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Completing the genetic puzzle of the reed warbler complex: insights from Italy

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ABSTRACT

Capsule: Analysis of mitochondrial DNA from reed warblers sampled in Italy reveals that they are most similar to the nominate race of European Reed Warbler *Acrocephalus scirpaceus scirpaceus*.

Aim: To fill gaps in our knowledge of the distribution of European/African Reed Warblers *Acrocephalus scirpaceus/baeticatus* in the Mediterranean region through genetic analysis of populations from the key biogeographic areas of Sicily, Sardinia and mainland Italy.

Methods: We assessed the genetic placement of Italian reed warbler populations within a comprehensive dataset of this species complex, by sampling these birds from principal peninsular and insular breeding populations (for a total of 17 samples) and by comparing their mitochondrial *cyt b* sequences with those available in GenBank-NCBI.

Results: The final dataset included 171 *cyt b* sequences (763 base pair long). The analysis showed samples from Italy had a high similarity with the European Reed Warbler *A. s. scirpaceus* subspecies. This fills an important information gap in the phylogeography of the group.

Conclusion: The genetic cohesion shown between Italian and other European populations may denote a structuring in at least three distinct refugia in the Western Palearctic: Iberia for *A. baeticatus ambiguus*, the Caucasus Mountains for *A. s. fuscus*, and both Italy and the Balkans for *A. s. scirpaceus*.

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The Mediterranean basin is ranked among the 25 global biodiversity hotspots (Myers *et al.* 2000). The higher conservation priority of this area is due both to its taxonomic richness and uniqueness, and to anthropogenic threats causing a severe loss of habitat (Cuttelod *et al.* 2008). Responses to paleo-climatic events, such as isolation in refugial areas during glacial periods, are documented as one of the drivers that led to the high phylogenetic diversity in this region (Bilton *et al.* 1998, Stewart *et al.* 2010, Voelker and Light 2011) and to the frequent occurrence of cryptic species in a wide range of taxa, including marine organisms (Calvo *et al.* 2009), freshwater arthropods (Bernini *et al.* 2016), amphibians (Carranza *et al.* 2008), reptiles (Ghielmi *et al.* 2016), mammals (Hulva *et al.* 2004) and birds (Brambilla *et al.* 2008, Pellegrino *et al.* 2017). Genetic tools have played a prominent role in our understanding of these processes and in assessing possible taxonomic delimitation, the implications of which extend into the

fields of biogeography and conservation (Lohman *et al.* 2010, Pertoldi *et al.* 2012, Galimberti *et al.* 2021).

Along with Iberia and the Balkans, the Italian peninsula and its islands represent a well-documented Pleistocene glacial refugium, which facilitated an evolutionary radiation area for many taxa, including several bird species (Brito 2007, Pellegrino *et al.* 2015, Drovetski *et al.* 2018). In birds, isolation dynamics and the subsequent evolution of unique genotypes within the Italian refugium are generally more common for sedentary or near-sedentary species (Förschler *et al.* 2009, Pellegrino *et al.* 2016). However, these divergence mechanisms also involve some long-distance migratory bird species, for which the Italian peninsula and the western Mediterranean islands have been a cradle of genetic isolation processes (Brambilla *et al.* 2008, Pons *et al.* 2016, 2019, Zuccon *et al.* 2020).

Among long-distance migrant species breeding extensively (but not necessarily exclusively) around

the Mediterranean, the European/African Reed Warbler *Acrocephalus scirpaceus/baeticatus* complex has attracted the attention of many researchers, who have investigated, through molecular and morphological methods, the species/subspecies boundaries and evolutionary relationships of the group (Procházka *et al.* 2011, Arbabi *et al.* 2014, Hering *et al.* 2016, Olsson *et al.* 2016, Pavia *et al.* 2018, Babbington *et al.* 2019). According to the International Ornithological Congress (Gill *et al.* 2020), this species complex is considered to be composed of two species: the European Reed Warbler *Acrocephalus scirpaceus* with four subspecies (*A. s. scirpaceus*, *A. s. fuscus*, *A. s. avicenniae*, and *A. s. ammon*), and the African Reed Warbler *Acrocephalus baeticatus* with six subspecies (*A. b. baeticatus*, *A. b. ambiguus*, *A. b. minor*, *A. b. cinnamomeus*, *A. b. suahelicus*, and *A. b. hallae*). This assessment is mostly in accordance with the mitochondrial cytochrome *b* analysis presented by Olsson *et al.* (2016), wherein they proposed to treat the complex as one polytypic species (*A. scirpaceus*) with seven well-supported clades divided in two main clusters: a Eurasian group that included the subspecies *Acrocephalus scirpaceus scirpaceus*, *A. s. fuscus* and *A. s. avicenniae*, and an African group encompassing not only the Sub-Saharan subspecies *A. s. baeticatus*, *A. s. minor*, *A. s. hallae*, *A. s. cinnamomeus*, but also the Iberian and Moroccan populations. These latter populations belong to the resurrected taxon *A. scirpaceus ambiguus*, which is characterized by intermediate morphometries between *A. s. scirpaceus* and *A. s. baeticatus* (Olsson *et al.* 2016).

