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Additional Information

**Does fragmentation of wetlands affect gene flow in sympatric *Acrocephalus* warblers with different migration strategies?**

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## 1 **Abstract**

2

3 Wetlands are naturally patchy habitats, but patchiness has been accentuated by the extensive  
4 wetlands loss due to human activities. In such a fragmented habitat, dispersal ability is especially  
5 important to maintain gene flow between populations. Here we studied population structure, genetic  
6 diversity and demographic history of Iberian and North African populations of two wetland  
7 passerines, the Eurasian reed warbler *Acrocephalus scirpaceus* and the moustached warbler  
8 *Acrocephalus melanopogon*. These species are closely related and sympatric in our study sites, but  
9 the reed warbler is a long-distance migrant and widespread bird while the moustached warbler is  
10 resident or migrates over short distances, and breeds across a more discontinuous range. Using  
11 microsatellites and mtDNA data, we found higher population differentiation in moustached than in  
12 reed warbler, indicating higher dispersal capability of the latter species. Our results also suggest that  
13 **the** sea limits dispersal in the moustached warbler. For both species, we found evidence of gene  
14 flow between study sites, indicating the capability of **compensating** for habitat fragmentation.  
15 However, in most cases gene flow was restricted, possibly because of the large distances between  
16 study sites (from c. 290 to 960 km) or breeding site fidelity. Haplotype diversity was higher for **the**  
17 reed warbler, possibly because of a more important contribution of dispersal from different  
18 populations and the higher population size. Studying demographic history, we obtained signs of  
19 postglacial population growth for both species, and evidences of a recent colonization or re-  
20 colonization of the Mallorca Island by the moustached warbler.

## 21 **Introduction**

22

23 Habitat fragmentation consists of reduction of the total habitat area and creation of separate patches  
24 from a wider continuous distribution (Frankham et al. 2010). The consequences of habitat  
25 fragmentation on the demographic and genetic structure of a natural population vary according to  
26 both landscape features (degree of habitat isolation, type of matrix between fragments) and species  
27 characteristics (population size and density, dispersal ability, stress tolerance; Matthysen et al. 1995,  
28 Newton 1998, Bohonak 1999, Desrochers et al. 1999, Galbusera et al. 2004). Wetlands are patchy  
29 habitats immersed in a terrestrial matrix, and this natural characteristic of discontinuity has been  
30 accentuated by the extensive habitat destruction caused by human activities (Finlayson et al. 1992,  
31 Van Vesseem et al. 1997, Paracuellos and Telleria 2004, Silva et al. 2007, Laurence 2010). In such a  
32 scattered habitat, the dispersal ability of a species is crucial to produce sufficient gene flow to  
33 reduce the impact of population fragmentation. Lack of gene flow between fragmented populations  
34 can lead to loss of genetic diversity (e.g. Kvist et al. 2011), inbreeding and consequently higher  
35 extinction risk compared to a continuous population (Frankham et al. 2010). Dispersal ability of  
36 birds is generally high (Koenig et al. 1996, Frankham et al. 2010), but detailed species-specific  
37 estimates are difficult to obtain. This is largely due to practical difficulties; studying dispersal over  
38 large distances requires large-scale marking schemes (Paradis et al. 1998, Hansson et al. 2002), and  
39 the use of satellite telemetry or geolocators is usually possible only on a reduced number of  
40 individuals due to the high costs. However, indirect genetic methods can provide useful information  
41 about gene flow and population differentiation.

42 Here we present new information of genetic diversity and population structure of two  
43 closely related and sympatric wetland passerines with different migration strategies, the moustached  
44 warbler *Acrocephalus melanopogon* and the Eurasian reed warbler *Acrocephalus scirpaceus*  
45 (hereafter reed warbler), based on both microsatellites and mitochondrial DNA data.

46           The moustached warbler is a polytypic species breeding across a discontinuous area ranging  
47 from SW Europe to Central Asia (Kennerley and Pearson 2010). Our study populations in Spain  
48 belong to the nominal subspecies *melanopogon*, which occupy the western part of the species range.  
49 These populations are mostly sedentary or migrate over short distances (Castany 2003, Castany and  
50 López 2006, Kennerley and Pearson 2010). In Spain, the distribution of **the** moustached warbler is  
51 discontinuous and most of the ~ 1000 breeding pairs are concentrated in a few marshlands along the  
52 Mediterranean Coast and **on** the Mallorca Island (Castany and López 2006). Until now, no  
53 information about genetic diversity and genetic population structure has been provided for this  
54 species.

55           The reed warbler is a common and largely widespread breeding bird in Europe. This species  
56 is a long-distance migrant wintering in sub-Saharan Africa (Kennerley and Pearson 2010); a  
57 migratory divide in Central Europe splits the European reed warbler population (subspecies  
58 *scirpaceus*) into SW- and SE-migrating populations (Procházka et al. 2008). Iberian reed warblers  
59 belong to the SW-migrating group, have more rounded and shorter wings (Cramp 1992, Peiró 2003)  
60 and winter more north than the other SW-migrating populations (Procházka et al. 2008). Procházka  
61 et al. (2011), using ten microsatellite loci, found no clear population structure and low genetic  
62 differentiation of reed warbler populations across Europe, indicating a high level of gene flow.  
63 Furthermore, the authors reported slight, but significant, differentiation of Iberian populations and  
64 suggested that they may have a different evolutionary history than other populations. North African  
65 reed warblers seem to be partly sedentary (Amezian et al. 2010, Kennerley and Pearson 2010) and  
66 birds breeding in Morocco differ from European reed warblers also in biometrics, coloration and  
67 moult strategy (Amezian et al. 2010, Jiguet et al. 2010). On this basis, some authors suggest that  
68 these birds could constitute a new taxon (Amezian et al. 2010, Jiguet et al. 2010), thus the status of  
69 this population is still unclear.

70           The aims of this study were: 1) to provide new information of genetic diversity, population

71 structure and population history of the study species in Iberia (and also in North Africa for the reed  
72 warbler), 2) to assess the possible effects of habitat fragmentation on the genetic diversity and  
73 structure of the study populations and 3) to compare the results obtained for the two species, taking  
74 into account especially their differences in migration strategy and population size.

75

76

## 77 **Methods**

78

### 79 **Sampling and DNA extraction**

80

81 We obtained blood samples from birds mist-netted during the breeding season of 2012-2013 at four  
82 study areas: the Pego-Oliva Natural Park (38°51' N - 0°03' W), S'Albufera de Mallorca (39°47' N -  
83 3°06' E) and Villafranca de los Caballeros (39°27' N - 3°19' W) in Spain and Larache (35°08' N -  
84 6°05' W) in Morocco (Fig. 1). At the last site, the breeding population of moustached warbler is  
85 small and we captured no individuals. Blood drops (5-15 µl) were obtained from the brachial vein  
86 and stored in 96% ethanol. Overall, we sampled 54 moustached warblers and 68 reed warblers. We  
87 extracted DNA using UltraClean™ Blood Spin™ Kit (MoBio Laboratories) according to the  
88 protocol.

89

### 90 **DNA amplification**

91

#### 92 **Microsatellites**

93

94 We amplified 16 polymorphic microsatellite loci from reed warbler DNA samples: Ase25, Ase34,  
95 Ase37, Ase48, Ase58 (Richardson et al. 2000, *Acrocephalus sechellensis*), Pocc2 (Bensch et al.

