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Morphometric versus genetic variation in the Eurasian Reed Warbler *Acrocephalus scirpaceus* in Italy

Renzo Ientile^a, Marcello Tagliavia^b, Natalino Cuti^c, Rosa Termine^d, Carlo Giannella^e, Sergio Nissardi^f, Carla Zucca^f, Vincenzo Cavaliere^g, Gabriella Lo Verde ^h, Daniela Campobello ⁱ and Bruno Massa ^{c,h}

^aDepartment of Biological, Geological and Environmental Sciences, University of Catania, Catania, Italy; ^bIRIB-CNR, Palermo, Italy; ^cStazione Ornitologica 'Aegithalos', Monreale, Italy; ^dLaboratory of Health and Environmental Engineering, University of Enna 'Kore', Enna, Italy; ^eStazione Ornitologica Modenese 'Il Pettazzurro', Mirandola, Italy; ^fAnthus snc, Cagliari, Italy; ^gVia Amendola 2 h, Portici, Italy; ^hDepartment of Agriculture, Food and Forest Sciences, University of Palermo, Palermo, Italy; ⁱDepartment of Biological, Chemical and Pharmaceutical Sciences and Technologies, University of Palermo, Palermo, Italy

ABSTRACT

Capsule: The Eurasian Reed Warbler *Acrocephalus scirpaceus* from breeding sites in Italy has little genetic variability in the mitochondrial cytochrome b gene but shows morphological variation suggesting isolation of some populations.

Aims: To investigate the morphometric and genetic trait variation of Eurasian Reed Warbler populations at a large temporal and spatial scale.

Methods: We analysed morphometric traits and the mitochondrial cytochrome b (cyt b) gene of samples collected over 25 years, from populations at seven sites across the Italian peninsula and islands.

Results: While we found no genetic differences, we detected significantly smaller body sizes in individuals breeding in Sicily and Sardinia compared to those from mainland Italy. There were also significant body size differences between two Sicilian populations: one larger breeding on the southern coast and the other smaller at an inner highland site.

Conclusion: The morphometric data, together with the site fidelity of individuals, are consistent with there being isolation of some populations. Specifically, our results are consistent with an example of polyphenism, morphological plasticity, in the populations of the main Italian islands, Sicily and Sardinia.

ARTICLE HISTORY

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The Eurasian Reed Warbler *Acrocephalus scirpaceus* is a trans-Saharan species breeding in the Palearctic region and wintering mostly in tropical Africa. It is linked to reedbeds dominated by *Phragmites australis*, where it is the most widespread breeding bird species (Catchpole 1974, Leisler & Schulze-Hagen 2011, Hering *et al.* 2016). Being very common, it has been adopted as an excellent species model for biological, eco-ethological and taxonomic studies (e.g. Green & Davies 1972, Leisler *et al.* 1997, Merom *et al.* 1999, García Peíro 2003, Procházka *et al.* 2011, Olsson *et al.* 2016, Campobello & Sealy 2018, 2020, Pollo *et al.* 2018, Ientile *et al.* 2022).

The Eurasian Reed Warbler is a philopatric (Pollo *et al.* 2018) and monogamous (Leisler & Catchpole 1992) species, two traits which probably drive population isolation and, in turn, accumulation of differences between populations over time.

Consequently, many subspecies have been described across the Palearctic region and Africa, although, to date, there is no complete agreement between studies investigating the variation. The European populations are considered stable (BirdLife International 2017) under the current definitions of the subspecies but the phylogeny of these is still subject to debate.

According to del Hoyo *et al.* (2006) and Kennerley & Pearson (2010), populations living in sub-Saharan Africa should be separated from the Eurasian Reed Warbler and be identified as the African Reed Warbler *Acrocephalus baeticatus* (online Table S1). This is in contrast to Olsson *et al.* (2016) who considers all included taxa as belonging to the Eurasian Reed Warbler. Recently, Olsson *et al.* (2016) confirmed that the Iberian and Moroccan populations form a taxonomically distinct clade, *A. scirpaceus ambiguus*, following reports from Thévenot *et al.*

CONTACT Daniela Campobello  daniela.campobello@unipa.it

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(2003) and Amezian *et al.* (2010) who described some wintering individuals in Morocco as belonging to this clade. Whether all populations from North Africa, thus from Morocco to Tunisia and north Libya, belong to *A. s. ambiguus* is yet unknown (Figure 1). Hering *et al.* (2016) described Libyan specimens, from the Siwa Oasis complex, as another subspecies of Eurasian Reed Warbler *A. scirpaceus ammon*, which is sedentary in the Egypt/Libya border region and characterized by small size. Finally, populations in northern Libya and Tunisia have not been clearly ascribed as belonging to any of the known subspecies.

There are significant morphometric differences between the Continental European-Mediterranean subspecies of the Eurasian Reed Warbler between geographic sites, with *A. s. scirpaceus* from northern and eastern European populations being characterized by longer wings (Wilson *et al.* 2001, Procházka *et al.* 2011) and *A. s. ambiguus* and *ammon* characterized by smaller biometrics (online Tables S1 and S2). Using ten polymorphic microsatellite loci and extensive sampling, Procházka *et al.* (2011) found three genetically different populations in Europe, with one of them in the Iberian Peninsula. Overall, they found small but significant genetic differentiation among populations across Europe. Furthermore, they interpreted their data as showing high levels of gene flow, suggestive of a possibly recent divergence of European populations after a rapid range expansion.

A recent investigation on Eurasian Reed Warblers in Italy concluded that Italian samples showed a high genetic similarity with the nominate subspecies of European Reed Warbler *A. s. scirpaceus* (Ilahiane *et al.* 2020). We question, however, whether populations breeding on the main Mediterranean islands (Sicily and Sardinia), might show morphometric and/or genetic differences to those breeding on the Italian mainland peninsula. The Mediterranean flyway is used by a minority of migratory breeders who cross the Mediterranean by stopping over on numerous small islands (e.g. Pantelleria, Ustica; Massa *et al.* 2015) and reaching Sicily from Tunisia and Libya, an area where South-Saharan birds stop to breed. We are however unaware whether, first, this North African population has different morphometric and genetic features in comparison with the other known subspecies and, second, some individuals cross the Mediterranean to breed on the main Mediterranean islands.

Here, we analysed data collected over 25 years from breeding and migratory individuals across seven breeding and stop-over sites. Our main aim was to highlight potential differences among populations by

investigating geographical variation in morphometric and genetic traits of reed warblers.

Methods

Study areas

We sampled at seven sites distributed across Italy and its main and minor islands as follows:

Mirandola, Mortizzuolo, Modena, Italy (44°51'40"N 11°06'25"E). Included in the Nature 2000 Network (SPA ITA 4040014), it is a protected area with marshland formed by several fresh-water ponds restored with European Union (EU) grants in the 1990s. The vegetation along the bank and open waters is mainly dominated by *Phragmites australis*, where many pairs of Eurasian Reed Warblers breed. Ringing activity has been conducted on the site since 1995 (Giannella & Gemmato 2003).

