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Short communication

Genetic differentiation between two subspecies of *Emberiza schoeniclus* and open forest bunting's evolution inferred from mitogenomes

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The reed bunting, *Emberiza schoeniclus* (Linnaeus 1758), is the only member of the genus adapted to Mediterranean wetlands, where some subspecies are critically endangered. The first complete mitogenome of the eastern Iberian reed bunting (*E. s. witherbyi*) is presented here and compared with an unpublished mitogenome obtained in northeast Asia (most likely *E. s. pyrrhulina*). Genetic distance analyses are consistent with the new reed bunting data corresponding to two distinct lineages of *E. schoeniclus*. A new fossil-calibrated phylogeny suggests that open forest buntings have suffered two rapid speciation events from Late Miocene to Pleistocene, that seem to be correlated with major climatic changes and habitat shifts. Adaptation to a new ecological niche (i.e. wetlands) could have favoured the reed bunting expansion across the Palearctic. The high intraspecific variation observed today could result from the establishment of resident populations within small areas, potentially acting as a climatic refuge.

Keywords: *Emberiza*, intraspecific evolution, phylogeny, rapid radiations, reed bunting

Introduction

Forest extent reduction and grasslands expansion due to the Middle Miocene Climate Transition opened new ecological niches for seed-feeding birds in Eurasia (Edwards et al. 2010). Thanks to their long-distance migration capabilities and preference for open areas, bunting species (i.e. *Emberiza* Linnaeus 1758) colonized the Old World from North America and diversified (Cai et al. 2021). By the Late Miocene, another radiation resulted on the 14 open forest bunting species found today (Clade II in Cai et al. 2021). Most of them are migratory species that inhabit open forests

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of east Asia (Winkler et al. 2020). However, there are exceptions to this trend such as reed buntings *Emberiza schoeniclus*, a Palaearctic partial migrant that can establish resident populations in wetlands all the way to the Iberian Peninsula (Vera et al. 2011).

The evolutionary events that lead to these secondary adaptations remain unexplored and are difficult to trace partly due to the questionable assignment of ecological traits to species in previous studies (Cai et al. 2021). Phylogeographic and discrete trait evolution studies have successfully been applied for resolving evolutionary histories in many bird groups when carried out accurately (Garcia-R et al. 2014). Given that different mitochondrial genes tend to have distinct substitution rates, generally faster than slowly-evolving nuclear genes, mitogenome sequences have been particularly useful in this kind of works as well as for intraspecific evolution studies (Ruiz-García et al. 2018, Lubbe et al. 2022).

Most of the open forest bunting clade species' mitogenomes have been published previously, but not that of the reed bunting. *E. schoeniclus* shows a complex geographical variation with up to 19 subspecies described across its wide distribution, having been recorded significantly differing in song, diet and morphology (Neto et al. 2013, Gordinho et al. 2015). Previous genetic studies suggested significant population structure among subspecies (Grapputo et al. 1998, Kvist et al. 2011), and three clades have been reported with some east Asian populations apparently in early stages of speciation (Zink et al. 2008).

The first complete mitogenome of reed buntings from Spain (*E. s. witherbyi*) is presented here and compared with unpublished data from China (presumably *E. s. pyrrhulina*). New genetic data is used to re-evaluate recent results from Cai et al. (2021) and characterize the evolutionary history of open forest buntings.

Material and methods

Sample collection and DNA extraction

DNA was extracted using the CleanNGS magnetic bead clean-up system (CleanNA, the Netherlands) from muscle tissue of a one young *E. s. witherbyi* male captured from s'Albufera de Mallorca (Balearic Islands, Spain) during an ongoing ex-situ conservation programme and died by natural causes. DNA was then quantified using Nanodrop and Qubit HS DNA and its integrity assessed using the HS Large Fragments kit in a Fragment Analyzer (Agilent, USA). Genomic libraries were prepared using the SMRTbell Express Template Preparation Kit v2.0 and sequenced using PacBio's SMRT (Single Molecule Real Time) Sequencing technology at the Central Service for Experimental Research (SCSIE) facilities of the University of Valencia.