96 1997, *Phylloscopus occipitalis*), Ppi2 (Martínez et al. 1999, *Pica pica*), Aar4, Aar5 (Hansson et al.  
97 2000, *Acrocephalus arundinaceus*), FhU2 (Ellegren 1992, *Ficedula hypoleuca*), Pca3 (Dawson et  
98 al. 2000, *Cyanistes caeruleus*), Pdo $\mu$ 1 (Neumann and Wetton 1996, *Passer domesticus*), Cu $\mu$ 28  
99 (Gibbs et al. 1999, *Catharus ustulatus*), Gf05 (Petren 1998, *Geospiza fortis*), Pdo5 (Griffith et al.  
100 1999, *P. domesticus*) and ZL54 (Frentiu et al. 2003, *Zosterops lateralis*). In the case of the  
101 moustached warbler, we successfully amplified eight polymorphic loci: Ase18 (Richardson et al.  
102 2000, *A. sechellensis*), Aar4, Pdo5, Ppi2, ZL54, Pocc2, Pca3 and Ase25. **Details on the Polymerase**  
103 **Chain Reaction (PCR) are available in the online supplementary material.** We ran the PCR products  
104 with ABI PRISM 3730 DNA Analyzer (Applied Biosystems) and scored them with GeneMapper  
105 4.0. We checked the data with the program MICROCHECKER 2.2.3 (van Oosterhout et al. 2004)  
106 for possible genotyping errors (null alleles, scoring errors due to stuttering, large allele dropouts).

107

108 Mitochondrial DNA

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110 For reed warblers we amplified 473 bp of the mitochondrial cytochrome oxidase CO1 using primers  
111 CO1F and CO1R (Hebert et al. 2004). For moustached warblers we amplified 623 bp of the CO1  
112 using primers CO1F and CO1R2 (Kerr et al. 2007). **Details on the PCR procedures are available in**  
113 **the online supplementary material.** For reed warblers, we sequenced both strands, while for  
114 moustached warblers we sequenced all individuals with forward and 26 individuals also with  
115 reverse primers. We used the BigDye™ Terminator 3.1 Cycle Sequencing Kit (Applied Biosystems)  
116 and ran the sequencing reactions with ABI PRISM 3730 automatic sequencer (Applied  
117 Biosystems). We obtained sequences of 50 reed warblers and 43 moustached warblers and we  
118 checked and aligned them with BioEdit 7.2.5 (Hall 1999).

119

120 **Statistical analysis**

121

122 Microsatellites

123

124 We tested the possible deviations from the Hardy-Weinberg and linkage equilibrium with  
125 GENEPOP 4.2 (Raymond and Rousset 1995, Rousset 2008), for each sampling site and for the total  
126 sample of both species. For the same groups we calculated the expected heterozygosity ( $H_e$ ) with  
127 Arlequin 3.5.1 (Excoffier et al. 2010) and inbreeding coefficient ( $F_{IS}$ ) and allelic richness ( $A$ ) with  
128 FSTAT 2.9.3 (Goudet 1995).

129 To infer the population genetic structure we used the program STRUCTURE 2.3.4  
130 (Pritchard et al. 2000, Falush et al. 2003). This program is based on a Bayesian approach and allows  
131 estimation of the most probable number of distinct genetic clusters ( $K$ ) in the data set. We chose a  
132 model with population admixture and correlated allele frequencies (Falush et al. 2003), and  
133 performed the analysis without any prior spatial information. We performed ten independent runs  
134 for each value of  $K$  between 1 and 10, with a burn-in period of 50000 iterations and 500000 Markov  
135 chain Monte Carlo (MCMC) replications. Furthermore, we calculated the ad hoc statistic  $\Delta K$  from  
136 the STRUCTURE results as described by Evanno et al. (2005), to better assess the real number of  
137 genetic clusters. We investigated population differentiation also by calculating pairwise  $F_{ST}$  values  
138 between each of the sampling sites using the program Arlequin.

139 We explored the spatial genetic structure with the program SPAGeDi 1.4 (Hardy and  
140 Vekemans 2002), using the Loiselle kinship coefficient (Loiselle et al. 1995) and four (reed  
141 warbler)/three (moustached warbler) distance classes. The spatial coordinates of the individuals  
142 corresponded to the coordinates of the four sampling sites, and we built distance classes to include  
143 one site per class. To obtain information about current dispersal between populations, we carried out  
144 an assignment analysis and looked for first generation migrants with the program GENECLASS 2  
145 (Piry et al. 2004). We used the Bayesian individual assignment methods by Rannala and Mountain

146 (1997) and the simulation algorithm of Paetaku et al. (2004). For both assignment analysis and first  
147 generation migrant detection, we used 1000 replicates, alpha level for the MCMC simulations at  
148 0.01 and assignment threshold at 0.05.

149 Genetic data from population samples carry also information about population history. First,  
150 we looked for past bottlenecks by means of **the** program BOTTLENECK 1.2.02 (Cornuet et al.  
151 1996, Piry et al. 1999), which tests for heterozygosity excess caused by recent reduction of the  
152 effective population size (Piry et al. 1999). We used the Wilcoxon test under the two-phase mutation  
153 model with 95% single-step mutations. We also estimated the Garza-Williamson index (Garza and  
154 Williamson 2001) with Arlequin, in order to search for strong past population bottlenecks. Using  
155 the genetic clusters identified by STRUCTURE, we studied the population history with **the** program  
156 DIY ABC 2.0.3 (Cornuet et al. 2008), based on approximate Bayesian computation (ABC). Using  
157 DIY ABC it is possible to compare different competing historical/demographic scenarios and to  
158 obtain parameter estimators for each of them. For reed warblers, we found no population  
159 differentiation with STRUCTURE ( $K = 1$ , see Results), thus we performed the analysis in DIY  
160 ABC only for moustached warbler ( $K = 2$ , see Results). We were interested to estimate the  
161 divergence time and the current effective population sizes for the two STRUCTURE populations  
162 (Spanish mainland and Mallorca Island, see Results) and for the ancestral population. We explored  
163 five scenarios, the simplest one containing one divergence event while the remaining four contained  
164 population size changes at different times after divergence in only one or both populations. We  
165 chose the default range of priors for effective population sizes and divergence times (10-10000) and  
166 set the conditions for the chronological order of historical events. We adopted the default  
167 Generalized Stepwise Mutation model (Estoup et al. 2002) and seven of 11 default summary  
168 statistics (four within- and three among-populations). With these settings, a total of 5000000  
169 simulated data sets were calculated (1000000 per scenario); among them, the 50000 sets closest to  
170 the observed data according to the summary statistics were used for parameter estimation.

171

172 Mitochondrial DNA

173

174 We calculated haplotype and nucleotide diversities of the entire sample and of each population for  
175 both species using DnaSP 5.10.01 (Librado and Rozas 2009). To build a statistical parsimony  
176 network, we used **the** program TCS 1.21 (Clement et al. 2000) with default settings of 95%  
177 parsimony connection limit. As outgroups, we included sequences from other European populations  
178 obtained from the GenBank (accession numbers and origin: moustached warbler - GQ481257,  
179 Russia; reed warbler - GU571698, Sweden).

180 We used **the** program Arlequin to obtain information about population differentiation, by  
181 performing **an analysis of molecular variance (AMOVA)** and calculating pairwise  $\phi_{ST}$  values  
182 between each of the sampling sites using pairwise differences and frequencies of haplotypes.