Molentargius, Saline Natural Park, Sardinia, Cagliari (39°12'28"N 9°08'59"E). The area is a Special Protection Area (SPA; ITB044022), a Special Area of Conservation (SAC; ITB040022) and a Regional Natural Park. Its habitats include freshwater ponds, saltwater and a sandy agricultural plain. The presence of areas with different salinity favours a rich variety of plant and animal species. There are over 200 ha of *Phragmites australis* reedbed, which form an important site for stopover, breeding and wintering of passerines, including a large population of Eurasian Reed Warblers. Ringing activity has been carried out discontinuously since 1992.

Persano Oasis, Campagna and Serre, Salerno, Campania, Italy (40°36'12"N 15°08'46"E). The Oasis of Persano lies in the high part of the Plain of Sele River. It is a wetland of international importance, and is included in the Ramsar Convention, within *Zone Spéciale de Conservation* (ZSC) and SPA IT8050021 'Medio corso del fiume Sele-Persano' and also part of the Nature Reserve 'Foce Sele-Tanagro'. It extends over 110 ha, of which 70 ha flood. Ringing activity has been conducted since 2000.

Gela Lake, Sicily, Italy (37°01'17"N 14°20'30"E). Since 2000, the area has been protected as a nature reserve and an SPA. This is a retrodunal humid area, approximately 120 ha wide. Generally, it contains freshwater, but in some summers the water may become brackish. The vegetation along the bank is dominated by *Phragmites communis* and much less by *Typha*. Also, *Arundo donax* and *Tamarix* sp. are present, the latter with thick formations. The surrounding areas are heavily cultivated, especially with greenhouses (Triolo *et al.* 2011). Ringing activity allowed sample collection between 2001 and 2014.

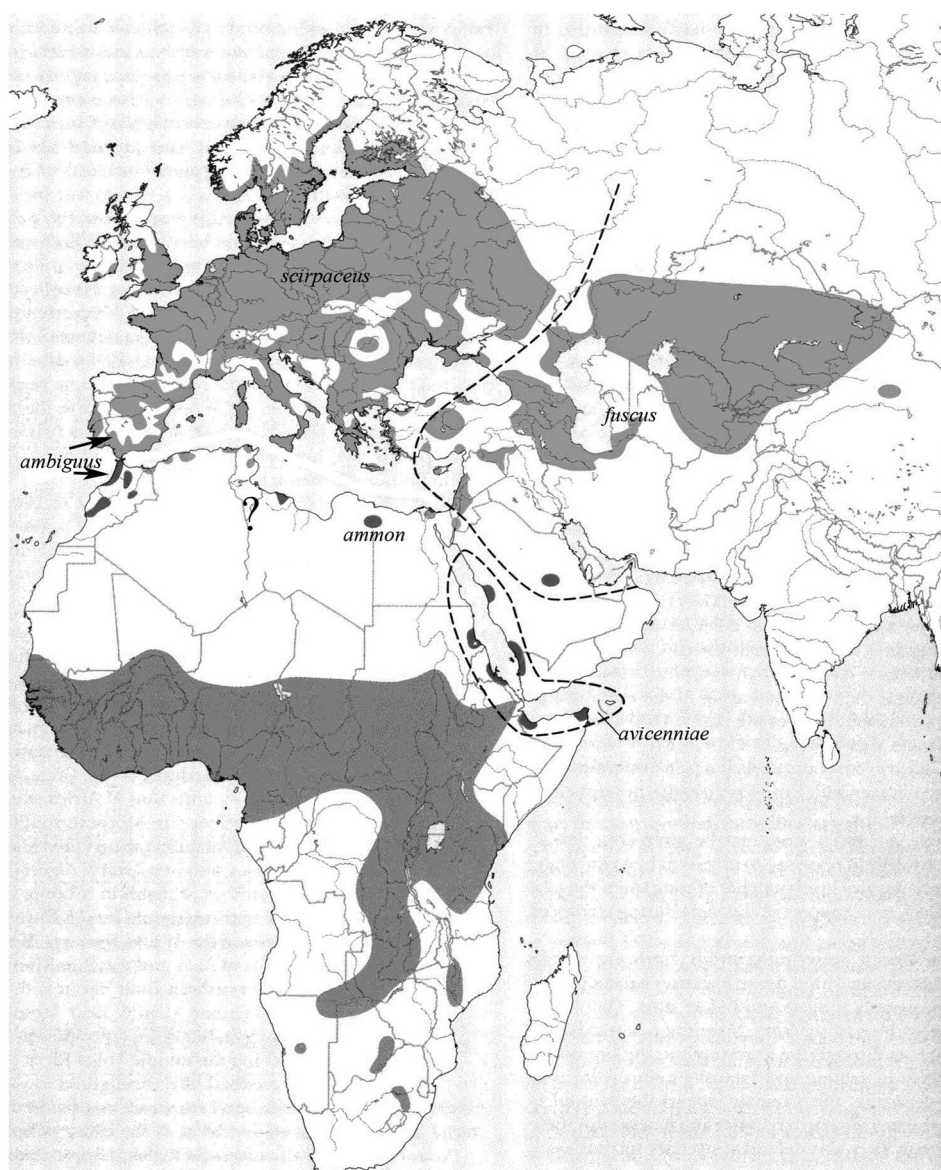


Figure 1. Distribution of main populations of Eurasian Reed Warbler subspecies (light grey = breeding areas, dark grey = wintering area).

Vendicari, Sicily, Italy (37°47'30"N 15°05'29"E). Since 1984 the area has been a nature reserve (*Oasi faunistica di Vendicari*) and also an SPA. The area is part of a complex of retrodunal marshes with salt waters, approximately 150 ha wide with most vegetation along the banks dominated by *Phragmites communis*. Ringing activity has been carried out since 2003.

Pergusa Lake, Enna, Sicily, Italy (37°30'50"N 14°18'18"E). The site in the centre of Sicily was established as Special Nature Reserve in 1995 (SPA). Pergusa is an endorheic lake 667 m above sea level (ASL) with a perimeter of 4.5 km and is surrounded by hills between 750 and 911 m ASL. The vegetation along the bank is dominated by

Phragmites australis, where many pairs of Eurasian Reed Warblers breed regularly. Due to a severe drought drastically reducing the lake size, the warbler population risked extinction at the end of the 1900s. At the beginning of the 2000s, the lake filled again resulting in increased reed vegetation and in turn, a renewed population of reed warblers. Ringing activity allowed sample collection between 2003 and 2019.

Ustica Island, south Tyrrhenian Sea, Sicily (38°42'12"N 13°10'07"E). The area is a nature reserve and an SPA. Its habitat is composed of a wide Mediterranean maquis dominated by *Pistacia lentiscus*. Since 1999, ringing activity allowed sample collection within the 'Small Islands' project.