Mitochondrial genome data and genetic distances

The complete mitogenome sequence was isolated from the PacBio reads by running a BLAST search using the reed bunting cytb sequence available in GeneBank (accession: KP877731). Protein-coding, rRNA and tRNA genes were annotated using the metazoan mitochondrial genome annotation pipeline MITOS2 (Donath et al. 2019). Newly generated data was used for phylogenetic reconstruction together with mitogenome data obtained from GenBank for all other open forest *Emberiza* (except *E. variabilis*) and 5 bunting species used as outgroups (Table 1). Data for a second

Table 1. Species analysed in this study, their GenBank accession and publication reference (if available). Last 4 columns indicate the category in which they were classified for the discrete traits analysed. In breeding distribution: SW Europe = southwest Europe, E Asia = east Asia, C/E Asia = central and east Asia and S/SE Asia = south and southeast Asia. In preferred habitat: Q = wetlands, T = forests, S = heathlands, scrub and tundra.

Species	Accession	Distribution	Habitat	Resident pop.	Subspecies
Emberiza chrysophrys	HQ896034	E Asia	T	No	1–2
Emberiza elegans	KJ813903	E Asia	T	Yes	3–5
Emberiza aureola	LC541464	Eurasia	T	No	1–2
Emberiza tristrami	HQ896035	E Asia	T	No	1–2
Emberiza rustica	KC831775	Eurasia	T	No	1–2
Emberiza pusilla	KC407232	Eurasia	T	No	1–2
Emberiza spodocephala	KC758647	E Asia	T	No	1–2
Emberiza rutila	KC952874	E Asia	T	No	1–2
Emberiza siemsseni	KX809695	E Asia	T	No	1–2
Emberiza yessoensis	LC541450	E Asia	Q	No	1–2
Emberiza pallasi	MK687386	E Asia	S	No	3–5
Emberiza schoeniclus witherybi	OQ274893	SW Europe	Q	Yes	> 5
Emberiza schoeniclus pyrrhulina	MT081419	E Asia	Q	No	> 5
Emberiza sulphurata	KY419885	E Asia	T	No	1–2
Emberiza lathami	KX702277	S/SE Asia	S	Yes	1–2
Emberiza cioides	KF322027	E Asia	S	Yes	1–2
Emberiza fucata	KT737824	E Asia	T	Yes	3–5
Emberiza jankowskii	KP738714	E Asia	S	Yes	1–2
Emberiza leucocephalos	KY349100	C/E Asia	T	Yes	1–2

subspecies of *E. schoeniclus* from China was released recently (22/03/2022; Genbank accession: MT081419.1). Although no information was provided on the subspecies, the specimen most likely belongs to *E. s. pyrrhulina* given that the sequencing was carried out in Qingdao, China, and that is the only subspecies reported from the area (Sullivan et al. 2009, Winkler et al. 2020). To analyse mitochondrial gene divergence between both lineages, Kimura 2-Parameter (K2P) genetic distances and their standard errors were estimated using MEGA ver. 11.0.13 (Tamura et al. 2021). Transitions and transversions were included in the K2P model with uniform rates among sites specified. Distances variance was estimated using 500 bootstrap replicates.

Phylogenetic reconstruction

Alignments were performed for each of the ribosomal (rDNA) and protein-coding sequences (CDS) using MAFFT ver. 7.490 with default parameters (Katoh and Standley 2013). Best nucleotide substitution models, including estimates for nucleotide frequencies per, discrete Gamma (+*G*) and evolutionarily invariable (+*I*) parameters, were selected according to the Bayesian information criterion as implemented in ModelTest-NG ver. 0.1.7 (Darriba et al. 2020). The evolutionary history was first inferred by using the maximum likelihood method as implemented in the randomized axelerated maximum likelihood – RaxML – computer software, selecting the topology with superior log likelihood value and running 500 bootstrap replicates (Stamatakis 2014). A Bayesian inference (BI) approach was also conducted using the BEAST ver. 1.10.4 software (Bouckaert et al. 2019). Partitioned analyses were carried out by specifying the best substitution models in Beauti ver. 2.6.6 (Drummond et al. 2012). Two independent runs starting from a random tree and a Calibrated Yule tree prior were set up with 10^8 Markov Chains Monte Carlo (MCMC) generations each, sampling every 10 000th tree. The MCMC convergence was assessed in Tracer ver. 1.7.2 (Rambaut et al. 2018) and, after removing a 10% burn-in, the resulting posterior distribution of trees and parameters were summarized on the maximum clade credibility tree (MCC) using TreeAnnotator ver. 2.6.6 (Bouckaert et al. 2019).