183 We studied demographic history by means of mismatch distribution analysis, which consists  
184 of computing the distribution of the observed number of differences between pairs of sequences in a  
185 sample. Unimodal distributions usually indicate an expansion event, whereas multimodal  
186 distributions are typical of populations at demographic equilibrium (Rogers and Harpending 1992).  
187 In addition, we carried out Tajima's D (Tajima 1989) and Fu's  $F_s$  (Fu 1997) neutrality tests, where a  
188 statistically significant negative value indicates a recent expansion event. We performed mismatch  
189 distribution analysis and the neutrality tests for the entire sample and for each sampling site using  
190 Arlequin and DnaSP.

191 The reed warbler mismatch distribution suggested an admixture of two previously isolated  
192 populations (see Results). Thus, we ran reed warbler data with DIY ABC to estimate the timing of  
193 this demographic event. We compared four scenarios, where the simplest one consisted of a  
194 population admixture at time  $t_a$  and a previous population divergence at time  $t_2$ . The remaining three  
195 scenarios followed the same basic setting but included population size changes at different times for

196 only one or both populations before admixture. Reed warbler populations in Iberia and across  
197 Europe are abundant (Carrascal and Palomino 2008, BirdLife International 2014), thus we set the  
198 maximum effective population size at 1000000 individuals in the model. As maximum time for the  
199 admixture and the previous divergence events we chose 10000 and 20000 generations  
200 (corresponding to 10000 and 20000 years for the study species), respectively. We used the Kimura  
201 two parameters mutation model (Kimura 1980) and all default summary statistics. A total of  
202 4000000 simulated data sets were calculated (1000000 per scenario); among them, the 40000 sets  
203 closest to the observed data according to the summary statistics were used for parameter estimation.

204

205

## 206 **Results**

207

### 208 **Reed warbler**

209

#### 210 **Microsatellites**

211

212 Using MICROCHECKER we found possible null alleles in loci Ase25, Ase37, Ase48 and ZL54.  
213 We excluded these loci from calculations for the Hardy-Weinberg equilibrium, linkage  
214 disequilibrium and inbreeding coefficient, as well as from BOTTLENECK analysis, while for the  
215 other analyses we used all 16 loci. We found no evidences of large allele dropouts or scoring errors  
216 in the data set.

217         We found no significant deviations from Hardy-Weinberg equilibrium for any sampling site  
218 or for the entire data set. Linkage disequilibrium was found only for FhU2 and Pca3 in the  
219 Villafranca population and in the entire sample. Basic polymorphism parameters (Table 1) showed  
220 similar values across the four sampling sites, ranging from the highest polymorphism in Villafranca

221 ( $H_e = 0.710$ ,  $A = 1.71$ ) to the lowest in Mallorca ( $H_e = 0.650$ ,  $A = 1.65$ ).

222         **The** program STRUCTURE attributed the highest likelihood (-2412) to  $K = 1$ , although  $K =$   
223 2 obtained a similar support (-2417).  $\Delta K$  showed a peak at  $K = 2$ , but as it is not possible to  
224 compute  $\Delta K$  for  $K = 1$ , we could not compare the two hypotheses based on this statistic. The  
225 STRUCTURE bar plot obtained for  $K = 2$  (Fig. 2) support the  $K = 1$  hypothesis, because all  
226 individuals showed approximately equal probability of belonging to both of the two clusters. To  
227 detect a possible weak population structuring we repeated the analysis adding geographical  
228 information (LOCPRIOR model), but we obtained similar results. Therefore, the most supported  
229 hypothesis is the lack of a clear population structuring in the sample. **Nonetheless, in two cases**  
230 **(Larache - Pego-Oliva and Villafranca - Mallorca) pairwise  $F_{ST}$  values between sampling sites were**  
231 **significant, though low (Table 2).**

232         Using **the** program SPAGeDi, the regression between the kinship coefficients of individual  
233 pairs and the logarithm of the distances between sampling sites was significantly negative ( $r =$   
234  $-0.058$ ,  $p = 0.042$ ). **However, given the low regression coefficient, this result does not represent a**  
235 **clear evidence for isolation by distance.** Out of 68 birds, 25 (38%) were not assigned to their  
236 sampling sites (Table 3) by the assignment test. Two birds sampled at Mallorca were identified as  
237 first generation migrants and assigned to Pego-Oliva. Both individuals (a male with evident cloacal  
238 protuberance and a female with brood patch) were breeding when sampled, thus this result indicates  
239 possible evidence of dispersal.

240         We found no evidence of past bottlenecks according to the shapes of allele frequency  
241 distributions or excess of heterozygotes for the entire data set and for Larache and Villafranca  
242 samples (Table 1). Pego-Oliva and Mallorca samples were too small to perform the analysis. The  
243 Garza-Williamson (G-W) index provides a sign of past bottleneck when is lower than 0.68 while  
244 excludes this event if exceeds 0.8 (Garza and Williamson 2001). Based on this, the entire and the  
245 Larache samples did not show evidence of past bottlenecks, in Villafranca the G-W index was

246 intermediate and Pego-Oliva and Mallorca values were lower than 0.68 (Table 1). However, the  
247 results of the latter two sites are poorly reliable due to the low sample size. Overall, we found no  
248 clear evidence of past bottlenecks.

249

250 Mitochondrial DNA

251

252 The 50 sequenced reed warblers belonged to 18 different haplotypes. The TCS cladogram (Fig. 3)  
253 show a star-like structure, with one dominant haplotype including 25 individuals (50% of the total)  
254 and 17 other haplotypes represented by few birds (1-4). This structure suggests past population  
255 expansion events. Four birds resulted to be more related to the outgroup individual from Sweden  
256 than to the remaining Iberian/African sample (Fig. 3). Haplotype and nucleotide diversities are  
257 reported in Table 4.

258 AMOVA analysis did not detect any population differentiation ( $\phi_{ST} = -0.0087$ ,  $p = 0.438$ ),  
259 with 100% variation ascribed to differences within sampling sites. We obtained no significant  
260 pairwise  $\phi_{ST}$  values between study sites (Table 5).

261 According to the neutrality tests, we found signs of population expansion for Larache and,  
262 less clearly, for the entire data set. In fact, Tajima's D and Fu's  $F_s$  were significantly negative for  
263 Larache sample ( $D = -2.203$ ,  $p < 0.01$ ;  $F_s = -7.063$ ,  $p = 0.001$ ), not significant for Pego-Oliva and  
264 Villafranca, while over the whole sample only Fu's  $F_s$  was significantly negative ( $D = -1.581$ ,  $p >$   
265  $0.05$ ;  $F_s = -8.590$ ,  $p < 0.001$ ). Mismatch distribution did not detect significant deviations from the  
266 expansion hypothesis. Furthermore, mismatch distribution of Larache, Villafranca and the whole  
267 data set showed a bimodal shape (Fig. 4), suggesting a past admixture of previously isolated  
268 populations. Exploring demographic history with DIY ABC, the scenario with populations size  
269 change in one population before admixture (hereafter A; Fig. 5) resulted to be the best. However,  
270 the simplest scenario with an admixture and a previous divergence event, without population size