Ringling and biometrics

We analysed data collected during the ringing activities carried out between 1993 and 2018 at the study sites. Specifically, we determined inter-annual fidelity to the breeding site and mist-net transects when we recaptured individuals (both adults and juveniles) ringed in June–July in Mirandola, Molentargius, Persano, Gela and Pergusa lakes. Birds were captured using four-shelf mist-nets then the following biometrics were recorded: wing chord (mm), length of Primary 8 (P8, primaries numbered in descending order, mm), tarsus length (mm) and body mass (g). All lengths were measured to the nearest 0.5 mm, except for tarsus which was measured to 0.01 mm, following Spencer (1972). The wing and tarsus measurements are taken during bird ringing and are considered to have high repeatability both within and between measurers (Alatalo & Lundberg 1986) which, in our case, also means within and between ringing sites. Despite there being no global consensus on the best practice to measure the tarsus (Caravaggi *et al.* 2021), in Italy all the ringers adopt the method standardized for contributing to the EURING scheme. Each individual bird was aged using the EURING age codes, namely ‘3’ for birds in their first calendar year, as indicated by a partial moult limited to a small part of the body plumage at most, or age ‘4’ for birds in at least their second calendar year, as indicated by post-breeding moult which was either partial, to be completed in the African wintering grounds, or, in very rare cases, complete (R. Ientile pers. obs.). Males and females were sexed only during the breeding season, when a cloacal protuberance or brood patch, respectively, are present. To determine biometrics of breeding individuals, our sample size included sexed adults captured only in May at the Persano Oasis and in June–July in all other sites. We excluded individuals captured in other months to avoid analysing migratory individuals, which could already be present in August. At Pergusa and Gela lakes, measurements were taken across months from breeding and non-breeding periods. On Ustica Island, measurements were from migratory unsexed and unaged individuals during the migration period (April–May). These data were compared to the unified dataset from the other six populations including both males and females.

Molecular analysis

DNA was extracted from feathers, using the procedure described in Tagliavia *et al.* (2016). We proceeded by comparing three samples from Pergusa (Sicily), and

three from Molentargius (Sardinia) with corresponding sequences from selected Mediterranean areas found in GenBank.

We amplified the mitochondrial cytochrome b (cyt b) gene, as indicated by Ilahiane *et al.* (2020) and following the protocol described in Olsson *et al.* (2016) with some modifications. The locus was chosen based on the existing literature on the studied species, whose sequences are available in public databases (i.e. GenBank). This allows for robust comparison of the obtained sequence data with those from previous studies, which include samples from many different geographical areas.

We modified the primers to improve performance without affecting the target specificity (L14995s: 5'-CAGGAAACAGCTATGACCAACATCTCAGCATGATGAAACTTCG-3' and H16065s: 5'-AAGGGTGGAGTCTTCAGTTTTTGGTTACAAGAC-3'). Polymerase chain reactions (PCR) were performed in a final volume of 50 µl using Phusion High-Fidelity DNA Polymerase (Thermo Scientific) in HF buffer, following the manufacturer's directions for reaction and cycling condition settings. The amplicons were verified by agarose gel electrophoresis on gel agarose using GelRed (Biotium) as DNA dye. PCR products were precipitated with 2.5M ammonium acetate plus 2.5 volumes of ethanol and then suspended into molecular biology-grade double distilled water and sequenced by Macrogen Europe (the Netherlands). High-quality sequences were manually checked and edited, then trimmed and aligned using either Clustal Omega or UGENE. Bioinformatic analyses were restricted to the sequence region corresponding to that of sequences available in GenBank. In particular, after multiple sequence alignments, sequences were clustered by the neighbour-joining (NJ) method using the software default settings, and a distance matrix was generated.

Statistical analysis

Morphometric data were normalized by means of a Box–Cox transformation prior to each analysis. Morphometric differences among populations were analysed using a one-way analysis of variance (ANOVA) where each of the biometric measures was treated as a dependent variable and study site as a factor with seven levels. To assess differences in the two Sicilian sites of Gela and Pergusa in the different months, a general linear model (GLM) was applied, in which the wing length was treated as a dependent variable and site and month as fixed factors. All significant differences were further analysed with

Tukey post hoc tests. We used Minitab software for all statistical analyses. We conducted a temporal analysis (from June to September, ANOVA with repeated measures followed by F statistics for significance assessment) of morphometric traits recorded in Pergusa and Gela to detect any possible difference between breeding and migratory individuals.

Results

Site fidelity

At Mirandola, 6692 birds were ringed (1978 adults aged '4', 4714 juveniles aged '3'). Of these, 840 (12.5%) were retrapped in the following years: 392 (19.8%) adults and 448 (10.1%) juveniles. At Molentargius, 866 birds were ringed (537 adults and 329 juveniles) and 77 (14.3%) adults and 6 (1.8%) juveniles were recaptured in the following years. At Persano, 221 birds were ringed, and 7 (3.2%) were retrapped in following years, all adults. At Gela Lake, 643 birds were ringed and 30 (4.7%) were recaptured in following years: 24 (80%) adults and 6 (20%) juveniles. Interestingly, a large influx of migratory birds was present in summer (August–September); among them, 11 were already ringed and came from other sites in Italy (Emilia-Romagna, Latium and Sicily) and Croatia. At Pergusa Lake, 1553 birds were ringed (478 adults and 1075 young), and 26 (5.4%) adults and 31 (2.9%) juveniles were recaptured in the following years. Out of all recaptures, 84.2% of adults and 68.4% of young were found in the same transect where they were originally trapped.

Biometrics

Body mass was highly variable throughout the seasons, so was not included in the analysis. Mean wing length and P8 length did not differ significantly between breeding males and females in the Italian peninsula (Table S3, Figures 2 and 3), but they were significantly different from Sicilian and Sardinian ones. Wing length of females from Molentargius (Sardinia) and Pergusa (Sicily) was significantly smaller than females from other Sicilian sites (Figure 3), although differences in the P8 length were not significant (Figure 4). There were no significant geographical differences in the tarsus length (Figure 4). Wing length measurements of unsexed individuals captured on Ustica Island were larger than male and female individuals captured at Sicilian and Sardinian breeding sites but did not differ significantly from those of peninsular Italy (Persano; one-way ANOVA, $F_{5, 2289} = 93.55$, $P < 0.01$).

At Gela Lake (Sicily), mean values of P8 length were fairly consistent during July but gradually increased in August and September, with the exception of a very low value, even lower than in July, recorded in the second period of August. Variation in P8 length for 10-day periods was significant for pairwise comparisons (one-way ANOVA: $F_{6, 1095} = 11.91$, $P < 0.01$; Tukey's HSD test, the majority $P < 0.05$). The temporal analysis of wing length at Pergusa and Gela (Sicily) over the breeding and migration months showed significant differences between the two sites ($F_{1,810} = 67.44$, $P < 0.01$) and between months ($F_{6,810} = 4.87$, $P < 0.01$). The site of Gela in August–September holds many migrating individuals coming from central Europe, as is evident from the significant increase in the mean wing length over the two previous months. At Pergusa Lake (only 52 km away from Gela), however, the mean wing length did not change significantly with month from June through to September, and showed only a small increase when migrant birds stopped at the site (September), suggesting that the site is not important for stopover on migration (Figure 5).

Genetic analyses

Sequence analyses showed only negligible differences among Sicilian and Sardinian samples, as well as between them and others coming from the distribution area of the nominate subspecies of the Eurasian Reed Warbler *A. s. scirpaceus*. Moreover, no new or unique haplotypes were detected (Figure 6, Table S4). Such a scenario made the genetic data set unsuitable for further phylogenetic analyses.

