

Multilocus approach reveals an incipient differentiation process in the Stone-curlew, *Burhinus oedicnemus* around the Mediterranean basin.

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Abstract The Stone-curlew *Burhinus oedicnemus*, a steppe bird species, is mainly distributed in the Mediterranean and Macaronesian regions, which are considered hotspots of biodiversity with priority for animal and plant species richness conservation. In this study, we investigated the genetic diversity of the Stone-curlew in the Mediterranean basin and in the Canary Islands by applying a multilocus approach. We analysed mitochondrial and nuclear markers in order to evaluate the genetic structure and the congruence between morphological subspecies and geographic samples. We found a significant level of genetic differentiation between Mediterranean and Canary Island populations with all markers. Both in the Mediterranean basin and in the Canary Islands, we found a significant level of genetic diversity with nuclear markers only. We identified seven population groups, including insular populations. The four subspecies described for the Western Palaearctic were confirmed with some changes in distribution range. In spite of habitat fragmentation and negative population trend, the Stone-curlew showed a significant level of genetic diversity and gene flow among continental populations. However, islands constitute important reservoirs of genetic diversity and a potential for the evolution of the species.

Keywords: avian, *Burhinus oedicnemus*, genetic diversity hotspot, Stone-curlew, steppe bird

Hotspots of intraspecific genetic diversity are regions of the utmost importance for species survival and conservation (Canestrelli et al. 2010). Intraspecific genetic diversity likely co-varies with species diversity in community (Vellend and Gebber 2005; Kahilainen et al. 2014), which is particularly high in hotspots of biodiversity. The Mediterranean biodiversity hotspot, which includes the Macaronesian islands (Quézel and Médail 2003), is a priority area for conservation worldwide in terms of both animal and plant species richness (Médail and Quézel 1999; Myers et al. 2000; Maiorano et al. 2013). This region's complex paleogeography has led to a high degree of endemism, which is concentrated in mountain chains and on islands (Cuttelod et al. 2008). In the Mediterranean biodiversity hotspot, hotspot islands are particularly important, as

39 the Tyrrhenian islands (Balearic Islands, Corsica, Sardinia, and Sicily) and the Macaronesian
40 islands (Canary Islands, and Madeira) (Médail and Quézel, 1999). Several endemic taxa, most
41 of them subspecies, have been recognized in the western Mediterranean islands, especially in
42 Corsica and the Balearics (Blondel et al. 2010; Maiorano et al. 2013), as well as in the Canary
43 Islands (Martín and Lorenzo 2001; Illera et al. 2012, and references therein). Although the
44 number of endemic Canarian species may be underestimated (Illera et al. 2016), the Canary
45 Islands are considered an Endemic Bird Area (Stattersfield 1998). Most of these taxa are
46 subspecies, included on the national list of threatened taxa (Madroño et al. 2004).

47 The Stone-curlew *Burhinus oedicnemus* exemplifies both the intraspecific diversity and the
48 conservation concern characteristic of species in the Mediterranean biodiversity hotspot. The
49 species is polytypic with five subspecies distributed in Africa and Western Eurasia (Cramp and
50 Simmons 1983; Fig. 1). *B. o. oedicnemus* is distributed in Western and Southern Europe and it
51 is generally migratory in temperate latitude. *B. o. saharae*, probably resident or partially
52 migratory, is distributed in Northern Africa and Eastern Mediterranean, whilst *B. o. harterti*,
53 mainly migratory, dwells Central Asia. *B. o. insularum* and *B. o. distinctus* are resident in the
54 Canary Islands (Fig. 1). The Stone-curlew mainly inhabits (pseudo) steppe and agricultural
55 lands. These habitats constitute a significant portion of the European region (European
56 Commission, 2009), and represent some of the most degraded and exploited habitats in the
57 region (Cremene et al. 2005). As a consequence, many steppe species have small, resident
58 populations isolated in small remnants of suitable habitat, which are often distantly located from
59 one another (Santos and Suarez 2005) and show decreasing population trends and an
60 unfavourable conservation status (Burfield 2005). Although Stone-curlew is listed as “Least
61 Concern” by the IUCN Red List, some populations are declining and are included in National
62 Red Lists as Vulnerable species (Italy: Peronace et al. 2012) or Endangered (Canary islands:
63 Madroño et al. 2004).