Divergence time estimates

Timing of clade origin and major evolutionary events were estimated using the relaxed molecular clock method implemented in BEAST ver. 1.10.4. The fossil of *Emberiza shaamarica* (Upper Pliocene of Shaamar, northern Mongolia) was previously used to calibrate the separation between *E. pallasi* and *E. schoeniclus* (Cai et al. 2021). Although Palastrova and Zelenkov (2020) mention that *E. shaamarica* and *E. schoeniclus* have similar orientation of trochlea metatarsi II, they state that *E. shaamarica* is closest to the species group including *E. cirlus*, *E. stewarti*, *E. citrinella* and *E. leucocephalos*. In fact, the trochlea metatarsi II orientation vary significantly in passerines and unrelated *Serinus* species also show this unusual character (Nguembock et al. 2009). Therefore, we used a normal prior distribution spanning the Upper Pliocene (95% HPD: 2.04–5.96 Ma), where *E. shaamarica* was found, to calibrate the node corresponding to the ancestor of *E. leucocephalos*, *E. cioides* and *E. jankowskii*. Following previous estimates of Emberizidae origins by Barker et al. (2015), we also calibrated the root of our tree on the Mid-Late Miocene using a normal prior distribution (95% HPD: 6.64–10.56 Ma). Uncorrelated lognormal relaxed clock model and Calibrated Yule speciation tree priors were selected for the analyses. MCMC analyses were run as indicated above, and the MCC tree was represented with its respective parameters using FigTree ver. 1.4.4 (Rambaut 2018). Lower and upper bounds of the 95% highest posterior density (95% HPD) interval were obtained for every node.

Discrete trait analyses

Some concerns must also be raised on the buntings' habitat assignments defined by Cai et al. (2021). Although Winkler et al. (2020) mention that reed buntings can be found in grasslands and farmlands, it must be pointed out that their preferred habitats are in fact wetlands (Pasinelli et al. 2008). Therefore, the assignment of the same habitat type to reed buntings and cirl buntings (*Emberiza cirlus*), which are typically found in shrubs and farmlands (Morelli et al. 2014), is considered here to be inaccurate. A revised habitat assignment, following the EUNIS Habitat Classification (Chytrý et al. 2020), is used here instead. Forests (T) include deciduous, coniferous and mixed forests and open forests; heathlands, scrub and tundra (S), include non-forested habitats with low humidity; and wetlands (Q), which include reed beds and other tall herbage within marshes. Besides habitat preference, other traits such as the number of described subspecies (using three categories: 1–2, 3–5 or > 5 subspecies), breeding distribution (Eurasia, east Asia, east and central Asia and south and southeast Asia) and migratory behaviour (presence or absence of resident populations) were also analysed using data from Winkler et al. (2020) and BirdLife Data Zone (http://datazone.birdlife.org). Another concern about Cai et al. (2021) trait analyses are related to ancestral state reconstruction methodology, which does not calculate each node's most likely state (Paradis et al. 2004). A discrete Bayesian phylogeographic approach is used here, based on a continuous time Markov chain (CTMC) over discrete trait specifications, and a Bayesian stochastic search variable selection (BSSVS) model (Lemey et al. 2009). Four independent runs were performed in BEAST ver. 1.10.4 for 10^8 generations and sampling every 10 000 steps. Convergence and effective sampling size were assessed by visual inspection using TRACER ver. 1.7.2, and TreeAnnotator ver. 2.6.6 was used, after a 10% burn-in, to obtain and annotate the MCC tree.