Discussion

Our genetic data confirmed the findings of Ilahiane *et al.* (2020), that there are no genetic differences among our study populations of Eurasian Reed Warblers in Italy, and they are similar to other European populations. Note that our genetic results refer to the analysis of a single mitochondrial locus. While this choice was to enable comparison with other studies (Ilahiane *et al.* 2021, Ollson *et al.* 2005), the possibility does remain that analyzing further loci, including nuclear loci, might result in a different outcome. However, to be properly informative, such an analysis would require the availability of sequences of other loci in public databases but these are currently unavailable. In fact, analysing cyt-b was the only way for a full and robust comparison of the sequence data obtained in our

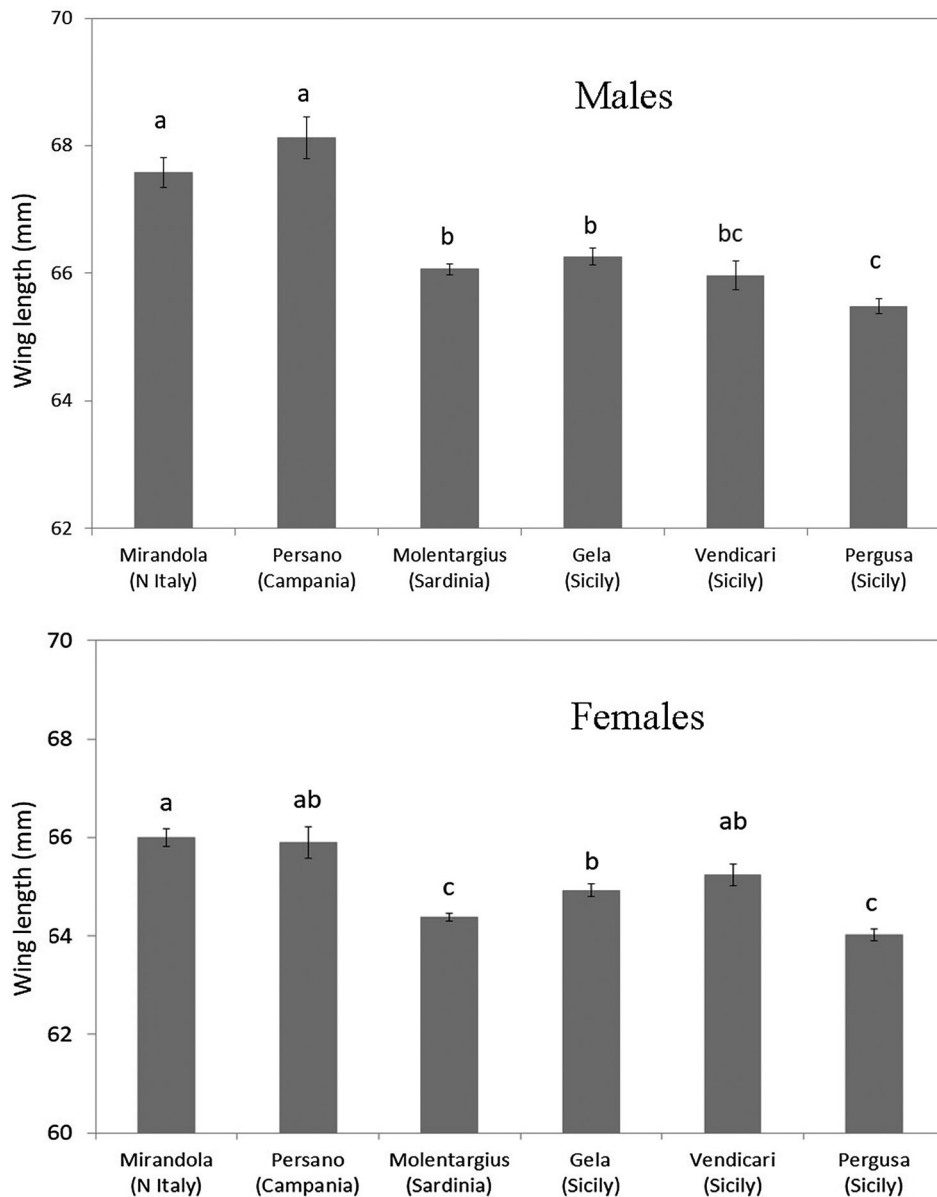


Figure 2. Wing length \pm SD (mm) of male and female Eurasian Reed Warblers and significant differences among populations from the Italian peninsula, Sardinia and Sicily. Letters on columns indicate the statistical differences among the different populations (one-way ANOVA followed by Tukey post hoc test; males: $F_{5,693} = 24.48$, $P < 0.01$; females: $F_{5,793} = 29.18$, $P < 0.01$).

study with those from previous ones, which included samples from many different geographical areas.

We did, however, find significant differences in morphometric traits between Italian breeding populations of Eurasian Reed Warblers. Sicilian and Sardinian breeding populations were smaller than those breeding on the Italian mainland. Migratory individuals stopping over the small island of Ustica in spring were larger than the other Sicilian breeders but more similar than breeders on the Italian mainland.

Interestingly, Sicilian and Sardinian breeding populations showed biometrics similar to those of the subspecies *A. s. ambiguus*, which breeds in the Iberian

Peninsula and Morocco. Nevertheless, the subspecies *ambiguus* has a more rounded wing than *A. s. scirpaceus*, completes the moult before migrating to sub-Saharan wintering areas, and in some cases may overwinter in Morocco, while Sicilian and Sardinian populations moult after their autumnal migration to the sub-Saharan wintering areas. Only exceptionally, in Sardinia and Sicily, a complete post-breeding moult in Eurasian Reed Warblers has been recorded (Nissardi & Zucca 2001, R. Ientile pers. obs.)

During our long-term study, we did not find any individual with plumage traits consistent with the subspecies *A. s. fuscus*: slightly greyer, and more olive-

