Ornis Fennica 94: 141–149. 2017

Evolutionary divergences in *Luscinia svecica* subspecies complex – new evidence supporting the uniqueness of the Iberian bluethroat breeding populations

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Received 12 August 2016, accepted 14 April 2017

The assessment of evolutionary divergences within subspecies complexes provide an effective short-cut for estimating intraspecific genetic diversity, which is relevant for conservation actions. We explore new evidence supplementing the existing knowledge about the singularity of Iberian bluethroats within the *Luscinia svecica* subspecies mosaic. We compared biometric traits of Iberian males (*L. s. azuricollis)* to the closest subspecies (*L. s. cyanecula, L. s. namnetum* and *L. s. magna*) using general linear models and analysed the correlations between biometric and genetic differentiation (based on nuclear microsatellites) among the target subspecies with a Mantel test. Biometric differences were calculated using 63 museum skins and 63 live specimens. Genetic distances were estimated in a sample of 136 individuals. An additional characterisation of the plumage of Iberian males was shaped from 22 live specimens. We highlight the distinctiveness of Iberian birds within the subspecies mosaic since *L. s. azuricollis* had longer wings than *L. s. cyanecula* and *L. s. namnetum*, but shorter wings than *L. s. magna.* Indeed, *L. s.* a*zuricollis* had longer tarsus and bill than *L. s. namnetum,* but shorter bill than *L. s. magna.* Biometric divergence was not significantly associated with genetic distance. Iberian males showed an all-blue plastron in 77% of specimens, a mostly non-marked black band and no white band, which distinguished them from males of *L. s. cyanecula* and *L. s. namnetum*. We conclude the importance of considering phenotypic and genotypic differences at subspecies level, which is essential for designing realistic conservation strategies addressed to preserve species genetic diversity patterns.

1. Introduction

The presence of endangered species is currently one of the main principles for establishing priorities of conservation and designing protected areas, like the Nature 2000 network in Europe. However, conservation strategies may benefit from targeting lower taxonomic levels of assessment to avoid widespread loss of genetic diversity (Meffe & Carrol 1997). Successful conservation policies should be explicitly focused on the preservation of multiple populations across the range of the species, that should be self-sustaining, healthy and genetically robust (Redford *et al.* 2011), since genetic variability determines the adaptive potential of the species and their resilience to environmental changes (Reed & Frankham 2003).

In the case of bird species, the assessment of intra-specific variation has been traditionally addressed at the taxonomic category of subspecies (Winker 2010). The evaluation of bird subspecies is critical since it contributes to explain the current distribution and the biogeographic history of species (Newton 2003). Indeed, according to Phillimore & Owens (2006), it offers an effective shortcut for estimating patterns of intraspecific genetic diversity, thereby providing a useful tool for the study of evolutionary divergence and conservation. However, it is important to have in mind that, despite the convenience of using this study level, the traditional subspecies limits, that have been based on phenotypic features, could sometimes be contradicted by the outcomes derived from modern molecular techniques (Zink *et al.* 2003, Rheindt *et al.* 2011), which makes the approach more challenging.

The bluethroat (*Luscinia svecica)* is a small migratory passerine (weight 14–20 g) with breeding populations distributed through the west and north of the Western Paleartic (Cramp 1988, Dement'ev & Gladkov 1968). It has been incorporated in the Annex I of the European Union's Birds Directive (Directive 2009/147/CE) that includes the most endangered species of Europe. The species shows a high sexual dichromatism, which influences inter and intrasexual communication. In the case of males, throat and breast are dominated by a large patch of ultraviolet (UV)/blue plumage (with or without a central spot of white/chestnut coloration) and a breast band of chestnut plumage below. Females are highly variable in the extent of their throat coloration, from entirely drab to almost male-like (Cramp 1988, Amundsen *et al.* 1997). The phenotypic variation of Bluethroats constitutes a complex mosaic associated to differences in geographical morphs and life history. Ten subspecies have been described according to both male throat ornament and general plumage patterns (Cramp 1988, Del Hoyo *et al*. 2005), although a unanimous consensus does not exist about subspecies classification, neither about phylogenetic relationships (Questiau *et al.* 1998, Eybert *et al.* 2003, Zink *et al.* 2003, Johnsen *et al.* 2006).