Results

Mitochondrial genome data and genetic distances

The complete mitogenome sequence of *E. s. witherbyi* was 16 776 bp long, 3 bp shorter than the unpublished sequence of presumed *E. s. pyrrhulina*. Both mitogenomes were composed of 13 protein-coding genes, 2 rRNA genes, 22 tRNA genes and the control region (Fig. 1), with minor gene length variations detected (Supporting information). Most mitochondrial genes were encoded in the heavy strand (H-strand), except for ND6 and eight tRNA genes (Supporting information). For every gene analysed, K2P genetic distances between *E. s. witherbyi* and presumed *E. s. pyrrhulina* were the lowest (see red asterisks in Fig. 2). Genetic distances between both subspecies were higher for protein coding genes (up to 0.026 in ND3) than for rRNA genes (< 0.010) (Supporting information). When average distance values were obtained considering all *Emberiza* species analysed, ribosomal genes remained the lowest diverging markers, while ND1 and ND2 showed the highest K2P distances.

Phylogenetic inference and divergence time estimates

The final concatenated alignment, including every protein-coding and rRNA genes, was 13 962 long. Nucleotide substitution models, gamma values and proportion of invariant sites varied notably, although genes within the same complex (e.g. ribosomes versus cytochromes or ATPases) had most similar models (Supporting information). The ML and MCC tree topologies were fully congruent, and most clades showed high support values. Our results confirm the monophyly of the so-called open forest buntings, which can be subdivided into three subclades (named A–C in Fig. 3). The first subclade to diverge (A) consists of *E. elegans* and *E. siemsseni*. Two well supported subclades appear then, one including species from forest areas (subclade B) and another consisting of species found in reed beds or tundra-like habitats (subclade C). All phylogenetic relationships within subclade C are strongly supported under both ML and BI analyses, with *E. yessoensis* diverging earlier and *E. schoeniclus* and *E. pallasi* as closest relatives. Although subclade B could be subdivided in three monophyletic groups, the relative position of *E. chrysophrys* and *E. tristrami* remained unresolved (Supporting information).

The Bayesian relaxed molecular clock analyses support a Late Miocene origin of open forest buntings, dated around 7.9 Ma (95% HPD: 6.2–9.5) (Fig. 3). The first subclade (A) would have diverged around 5.8 Ma (95% HPD: 4.6–7.1), quickly followed by the other two subclades around 5.7 Ma (95% HPD: 4.5–6.9). Four divergence events occurred within subclade B in a very short period between 3.6 Ma (95% HPD: 2.8–4.3) and 3.2 Ma (95% HPD: 2.5–3.9). While *E. yessoensis* would have diverged soon after the origin of subclade C, around 5.1 Ma (95% HPD: 4.0–6.2), the