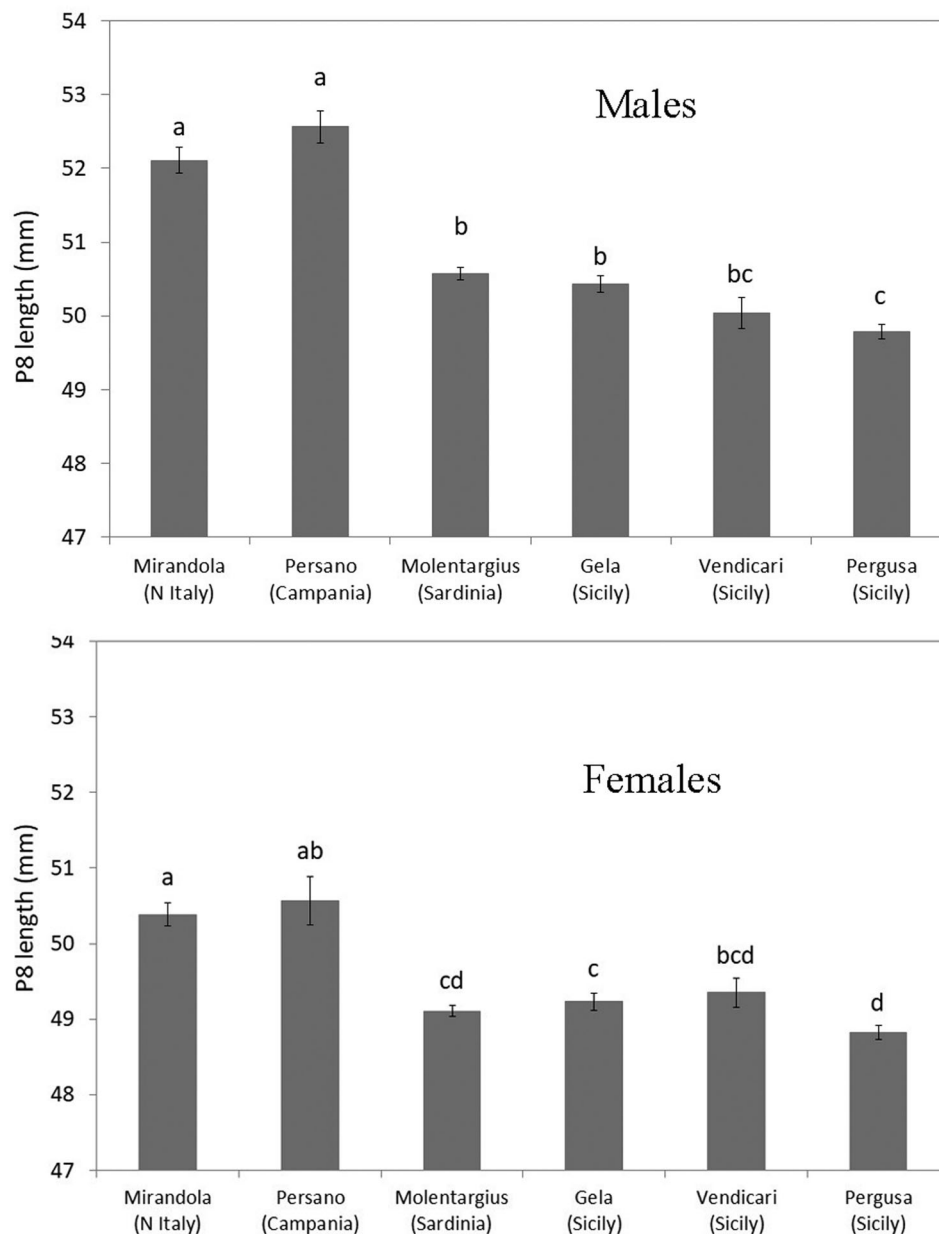


Figure 3. Length of 8th primary \pm SD (mm) of male and female Eurasian Reed Warblers and significant differences among populations from the Italian peninsula, Sardinia and Sicily. Letters on columns indicate the statistical differences among the different populations (one-way ANOVA followed by Tukey post hoc test; males: $F_{5,749} = 37.45$, $P < 0.01$; females: $F_{5,844} = 21.86$, $P < 0.01$).

brown than nominate, less tinged rufous above and slighter whiter, less creamy-buff, on the sides of the breast and on the flanks; in worn plumage, paler than nominate. We may conclude, therefore, that Sicilian and Sardinian breeding populations belong to the subspecies *scirpaceus*; however, this *fuscus* has been found in central Europe during migration (Arbabi *et al.* 2014). These results are consistent with breeding Eurasian Reed Warblers in Sicily and Sardinia displaying polyphenism, a morphological plasticity that can be attributed to external influences and therefore, which takes place in the absence of genetic

differences (Fusco & Minelli 2010, Pigliucci 2010). Very likely it is dependent on adult philopatry (4.7%–19.8%), which is a prerequisite of an isolation process.

We do not know if different populations of Eurasian Reed Warblers have different wintering areas (we obtained only one winter recovery in the Central African Republic of one breeding individual from Pergusa), but morphometric differences reported in this paper demonstrate that there is an isolation in progress, likely due to absence or scarcity of gene flow between different populations. Statistical differences detected between populations breeding at Gela and

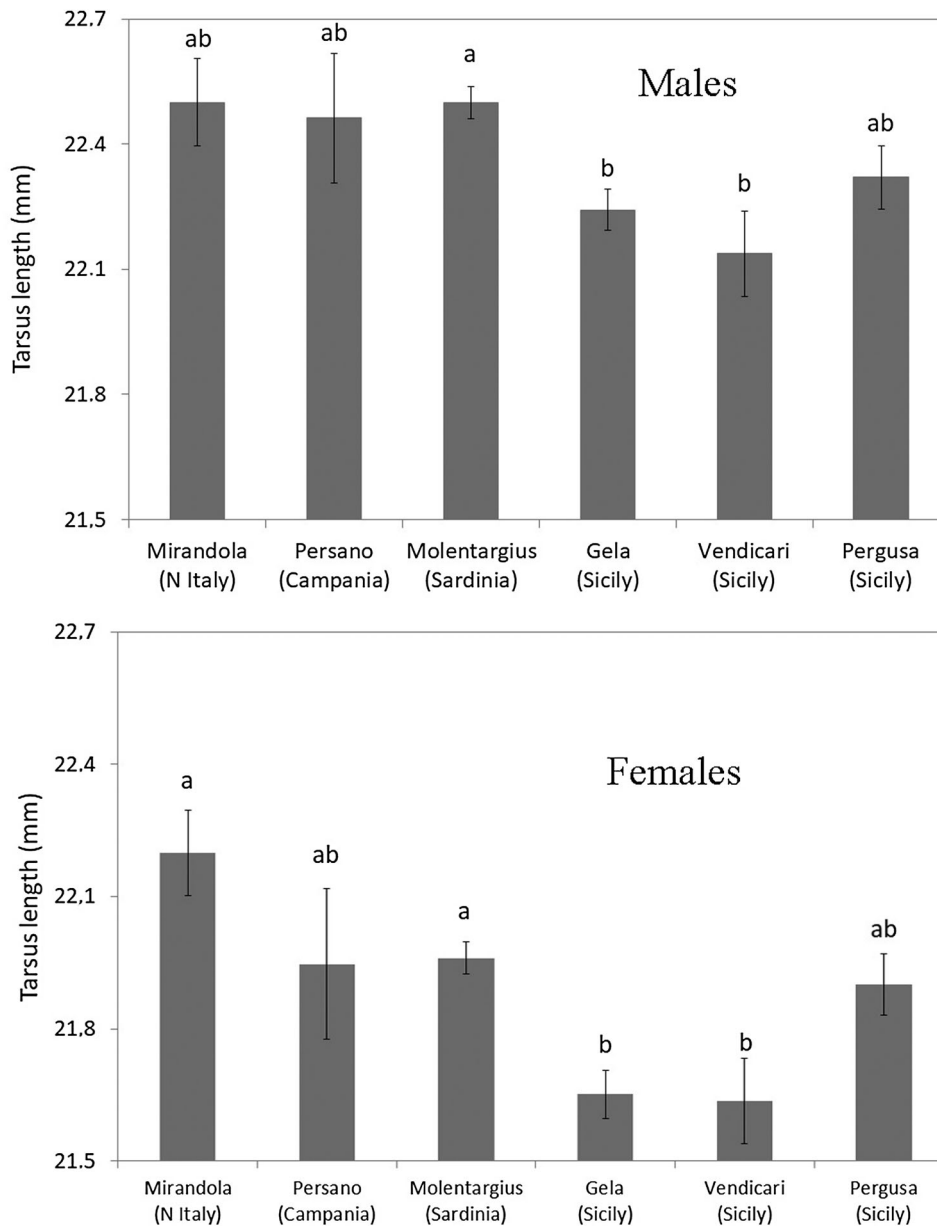


Figure 4. Tarsus length \pm SD (mm) of male and female Eurasian Reed Warblers and significant differences among populations from Italian peninsula, Sardinia and Sicily. Letters on columns indicate the statistical differences among the different populations (one-way ANOVA followed by Tukey post hoc test; males: $F_{5,546} = 4.98$, $P < 0.01$; females: $F_{5,577} = 8.85$, $P < 0.01$).