According to Zink *et al.* (2003), only two clades can be differentiated in Eurasia based on mitochondrial DNA studies. One of these groups is located in northern Eurasia and includes chestnut-spotted subspecies (*L. s. svecica, L. s. volgae* and *L. s. pallidogularis*). The other comprises southern subspecies with or without a white throat spot (*L. s. azuricollis*, *L. s. namnetum*, *L. s. cyanecula* and *L. s magna*). Johnsen *et al.* (2006) found evidence for low gene flow among northern and southern groups, the latter being more differentiated than the former. These authors also showed that Bluethroat genetic structure (based on microsatellite marker analysis) was consistent with the subspecies classification (based on phenotypic features). They found significant qualitative variation in throat spot coloration and quantitative variation in hue, chroma and brightness of the UV/blue throat coloration that possibly evolved by sexual selection through female choice, in turn leading to subspecies diversification (Peiponen 1960, Andersson & Amundsen 1997, Johnsen *et al.* 1998, 2001).

Indeed, Hogner *et al.* (2013), on the basis of sperm characteristics, showed a significant differentiation between *L. s. svecica, L. s. namnetum, L. s. cyanecula* and *L. s. azuricollis* that was consistent with the findings of Johnsen *et al.* (2006) and, therefore, supported the status of these subspecies as independent taxa. The high number of differences existing between subspecies and their geographic isolation indicate that Bluethroats are currently in an advanced stage of the speciation process, when compared with other subspecies complexes (Johnsen *et al.* 2006).

Iberian breeding Bluethroats have been traditionally misclassified within one of the white-spotted subspecies, *L. s. cyanecula* (Cramp 1988, Del Hoyo *et al*. 2005), although decades ago other authors as Mayaud (1958) and Corley-Smith (1959) had highlighted that they presented sufficient plumage differences to be considered as a different subspecies (*L. s. azuricollis)*. Recently, Johnsen *et al.* (2006, 2007) and Hogner *et al.* (2013) have shown that Iberian populations are genetically well defined, both in nuclear microsatellites and mtDNA (but see Zink *et al*. 2003). In