While the taxonomy and evolutionary history of the complex are becoming clearer, there are several aspects still in need of further study. One important aspect is increased sampling, as genetic information from key biogeographic regions (e.g. the Italian peninsula, Sardinia and Sicily) is still missing. Here, we aim to assess the genetic relationships of Italian populations of reed warblers relative to other populations, by adding new genetic data to that of Olsson *et al.* (2016). We specifically seek to address several questions. First, are some or all of the Italian populations genetically differentiated from non-Italian populations? We hypothesize that the population most likely to be differentiated is the Sardinian one, where numerous endemic bird taxa (Giglio-Tos 1918) and unique endoparasite lineages (Pellegrino *et al.* 2021) have been described. Second, if some or all of the Italian populations are not distinct, to which other populations are they most closely related? In this case we are unable to make predictions, as Italian

bird populations have variably been most closely related to populations in Spain and France (Hourlay *et al.* 2008), and the Balkans and Europe more broadly (Drovetski *et al.* 2018, Raković *et al.* 2019). Further, while connections to northern African populations are possible, and have been observed in other taxa, including butterflies (Habel *et al.* 2009), shrews (Cosson *et al.* 2005) and frogs (Stöck *et al.* 2008), such a link has not as yet been established for populations of Italian avifauna. Nevertheless, several bird taxa endemic to the Western Palearctic include distributions both on Sardinia and Sicily (e.g. Spotless Starling *Sturnus unicolor* and Purple Swamphen *Porphyrio porphyrio*; Keller *et al.* 2020), as well as northwest Africa, suggesting a possible biogeographic connection between these southwestern Mediterranean areas.

Considering that (i) the Mediterranean and Italian areas have a well-established biogeographic potential as a diversity incubator, even for long-distance migrant birds; (ii) there is a lack of genetic assessment of reed warblers of some Mediterranean areas, such as Italy and its islands, (iii) taxonomic studies show genetic affinities between Italy and other European refugia, as well as northern Africa, we aim to assess the genetic affinities of Italian reed warbler populations by analysing the most comprehensive genetic dataset of the Eurasian/African species complex to date.

Methods

Sampling, DNA amplification and sequencing

Sampling of Reed Warblers took place in four geographic areas (i.e. northern Italy, southern Italy, Sardinia and Sicily), which generally correspond to areas of intraspecific genetic divergence previously identified in a wide range of taxa, such as in dragonflies and damselflies (Galimberti *et al.* 2021), butterflies (Scalercio *et al.* 2020), amphibians (Mattocchia *et al.* 2005), reptiles (Kindler and Fritz 2018), mammals (Wauters *et al.* 2017) and birds (Pellegrino *et al.* 2015, Zuccon *et al.* 2020) (see Table 1 for sampling details). Birds were trapped using mist-nets set in Common Reed *Phragmites australis* dominated wetlands. Blood was drawn via brachial venipuncture using a small needle to puncture the vein, and a capillary tube to collect the blood. Blood was stored in 2 ml tubes filled with Queen's lysis buffer and samples were vouchered and placed in the collection of the University of Eastern Piedmont, Italy. For molecular analyses we included a subsample of 17

Table 1. Sampling details of reed warbler specimens collected from Italy for this study. The identifier of sampling sites in the map of Figure 1, the *cyt b* haplotypes and the GenBank accession numbers of the sequenced samples are also reported (* haplotypes discovered in this study).