Pergusa, only 52 km away from each other, show that inside the same regional area, there is little or no gene flow between populations. Additionally, Kralj *et al.* (2010) found differences in the wing length and indications of limited gene flow between coastal and inland breeding populations of Eurasian Reed Warblers in Croatia. In particular, Pergusa is located at 667 m ASL and is scarcely used by autumn migrants, while Gela is on the coast and gets an annual influx of migrants in the late summer and autumn. Furthermore, in the 1990s the lake of Pergusa completely dried up, and the population of Eurasian Reed Warblers was close to local extinction. Most

likely a bottleneck has occurred and when, in the first years of 2000s, the water filled the lake again, the reed vegetation could support a new population of birds starting from the few surviving individuals, which likely were smaller in size than the average breeders in Sicily. This is only a hypothesis, probably reinforced by the site fidelity of the Pergusa population (5.4% of ringed adults recovered in the following years).

Previously, Cramp (1992) and Kennerley & Pearson (2010) highlighted the geographical variation in the measurements of Eurasian Reed Warblers in the Palearctic region. Present data show that, in contrast to the proposal by Procházka *et al.* (2011), the species

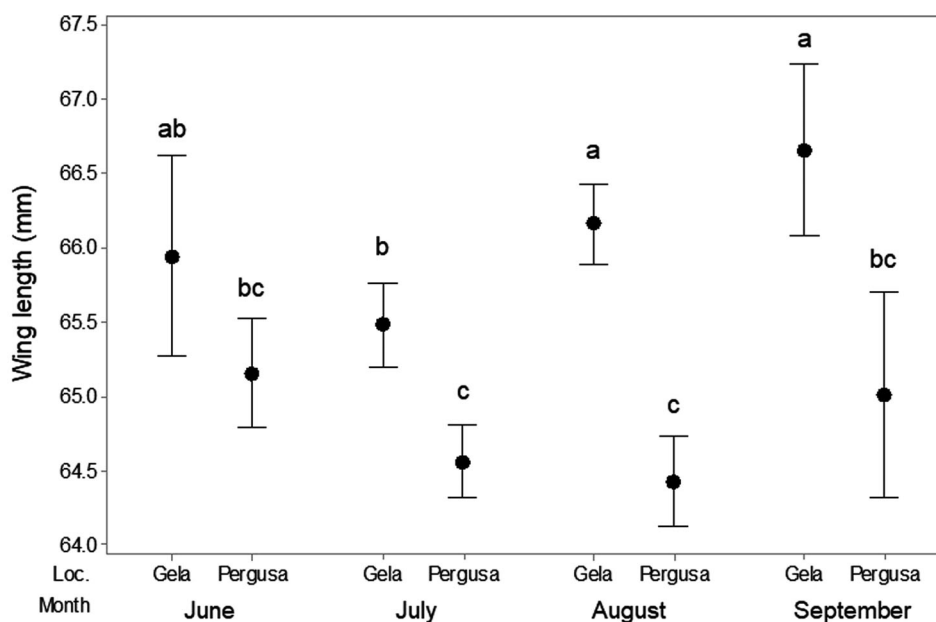


Figure 5. Mean wing length \pm SD (mm) of Eurasian Reed Warblers from June to September in two Sicilian breeding sites, Gela and Pergusa lakes. Letters on columns indicate the statistical differences among the different populations and months (one-way ANOVA followed by Tukey post hoc test; males: $F_{6,810} = 4.87$, $P < 0.01$).

does not have high levels of gene flow, but they express a phenotypic plasticity, in the sense of a ‘trait- and environment-specific property of the genotype which

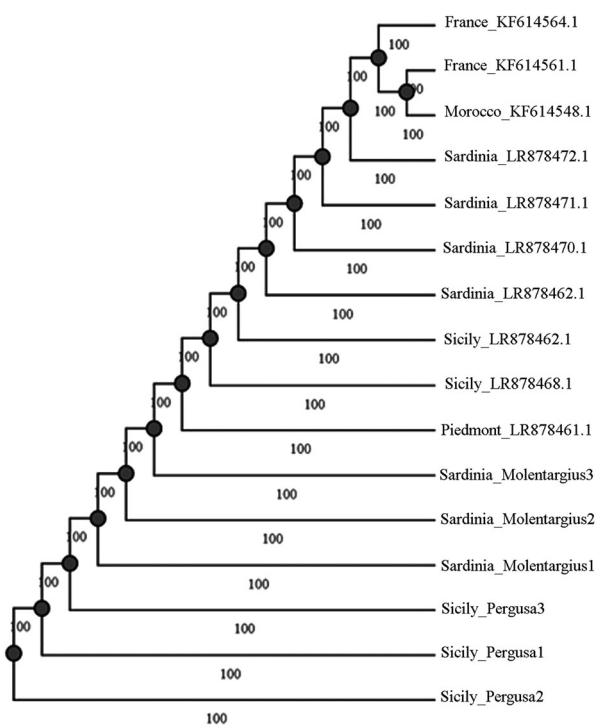


Figure 6. Neighbour-joining tree with bootstrap values created from the sequences obtained by mitochondrial cytochrome b (cyt b) gene in Sicily and Sardinia compared with corresponding sequences from selected Mediterranean areas found in GenBank. Details on Percent Identity Matrix and GenBank Access Numbers are provided in online Table S4.

may or may not be advantageous and may or may not be the result of adaptive evolution’ (Pigliucci 2010).

Sedentary passerine species are known to vary clinically in size from north to south, but also most trans-Saharan species have differentiated populations, recognizable as geographic subspecies. Following Vaurie (1959), at least 33/44 (75%) passerine species migrating from tropical Africa to Europe were subdivided into subspecies; this means that they were recognized as populations living in different parts of the geographic breeding range that generally do not meet. However, there are many cases of overlapping distributions and the consequent gene flow between different populations, such as the case of the wagtails *Motacilla* (Harris *et al.* 2018). We do not know any case of trans-Saharan passerines where their clinal variation is described. However, in most cases, subspecies are only the extremes of a gradual latitudinal variation, with larger size specimens in the north and smaller specimens in the south, such as in the case of the Northern Wheatear *Oenanthe oenanthe* (Svensson 1992).

Interestingly, however, Eurasian Reed Warblers in Italy, even if variable, do not vary clinically, but populations elsewhere in Europe are biometrically similar. Overall, small-sized Eurasian Reed Warblers living in Sicily and Sardinia could be the result of a long isolation, due to scarce gene flow and site fidelity, indicating that most likely they are not the extremes of a clinal variation, but the product of isolated populations.