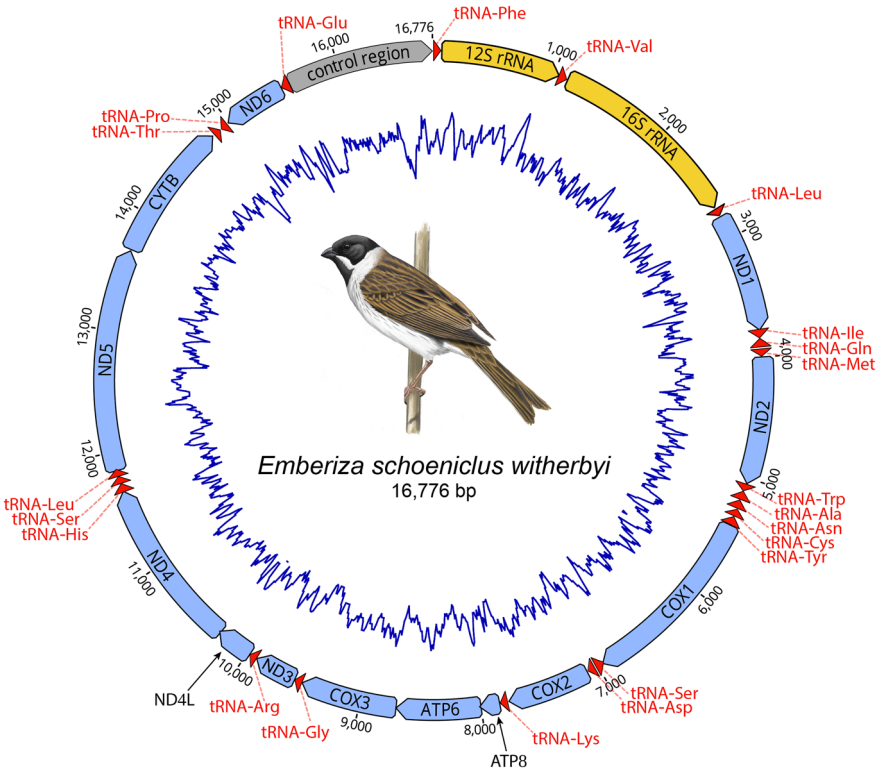

Figure 1. Circular mitochondrial genome of *E. s. witherbyi*. Blue line represents variation in GC content. Illustration of *E. s. witherbyi* by Àlex Mascarell.

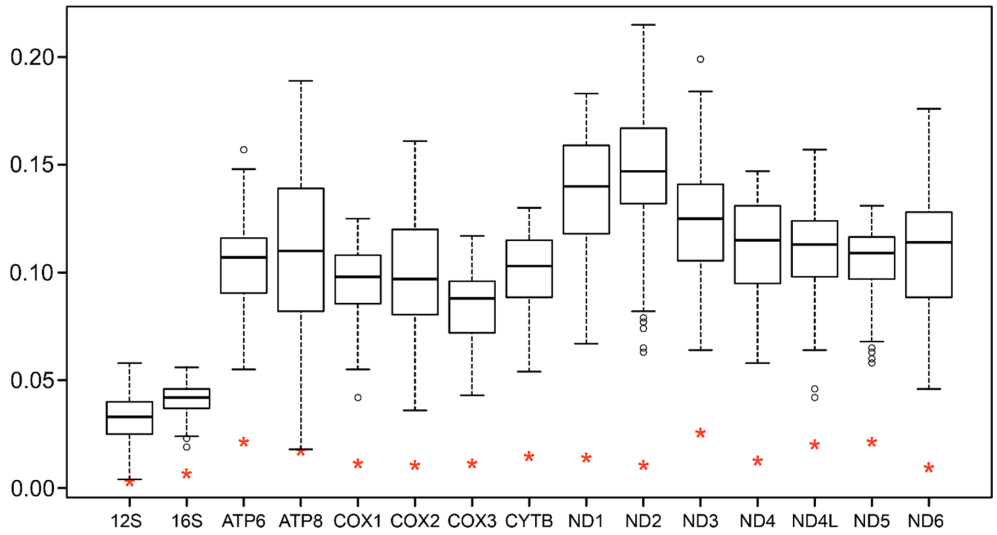

Figure 2. Boxplot of the Kimura 2-Parameter (K2P) genetic distances values obtained in all pair comparisons of the taxa analysed for each CDS and rRNA. Red asterisks indicate the genetic distance resulting from the comparison of both reed bunting sequences.

divergence between *E. schoeniclus* and *E. pallasi* is estimated to have occurred around 3.2 Ma (95% HPD: 2.5–4.1). As for *E. schoeniclus* subspecies analysed diverge time is estimated to be around 0.5 Ma (95% HPD: 0.37–0.64).