271 changes (hereafter B; Fig. 5), obtained high support too, thus we report the parameters estimates  
272 obtained from both scenarios (Fig. 5). Scenario A obtained lower posterior probability than B  
273 according to direct estimation ( $A = 0.386$ ,  $B = 0.440$ ), but the logistic approach clearly supported  
274 scenario A ( $A = 0.841$ ,  $B = 0.159$ ). Thus, we performed the analysis of confidence in scenario  
275 choice over all scenarios as implemented in DIY ABC, which confirmed scenario A as the best but  
276 with high Type I error (56.8% according to direct approach, 55.4% to logistic approach) due to the  
277 good performance of scenario B. Both scenarios fitted the data well according to model checking,  
278 with no significant deviation in summary statistics between posterior distributions and observed  
279 data. Timing of admixture was estimated to be 5690 generations ago (CI 95% 583 – 9610) from  
280 scenario A and 4750 generations (CI 95% 505 - 9420) from scenario B. **In both species, the first**  
281 **breeding occurs at the age of 1 year (like in most passerines, Noon and Sauer 1992), thus we**  
282 **assumed that this time corresponded to one generation.**

283

#### 284 **Moustached warbler**

285

286 Microsatellites

287

288 MICROCHECKER detected possible null alleles in loci Ase18 and Ase25, thus we excluded them  
289 from the same analyses as listed for **the** reed warbler. For the remaining analyses, we used all eight  
290 loci. We found no evidence of large allele dropouts or scoring errors in the data set.

291 The whole sample was not in Hardy-Weinberg equilibrium ( $\chi^2_{32} = \infty$ ,  $p =$  highly significant),  
292 nor was Pego-Oliva ( $\chi^2_{12} = \infty$ ,  $p =$  highly significant) and Mallorca samples ( $\chi^2_{12} = 47.18$ ,  $p <$   
293 **0.001**), while for Villafranca the test was not significant ( $\chi^2_{12} = 20.01$ ,  $p = 0.067$ ). We found no  
294 significant linkage disequilibrium between loci. We obtained higher expected heterozygosity  $H_e$  and  
295 allelic richness  $A$  values and lower inbreeding coefficients  $F_{IS}$  for the mainland sites of Pego-Oliva

296 and Villafranca than for the Mallorca sample (Table 1).

297 Using **the** program STRUCTURE, we obtained the highest support in terms of likelihood for  
298  $K = 3$  (-919). However, at  $K = 2$  (likelihood = -935) we observed a clear peak of  $\Delta K$ , indicating that  
299 the most reliable structuring is the occurrence of two clusters. In the STRUCTURE bar plot (Fig. 2)  
300 the Mallorca sample is clearly distinct from the remaining individuals, thus we identified two  
301 clusters corresponding to Mallorca and the Spanish mainland (Pego-Oliva + Villafranca sampling  
302 sites). Pairwise  $F_{ST}$  values were significant between all sampling sites, as well as between the  
303 STRUCTURE populations (**Table 2**).

304 We did not find any correlation between the Loiselle kinship coefficients and logarithms of  
305 distances ( $r = 0.000$ ,  $p = 0.825$ ) when studying isolation by distance with **the** program SPAGeDi.  
306 The assignment analysis did not assign 12 out of 54 (22%) individuals to their sampling sites (Table  
307 3). Considering the STRUCTURE populations, 6 out of 54 birds (11%) were not assigned to their  
308 cluster (Table 3). One bird sampled in Mallorca and one in Villafranca were identified as first  
309 generation migrants and assigned to Pego-Oliva. The first was a female with regressing brood patch  
310 and the second a male with evident cloacal protuberance (i.e. breeding individuals), thus our results  
311 suggest dispersal between the study sites.

312 We found signs of recent population reduction in Mallorca with **the** program  
313 BOTTLENECK (Wilcoxon test, heterozygosity excess,  $p = 0.023$ ; shifted allele frequency class  
314 mode) and less clearly for the Spanish mainland STRUCTURE population (Wilcoxon test,  
315 heterozygosity excess,  $p = 0.039$ ; normal L-shape of allele frequency distribution). No evidence of  
316 past bottlenecks was found for Pego-Oliva and Villafranca samples. G-W indexes were always  $>$   
317 0.8, indicating no bottleneck history in a more remote past. We obtained further information about  
318 demographic history using **the** program DIY ABC. Out of the five simulated scenarios, the best was  
319 the simplest one (Fig. 6), with divergence at time  $t_1$  and no effective population size ( $N_e$ ) change  
320 after divergence. Posterior probabilities of this scenario were 0.970 according to direct estimation

321 and 0.991 according to logistic approach. The model checking based on seven summary statistics  
322 and the PCA analysis supported the reliability of the scenario, with the exception of two summary  
323 statistics (mean M index, Garza and Williamson 2001;  $(\delta\mu)^2$  distance, Goldstein et al. 1995) where  
324 posterior distributions strongly deviated ( $0.01 > p > 0.001$ ) from the observed data. The estimate of  
325 divergence time was 230 generations (95% CI 41 - 449), corresponding to 230 years.  $N_e$  estimate  
326 for Spanish mainland was 4460 (95% CI 1240 - 6360), and 1500 for Mallorca (95% CI 290 - 2740).  
327 The ancient population size (before divergence) was estimated to be 5890 (95% CI 1430 - 7820).

328

329 Mitochondrial DNA

330

331 The 43 sequenced moustached warblers belonged to seven different haplotypes. Similarly to the  
332 reed warbler, TCS cladogram (Fig. 3) showed a star-like structure, suggesting past population  
333 expansion. A dominant haplotype included 35 individuals (81% of the total), while the other six  
334 haplotypes were represented by one or two birds. Compared to the reed warbler, haplotype and  
335 nucleotide diversity (Table 4) are markedly lower.

336 We did not find significant differentiation between sampling sites with AMOVA analysis  
337 ( $\phi_{ST} = 0.0363$ ,  $p = 0.108$ ), while using the populations identified by STRUCTURE, we obtained a  
338 significant result ( $\phi_{ST} = 0.0862$ ,  $p = 0.022$ ), with 8.62% variation due to differences among  
339 populations and 91.38% within populations. Pairwise  $\phi_{ST}$  values were significant between  
340 STRUCTURE populations and between Pego-Oliva and Mallorca samples, and not significant  
341 between the other sampling sites (Table 5).

342 The results of neutrality tests suggested past expansion for the whole sample ( $D = -1.954$ ,  $p$   
343  $< 0.05$ ;  $F_s = -5.308$ ,  $p = 0.004$ ), but not for the single sampling sites or for the STRUCTURE  
344 populations. Mismatch distribution did not detect significant deviations from the expansion  
345 hypothesis.

346

347

## 348 **Discussion**

349

### 350 **Reed warbler**

351

352 Our results indicate high gene flow between the sampling sites, suggesting high dispersal capability  
353 for the reed warblers. However, a weak but significant differentiation among some of the sampling  
354 sites (Table 2) indicates that gene flow is partly restricted. Other researchers obtained similar results  
355 studying the reed warbler across the whole Europe (Procházka et al. 2001) and in Croatia (Kralj et  
356 al. 2010). A possible cause of gene flow restriction in our study is the relatively large distance  
357 between sampling sites (from c. 290 to 960 km), but testing for isolation by distance did not clearly  
358 support this hypothesis. Breeding site fidelity, reported for this species especially for adult birds  
359 (Vadász et al. 2008), could also explain limitations to gene flow. Given the lack of a clear  
360 structuring in a sample including birds breeding in Spain (subspecies *scirpaceus*) and in Morocco,  
361 our results do not support the hypotheses that the latter belong to a new taxon (see Introduction;  
362 Amezian et al. 2010, Jiguet et al. 2010). Besides the geographical proximity of the two areas,  
363 Morocco is crossed by the migration routes of the European SW-migrating populations, which  
364 includes the Iberian reed warblers (Procházka et al. 2008), and this is likely to facilitate the  
365 exchange of individuals between Moroccan and Iberian populations.