Finally, our results show that even if genetic studies are useful to understand differences between populations of several bird species, the study of phenotypic traits is always indispensable. Biodiversity is not only related to the number of species but also their variability in space and the evolutionary processes that contribute to that variability. In other words, investigations on phenotypic traits still remain a crucial tool for biodiversity conservation although further investigations should aim to verify whether the use of a single gene provides a robust protocol for this type of genetic data investigation.




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Disclosure statement

No potential conflict of interest was reported by the author(s).

ORCID

Gabriella Lo Verde  <http://orcid.org/0000-0001-8207-1416>
Daniela Campobello  <http://orcid.org/0000-0003-4094-9395>
Bruno Massa  <http://orcid.org/0000-0003-2127-0715>

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Supplemental Materials

Morphometric versus genetic variation in the Eurasian reed warbler *Acrocephalus scirpaceus*, in Italy

Renzo Ientile¹, Marcello Tagliavia², Natalino Cuti³, Rosa Termine⁴, Carlo Giannella⁵, Sergio Nissardi⁶, Carla Zucca⁶, Vincenzo Cavaliere⁷, Gabriella Lo Verde⁸, Daniela Campobello^{9*}, Bruno Massa^{3,8}

¹Department of Biological, Geological and Environmental Sciences, University of Catania, via Androne 81, 95124 Catania (Italy); ²IRIB-CNR, via La Malfa 153, 90146 Palermo (Italy); ³Stazione Ornitologica 'Aegithalos' (Italy); ⁴Laboratory of Health and Environmental Engineering, University of Enna 'Kore', 94100 Enna (Italy); ⁵Stazione Ornitologica Modenese 'Il Pettazzurro', via Montirone, 41037 Mirandola (Modena, Italy); ⁶Anthus snc, Via Luigi Canepa 22, 09129 Cagliari (Italy); ⁷Via Amendola 2h, 80055 Portici (Napoli, Italy); ⁸Department of Agriculture, Food and Forest Sciences, University of Palermo, Viale delle Scienze Bd 5A, 90128 Palermo (Italy); ⁹Department of Biological, Chemical and Pharmaceutical Sciences and Technologies, University of Palermo, Via Archirafi 18, 90123 Palermo (Italy); *corresponding author daniela.campobello@unipa.it

Subspecies	Distribution	References
<i>Acrocephalus scirpaceus</i>		
<i>scirpaceus</i> (Hermann, 1804)	Europe, north of Scandinavia and east of western Russia, Ukraine, Crimea, western Asia Minor, north-west Africa (?)	Kennerley & Pearson 2010
<i>ambiguus</i> (Brehm, 1857)	South France, Spain, south of Catalonia, Morocco, probably Algeria and Tunisia	Jiguet <i>et al.</i> 2010, Procházka <i>et al.</i> 2011, Olsson <i>et al.</i> 2016; but see Winkler <i>et al.</i> 2013 and Gill <i>et al.</i> 2020
<i>fuscus</i> (Hemprich and Ehrenberg, 1833)	Middle East, Cyprus, Levant, northern Caspian area, east of Kazakhstan and extreme north-west China, south of Iran and north-west Afghanistan, and presumably Egypt	Goodman & Meininger 1989, Cramp 1992, Svensson 1992, Kennerley & Pearson 2010
<i>ammon</i> Hering, Winkler & Steinheimer, 2016	Egypt/Libya, at the border with northern Sahara	Hering <i>et al.</i> 2016
<i>avicenniae</i> Ash, Pearson, Nikolaus & Colston, 1989	Northern Somalia, Eritrea, northern Yemen and western Saudi Arabia	Kennerley & Pearson 2010, Olsson <i>et al.</i> 2016, Babbington <i>et al.</i> 2019; but see del Hoyo <i>et al.</i> 2006
<i>Acrocephalus baeticatus</i>		
<i>baeticatus</i> (Vieillot, 1817)	From South, Central and South Africa to Zimbabwe and Botswana	Kennerley & Pearson 2010
<i>minor</i> Lynes, 1923	Mauritania and Lake Chad	Olsson <i>et al.</i> 2016
<i>hallae</i> White, 1960	From West South Africa to Namibia, North-West Botswana, South Angola and South-West Zambia	Kennerley & Pearson 2010
<i>guiersi</i> Colston & Morel, 1984	Northern Senegal and perhaps Mali	Kennerley & Pearson 2010; but see Gill <i>et al.</i> 2020
<i>hopsoni</i> Fry, Williamson & Ferguson-Lees, 1973	Lake Chad	Kennerley & Pearson 2010; but see Gill <i>et al.</i> 2020
<i>suhaelicus</i> Grote, 1926	From coastal Tanzania to East Mozambique and East South Africa	Kennerley & Pearson 2010
<i>cinnamomeus</i> Reichenow, 1908	South, West and East Africa in South Zambia, Malawi and inland Mozambique, north of South Sudan, South Somalia and Ethiopia and west of Nigeria and North Niger, Cameroon, Gabon, Chad and Congo	Leisler <i>et al.</i> 1997, Kennerley & Pearson 2010, Olsson <i>et al.</i> 2016

Table S1. Distribution of all subspecies described within the *Acrocephalus scirpaceus* and *A. baeticatus* species.

	Wing chord	Tarsus length	Reference
<i>A. s. scirpaceus</i>			
<u>North-West Europe</u>			
Males Age 4	66.8 ± 1.36 (n = 22)	23.1 ± 0.71 (n = 38)	Cramp 1992
Females Age 4	66.0 ± 2.07 (n = 29)	22.8 ± 0.80 (n = 29)	Cramp 1992
<u>North-West Europe</u>			
Males Age 4	66.7 (range: 64.0-69.0, n = 43)	22.9 (range: 21.5-24.5, n = 38)	Kennerley & Pearson 2010
Females Age 4	65.8 (range: 63.0-69.0, n = 28)	22.6 (range: 21.5-24.0, n = 37)	Kennerley & Pearson 2010
<i>A. s. ambiguus</i>			
Unsexed	60.3 (range: 55-66, n = 77)	21.9 (range: 20.2-23.7, n = 77)	Jiguet <i>et al.</i> 2010
Unsexed	63.7 ± 1.9 (range: 59-67.5, n = 76)	22.3 ± 0.8 (range: 19.1-23.8, n = 76)	Olsson <i>et al.</i> 2016
<i>A. s. ammon</i>			
Unsexed	52.3-56.3 (n = 2)	20.0-20.8 (n = 2)	Hering <i>et al.</i> 2016

Table S2. Biometrics (mean ± S.E., mm) of Eurasian Reed Warblers breeding across the Palaearctic Region.