64 Data about the ecology and behaviour of the Stone-curlew are difficult to collect because of
65 its secretive and crepuscular habits; instead, molecular methods may be the most efficient way
66 in which to investigate population structure at different temporal scales. A preliminary genetic
67 study (Mori et al. 2014a) provided the first information about the genetic structure of the
68 species. In this paper, we investigated the level of intraspecific genetic diversity of the Stone-
69 curlew by using a more representative population sample and by utilizing specific microsatellites
70 (Mori et al. 2014b). Our aim was threefold: a) to characterize the genetic identity of the
71 subspecies in the Mediterranean basin and in the Canary Islands by using two different types of
72 molecular markers, mitochondrial and nuclear, in particular 20 species-specific microsatellites;
73 b) to investigate the genetic diversity level and connectivity among populations of the Stone-

74 curlew in the Mediterranean hotspot; c) to evaluate the conservation status of the Stone-curlew
75 at present and to provide some indications for future studies directed to preserve fragmented
76 populations.

78 **Materials and methods**

79 **Population sampling and DNA extraction**

80 We collected whole blood, muscle or feathers (plucked or shed) from a total of 294 individual
81 Stone-curlews from 14 regions across the species' western distribution (Fig. 1; Table 1), of
82 which 155 samples were previously analysed in Mori et al. (2014a). All samples were collected
83 during the breeding period (April – August) from known breeding areas or wildlife rehabilitation
84 centres. Stone-curlews were trapped using fall traps, mist nets, or dip nets. In the case of shed
85 feathers, we used only one feather per site. Samples were stored in absolute ethanol until DNA
86 extraction.

87 DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA) following
88 the manufacturer's protocol. DNA quality and quantity were tested by spectrophotometric
89 measurements. We obtained concentration of 15ng/μl for feathers and 100ng/ μl for blood
90 samples. 260/280 rates ranged from 1.6 to 1.8, with the lowest values in some shed feathers
91 collected on the ground during summer. Not all DNA samples gave good amplifications with
92 both nuclear and mitochondrial markers and frequently not for all 26 microsatellites. As a
93 consequence, we obtained N = 214 sequences that could be used in the analyses, while
94 nuclear markers were successfully amplified in N = 198 samples.

95 Individuals were sexed using PCR amplification of the CHD fragment as described in Griffiths
96 et al. (2008) and/or amplification of two Z-linked microsatellites (Z-002) (Dawson 2007).

98 **Mitochondrial DNA sequencing and analysis**

99 A 652 bp fragment of the NADH2 dehydrogenase was amplified using primers L5216 and
100 H6313 (Sorenson et al. 1999). PCR-reaction conditions were reported in Online Resource
101 Appendix 1. The sequences were obtained using a BigDye Terminator v.3.1 kit and run on an
102 ABI 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). All sequences were
103 visualised, manually corrected and aligned by MEGA5 (Tamura et al. 2011). Unique haplotypes
104 were identified using DNAsp v.5 (Librado and Rozas 2009).

105 A haplotype network was constructed to determine the relationships and relative frequencies
106 in each cluster (see STRUCTURE Result) using median-joining algorithm (Bandelt et al. 1999),
107 implemented in the software PopART (Leigh and Bryant 2015).

108 We used DNAsp (Rozas et al. 2003) to calculate number of haplotypes (NH), haplotypic

diversity (h) and nucleotide diversity (π) for each sampled populations. ARLEQUIN v.3.5 (Excoffier and Lischer 2010) was applied to the dataset to characterize the genetic differentiation among populations by using pairwise Φ_{ST} .

JMODELTEST 2 (Darriba et al. 2012) was used to determine the most accurate model of substitution using the Bayesian Information Criterion (BIC; Schwarz 1978) and the Akaike's Information Criterion. (AIC; Akaike 1974). Significance of pairwise tests was assessed using 10 000 permutations and was corrected for multiple tests using the Benjamini-Yekutieli correction (Benjamini and Yekutieli 2001).

Microsatellite genotyping and analysis

Twenty autosomal microsatellites isolated for the Stone-curlew (Mori et al. 2014b) and six published microsatellites designed for a wide range of birds (Dawson et al. 2010) were amplified in multiplex reactions using QIAGEN Multiplex PCR following the manufacturer standard protocol with an annealing temperature (T_a) of 57°C (see Online Resource Appendix 2 for Multiplex sets). PCR products were analysed by an ABI 310 DNA Genetic Analyzer and scored against an internal lane standard (GeneScan ROX 500) in GENEMAPPER v.3.7 (Applied Biosystems Inc.). Genotyping errors were checked with multiple blind runs of 7% of samples (Pompanon et al. 2005), with the aim to get an error rate lower than 0.8-1% (Bonin et al. 2004).