366 Our results about demographic history indicate past population growth and mixing of  
367 previously isolated populations. The dating of this population admixture calculated in DIY ABC  
368 (Fig. 5) is compatible with a postglacial expansion from a refugium area. In fact, during and before  
369 this time the climate became warmer and wetter, causing the expansion of the suitable habitat also  
370 for many other species (Murray Gates 1993, Wright et al. 1993, Hewitt 2000, Dubey et al. 2006).

371 Arbabi et al. (2014) investigated phylogeography of reed warblers with mtDNA data and identified  
372 three evolutionary lineages corresponding to three subspecies (*scirpaceus*, *fuscus* and *avicenniae*),  
373 which had remained isolated in three different glacial refugia during the Middle Pleistocene  
374 glaciations. Procházka et al. (2011) suggested that it is possible that the Iberian Peninsula was one  
375 of the refugium areas for reed warblers, as has been documented for many other species (Hewitt  
376 2004). As we did, also Arbabi et al. (2014) found evidence of population growth, but their samples  
377 were mainly from Germany, representing the subspecies *scirpaceus*. Comparing diversity indices,  
378 both haplotype and nucleotide diversities were higher in our study ( $N = 50$ ;  $h = 0.745$ ;  $\pi = 0.006$ )  
379 than in Germany ( $N = 347$ ;  $h = 0.544$ ;  $\pi = 0.002$ ) or for the whole sample of subspecies *scirpaceus*  
380 ( $N = 380$ ;  $h = 0.558$ ;  $\pi = 0.002$ ) in Arbabi et al. (2014). The higher diversity in our study area can be  
381 a sign of more ancient refugium area (Taberlet et al. 1998, Comes and Kadereit 1998) than  
382 suggested by Arbabi et al. (2014), or, more likely, a result of the relatively recent admixture event.  
383 The population divergence preceding the admixture, according to both DIY ABC scenarios (Fig. 5),  
384 is posterior to the Last Glacial Maximum and could have been caused by the colonization of  
385 separated areas by individuals originating from the same glacial refugium. Due to the drier climate,  
386 wetland areas were probably scarcer than currently, thus making it plausible that habitats suitable  
387 for reed warblers were sparse and isolated enough to promote population divergence.

388

### 389 **Moustached warbler**

390

391 The results obtained from microsatellite data indicate that gene flow is limited between the Spanish  
392 mainland and Mallorca Island (also according to the mtDNA results, see Table 5) and partly  
393 restricted among the two mainland sites. Approximately the same distance (c. 290 km) separates  
394 Pego-Oliva from Villafranca and from Mallorca, but only the Mallorcan sample was clearly distinct  
395 according to STRUCTURE analysis. This fact suggests that gene flow between the Spanish

396 mainland and Mallorca is likely to be limited not only by the distance, but also by the sea (c. 200  
397 km wide) between the coast and the island. Thus, our results suggest that the sea forms an effective  
398 dispersal barrier and limits gene flow in moustached warblers, at least for our study populations.  
399 For a warbler, crossing the sea implies the lack of resting and foraging opportunities until reaching  
400 land, resulting to more risky and difficult displacement than over the solid ground. **The**  
401 **GENECLASS results** suggest that limited dispersal from the mainland to Mallorca can occur.  
402 However, it is possible that the classification **of a breeding bird** as a first generation migrant is not  
403 due to a real dispersal event, but to false detection due to small sample size or due to the sampling  
404 sites not being in H-W equilibrium (Excoffier and Heckel 2006). **The low differentiation among the**  
405 **mainland sites indicates** the occurrence of gene flow between the important breeding populations of  
406 the Mediterranean coast and the small and scattered inland populations breeding in Castilla-La  
407 Mancha (estimated to be only 10 pairs by Castany and López 2006), represented respectively by  
408 Pego-Oliva and Villafranca samples in our study. The breeding bird sampled in Villafranca and  
409 classified as first generation migrant from Pego-Oliva can be a further sign of dispersal. The gene  
410 flow, however, is partly restricted, possibly because the two populations are divided by a wide area  
411 without any other known breeding populations (Castany and López 2006) and without suitable  
412 habitat. Like for reed warblers, breeding site fidelity in moustached warblers (Vadász et al. 2008)  
413 could also reduce dispersal.

414 mtDNA showed evidence of past population growth, probably a sign of postglacial  
415 expansion. As for reed warblers, it is likely that moustached warbler populations were confined in  
416 glacial refugia and extended their range across Europe as the climate became warmer and wetter.  
417 Taking into account the population structuring and the results of BOTTLENECK and coalescence  
418 analysis, we conclude that Mallorca has been recently colonized or re-colonized by individuals  
419 originating from the mainland. After the settlement of the breeding population, the limited gene  
420 flow produced the population structuring we observed.

421

422 **Differences in population structure and demographic history between the two species –**  
423 **possible explanations**

424

425 The lower genetic differentiation found in reed warblers indicates a higher dispersal ability in them  
426 than in moustached warblers. This is consistent with Paradis et al. (1998), who found long-distance  
427 migrants to have higher dispersal ability than sedentary or short-distance migrants. Also other  
428 researchers have reported lower intraspecific differentiation in migratory than in sedentary bird  
429 species (e.g. Gill et al. 1993, Lovette et al. 1998, Arguedas and Parker 2000). Our results suggest  
430 that differences in dispersal ability between reed and moustached warblers also can result from  
431 different capability in crossing natural barriers, in this case stretches of sea. Migration routes of reed  
432 warblers include crossing the Mediterranean Sea, thus crossing this kind of a barrier is a necessary  
433 ability for this species. Conversely, migration of Spanish and French populations of moustached  
434 warblers mostly follow the Mediterranean coast (Castany 2003) and do not necessarily require  
435 crossing the sea, thus overcoming such a barrier is probably less common for these birds. Higher  
436 differentiation in moustached warblers could be also due to the smaller population size and the  
437 more discontinuous breeding range than in reed warblers. In fact, breeding areas of moustached  
438 warblers are more isolated from each other, making the exchange of individuals more difficult. In  
439 addition, Vadász et al. (2008), studying several warbler species in Hungary, reported higher  
440 breeding site fidelity in moustached than in reed warblers. The authors hypothesized that the more  
441 specialized habitat preferences of moustached warblers limit the opportunities of finding new  
442 suitable areas, and that would result in reduced dispersal rates (Vadász et al. 2008). Similar studies  
443 of the West Mediterranean moustached and reed warbler populations would be needed to assess if  
444 breeding site fidelity is higher for moustached warblers also in this area. Differences in wing  
445 morphology could also partly explain the difference in dispersal capability between the two species.

446 In fact, reed warblers have more pointed wings than moustached warblers (Kennerley and Pearson  
447 2010) allowing greater flight efficiency (e.g. Norbert 1989).

448 Both species showed signs of postglacial expansions, consistently with the findings of other  
449 researchers studying European populations of wetland passerines (e. g. Hansson et al. 2008, Neto et  
450 al. 2012, Arbabi et al. 2014). Haplotype diversity was higher for reed warblers, possibly because of  
451 a more important contribution of dispersal from different populations and the higher population  
452 size. Unlike in reed warblers, we did not find evidences of past population admixtures in  
453 moustached warblers, indicating that the postglacial expansion of these species evolved in different  
454 ways.