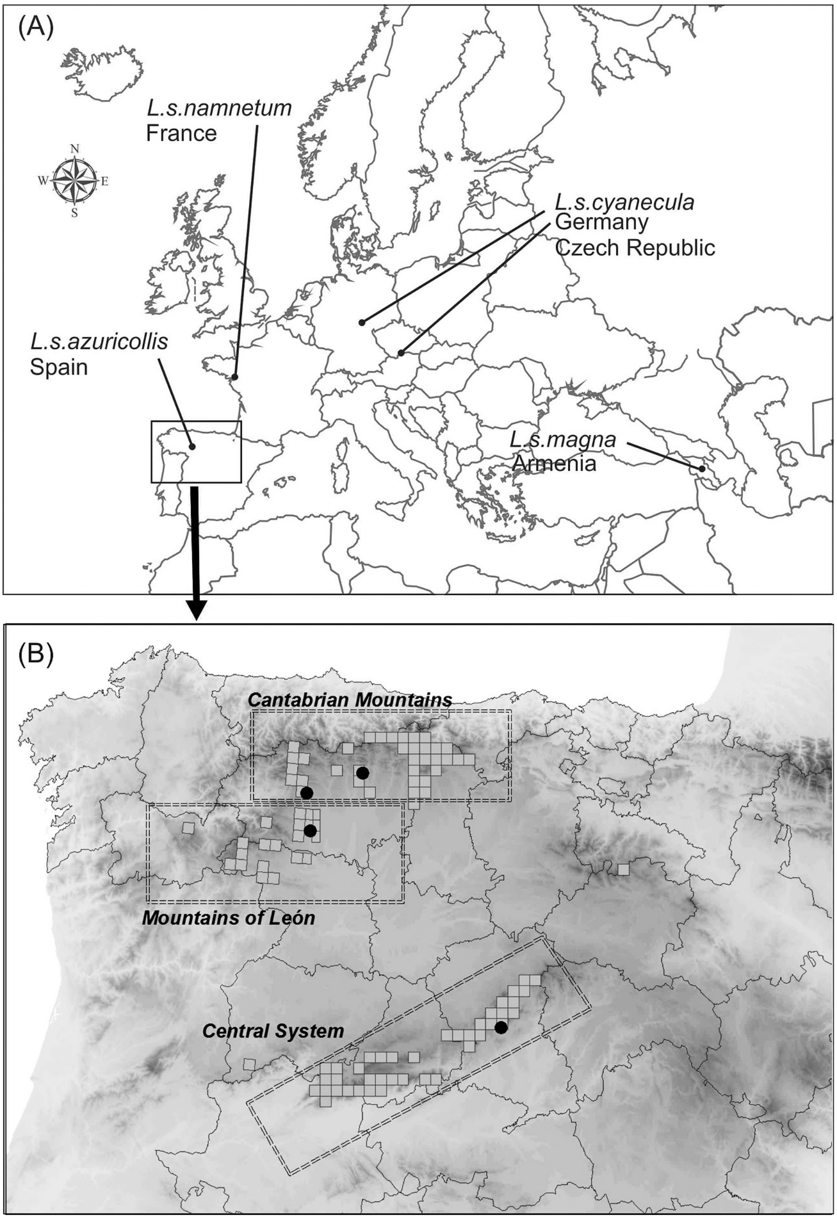

Fig. 1. Study area. A) Map of Eurasia showing the geographic location where Bluethroat specimens from different subspecies were captured. B) Breeding distribution of Bluethroat in Spain (10 × 10 km UTM squares; Martí & Del Moral, 2003) and sampling localities.

this sense, the accurate assessment of subspecies taxonomic position, as well as the full characterisation of phenotypic and genotypic distinctiveness of subspecies, is essential for implementing adequate measures of conservation addressed to protect the Eurasian Bluethroats as a whole.

The objective of this study was to add new evidence that supplements the existing knowledge about the relevance and singularity of the Iberian breeding bluethroats in the context of the *Luscinia svecica* subspecies complex. In particular, we evaluated biometric differences of Iberian males (*L. s. azuricollis)* against those of the closest subspecies, according to geographic (*L. s. cyanecula* and *L. s. namnetum*) and plumage (*L. s. magna*) criteria, and tested the correlation between biometric and genetic differentiation among the target subspecies. Additionally, we aimed to carry out a specific characterisation of Iberian males according to their plumage.

2. Material and methods

In order to evaluate the biometric differences among subspecies, we have considered three variables: wing chord length (± 0.5 mm), tarsus length, (± 0.1 mm) and bill length (± 0.1 mm) (Svensson 1992). These data have been collected in a sample of 63 male museum skins: seven *L. s. azuricollis* (housed in the Natural History Museum [NHM] of London), 28 *L. s. cyanecula* (NHM of London, Paris and Oslo), 10 *L. s. magna* (NHM of London) and 18 *L. s. namnetum* (NHM of Paris). The sample had been used previously in Johnsen *et al*. (2006) but, whereas in that study the morphological measurements were combined into a principal component analysis (PCA), here they have been analysed separately. Additionally, we have considered the wing chord length (± 0.5 mm) measured on a sample of 63 live male specimens captured using mistnets and spring traps in their breeding areas: 24 *L. s. azuricollis,* 13 *L. s. cyanecula*, 15 *L. s. magna* and 11 *L. s. namnetum* (Fig. 1). Biometric comparisons among subspecies were performed by applying univariate General Linear Models (GLMs) with a Tukey's Honest Significant Difference test (Tukey-HSD), which incorporates an adjustment for dealing with unbalanced sample sizes. We avoided comparisons between measurements made on museum skins and live birds, due to eventual differences in the preparation of the museum specimens that could affect results (Jenni & Winkler 1989, García *et al*. 2000).

To analyse the relationship between both biometric and genetic differentiation among the four subspecies, we applied a nonparametric Mantel test (Mantel 1967). Biometric differentiation was estimated as the Euclidean distance of the wing chord length mean values (measured on the 63 live male specimens above mentioned) among subspecies. Genetic differentiation was calculated as the pairwise genetic distance among subspecies (F_{ST} values; Weir & Cockerham 1984) using a sample of 135 individuals (36 *L. s. azuricollis*, 54 *L. s cyanecula*, 21 *L. s. namnetum* and 24 *L. s. magna;* Fig. 1). Genotypic characterisation had been accomplished by Johnsen *et al*. (2006) on the basis of 11 heterologous microsatellite markers using standard PCR conditions.

Finally, we described the plumage of the Iberian subspecies using a sample of 22 live males collected in Spain between 1999 and 2002 (Fig. 1b). We evaluated the tone of the breast blue plastron (light, intermediate or dark), the colour of the spot (white, chestnut or blue if it were absent) and the presence of black, white and chestnut breast bands (absent, not marked, marked or very marked).