Besides, we checked for scoring errors and large allele dropouts using MICROCHECKER v.2.2.3 (Van Oosterhout et al. 2004), and calculated the frequency of null alleles for each locus and each population using the software CERVUS v.3.0 (Kalinowski et al. 2007). The significant threshold of frequency (p) for null alleles is 20% ($p > 0.20$, following Dakin and Avise 2004). We tested for significant departures from Hardy–Weinberg equilibrium (HWE) proportions using the procedure described in Guo and Thompson (1992), and measured linkage disequilibrium (LD) using Fisher's exact tests. Both tests were performed in GENEPOP v.4.2 (Rousset 2008) using 10 000 dememorizations, 1000 batches and 10 000 iterations per batch and were conducted for each population. The level of significance was corrected for multiple tests using the Benjamini-Yekutieli technique. For each locus, we calculated the number of alleles (n_A), polymorphic information content (PIC), observed (H_O) and expected heterozygosity (H_E).

In order to evaluate the levels of genetic diversity, we calculated for each population: observed (H_O) and estimated heterozygosity (H_E), inbreeding coefficient (F_{IS}) and its significance after 1000 permutations in GENETIX v.4.05 (Belkhir et al. 1996–2004) and allelic richness (K_{AR}), private allelic richness (K_{PAR}) in HP-RARE (Kalinowski 2005). In order to infer the genetic differentiation among populations, pairwise F_{STs} between populations were estimated using ARLEQUIN. The level of significance was assessed using 10 000 permutations and corrected

144 with the Benjamini-Yekutieli correction.

145 An Isolation By Distance (Wright 1943) test was permuted in SPAGeDI software (Hardy et al.
146 2002) on the entire dataset and on the only Mediterranean cluster (see Results section), to
147 analyse the effect of geographical distance on genetic distance (F_{ST}). An estimate of the
148 correlation between the matrix of genetic distance between pairs of populations ($F_{ST}/1 - F_{ST}$)
149 and the matrix of the natural logarithm of geographic distances was calculated. The slope of this
150 relationship was tested for a significant difference from zero by 10 000 permutations and a
151 jackknife procedure over loci was used to estimate standard errors.

152 We used two Bayesian clustering softwares to infer population structure: STRUCTURE
153 v.2.3.3 (Pritchard et al. 2000) and TESS v.2.3 (Durand et al. 2009), which incorporates
154 geographic coordinates of the capture/collection sample sites, as *a priori* information. Analysis
155 run in STRUCTURE were conducted using the admixture and correlated allele frequencies
156 models and no *a priori* information. The number of clusters (K) tested ranged from 1 to 10, with
157 five replicates (runs) per K. All runs consisted of 2×10^6 Markov chain Monte Carlo (MCMC)
158 steps after a burn-in of 2×10^5 steps. The best K (given the data) was determined using the
159 Evanno method (Evanno et al. 2005), based on ΔK calculated using STRUCTURE
160 HARVESTER v.0.5 (Earl and von Holdt 2012), after having ruled out $K = 1$ by examining the
161 mean log probability. Once the best K was determined, each individual was assigned to a
162 cluster based on maximum membership proportions. In order to accurately identify a possible
163 subpopulation structure, the analysis was re-run in each of the identified clusters. Our initial
164 tests showed that the program was unable to find any structure without *a priori* knowledge
165 within the Mediterranean cluster (see Results section). Therefore we included the sampling
166 location as prior in order to assist clustering (LOCPRIOR model, Hubisz et al. 2009). This model
167 is useful to assist clustering when the signal of structure is relatively weak (for example,
168 significant but low F_{ST}). Furthermore, it does not tend to find structure when none is present and
169 it can ignore sampling information when ancestry of individuals is uncorrelated with sampling
170 locations (Hubisz et al. 2009).

171 TESS was run using the admixture model where the prior distribution on cluster labels is
172 determined by a hidden Gaussian random field (CAR model). This approach may provide lower
173 error rates than other clustering methods when low levels of genetic structure are observed
174 (Chen et al. 2007). As individuals of some populations shared the same coordinates, we
175 randomized geographic coordinates for each individual using the specific command in the
176 software. We ran ten replicate runs for each K set from 2 to 10 using a burn-in of 50 000 steps
177 followed by 500 000 MCMC steps. The best K_{max} was chosen based on the largest drop in mean
178 Deviance Information Criterion (DIC) value between successive K_{max} values, after averaging the

179 DIC across the ten replicate runs for each K (Durand et al. 2009) and by examining plots of
180 individual membership probabilities. Once the best $K_{\max}$ was determined, 50 runs were
181 conducted and from these the 20% with the lowest DIC values were used to calculate average
182 individual admixture proportions.