455

## 456 **Conclusion**

457

458 Despite the large distances between our sampling sites, we detected gene flow between sampling  
459 sites for both reed and moustached warblers, although partly restricted or even limited in the case of  
460 moustached warblers of Mallorca. These results suggest that both species are able to avoid the risk  
461 of isolation derived from breeding in a fragmented habitat. We found lower differentiation in reed  
462 warblers than in moustached warblers, indicating higher dispersal capability of reed warblers, a  
463 species migrating over longer distances, with higher population size and more continuous breeding  
464 range than moustached warblers. We did not find support for the occurrence of a new taxon of reed  
465 warbler breeding in Morocco. Additional information from other breeding areas is needed for  
466 moustached warblers in order to investigate population structuring and demographic history on a  
467 larger scale. For both species, further information about dispersal is needed from large-scale  
468 marking schemes.

469

470

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Table 1. Sample sizes, expected heterozygosity ( $H_e$ ), allelic richness (A), inbreeding coefficient ( $F_{IS}$ ), Wilcoxon p – values (Wil. Test, one-tailed for heterozygosity excess) and Garza-Williamson index (G-W) of (a) reed warbler and (b) moustached warbler, calculated from microsatellite data for each sampling site and populations defined by STRUCTURE (Str).

a)

Sample	N	$H_e$ (SD)	A (SD)	$F_{IS}$ (SD)	P (Wil. Test)	G-W
Larache	38	0.696 (0.316)	1.70 (0.32)	0.127 (0.314)	0.207	0.84
Pego-Oliva	7	0.681 (0.366)	1.68 (0.37)	0.049 (0.281)		0.67
Mallorca	3	0.650 (0.375)	1.65 (0.38)	-0.153 (0.413)		0.62
Villafranca	20	0.710 (0.249)	1.71 (0.25)	-0.022 (0.257)	0.661	0.73
Total	68	0.716 (0.272)	1.68	0.014 (0.316)		1.00

b)

Sample	N	$H_e$ (SD)	A (SD)	$F_{IS}$ (SD)	P (Wil. Test)	G-W
Pego-Oliva	30	0.602 (0.184)	3.76 (2.16)	-0.275 (0.404)	0.078	0.96
Villafranca	9	0.590 (0.185)	3.47 (2.22)	-0.344 (0.477)	0.078	0.85
Mallorca	15	0.538 (0.202)	2.96 (1.60)	-0.500 (0.454)	0.023	0.82
(= Str - Mallorca)						
Str - Spanish mainland	39	0.611 (0.172)	4.28 (3.01)	-0.240 (0.394)	0.039	0.96
Total	54	0.621 (0.182)	3.40	-0.250 (0.115)		0.88

Table 2. Pairwise  $F_{ST}$  values (p-values in parentheses) between sampling sites and between STRUCTURE (Str) populations of (a) reed warbler and (b) moustached warbler, calculated from microsatellite data.

a)

Sample	Pego-Oliva	Mallorca	Villafranca
Mallorca	0.0030 (0.189)	-	-
Villafranca	-0.0211 (0.505)	0.0732 (0.018)	-
Larache	0.0189 (0.027)	-0.0029 (0.351)	-0.0193 (0.892)

b)

Sample	Pego-Oliva	Mallorca (= Str - Mallorca)
Mallorca	0.1146 (0.000)	-
Villafranca	0.0609 (0.000)	0.1352 (0.000)
Str - Spanish mainland	-	0.1078 (0.000)

Table 3. Assignment of (a) reed warblers and (b) moustached warblers to the sampling sites and the STRUCTURE (Str) populations, on the basis of microsatellite data; individuals which have not been assigned to their site/population of origin are reported in bold.

a)

Sampling site of origin	Assigned to			
	Larache	Pego-Oliva	Mallorca	Villafranca
Larache	35	<b>1</b>	0	<b>2</b>
Pego-Oliva	<b>3</b>	0	0	<b>4</b>
Mallorca	<b>2</b>	0	0	<b>1</b>
Villafranca	<b>12</b>	0	0	8

b)

Sampling site of origin	Assigned to		
	Pego-Oliva	Mallorca	Villafranca
Pego-Oliva	30	0	0
Mallorca	<b>5</b>	9	<b>1</b>
Villafranca	<b>6</b>	0	3

Str population of origin	Assigned to	
	Str - Spanish mainland	Str - Mallorca
Str - Spanish mainland	39	0
Str - Mallorca	<b>6</b>	9

Table 4. Sample sizes, number of segregating sites, number of haplotypes, haplotype diversity (h) and nucleotide diversity ( $\pi$ ) of (a) reed warbler and (b) moustached warbler, calculated from mitochondrial DNA data for each sampling site and STRUCTURE (Str) population (Str populations were identified on the basis of microsatellite data).

a)

Sample	N	N segregating sites	N haplotypes	h	$\pi$
Larache	29	21	13	0.823	0.004
Pego-Oliva	5	11	3	0.700	0.010
Mallorca	3	0	1	0.000	0.000
Villafranca	13	14	6	0.718	0.008
Total	50	23	18	0.745	0.006

b)

Sample	N	N segregating sites	N haplotypes	h	$\pi$
Pego-Oliva	22	3	4	0.333	0.001
Villafranca	8	0	1	0.000	0.000
Mallorca	13	4	4	0.526	0.001
(= Str - Mallorca)					
Str - Spanish mainland	30	3	4	0.251	0.000
Total	43	7	7	0.339	0.001

Table 5. Pairwise  $\phi_{ST}$  values (p-values in parentheses) between sampling sites and between STRUCTURE (Str) populations of (a) reed warbler and (b) moustached warbler, calculated from mitochondrial DNA data. STRUCTURE population were identified on the basis of microsatellite data.

a)

Sample	Pego-Oliva	Mallorca	Villafranca
Mallorca	-0.0918 (0.802)	-	-
Villafranca	-0.0997 (0.685)	-0.1149 (0.991)	-
Larache	0.0486 (0.207)	-0.1742 (0.991)	0.0302 (0.072)

b)

Sample	Pego-Oliva	Mallorca (= Str - Mallorca)
Mallorca	0.0660 (0.036)	-
Villafranca	-0.0402 (0.739)	0.0065 (0.541)
Str - Spanish mainland	-	0.0862 (0.000)



Figure 1. Location of the sampling sites: 1) Larache, 2) Villafranca de los Caballeros, 3) Pego-Oliva Natural Park and 4) S'Albufera de Mallorca.