Discrete trait analyses

Our results confirm that the most recent common ancestor (MRCA) of all extant open forest buntings had an east Asian breeding distribution (Fig. 4). Shifts on distribution would have occurred on both the MRCA of *E. aureola, E. pusilla* and *E. rustica*, and the ancestor of *E. s. witherbyi*, which expanded towards Eurasia. The MRCA subclade C underwent a shift in habitat requirements towards wetlands while tundra-like habitats would have been later occupied by the *E. pallasi* lineage (although support values were lower than 0.9). High migration rates would be the dominant trait within open forest bunting evolution, with the establishment of resident populations occurring only twice (in *E. elegans* and *E. witherbyi* lineages). Finally, subspeciation rates would have been low in most ancestors (> 95% probability), except for two lineages (*E. elegans* and the clade including *E. pallasi–E. schoeniclus*).

Discussion

Gene order and overall mitogenome size are conserved across *Emberiza*, and agree with the ancestral avian mitochondrial arrangement (Caparroz et al. 2018). Sequencing errors were

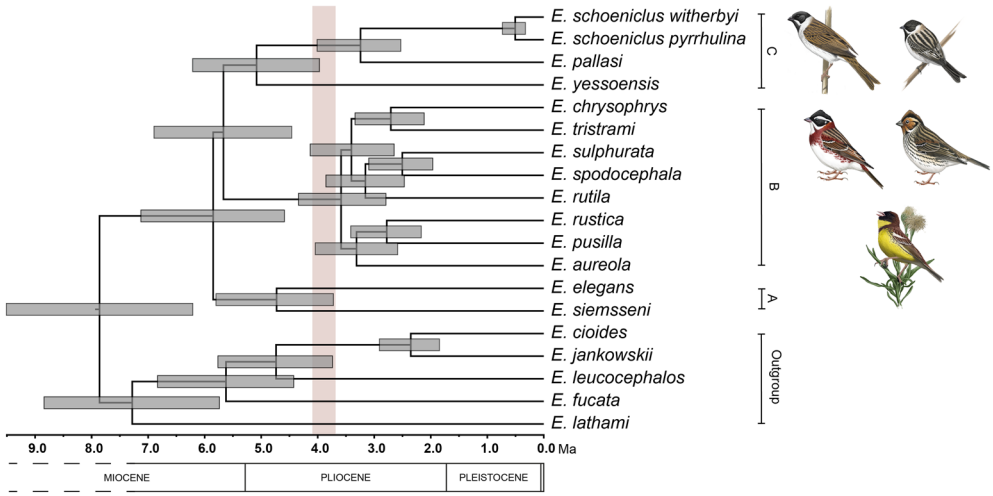

Figure 3. Calibrated phylogenetic tree for the complete mitogenomes dataset indicating divergence time among *Emberiza* species analysed. The different subclades within the open forest buntings are labelled as A, B and C. Grey bars show the lower and upper bounds of the 95% highest posterior density (95% HPD) interval for the time estimates. Shaded red line mark potentially significant weather shift (shift in the east Asian monsoon and start of North Hemisphere glaciations) near radiation events. Bunting's illustrations by Àlex Mascarell.