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	Wing Length Mean \pm SE (min – max; sample size)	P8 Length Mean \pm SE (min – max; sample size)	Tarsus Length Mean \pm SE (min – max; sample size)
<i>MALES</i>			
Mirandola (N Italy)	67.58 \pm 0.23 (61.0-70.0; n = 46)	52.11 \pm 0.17 (49.0-54.5; n = 46)	22.50 \pm 0.10 (21.0-24.3; n = 45)
Molentargius (Sardinia)	66.06 \pm 0.09 (61.5-70.0; n = 268)	50.58 \pm 0.08 (46.5-54.0; n = 268)	22.50 \pm 0.04 (20.7-24.3; n = 232)
Persano (Campania)	68.12 \pm 0.32 (65.5-71.5; n = 24)	52.56 \pm 0.22 (50.5-54.5; n = 24)	22.46 \pm 0.15 (21.0-24.2; n = 24)
Gela (Sicily)	66.26 \pm 0.13 (60.0-70.0; n = 151)	50.43 \pm 0.11 (46.5-53.5; n = 156)	22.24 \pm 0.05 (20.6-23.7; n = 136)
Vendicari (Sicily)	65.96 \pm 0.23 (63.0-68.5; n = 39)	50.04 \pm 0.21 (46.5-53.0; n = 40)	22.14 \pm 0.10 (20.6-23.5; n = 40)
Pergusa (Sicily)	65.48 \pm 0.12 (61.0-69.5; n = 171)	49.79 \pm 0.10 (46.0-53.5; n = 221)	22.32 \pm 0.07 (20.5-23.6; n = 75)
<i>FEMALES</i>			
Mirandola (N Italy)	65.99 \pm 0.179 (62.0-71.0; n = 104)	50.38 \pm 0.15 (47.0-54.5; n = 104)	22.20 \pm 0.10 (20.0-28.0; n = 104)
Molentargius (Sardinia)	64.39 \pm 0.08 (60.5-68.5; n = 326)	49.10 \pm 0.07 (46.0-53.0; n = 328)	21.96 \pm 0.036 (20.1-23.5; n = 254)
Persano (Campania)	65.90 \pm 0.3170 (64.0-68.0; n = 15)	50.57 \pm 0.32 (48.0-52.0; n = 15)	21.95 \pm 0.17 (21.0-23.6; n = 15)
Gela (Sicily)	64.93 \pm 0.13 (60.5-69.0; n = 129)	49.23 \pm 0.11 (46.5-52.5; n = 134)	21.65 \pm 0.055 (19.9-23.2; n = 120)
Vendicari (Sicily)	65.24 \pm 0.22 (63.0-68.0; n = 35)	49.35 \pm 0.19 (47.5-51.5; n = 37)	21.63 \pm 0.097 (20.8-23.2; n = 37)
Pergusa (Sicily)	64.38 \pm 0.07 (60.5-68.5; n = 327)	49.10 \pm 0.07 (46.0-53.0; n = 329)	21.96 \pm 0.036 (20.1-23.5; n = 255)
<i>UNSEXED</i>			
Molentargius (Sardinia)	65.50 \pm 0.11 (62.0-69.5; n = 213)	49.98 \pm 0.10 (46.5-53.5; n = 215)	22.36 \pm 0.05 (20.8-23.6; n = 159)
Persano (Campania)	67.396 \pm 0.12 (63.0-74.5; n = 211)	51.72 \pm 0.10 (47.5-56.5; n = 226)	22.20 \pm 0.04 (20.2-24.2; n = 227)
Gela (Sicily)	66.36 \pm 0.06 (60.0-72.5; n = 1028)	50.34 \pm 0.05 (43.0-58.0; n = 1105)	22.01 \pm 0.02 (19.8-24.7; n = 1067)
Vendicari (Sicily)	66.18 \pm 0.14 (60.0-71.0; n = 177)	50.19 \pm 0.12 (45.5-54.5; n = 182)	22.06 \pm 0.05 (19.8-23.8; n = 179)
Pergusa (Sicily)	64.73 \pm 0.08 (60.0-72.0; n = 471)	49.28 \pm 0.07 (45.0-55.0; n = 554)	22.03 \pm 0.048 (20.5-24.0; n = 181)
Ustica (Sicily)	67.05 \pm 0.15 (58.0-74.5; n = 195)	51.11 \pm 0.12 (47.0-56.5; n = 214)	22.50 \pm 0.10 (20.8-24.1; n = 63)

Table S3. Biometrics (in mm) of Eurasian Reed Warblers breeding or migrating (Ustica Is., Age 4) in different Italian areas.

	Morocco KF614548.1	France KF614561.1	Sicily 3	Sardinia 2	Sardinia 3	Sicily LR878468.1	Sicily 2	Sardinia LR878470.1	Sardinia LR878471.1	Sardinia 1	Sicily 1	Sicily LR878469.1	Piedmont LR878461.1	Sardinia LR878462.1	France KF614564.1
Sardinia LR878472.1	99.1	99.2	99.1	99.1	99.1	99.1	99.1	99.3	99.3	99.3	99.3	99.3	99.4	99.4	99.4
Morocco KF614548.1	100.0	99.9	99.5	99.5	99.5	99.5	99.5	99.5	99.5	99.5	99.5	99.5	99.6	99.6	99.6
France KF614561.1	99.9	100.0	99.6	99.6	99.6	99.6	99.6	99.6	99.6	99.6	99.6	99.6	99.8	99.8	99.8
Sicily 3	99.5	99.6	100.0	100.0	100.0	100.0	99.5	99.5	99.5	99.5	99.5	99.5	99.6	99.6	99.6
Sardinia 2	99.5	99.6	100.0	100.0	100.0	100.0	99.5	99.5	99.5	99.5	99.5	99.5	99.6	99.6	99.6
Sardinia 3	99.5	99.6	100.0	100.0	100.0	100.0	99.5	99.5	99.5	99.5	99.5	99.5	99.6	99.6	99.6
Sicily LR878468.1	99.5	99.6	100.0	100.0	100.0	100.0	99.5	99.5	99.5	99.5	99.5	99.5	99.6	99.6	99.6
Sicily 2	99.5	99.6	99.5	99.5	99.5	99.5	100.0	99.5	99.5	99.8	99.5	99.5	99.6	99.6	99.6
Sardinia LR878470.1	99.5	99.6	99.5	99.5	99.5	99.5	99.5	100.0	100.0	99.8	99.8	99.8	99.9	99.9	99.9
Sardinia 1	99.5	99.6	99.5	99.5	99.5	99.5	99.8	99.8	99.8	100.0	99.8	99.8	99.9	99.9	99.9
Sicily 1	99.5	99.6	99.5	99.5	99.5	99.5	99.5	99.8	99.8	99.8	100.0	100.0	99.9	99.9	99.9
Sicily LR878469.1	99.5	99.6	99.5	99.5	99.5	99.5	99.5	99.8	99.8	99.8	100.0	100.0	99.9	99.9	99.9
Piedmont LR878461.1	99.6	99.8	99.6	99.6	99.6	99.6	99.6	99.9	99.9	99.9	99.9	99.9	100.0	100.0	100.0
Sardinia LR878462.1	99.6	99.8	99.6	99.6	99.6	99.6	99.6	99.9	99.9	99.9	99.9	99.9	100.0	100.0	100.0
France KF614564.1	99.6	99.8	99.6	99.6	99.6	99.6	99.6	99.9	99.9	99.9	99.9	99.9	100.0	100.0	100.0

Table S4. Percent Identity Matrix created from the sequences obtained by mitochondrial cytochrome b (cyt b) gene in Sicily (Pergusa) and Sardinia (Molentargius), both of them in bold, compared with corresponding sequences from some selected Mediterranean areas found in GenBank, whose Access Number is indicated below each label.