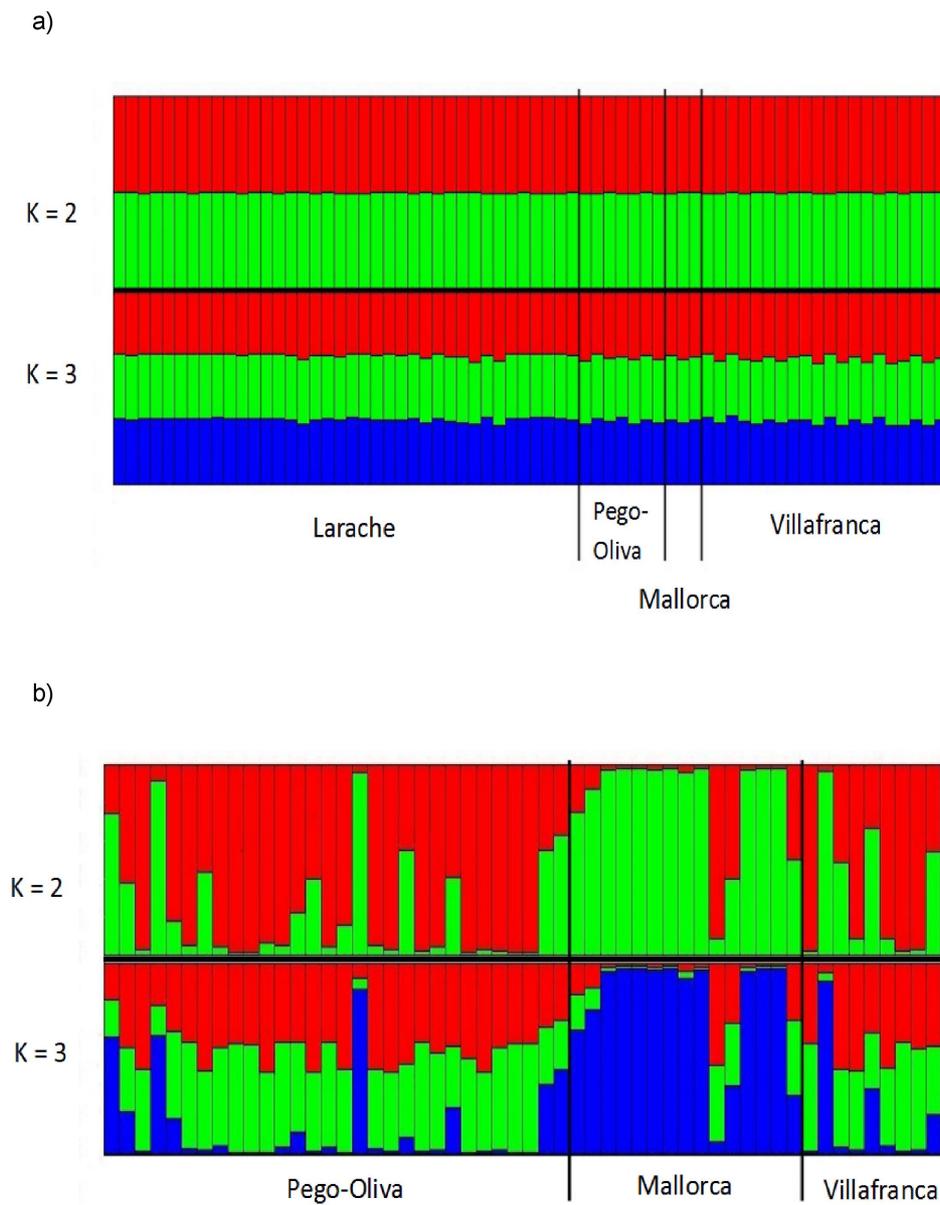


Figure 2. Bar plots of the STRUCTURE results based microsatellite data of (a) reed warbler and (b) moustached warbler for  $K = 2$  and  $K = 3$ . Each column corresponds to an individual and represents its probability to belong to one of the  $K$  clusters.

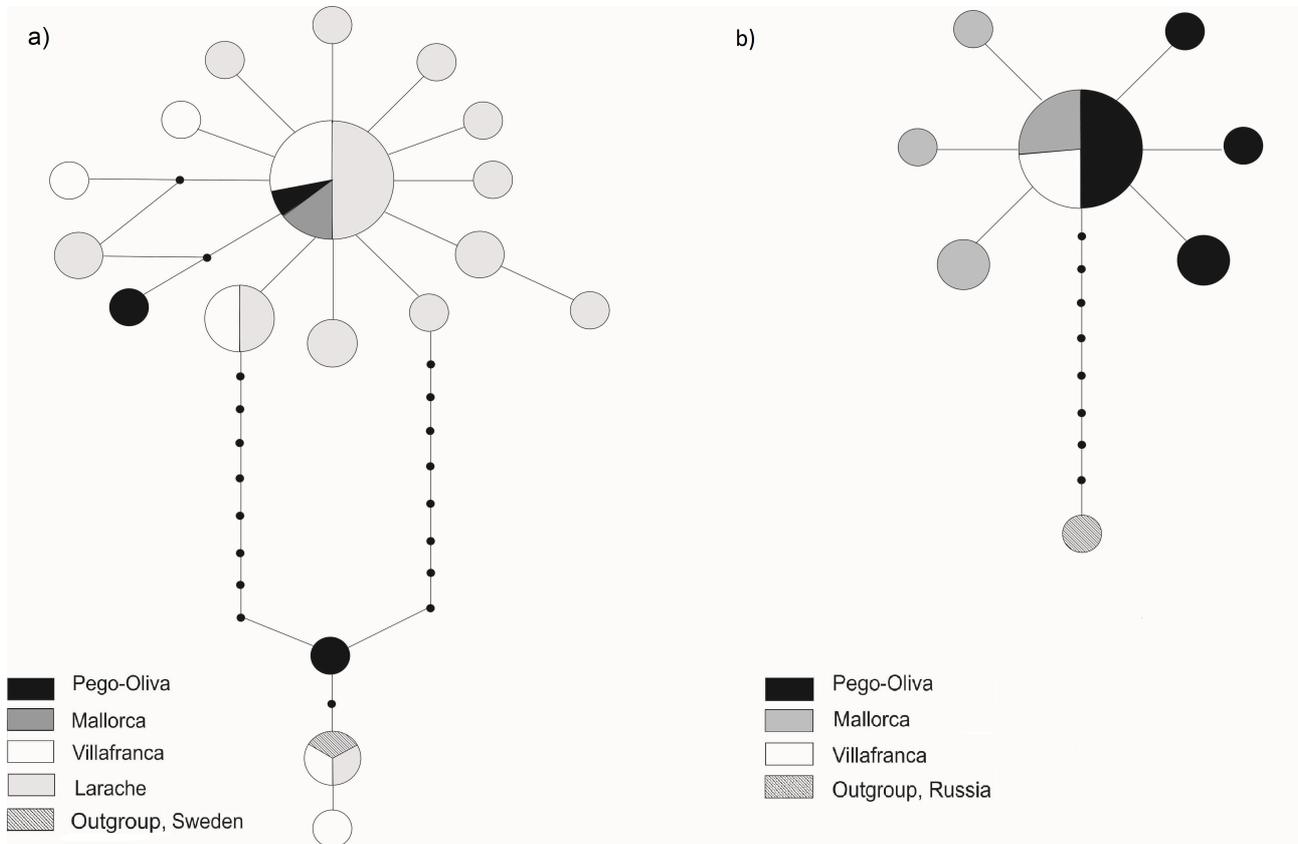


Figure 3. Statistical parsimony network based on (a) reed warbler and (b) moustached warbler mtDNA data. Each haplotype is represented by a circle, whose area is proportional to the number of individuals belonging to the haplotype. Each connecting bar stands for one substitution.