Individual Id	Haplotype	Sampling Area	Map Id	Sample Site Name	Latitude	Longitude	Accession Number
IT 0985	ACR1	Southern Italy	2	Pignola	40.5838	15.7486	LR878457
IT 0887	ACR1	Sardinia	3	Terralba	39.7099	8.5506	LR878456
IT 1280	ACR9	Sicily	4	Augusta	37.2072	15.1810	LR878458
AJAH222	ACR10	Northern Italy	1	Fontanetto Po	45.2193	8.1772	LR878459
AJAH228	ACR10	Northern Italy	1	Fontanetto Po	45.2193	8.1772	LR878460
AJAH289	ACR10	Northern Italy	1	Fontanetto Po	45.2193	8.1772	LR878461
IT 0897	ACR10	Sardinia	3	Terralba	39.7099	8.5506	LR878462
IT 0903	ACR10	Sardinia	3	Terralba	39.7099	8.5506	LR878463
IT 1322	ACR10	Sicily	5	Ispica	36.7166	15.0175	LR878464
IT 0902	ACR67*	Sardinia	3	Terralba	39.7099	8.5506	LR878465
IT 0984	ACR68*	Southern Italy	2	Pignola	40.5838	15.7486	LR878466
IT 0987	ACR69*	Southern Italy	2	Pignola	40.5838	15.7486	LR878467
IT 1281	ACR70*	Sicily	4	Augusta	37.2072	15.1810	LR878468
IT 1321	ACR71*	Sicily	5	Ispica	36.7166	15.0175	LR878469
IT 0890	ACR72*	Sardinia	3	Terralba	39.7099	8.5506	LR878470
IT 0983	ACR72*	Sardinia	3	Terralba	39.7099	8.5506	LR878471
IT 0984	ACR73*	Sardinia	3	Terralba	39.7099	8.5506	LR878472

breeding individuals, as determined via the presence of a cloacal protuberance or brood patch (Svensson 1992) (Table 1).

DNA was extracted from approximately 25 to 50 µL of blood using the E.Z.N.A. Tissue DNA extraction kit (Omega Bio-Tek, Norcross, GA) following the manufacturer's instructions. A 1022 base pair (bp) region of the mitochondrial cytochrome *b* (*cyt b*) gene was amplified using the primer pair L-14995/H-16065 (Helbig *et al.* 1995). Polymerase chain reaction (PCR) conditions consisted of an initial denaturation step at 94 °C for 5 min, followed by 35 cycles at 94 °C for 1 min, 52 °C for 1 min and 72 °C for 1 min, with a final extension step at 72 °C for 10 min. Sequencing was performed bi-directionally at Eurofins Genomics (Milan, Italy) using the same PCR primers. Consensus sequences were obtained by editing the electropherograms with Bioedit 7.2 (Hall 1999). After primer trimming, the presence of an open reading frame was verified for the obtained consensus sequences by using the on-line tool EMBOSS Transeq (http://www.ebi.ac.uk/Tools/st/emboss_transeq/). This analysis was conducted to exclude the amplification of pseudogenes that could affect identification results or the discovery of divergent intraspecific lineages. Consensus sequences were deposited in EMBL GenBank (accession numbers are listed in Table 1).

Dataset assembly and haplotype structure analysis

Orthologous *cyt b* sequences, belonging to reed warbler subspecies breeding in the Western Palearctic (i.e. *A. s. scirpaceus*, *A. s. fuscus*, *A. s. avicenniae*, and *A. b. ambiguus*) were downloaded from GenBank-

NCBI; no sequences were available for *A. s. ammon* (Hering *et al.* 2016). Moreover, we also considered reference *cyt b* sequences of *A. baeticatus* subspecies breeding in sub-Saharan Africa (i.e. *A. b. baeticatus*, *A. b. minor*, *A. b. hallae* and *A. b. cinnamomeus*), to calculate their genetic distance values from Western Palearctic subspecies and verify their possible genetic affinity to the Italian populations. Sequences showing insertions/deletions, that were missing more than 1% of sites or that were overlapping less than 750 bp with the region amplified and sequenced for the Italian samples, were discarded. Overall, a total of 154 *cyt b* sequences were retrieved from the public repository and combined with the 17 sequenced Italian reed warblers to constitute a unique dataset (see online Table S1).

