8.51	0.001
	lus ≠ wit		sch ≠ wit; sch ≠ lus		sch ≠ wit; lus ≠ wit	
Song length	4.25	0.024	1.07	0.351	9.99	<0.001
	sch ≠ wit; sch ≠ lus		sch ≠ wit; lus ≠ wit		None	
Number of syllables	2.98	0.066	0.28	0.755	5.71	0.005
	sch ≠ wit		sch ≠ lus; lus ≠ wit		None	
Number of different syllables	11.56	<0.001	2.78	0.073	16.01	<0.001
	sch ≠ wit; sch ≠ lus		sch ≠ wit; lus ≠ wit		None	

The table shows the *F* tests with associated probability (*P*), as well as the subspecies pairs that differed significantly in their estimated marginal means according to log-likelihood tests. lus = *lusitânica*, sch = *schoeniclus*, wit = *witherbyi*. Significant results are shown in bold.

variables, especially in fast songs, as they produce the longest and more varied songs; whereas *lusitânica* is intermediate in most acoustic variables relative to the other two subspecies.

Reed buntings can increase their repertoire, and thus song diversity, between successive years (Suter et al., 2009), but it is unlikely that variation in the proportion of older males affected our results. Plastic changes in some song traits due to seasonal or environmental effects (e.g. increased minimum frequency in response to anthropogenic noise; Gross, Pasinelli, & Kunc, 2010) are also unlikely to have affected our results because birds were sampled only during the breeding season and all study sites were in natural habitats, distant from loud sources of anthropogenic noise. Additionally, the rather small differences in timing of sampling of each population should not have affected our results because the breeding season is very long, and the singing style potentially showing a greater seasonal variation (fast; for which we have the largest sample size) is actually used later on by mated males in secondary territories (where they try to attract additional social females; L.O.G. & J.M.N., personal observation).

The substantial song divergence found among subspecies (as well as the morphological differences) must have evolved rapidly, as indicated by the star shape of the haplotype network presented by Kvist et al. (2011) supporting a recent expansion from a single glacial refugium of the reed buntings occurring in Europe. Interestingly, our findings oppose the expected latitudinal gradient resulting from higher rates of evolution of syllable diversity and song length in north European passerines than in Mediterranean passerines (Cardoso, Hu, & Mota, 2012; Weir & Wheatcroft, 2011). Bill morphology has also been shown to affect song output (Christensen et al., 2006; Huber & Podos, 2006; Podos, 1997; but see Grant, B.R. & Grant, 2002; Grant, P.R. & Grant, 2002; Slabbekoorn & Smith, 2000), so this trait could potentially explain the differences found between subspecies, especially the generally higher song divergence of *witherbyi*, which has the most differentiated bill (Neto et al., 2013). In our study, we found that the thick-billed subspecies *witherbyi* sang songs with the lowest minimum frequency, thin-billed *schoeniclus* sang with the highest minimum frequency, and the intermediate-billed subspecies *lusitânica* used intermediate frequencies. Similar results have been described for Darwin's finches (Huber & Podos, 2006; Podos, 2001). However, *witherbyi* songs also have a higher maximum frequency than those of *schoeniclus* and, consequently, for dawn and fast