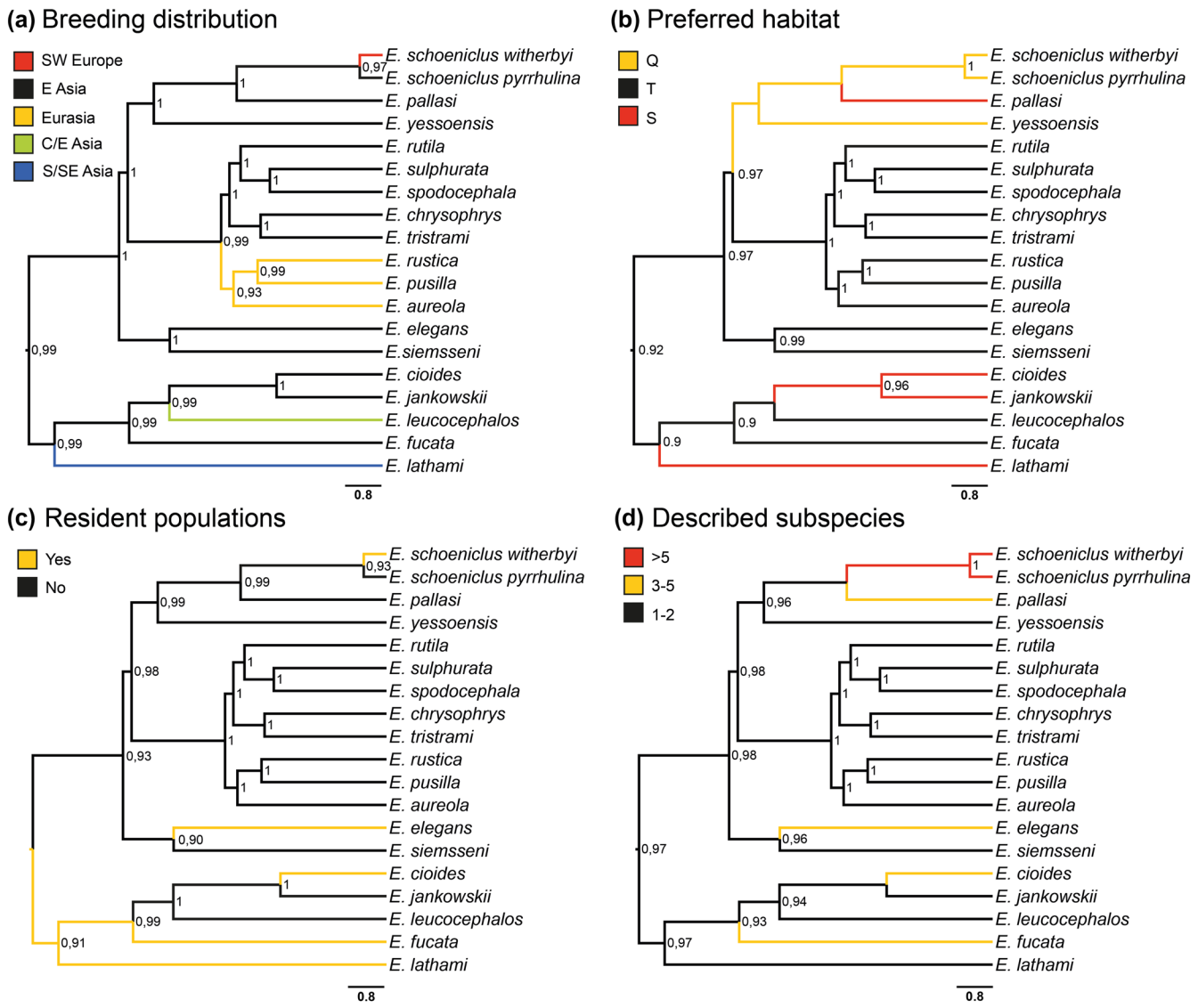

Figure 4. Results of the discrete trait analyses. Branch support value (Bayesian posterior probabilities) is absent when values are < 90. In breeding distribution: SW Europe = southwest Europe, E Asia = east Asia, C/E Asia = central and east Asia and S/SE Asia = south and southeast Asia. In preferred habitat: Q = wetlands, T = forests, S = heathlands, scrub and tundra.

observed in available mitogenomes (e.g. four guanine insertion and three missing nucleotides in the ND4L gene were detected in *E. yessoensis*), so phylogenetic studies are confirmed to improve error detection from public databases (Sangster and Luksenburg 2021). Interspecific divergence values among buntings were like those previously reported for mitochondrial genes in birds, with protein-coding regions evolving faster than rRNA sequences (Aliabadian et al. 2009). This significant variation in evolutionary rates makes mitochondrial genes particularly useful to unravel multiple speciation phenomena occurring in narrow time ranges (rapid radiations) (Morin et al. 2010).