Sequences were aligned with MAFFT 7.110 (Katoh and Standley 2013) using the *E-INS-I* option and cut to a final alignment length of 763 bp. This length was a compromise between including the highest number of taxa in the European/African Reed Warbler complexes and conserving the highest nucleotide information. As no phylogenetic analyses were necessary for the purpose of this study, we did not estimate the best-fit model for the dataset. However, to provide a measure of genetic distance among the Italian populations and the other subspecies known for the Mediterranean and the African Reed Warbler taxa, we calculated average uncorrected pairwise genetic distances (p-distance) using MEGA X (Kumar *et al.* 2018). The average within-group genetic distances (inferred with MEGA X) for each subspecies and country of origin were also calculated (online Table S2).

Identical sequences were collapsed into unique haplotypes using FaBox 1.5 online (Villesen 2007)

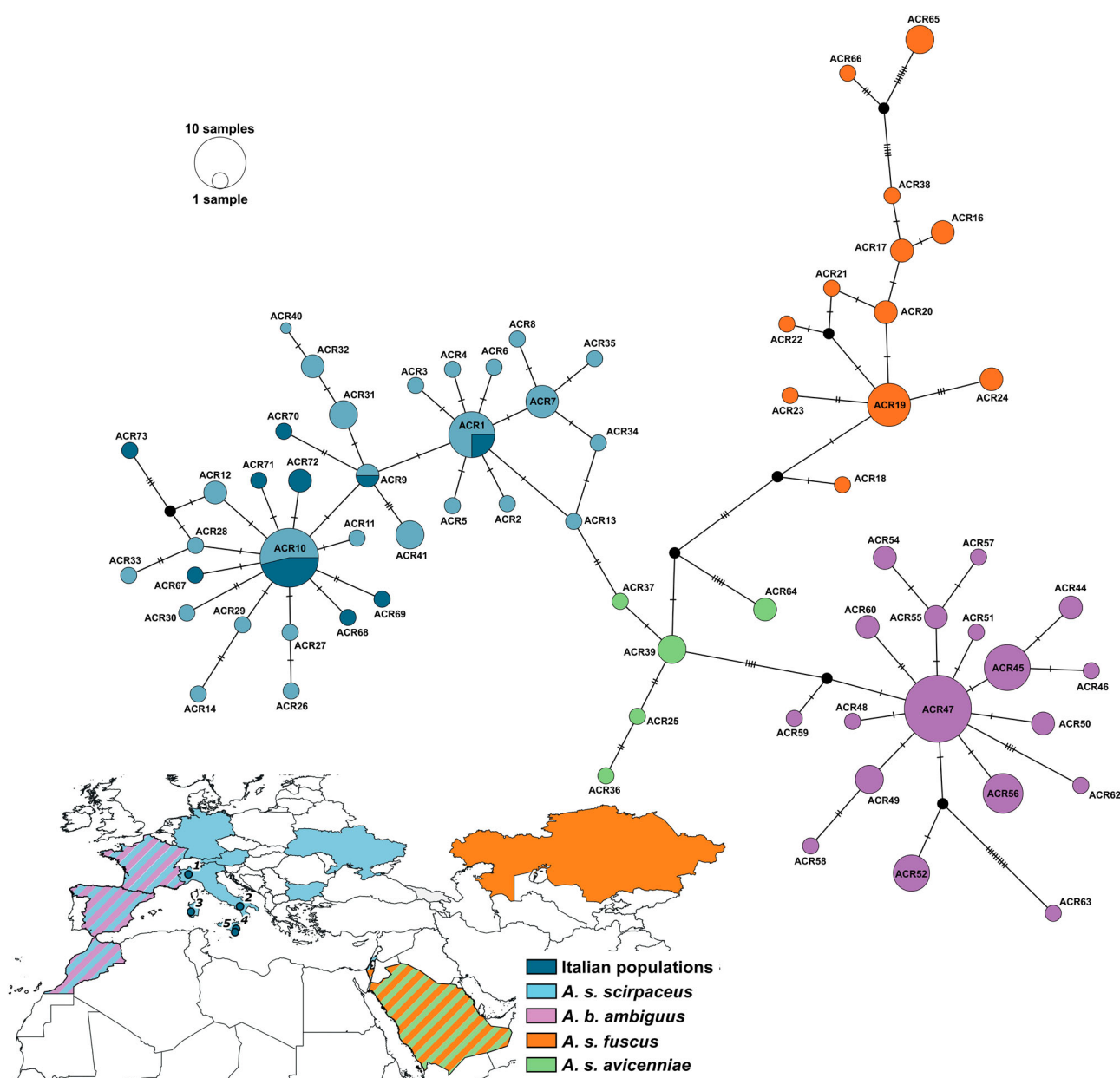


Figure 1. Median-joining network of *cyt b* haplotypes of Western Palearctic *Acrocephalus scirpaceus*/*A. baeticatus* species complex populations (Table 1, Table S1). Each circle represents a haplotype and circle size is proportional to haplotype frequency. Colours indicate different subspecies and blue dots also account for Italian sampling sites. Dashes represent substitutions. Distribution map is shown on the countries boundaries basis.