styles, a wider frequency bandwidth that does not fit with the expectations from the differences in bill morphology (although this expectation is specific to trilled vocalizations, which are common in this species but were not studied here in detail). In swamp sparrows, *Melospiza georgiana*, it has been suggested that males with larger bills and lower 'song performance' (ability to produce rapid, broadband trills) may compensate for that by increasing song complexity (Ballentine, 2006; Cardoso & Hu, 2011). Because higher song complexity (larger number of different syllables) was also found in the 'thick-billed' *witherbyi* and was positively correlated with frequency bandwidth (Pearson correlation: $r = 0.470$), it is possible that this subspecies has been selected for higher song complexity in a similar way. A detailed study of song performance, comparing trill rates and frequency bandwidth in the three subspecies, is needed to clarify this issue. In general, evolutionary changes in the quantity of syllables are thought to be due to changes in pressure from sexual selection (Baker, 1996; Lynch, 1996; Read & Weary, 1992). Perhaps the larger number of syllables found in *witherbyi* is the result of a shift in female preferences from vocal performance to song complexity, as suggested for coastal swamp sparrows (Ballentine, 2006). In a study restricted to the fast singing style, Matessi, Dabelsteen et al. (2000) also found higher syllabic complexity in thick-billed subspecies (*intermedia* and *witherbyi*) and a strong tendency for higher minimum frequency in the thin-billed *schoeniclus*. The fact that dawn and fast songs are relatively complex agrees with these styles being under sexual selection, because the production of complex songs has been associated with female mate choice in many previous studies (e.g. Brunner & Pasinelli, 2010; Catchpole & Leisler, 1996; Forstmeier & Balsby, 2002; Hasselquist, Bensch, & von Schantz, 1996; Neubauer, 1999).

Nevertheless, the causes of the differential song divergence relative to singing style in reed buntings are unclear. The relative importance of imitation and improvisation could vary with singing style, and this cultural evolution may be dependent on whether the particular singing style is constrained by selection. Indeed, the song of two species of *Cistothorus* wrens, which differ in the relative importance of imitation and improvisation at the syllable level, generated different geographical patterns of song variation (Kroodsma & Verner, 1978). In addition, a population of chestnut-sided warblers, *Dendroica pensylvanica*, studied across time showed a high rate of cultural evolution in the singing style