Statistical analyses were done with the packages "hierfstat" and "multcomp" of R 3.0.0 statistical software (R Development Core Team 2013).

3. Results

The comparison among male museum skins for the target subspecies showed that *L. s. azuricollis* had significantly longer wings than *L. s. cyanecula* whereas there was no difference in tarsus or bill length among these subspecies. In contrast, *L. s. azuricollis* had longer wings, tarsus and bill than *L. s. namnetum*, as well as shorter wings and bill than *L. s. magna*. In the case of live males, *L. s. azuricollis* had significantly longer wings than *L. s. namnetum* and shorter wings than *L. s. magna,* while there were no differences in this biometric trait with *L. s. cyanecula* (Table 1).

Biometric divergence (based on the wing length of live specimens) was not significantly correlated with genetic differentiation among subspecies (Mantel test, $r = -0.143$, $p = 0.661$).

The analysis of the plumage made on the Iberian live birds showed that the blue tone of the plastron was mostly intermediate, while only in a few cases it was light or dark. A 77% of specimens showed an all-blue plastron (in three individuals, we found some white feathers completely covered by blue feathers) and the remaining 23% had a white spot in the blue plastron. Regarding the breast bands, the white band was not detected in any specimen, while the chestnut one was marked or very marked in all birds. The black band was absent in 22.7% of the birds, not marked in 68.2% and marked in the remaining 9.1%.

4. Discussion

A proper knowledge of subspecies differentiation is crucial to understand the species adaptation to different environments, the consequences of geo-

Table 1. Comparison of biometric parameters among the males belonging to *L. s. azuricollis (azu), L. s. cyanecula (cya), L. s. namnetum (nam)* and *L. s. magna (mag):* mean ± SD (sample size). Table also shows the result of univariate GLMs with a Tukey-HSD post hoc analysis (*$p<0.05$, **$p<0.01$, ***$p<0.001$, n.s. = non-significant).

	L. s. azuricollis	*L. s. cyanecula*	*L. s. magna*	*L. s. namnetum*	*azu-cya*	*azu-mag*	*azu-nam*	*cya-mag*	*cya-nam*	*nam-mag*
Tarsus length (museum skins)	26.77 ± 0.84 (7)	26.23 ± 0.72 (26)	27.07 ± 0.66 (10)	24.52 ± 0.62 (18)	n.s.	n.s.	***	**	***	***
Bill length (museum skins)	8.76 ± 0.32 (7)	8.74 ± 0.44 (25)	9.53 ± 0.45 (8)	7.98 ± 0.28 (18)	n.s.	**	***	***	***	***
Wing length (museum skins)	76.86 ± 1.95 (7)	75.11 ± 1.29 (27)	80.70 ± 1.49 (10)	68.20 ± 2.62 (18)	*	***	***	***	***	***
Wing length (live specimens)	75.23 ± 1.41 (24)	75.84 ± 1.74 (13)	77.77 ± 2.93 (15)	67.55 ± 1.29 (11)	n.s.	**	***	n.s.	***	***

graphic isolation and the risks of population declines. In this context, subspecies classification should correctly reflect the evolutionary diversity of the target species (Zink 2004). In the case of Bluethroats, some controversy about the correct assignation of Iberian breeding birds (*L. s. azuricollis*) within the subspecies complex still exists. Currently, *L. s. azuricollis* is recognized as a separate subspecies by leading ornithologists (e.g., Clements *et al.* 2016), but some discrepancies arise (Zink *et al*. 2003, Del Hoyo *et al*. 2005).

Our results highlighted the relevance and singularity of the Iberian subspecies within the European subspecies complex. We found that *L. s. azuricollis* is phenotypically well differentiated from the other evaluated subspecies (*L. s. cyanecula, L. s. namnetum* and *L. s. magna),* showing important differences in biometry and plumage features. However, despite the microsatellite analysis made by Johnsen *et al*. (2006) revealed that *L. s. azuricollis* is genetically well differentiated from the other evaluated subspecies, we noticed that biometric differences in wing length are not significantly correlated to genetic distance among subspecies. A reason could be that phenotypic features may be the product of gradual geographic variations and clines, reflecting phenotypic plasticity in response to environmental contingencies, rather than genetic differences (Willoughby 2007). In fact, variability in biometric measurements can be related not only to genotypic features, but also to epigenomic differences. That is, birds with a similar genome may have different morphological traits depending on their habitat (Geist 1978).