(Table S1). In order to investigate the haplogroup assignment of Italian reed warbler *cyt b* haplotypes, a Median Joining network, encompassing all the Italian and Palearctic populations (Figure 1, Table S1), was built using the median-joining algorithm implemented in PopART 1.7 (Leigh and Bryant 2015).

Results

We obtained high-quality full-length *cyt b* sequences for all 17 Italian samples. No sequences contained insertions or deletions, stop codons, or were biased by

nuclear mitochondrial DNA interference. The comprehensive *cyt b* dataset assembled in this study included 171 sequences 763 bp long, which were collectively assigned to 82 haplotypes, 10 of which were found in reed warblers from Italy (Table 1, Figure 1, Appendix Table S1). Of the 10 haplotypes found in Italian reed warblers, seven were exclusive to the country: three were found in Sardinia (ACR67, ACR72, ACR73), two in Sicily (ACR70, ACR71) and two in Southern Italy (ACR68, ACR69) (Figure 1). The other sequences from Italian individuals coincide with three previously described haplotypes: ACR1,

Table 2. Between groups genetic divergence values of *cyt b* sequences, based on the uncorrected p-distance calculation (bottom left), for the taxa of European and African Reed Warblers considered in this study. Standard error values are reported above the diagonal.

	<i>A. b. ambiguus</i>	<i>A. b. baeticatus</i>	<i>A. b. minor</i>	<i>A. b. hallae</i>	<i>A. s. avicenniae</i>	<i>A. s. fuscus</i>	<i>A. s. scirpaceus</i>	Italian populations
<i>A. b. ambiguus</i>		0.0039	0.0034	0.0052	0.0032	0.0045	0.0042	0.0045
<i>A. b. baeticatus</i>	0.0144		0.0025	0.0031	0.0035	0.0049	0.0045	0.0047
<i>A. b. minor</i>	0.0091	0.0091		0.0051	0.0036	0.0050	0.0044	0.0047
<i>A. b. hallae</i>	0.0198	0.0115	0.0186		0.0041	0.0049	0.0050	0.0052
<i>A. s. avicenniae</i>	0.0112	0.0163	0.0120	0.0205		0.0028	0.0028	0.0033
<i>A. s. fuscus</i>	0.0185	0.0229	0.0193	0.0254	0.0131		0.0036	0.0039
<i>A. s. scirpaceus</i>	0.0171	0.0222	0.0180	0.0252	0.0116	0.0171		0.0011
Italian populations	0.0178	0.0230	0.0187	0.0261	0.0122	0.0177	0.0039	

sampled in Sardinia and Southern Italy, was also observed in France and Israel; ACR9, known from Morocco, was shared by a Sicilian individual; and ACR10, the most common haplotype in our populations (Sardinia, Sicily and Northern Italy), is widely distributed, having been found in the Canary Islands, Spain, Germany, France and Israel (Olsson *et al.* 2016) (Table 1).

Our Median Joining haplotypes network (Figure 1, Figure S1) shows that the different subspecies of the European Reed Warbler complex are distinct from the African Reed Warbler subspecies, as they have no shared haplotypes. On the whole, all the Italian haplotypes fall in the haplogroups representing *A. scirpaceus scirpaceus* (Figure 1).

The genetic distance (*p*-distances) calculated between our Italian samples and other sequences of *A. s. scirpaceus* (0.0039 ± 0.0011) was lower than those obtained between Italian samples and the other taxa (*A. s. avicenniae*, *A. s. fuscus*, *A. b. ambiguus*, *A. b. baeticatus*, *A. b. minor*, and *A. b. hallae*), which ranged from 0.0122 ± 0.0033 and 0.0261 ± 0.0052 (Table 2). For intragroup genetic distances, the Italian population showed a value falling in the range of other *A. s. scirpaceus* populations (0.0029 ± 0.0008 ; range 0 ± 0 – 0.0066 ± 0.0030) (Table S2).