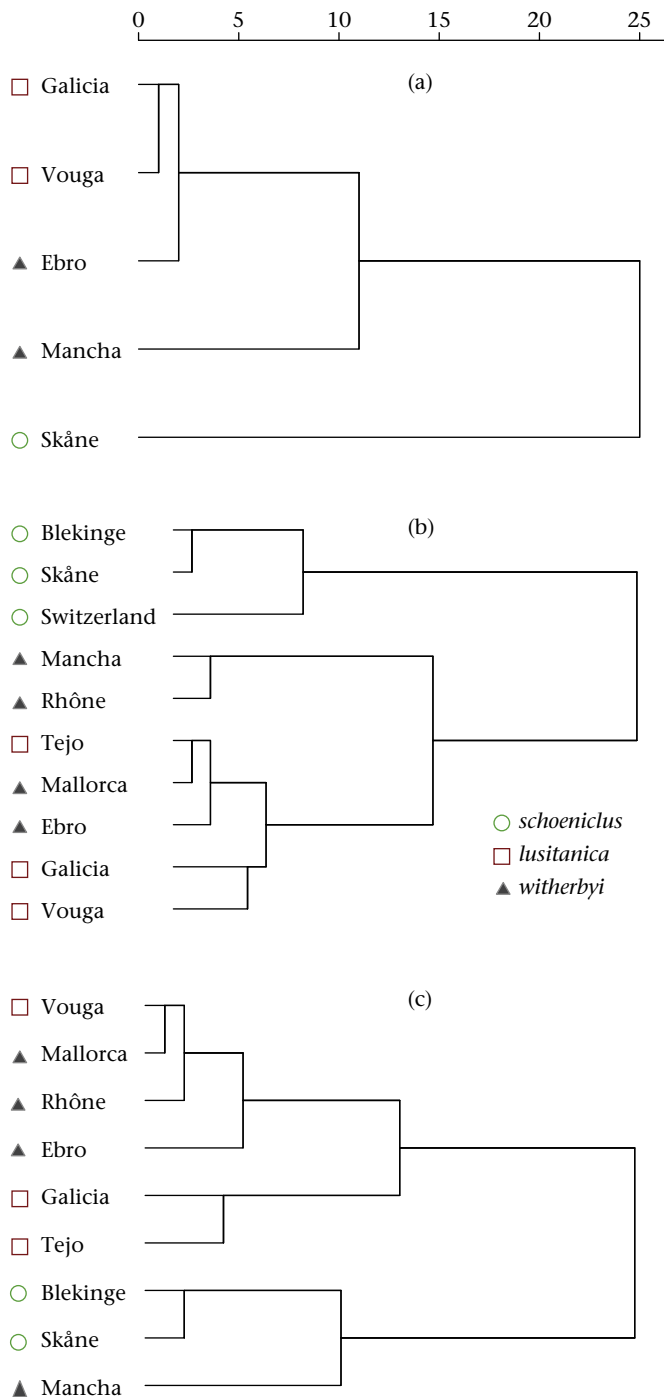


Figure 3. Dendrograms resulting from hierarchical cluster analysis using the between-groups linkage cluster method and squared Euclidean distances to group the sites where (a) dawn, (b) fast and (c) slow songs were recorded, according to the seven raw song variables. White circles = *Emberiza schoeniclus witherbyi*, black triangles = *E. s. lusitanica*, white triangles = *E. s. schoeniclus*. Dawn songs came from only five sites because this singing style was not described until 2009 and we used only fast songs from Switzerland to even the sample size across styles.

unconstrained by sexual selection, and a low rate in a sexually selected style (Byers et al., 2010). Other Nearctic warblers (e.g. Bay, 1999; Janes & Ryker, 2006, 2011; Moldenhauer, 1992), however, fit our prediction of greater divergence in singing styles under sexual selection. Overall, this prediction does not seem to hold for species in which such singing styles have a highly variable syntax (Martens, 1996; Salomon & Hemim, 1992) or contain syllables not used for

intrasexual countersinging (Byers, 1996; Byers et al., 2010; Kroodsmas, 1981; Kroodsmas, 1996; Lein, 2008; Nelson, 1992; Nelson & Croner, 1991; Soha, Lohr, & Gill, 2009). Different types of sound analysis can generate different patterns (e.g. Baker & Logue, 2003) and distinct methods have been used in the above-mentioned studies, so comparisons with our study may have limited value.

Geographical Congruence Between Morphology and Song

In the hierarchical cluster dendrogram for dawn songs (Fig. 3a) the grouping can be explained by either geographical or subspecies divergence. However, the dendrogram for fast singing style shows that songs from birds in central Iberia are more similar to songs of geographically distant birds from southern France than to songs of birds from the nearby western Iberia (Fig. 3b). This suggests an abrupt acoustic change, rather than clinal variation, although the location of sampling sites is not ideal to distinguish these patterns. The island population of *witherbyi* clustered within *lusitanica*, possibly due to undersampling or to the effects of insularity (Baker, Baker, & Baker, 2001; Griffith, 2000; Marler & Slabbekoorn, 2004). The fact that the well-sampled Ebro population of *witherbyi* also clustered within *lusitanica* is harder to explain, but may be due to side-effects of the small and undersampled *lusitanica* population of Tejo on clustering. Like geographical distance, habitat connectivity is probably not a confounding parameter, because reed beds are a rare and highly fragmented habitat in southwestern Europe, so connectivity between all 10 populations sampled is low to non-existent (see also Gammon, Baker, Tipton, & Yasukawa, 2005; Laiolo, Vögeli, Serrano, & Tella, 2008). However, many *schoeniclus* migrate to spend the winter in the Mediterranean area, using many reed bed patches during their annual cycle and co-occurring with *lusitanica*, *witherbyi* and other subspecies at their wintering quarters (Byers et al., 1995; Cramp & Perrins, 1994; Neto et al., 2013). Hence, *schoeniclus* males could potentially learn the songs of *lusitanica* and *witherbyi* among others, especially the fast songs, which start to be used by local residents before *schoeniclus* departs to the breeding areas (J.M. Neto, personal observation). Nevertheless, large differences between the subspecies were found, especially between *schoeniclus* and the two resident subspecies (Fig. 3b).