Any attempt to define biologically meaningful units for conservation at intra-specific level should consider genetic, phenotypic and life history characteristics. The identification of “evolutionarily significant units” (ESUs; Ryder 1986, Moritz 1994, Crandall *et al*. 2000, Luck *et al*. 2003) can be useful for designing realistic conservation strategies. When conservation measures are based on units with evolutionary significance, areas holding high levels of genetic differentiation must be identified, giving priority to the conservation of spaces including environmental gradients that assure the viability of populations/subspecies and its adaptive potential (Meffe & Carrol 1997, Moritz 2002, Holderegger *et al*. 2006). In this sense, southern populations of Bluethroats, where the Iberian subspecies is included, are genetically well differentiated, while Northern populations (included in subspecies *L. s. svecica*, *L. s. pallidogularis* and *L. s. tianshanica*) have a relatively reduced degree of genetic differentiation (Zink *et al.* 2003, Johnsen *et al*. 2006).

Within the former group, *L. s. azuricollis* and *L. s. namnetum* may be the most ancestral forms of the species (Johnsen *et al*. 2006). Despite their ecological value, they present important conservation problems that should be specifically addressed. In particular, they show a low genetic diversity that could be caused either by genetic drift (Johnsen *et al*. 2006) or by a recent “bottleneck”, resulting in an increased frequency of some rare alleles and a strong differentiation from the rest of the subspecies complex (Questiau *et al*. 1998). A low level of heterozygosity is particularly frequent in threatened species, being generally associated with low breeding success and survival rates (Frankham 1995, Haig & Avise 1996, Roques & Negro 2005). Additionally, Iberian Bluethroats are located in the southern edge of the species range in Eurasia, where available habitat is fragmented and relatively reduced due to threats related to land use (forestry, land clearing infrastructure, fires) and climate change (Martí & Del Moral 2003, Huntley *et al.* 2007).

Regarding biometry, significant differences were found among subspecies in tarsus, bill and wing length, in line with the findings of Johnsen *et al*. (2006). Dissimilarities in wing length between *L. s. azuricollis* and *L. s. cyanecula* are probably related to migration strategies. The Iberian subspecies hold birds with longer and more pointed wings that can be considered as longer distance migrants (Arizaga *et al*. 2006). This wing morphology is usually associated with a greater flight capacity to migrate further (Mönkkönen 1995) and, therefore, could imply the exploitation of different wintering areas (Arizaga *et al*. 2006).

In fact, according to Arizaga *et al.* (2015), at least some birds (or populations) of *L. s. azuricollis* could spend the winter in tropical Africa (i.e. Senegal), while *L. s. cyanecula* bluethroats would have their wintering quarters within the circum-Mediterranean region (specifically in southern Europe, northern Africa and the Sahel; Cramp, 1988). In these areas, suitable habitat could be affected by different environmental threats (e.g. drought, land use change, human disturbances), which would mean new challenges for designing global strategies addressed to the conservation of the *L. s. azuricollis* subspecies.

The comparison of biometric data for Iberian males measured in the two evaluated samples (i.e., museum skins and live birds) showed a difference in the mean values recorded for wing length. One reason may be that the study carried out at a European scale using museum skins was only based on birds collected in central Spain, while the analysis based on live specimens included birds from the north of the country, where the level of genetic differentiation of populations is high, as described by Alda *et al*. (2013). These authors found three different genetic clusters in this area, with F_{ST} values comparable to those among several of the recognized Eurasian subspecies (Johnsen *et al.* 2006). Due to the complex genetic patterns of Iberian populations, we highlight the importance of sampling across the subspecies range for improving the reliability of ecological studies focused on this species.

The assessment of plumage characteristics showed that both the presence of a distinct white spot and the breast band patterns widely change among the four subspecies. The most distinctive plumage traits of *L. s. azuricollis*, in relation to the other subspecies, are: (i) the absence of a white spot in a large number of birds, that was present (white or chestnut) in the rest of the subspecies, with the exception of *L. s. magna* (Johnsen *et al.* 2006); (ii) the complete absence of a white breast band, which is actually a distinguishing feature that is not usually considered (Clement & Rose 2015). Breast ornamentation is strongly associated with genetic differentiation within the Bluethroat subspecies mosaic in Eurasia and, therefore, it could be useful to discriminate among subspecies in areas of secondary contact (Johnsen *et al*. 2006). Currently, Iberian populations are geographically isolated from the other subspecies.