Discussion

The placement of Italian samples, from northern and southern populations as well as from the two major islands of Sicily and Sardinia, indicates that all belong to the *A. scirpaceus* nominal subspecies, the European Reed Warbler taxon that occurs throughout Europe, with the exception of most of the Iberian Peninsula (Olsson *et al.* 2016). This result fills a significant gap in the sampling framework of reed warbler phylogeography in the Mediterranean basin, as the Italian reed warbler populations have not been included prior to this new study (Hering *et al.* 2016, Olsson *et al.* 2016, Babbington *et al.* 2019).

The lack of unique haplotype clustering of our samples relative to other reed warbler samples (Figure

1) supports a previous study based on microsatellite markers (Procházka *et al.* 2011) that revealed high levels of gene flow among *A. s. scirpaceus* populations. Despite the occurrence of seven unique haplotypes from Sicilian, Sardinian and southern Italian individuals, our findings do not support the co-occurrence of divergent lineages typical of other European/African Reed Warbler subspecies in Italy. Furthermore, while the ACR9 haplotype is shared only by a single Sicilian (this study) and a single Moroccan individual (sampled in Olsson *et al.* 2016), this relationship suggests a possible similarity in *A. s. scirpaceus* populations from the southern Mediterranean basin. However, a greater sampling effort of reed warblers from northwest Africa will be necessary to establish this pattern.

On the whole, based on the reliability of *cyt b* in characterizing the principal clades of this passerine group (Olsson *et al.* 2016), genetic cohesion between Italian reed warblers and other Central European individuals belonging to *A. s. scirpaceus* populations is evident. On the other hand, the distance between Italian individuals and *A. s. fuscus* is similar to that calculated between Italian *A. s. scirpaceus* and Iberian *A. baeticatus ambiguus*. This phylogeographic pattern (i.e. the genetic homogeneity occurring among the Italian peninsula, the Balkans and the rest of Europe, vs. the divergent haplogroups shown by Iberian and Caucasus populations) is also known from studies of other passerine species (e.g. Dunnock *Prunella modularis*, Drovetski *et al.* 2018 and Common Chiffchaff *Phylloscopus collybita*, Raković *et al.* 2019), and may denote a population structuring of *Acrocephalus scirpaceus* through distinct glacial refugia within the Western Palearctic: *A. s. fuscus* and *A. s. avicenniae* in refugia in Saudi Arabia and the south Caucasus, and *A. s. scirpaceus* in refugia in the Italian and Balkan peninsulas.

The presence of two distinct clades of reed warblers in the Iberian Peninsula, with a clear genetic delimitation between this area and the rest of Europe, is comparable with that observed in Savi's Warbler *Locustella luscinoides*, another long-distance migrant associated

with wetland habitats (Neto *et al.* 2012). This convergence may indicate an important role of Iberia (and possibly of northwest Africa) as a disconnected refugium from the rest of the Mediterranean region for wetland passerines. Our results likely exclude the occurrence of African Reed Warbler haplotypes from the geographic areas we sampled in Italy, Sardinia and Sicily.

Interestingly, among European Reed Warblers from Sardinia, there are reported cases of individuals showing complete post-breeding moult (Nissardi and Zucca 2001), as well as of individuals wintering on the island (Nissardi 1998). These patterns of complete post-breeding moult and overwintering in the Palearctic also occur in African Reed Warbler *A. b. ambiguus*, and involve populations or individuals from both Morocco and Spain (Amezian *et al.* 2010, Nieto *et al.* 2018). However, Nieto *et al.* (2018) has suggested over the past few decades that European Reed Warbler individuals belonging to different populations are overwintering in Iberia, and that this pattern is related to increasingly warmer winters. In the light of the genetic assessment presented here, such modifications of trait phenology are likely not exclusive to a specific haplogroup, but could be due to the influence of shared environmental and climatic factors within the Mediterranean basin that deserve to be further addressed.

While this study adds an important piece to the complex puzzle of the genetic structure and taxonomy of the reed warbler group, further studies are still needed to disentangle remaining biogeographic knowledge gaps. In particular, sampling from transition areas between the different taxa in Turkey and the Near East, and from isolated populations (e.g. Cyprus, Crete, northeastern Africa) would be valuable; data from these regions are currently lacking.

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