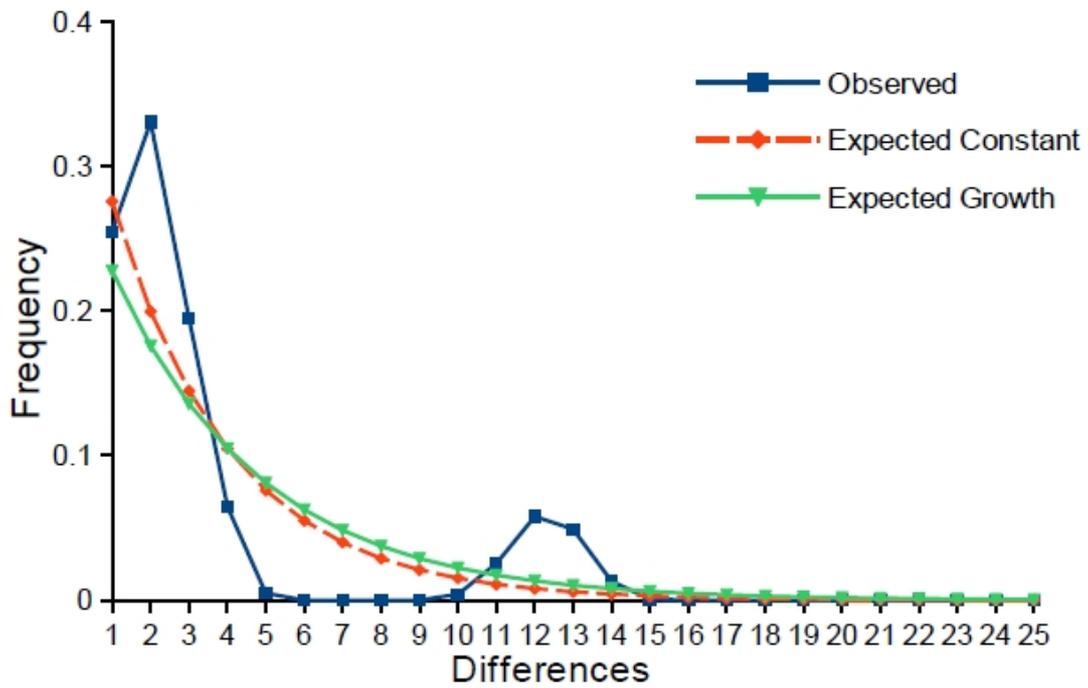


Figure 4. Mismatch distributions calculated over the whole reed warbler sample using mitochondrial DNA data. The bimodal shape of the observed frequencies indicates a past admixture of two previously isolated populations.

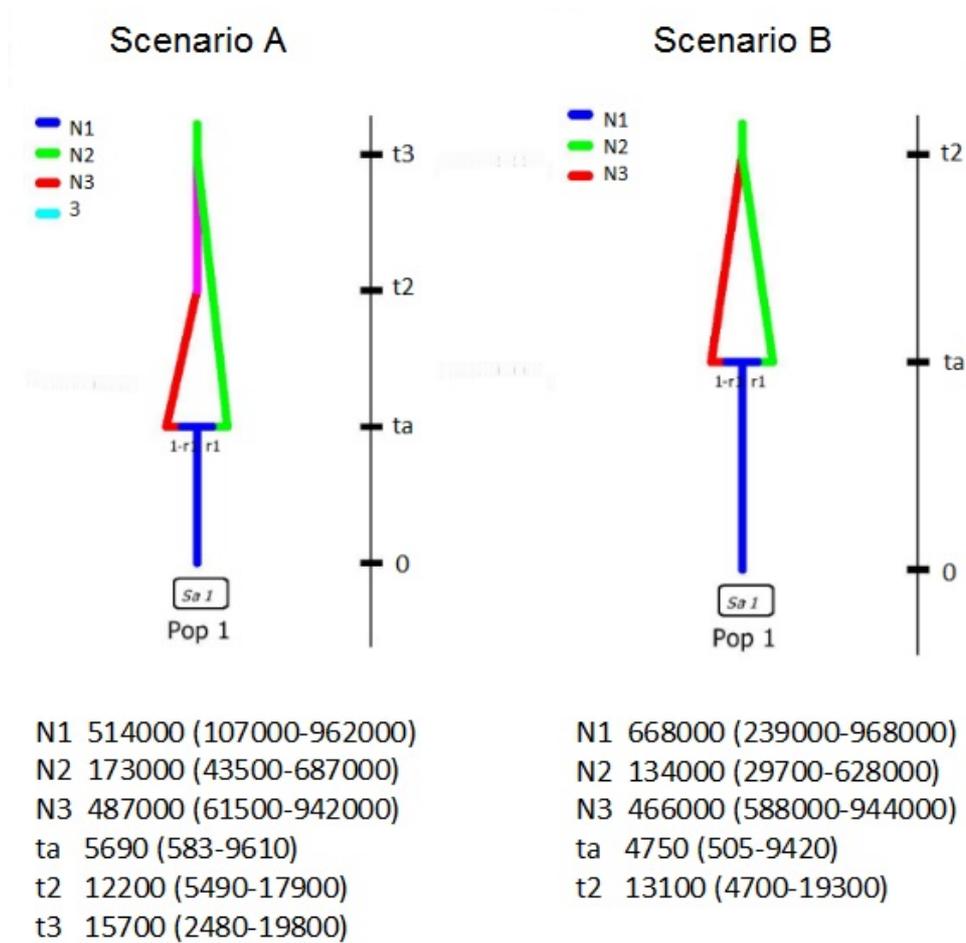


Figure 5. Demographic history of reed warbler according to the two best DIY ABC scenarios obtained from mitochondrial DNA data. The parameters estimates are provided as median (95% CI) and include the current (N1) and the ancient (N2, N3) effective population size, and timing (in terms of generations) of the admixture event (ta), population size variation (t2 scenario A) and population divergence (t3 scenario A, t2 scenario B).

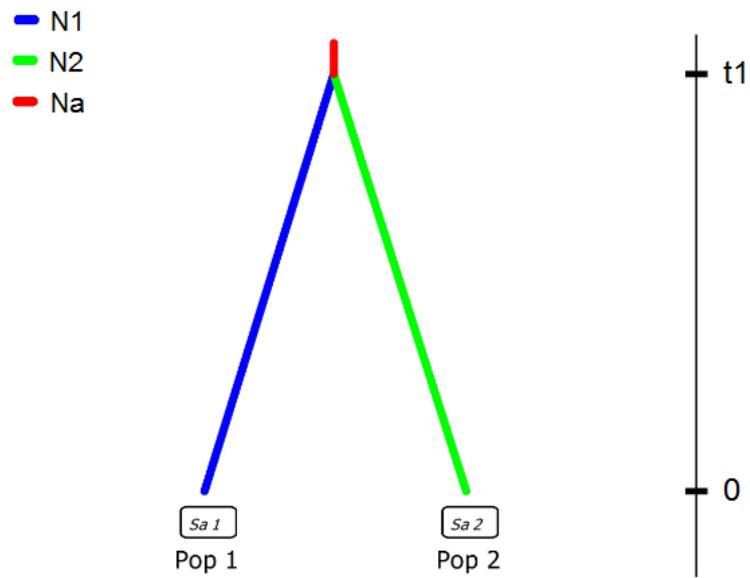


Figure 6. Demographic history of moustached warbler: best DIY ABC scenario obtained from microsatellite data. Divergence time ( $t_1$ ) of the two STRUCTURE populations (Pop 1 - Spanish mainland and Pop 2 - Mallorca) has been estimated to be 230 generations (95% CI 41 – 449).