183 For both STRUCTURE and TESS, we calculated the average of the run replicates and
184 averaged the results of the replicate runs using CLUMPP v.1.1.2 (Jakobsson and Rosenberg
185 2007).

186 We used factorial correspondence analysis (FCA) to examine genetic differentiation of
187 multilocus genotypes with GENETIX v.4.05. FCA is a multidimensional statistical method to
188 visualize data that is superior to principal component analysis when discrete variables such as
189 codominant microsatellite loci are involved.

190

191 **Demographic tests**

192 We used DNA-sp to investigate demographic history by mtDNA data, by calculating
193 deviations from neutrality with Tajima's D (Tajima 1989), Fu and Li's F (Fu and Li 1993), Fu's F_s
194 (Fu 1997) tests for each cluster resulting from the STRUCTURE analysis (see Results). Canary
195 Islands that showed a single fixed mtDNA haplotype (see Results), was excluded. Significant
196 negative D , F and F_s values were considered indicative of a population expansion.

197 We used two approaches to test for genetic bottlenecks at the microsatellite loci: 1) the "M-
198 ratio test" developed by Garza & Williamson (2001), based on the ratio of the number of
199 microsatellite alleles (K) to the range in allele size (r), i.e. $M = K/r$. The M value is expected to be
200 smaller in bottlenecked populations than in equilibrium populations. 2) the 'heterozygosity-
201 excess' test developed by Cornuet & Luikart (1996) that contrasts heterozygosity expected
202 under Hardy–Weinberg equilibrium with heterozygosity expected under mutation-drift
203 equilibrium calculated from observed number of alleles. In a recently bottlenecked population,
204 the observed heterozygosity is higher than the heterozygosity expected from the observed
205 number of alleles under the assumption of a population at mutation–drift equilibrium (i. e.
206 heterozygosity excess is detected).

207 M values were calculated for each cluster in Arlequin and tested by using a critical value of
208 0.68, derived from putatively stable wild populations (Garza & Williamson 2001). The
209 'heterozygosity-excess' test was conducted for each cluster using the software BOTTLENECK v
210 1.2.02 (Piry et al. 1999). A one-tail Wilcoxon's test (Luikart 1997) was performed with 1000
211 replicates, under the two-phase mutation model (TPM) with 95 % of single-step mutation and
212 5% of multi-step changes and a variance among multiple steps of 12, as recommended for
213 microsatellites loci (Piry et al. 1999). In addition, a descriptor of the allele frequency distribution

214 “mode-shift” was used to discriminate stable populations from populations that have undergone
215 a recent bottleneck (Luikart et al. 1998).

217 **Estimating recent migration rate**

218 In order to estimate rates of recent migration (within a few generations) among
219 Mediterranean populations (Mediterranean cluster, see “Results” section), the test for first-
220 generation migrants (F_0) was performed using a Bayesian approach (Rannala and Mountain
221 1997). The test was paired with a Monte Carlo re-sampling method for probability computation
222 of Paetkau et al. (2004) using a 10 000 simulated individuals and an assignment threshold
223 (α) set at 0.05. For the likelihood computation we used the likelihood ratio L_{home} (L_h), that
224 is the most appropriate estimation when it is not sure that all source populations were sampled
225 (Paetkau et al. 2004). The analysis was performed in the software GENECLASS v.2 (Piry et al.
226 2004).

228 **Results**

229 **Mitochondrial data**

230 The 214 NADH2 sequences obtained contained 36 variable sites defining 41 haplotypes, 26
231 of which come from a previous study (Mori et al. 2014a). Results from the median-joining
232 haplotype network showed a low level of phylogeographic structure among the Mediterranean
233 samples, with some reticulations probably explained by migration events. H1 is the most
234 common haplotype, shared by 50.5% of individuals from all the other populations (Fig. 2), with
235 the exception of Canary Island populations that shared an exclusive and single fixed haplotype
236 (H2).

237 Mitochondrial genetic diversity was zero in the Canary Islands due to the presence of a single
238 fixed haplotype, while the other populations present very different values of haplotype and
239 nucleotide diversity. Global haplotype diversity h is 0.710 and nucleotide diversity π is 0.00378
240 (Table 2).

241 JMODELTEST 2 selected the TNR3 + I as model of substitution and the Tamura and Nei
242 (1993) (the closest model to TNR3 + I) was used to calculate Φ_{ST} pairwise comparison. Table 3
243 shows that no comparison were significant, with the exception of the three Canary Island
244 populations versus all the Mediterranean populations.