Evolutionary Implications

Given that reed bunting subspecies seem to be at an incipient stage of speciation (Matessi, Pilastro et al., 2000; Neto et al., 2013, 2015), the finding of greater divergence in sexually selected singing styles is interesting, suggesting that sexual selection may be playing an important role in this process (probably in conjunction with natural selection). Moreover, some of the song traits measured in this study do not seem to rely on social experience for development (Ewin, 1978; Glutz von Blotzheim & Bauer, 1997; Stewart, 1955; Thorpe, 1964), and are likely to be targets of selection potentially leading to an evolutionary response (i.e. of song traits within populations/subspecies). Several studies suggest that sexual selection promotes the evolution of reproductive isolation and two recent meta-analyses found small but significant overall trends (Kraaijeveld et al., 2011; Seddon et al., 2013). For instance in antbirds (Thamnophilidae), a positive relationship was found between species diversity and the intensity of sexual selection, measured by the production of lower pitched and more complex songs (Seddon, Merrill, & Tobias, 2008). When divergence between populations in singing styles related to female attraction is greater than in other styles, as shown in our study and for some Nearctic warblers (Bay, 1999; Janes & Ryker, 2006, 2011; Moldenhauer, 1992), there seems to be an enhanced potential for premating reproductive isolation to

evolve. Indeed, population differences in sexually selected singing styles seem to be associated with different signal perceptions in reed buntings, and may be causing some level of reproductive isolation between the subspecies (Gordinho et al., n.d.).

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APPENDIX

Table A1

Factor loadings on principal components (PC) for acoustic measurements

	PC1	PC2
Eigenvalue	2.601	2.112
% Variance	37.159	30.166
Minimum frequency	−0.577	0.080
Maximum frequency	0.678	−0.182
Song length	0.678	0.632
First interval	−0.188	0.862
Number of syllables	0.705	−0.397
Number of different syllables	0.895	0.083
Log ₁₀ (song interval)	−0.124	−0.874

Acoustic traits were taken from song spectrograms of *Emberiza schoeniclus lusitanica*, *E. s. schoeniclus*, and *E. s. witherbyi* (143 males, up to 20 songs per male).

Table A2

Estimated marginal means (EMM) of the GLMMs comparing subspecies and singing styles for each song trait

Variable	Style	Subspecies	EMM	SE	df	95% Confidence interval	
						Lower bound	Upper bound
PC1	Dawn	<i>lusitanica</i>	0.407	0.159	132.279	0.092	0.723
		<i>schoeniclus</i>	−0.474	0.276	131.019	−1.019	0.071
		<i>witherbyi</i>	0.868	0.159	131.597	0.553	1.183
	Fast	<i>lusitanica</i>	−0.126	0.146	133.287	−0.414	0.163
		<i>schoeniclus</i>	−0.436	0.155	133.943	−0.743	−0.13
		<i>witherbyi</i>	0.739	0.119	134.569	0.503	0.976
Slow	<i>lusitanica</i>	−0.544	0.142	131.851	−0.824	−0.264	
	<i>schoeniclus</i>	−0.916	0.156	137.318	−1.224	−0.608	
	<i>witherbyi</i>	−0.396	0.179	133.844	−0.75	−0.043	
PC2	Dawn	<i>lusitanica</i>	1.102	0.095	132.014	0.914	1.291
		<i>schoeniclus</i>	1.18	0.164	130.835	0.855	1.505
		<i>witherbyi</i>	1.076	0.095	131.376	0.888	1.264
	Fast	<i>lusitanica</i>	−0.922	0.087	132.956	−1.094	−0.749
		<i>schoeniclus</i>	−1.022	0.092	133.574	−1.205	−0.839
		<i>witherbyi</i>	−1.022	0.071	134.161	−1.162	−0.881
	Slow	<i>lusitanica</i>	0.523	0.084	131.613	0.356	0.69
		<i>schoeniclus</i>	0.304	0.093	136.742	0.12	0.488
		<i>witherbyi</i>	0.026	0.107	133.497	−0.185	0.237
MinF	Dawn	<i>lusitanica</i>	2598.736	66.365	132.299	2467.463	2730.01