Therefore, it would be interesting to evaluate, in a hypothetic scenario of *L. s. namnetum* expansion (Eybert *et al.* 1999), to what extent the mechanisms of reproductive isolation, based on plumage or song (Turcokova 2011), could limit the hybridization among subspecies. It has been hypothesized that the evolution of the throat spot started from a phase of an entirely blue throat plastron, followed by an increase in the complexity of breast ornamentation (Johnsen *et al*. 2006). Nevertheless, genetic data suggest that *L. s. azuricollis* and *L. s. magna* are not as closely related as suggested by their similar phenotypic (absence of white spot) appearance (Johnsen *et al*. 2006).

We conclude the importance of considering explicitly the phenotypic and genotypic differences existing at subspecies level, since this information is essential for designing realistic conservation strategies at different geographic scales (from local to continental), specifically for the case of polytypic species. Our findings reinforce the idea that the Iberian breeding subspecies should be a conservation priority to preserve the intraspecific variation in genetic diversity of the Eurasian Bluethroats. A loss of species genetic diversity would imply a reduction on its resilience against environmental changes.

Acknowledgements. We want to thank GIA (Grupo Ibérico de Anillamiento, León, Spain), in particular E. Álvarez, H. Astiárraga, F. de la Calzada, J. Casado, J. M. Colino, J. Falagán, R. González, M. Juan, D. Miguélez, I. Roa, N. Rodríguez and C. Zumalacárregui, for collecting *L. s. azuricollis* samples, Martin Adamjan for collecting *L. s. magna* samples, Sophie Questiau for collecting *L. s. namnetum* samples and Dieter Franz, Václav Pavel and Bohumír Chutný for collecting *L. s. cyanecula* samples. The study was financed by a grant to A.J. from the Norwegian Research Council. This study was carried out in full compliance with the laws in each respective country.

Sinirinnan *Luscinia svecica* -alalajien eriytyminen: uusia todisteita Iberian sinirintapopulaatioiden eriytymisestä

Lajisisäisen geneettisen vaihtelun ymmärtäminen on tärkeää lajien suojelun kannalta. Alalajikompleksien eriytymistä voidaan käyttää lajinsisäisen geneettisen vaihtelun tutkimiseen. Selvitimme Iberian sinirintapopulaatioiden eriytymistä *Luscinia svecica* -alalajikompleksissa. Vertasimme Iberian koiraiden (*L. s. azuricollis)* biometrisiä mittauksia sen läheisimpiin alalajeihin (*L. s. cyanecula, L. s. namnetum* ja *L. s. magna*). Analysoimme fenotyyppisen ja geneettisen eriytymisen korrelaatiota alalajikompleksin lajien välillä mikrosatelliittien avulla. Biometrisiin mittauksiin käytettiin aineistoa 63 yksilöstä sekä 63 museonäytteestä, ja geneettiset erot laskettiin 136 yksilön aineistosta. Höyhenpuvun piirteet kuvattiin lisäksi 22 yksilöstä.

Aineistomme tukee Iberian populaatioiden eriytymistä: *L. s. azuricollis* oli pitkäsiipisempi kuin *L. s. cyanecula* ja *L. s. namnetum*, mutta sen siivet olivat lyhyemmät kuin *L. s. magna* -alalajilla. Tarsus ja nokka olivat pidemmät *L. s.* a*zuricollis* -alalajilla kuin *L. s. namnetum:*illa. *L. s.* a*zuricollis* oli lisäksi lyhytnokkaisempi kuin *L. s. magna.* Erot biometrisissa mittauksissa eivät olleet yhteydessä geneettiseen eriytymiseen. Suurimmalla osalla (77 %) Iberian koiraista oli täyssininen kurkkulappu ja musta, mutta ei valkoista vyötä: tämä erottaa ne *L. s. cyanecula* ja *L. s. namnetum* -alalajien koiraista. On tärkeää selvittää alalajien fenotyyppisiä ja geneettisiä eroja, sillä se auttaa ymmärtämään lajin geneettistä vaihtelua, ja mahdollistaa suojelutoimien paremman kohdentamisen.

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