The divergence time estimates obtained place the origin (7.9 Ma; 95% HPD: 6.2–9.5) and first radiation of open forest buntings (5.8 Ma; 95% HPD: 4.6–7.1) at the time when C4 plant expansion occurred (Edwards et al. 2010). An increase in open habitat availability has been correlated to diversification events of other palearctic bird genera (Fuchs et al. 2019), and might have affected the bunting's lineage as well. Early fossil records of waterbirds in Eurasia coincide with the spread of reeds, sedges, rushes and grasses (Greb et al. 2006). Furthermore, our results also agree with the fact that wetland development and establishment of recent plant genera used by reed buntings (e.g. *Phragmites*, *Cladium*, *Typha*) (Worobiec and Worobiec 2019, Alambiaga et al. 2021) should pre-date their expansion.

A second radiation would have occurred during the Early/Late Pliocene transition when North Hemisphere glaciations started (De Schepper et al. 2014) and a shift in the east Asian monsoon changed forest extent and composition (Salzmann et al. 2008, Wang et al. 2019). The ancestor of *Emberiza* species from humid forests (i.e. *E. aureola*,

E. rustica and *E. pusilla*), would have diversified and expanded westwards with the spread of suitable habitats during interglacial periods (Birkenmajer et al. 2010). In parallel, separation between *E. schoeniclus* and *E. pallasi* would have occurred because of a shift in habitat requirements of the latter towards expanding tundra systems (Wolfe 1985).

Divergence between the two reed bunting lineages from Spain and China would have occurred in the Late Pleistocene (~0.5 Ma), coinciding with important glacial periods that could have isolated populations in climatic refugia (e.g. Iberian peninsula) and favoured differentiation, as previously suggested in reed buntings (Zink et al. 2008). The loss of migratory behaviour and phenological mismatch between resident and migratory populations would have maintained the genetic isolation after geographic barriers disappeared (Levey and Stiles 1992), and could explain the high number of described subspecies. Nevertheless, this hypothesis requires more data and future research should focus on sampling further subspecies from eastern Europe and central Asia.

Genetic differentiation values between *E. s. witherbyi* and the presumed *E. s. pyrrhulina* were significantly lower than those obtained from comparisons between valid species, and agree with intraspecific divergence values reported in Passeriformes (K2P: 0.0–0.025) (Arida et al. 2021). To confirm the subspecies status, however, further efforts should be made to determine reproductive isolation and/or diagnosability between the two linages. Our genetic distances also agree with Grapputo et al. (1998) analyses (Supporting information), with European populations showing low genetic divergences when compared with east Asia subspecies. An incipient speciation process cannot be discarded though, as has previously been suggested (Zink et al. 2008, Gordinho et al. 2016). Given the critical status of Mediterranean wetlands, endangered populations such as *E. s. witherbyi* urge for conservation measures, and fast-evolving mitochondrial markers analysed there could be used to better understand their situation and implement effective measures.

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Permits – The DNA reference sample was obtained with the necessary permissions from the S'Albufera Natural Park (Mallorca, Spain) and the regional government of the Balearic Islands.

Author contributions

Iván Alambiaga: Conceptualization (equal); Data curation (supporting); Formal analysis (equal); Investigation (equal); Methodology (equal); Resources (supporting); Software (equal); Visualization (supporting); Writing – original draft (equal). **Roberto González**: Funding acquisition (equal); Project administration (supporting); Resources (equal). **Pablo Vera**: Funding acquisition (equal); Project administration (supporting); Resources (equal). **Juan Monrós**: Conceptualization (supporting); Funding acquisition (equal); Investigation (equal); Project administration (equal); Resources (equal); Supervision (equal); Writing – review and editing (supporting). **Ferran Palero**: Conceptualization (equal); Data curation (lead); Formal analysis (lead); Funding acquisition (equal); Investigation (equal); Methodology (equal); Project administration (equal); Resources (equal); Software (lead); Supervision (lead); Validation (equal); Visualization (equal); Writing – original draft (equal); Writing – review and editing (lead).

Transparent peer review

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Data availability statement

Data are available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.xksn02vk1 (Alambiaga et al. 2023). GenBank accession for the *Emberiza schoeniclus witherbyi* mitogenome: OQ274893.

Supporting information

The Supporting information associated with this article is available with the online version.

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