(continued on next page)

Table A2 (continued)

Variable	Style	Subspecies	EMM	SE	df	95% Confidence interval		
						Lower bound	Upper bound	
MaxF	Fast	<i>schoeniclus</i>	2592.802	114.68	131.11	2365.94	2819.664	
		<i>witherbyi</i>	2321.613	66.268	131.559	2190.523	2452.703	
		<i>lusitanica</i>	2471.605	60.643	132.82	2351.655	2591.555	
	Slow	<i>schoeniclus</i>	2739.541	64.457	133.872	2612.055	2867.028	
		<i>witherbyi</i>	2375.585	49.596	133.655	2277.49	2473.681	
		<i>lusitanica</i>	2662.945	58.91	131.817	2546.413	2779.477	
	Dawn	<i>schoeniclus</i>	2776.751	64.862	137.066	2648.491	2905.011	
		<i>witherbyi</i>	2486.556	74.36	133.337	2339.479	2633.633	
		<i>lusitanica</i>	7856.514	120.308	134.146	7618.569	8094.46	
	Fast	<i>schoeniclus</i>	6994.513	207.956	133.089	6583.187	7405.839	
		<i>witherbyi</i>	8021.11	120.155	133.488	7783.456	8258.765	
		<i>lusitanica</i>	7848.267	109.921	134.608	7630.872	8065.661	
	Slow	<i>schoeniclus</i>	7336.409	116.805	135.552	7105.413	7567.406	
		<i>witherbyi</i>	8212.446	89.88	135.354	8034.695	8390.197	
		<i>lusitanica</i>	7603.011	106.807	133.717	7391.762	7814.26	
	SL	Dawn	<i>schoeniclus</i>	7183.558	117.45	138.41	6951.33	7415.786
			<i>witherbyi</i>	7556.62	134.767	135.088	7290.094	7823.145
			<i>lusitanica</i>	2.81	0.125	131.411	2.563	3.057
Fast		<i>schoeniclus</i>	2.26	0.215	129.166	1.833	2.686	
		<i>witherbyi</i>	3.003	0.125	130.023	2.757	3.25	
		<i>lusitanica</i>	1.319	0.114	132.437	1.093	1.545	
Slow		<i>schoeniclus</i>	1.278	0.122	134.281	1.037	1.518	
		<i>witherbyi</i>	1.686	0.094	133.949	1.501	1.872	
		<i>lusitanica</i>	1.906	0.111	130.53	1.686	2.125	
FI		Dawn	<i>schoeniclus</i>	1.654	0.123	140.053	1.411	1.898
			<i>witherbyi</i>	1.631	0.14	133.105	1.353	1.908
			<i>lusitanica</i>	0.468	0.028	132.005	0.412	0.523
	Fast	<i>schoeniclus</i>	0.581	0.048	130.41	0.486	0.677	
		<i>witherbyi</i>	0.437	0.028	131.015	0.381	0.492	
		<i>lusitanica</i>	0.14	0.026	132.717	0.089	0.191	
Slow	<i>schoeniclus</i>	0.178	0.027	134.09	0.124	0.231		
	<i>witherbyi</i>	0.137	0.021	133.82	0.095	0.178		
	<i>lusitanica</i>	0.54	0.025	131.367	0.491	0.589		
LogSI	Dawn	<i>schoeniclus</i>	0.477	0.027	138.307	0.423	0.532	
		<i>witherbyi</i>	0.353	0.031	133.325	0.291	0.415	
		<i>lusitanica</i>	-0.235	0.048	132.007	-0.331	-0.139	
	Fast	<i>schoeniclus</i>	-0.161	0.084	130.97	-0.327	0.004	
		<i>witherbyi</i>	-0.28	0.048	131.446	-0.376	-0.184	
		<i>lusitanica</i>	0.869	0.044	132.834	0.782	0.957	
	Slow	<i>schoeniclus</i>	0.982	0.047	133.385	0.889	1.075	
		<i>witherbyi</i>	0.907	0.036	133.902	0.836	0.979	
		<i>lusitanica</i>	0.4	0.043	131.652	0.315	0.486	
	NS	Dawn	<i>schoeniclus</i>	0.479	0.047	136.186	0.385	0.573
			<i>witherbyi</i>	0.45	0.054	133.341	0.343	0.558
			<i>lusitanica</i>	5.14	0.417	133.207	4.317	5.964
Fast		<i>schoeniclus</i>	4.66	0.72	132.37	3.235	6.085	
		<i>witherbyi</i>	5.525	0.416	132.685	4.701	6.348	
		<i>lusitanica</i>	5.656	0.381	133.569	4.904	6.409	
Slow	<i>schoeniclus</i>	7.466	0.404	134.325	6.666	8.265		
	<i>witherbyi</i>	7.735	0.311	134.163	7.12	8.35		
	<i>lusitanica</i>	4.012	0.37	132.865	3.28	4.744		
NDS	Dawn	<i>schoeniclus</i>	4.201	0.406	136.603	3.398	5.004	
		<i>witherbyi</i>	4.373	0.466	133.971	3.45	5.295	
		<i>lusitanica</i>	4.636	0.251	131.83	4.138	5.133	
	Fast	<i>schoeniclus</i>	3.29	0.435	130.79	2.43	4.15	
		<i>witherbyi</i>	5.117	0.251	131.182	4.621	5.614	
		<i>lusitanica</i>	3.503	0.23	132.284	3.049	3.957	
	Slow	<i>schoeniclus</i>	2.972	0.244	133.213	2.489	3.454	
		<i>witherbyi</i>	4.97	0.188	133.019	4.598	5.341	
		<i>lusitanica</i>	3.095	0.223	131.407	2.654	3.537	
	NDS	Slow	<i>schoeniclus</i>	2.617	0.245	136.025	2.132	3.102
			<i>witherbyi</i>	3.241	0.282	132.756	2.684	3.798

Results are shown for each acoustic variable, subspecies and singing style resulting from the general linear mixed models, where male identity was included as a random effect (see also Table 2). MinF = minimum frequency, MaxF = maximum frequency, SL = song length, FI = first interval, SI = song interval, NS = number of syllables; NDS = number of different syllables.

Table A3

General rules for separating typical songs of the three subspecies (ssp.) for each singing style

Style/ssp. ≠	lus ≠ sch	lus ≠ wit	Sch ≠ wit
Dawn	NS < 4 = sch, FI < 0.5 = lus	NDS & NS < 5 = lus	MaxF < 7500 = sch, NDS < 4.5 = sch
Fast	MinF > 2600 Hz = sch	NDS < 4 & NS < 7 = lus	MaxF < 7600 = sch, NDS < 4 = sch
Slow	FI < 0.5 = sch	FI > 0.5 = lus	MaxF < 7300 = sch, NDS > 3 = wit

The table shows rules based on the GLMM results summary (Table 2) and on 95% confidence intervals built for our sample (Table A2). lus = *lusitanica*, sch = *schoeniclus*, wit = *witherbyi*. For dependent variable abbreviations see Table A2. In all styles, *schoeniclus* songs can be distinguished from those of *witherbyi* by a combination of MaxF lower than 7.3 kHz and NDS lower than 3. For slow songs, FI above 0.5 s is typical of *lusitanica*, whereas FI under 0.4 s is exclusive of *witherbyi*. Finally, dawn and fast songs of *lusitanica* can be separated from those of *witherbyi* by the lower NS and NDS, and from those of *schoeniclus* by the FI (<0.5 = *lusitanica* for dawn) and MinF (>2.6 kHz = *schoeniclus* for fast). Accurate identification of less typical songs using multivariate discriminant functions is possible for fast and, especially, for dawn songs, but not for slow songs.