246 **Microsatellite data**

247 We successfully genotyped 198 individuals. Polymorphism at the 26 microsatellite loci was
248 from moderate to high, with nA varying from 2 to 66 and PIC varying from 0.087 to 0.973 (see

Online Resource Appendix 3). After multiple runs, we calculated a genotyping error rate extensively less than 0.8%. No evidence for the presence of scoring errors, large allele dropouts or null alleles was detected, with the exception of locus TG0-05053 where CERVUS detected a frequency of null alleles higher than 20%, but only in three populations. Tests of Hardy-Weinberg equilibrium conducted within populations, detected heterozygosity deficiencies at locus Boe03 in TAR, at Boe10 in SIC, BAL and GC, at Boe14 in GC, at Boe16 in TAR, VEN, SIC and TUN and at TG0-05053 at VEN, SIC, BAL and GC (see Online Resource Appendix 4 for detailed table). Significant LDs were found for three pairs of loci in four sites: Boe06-Boe13 in SIC and VEN, Boe12-Boe19 in TUN, Boe02-Boe09 in TEN. As the three pairs of loci never had a significant LD more than twice, we considered the 26 loci to be independent. As deviation from HWE and linkage were inconsistent across loci and locus pairs, as well as null alleles, we retained all loci in subsequent analyses.

Expected heterozygosity (H_E), observed heterozygosity (H_O), inbreeding coefficient (F_{IS}), allelic richness (K_{AR}) and private allelic richness (K_{PAR}) were reported in Table 2. Inbreeding coefficient was positive and significant in Sicily, Cyprus and Greek Island populations, indicating an excess of homozygotes in these populations.

Differently from the mtDNA pairwise Φ_{STs} , all nuclear F_{ST} pairwise comparisons were significant. Significant differentiation was found among the three Canary Island populations and all Mediterranean populations, and among populations within Mediterranean area (Table 3).

The test for isolation by distance revealed a positive correlation between geographical distance and genetic distance between populations ($r = 0.22$, $p = 0.005$), suggesting that genetic differentiation increases with increasing distances between populations, while the same test on the only Mediterranean cluster was not significant ($r = 0.02$, $p = 0.40$).

Applying STRUCTURE and the Evanno method to the entire data set, the best supported number of clusters data was two ($K = 2$) (see Online Resource Appendix 5 for Evanno results). High ancestry proportions ($q > 0.8$) for one of the two clusters were seen in all individuals, with few exceptions. Individuals from the “Mediterranean cluster” were sampled in the Mediterranean region, while individuals from the “Canary Island cluster” were sampled in the Canary Islands (Fig. 3a). The analyses of the Mediterranean subdivision identified six genetically distinct clusters ($K = 6$) (see Online Resource Appendix 6 for Evanno results): most individuals from Taro, Tuscany and Veneto were allocated to cluster M-I, individuals belonging to the three insular populations of Corsica, Sicily and Balearic Islands were allocated to three different clusters (Cluster M-II, M-III and M-IV). Individuals from Southern Mediterranean populations, Tunisia, Greek Islands, Israel and Cyprus, were assigned to Cluster M-V, while Sardinia individuals resulted with moderate proportions assigned to both Cluster M-III and Cluster M-VI,

284 with some individuals exclusively assigned to cluster M-VI (Fig. 3b) (see Online Resource
285 Appendix 7 for proportion of individuals assigned to each clusters). Since the proportion of
286 individuals assigned to the Cluster M-III was greater than the same proportion assigned to
287 Cluster M-VI, for the analyses considering the cluster subdivision, Sardinia was included in the
288 Cluster M-III together with Sicily. The Canary Island cluster presented two sub-groups ($K = 2$)
289 (see Online Resource Appendix 8 for posterior probability plot) with all Fuerteventura individuals
290 assigned to one cluster (cluster C-I) and Gran Canaria and Tenerife samples composed of a
291 mixture of individuals assigned to both cluster C-I and cluster C-II (Fig. 3c).

292 TESS produced results very similar to STRUCTURE, but failed to reveal structure within the
293 Mediterranean cluster, while clearly identified two groups within the Canary Island cluster (data
294 not shown).

295 The factorial correspondence analysis (FCA) confirm the Bayesian analyses results. Although
296 the first two axes described only the 4.66% of the genetic variation, a clear separation was
297 observed between the Canary Islands and the Mediterranean populations (see Online Resource
298 Appendix 9 for the figure).

299

300 **Demographic test**

301 Neutrality indices (D , F and F_s) calculated on the different Stone-curlew genetic populations
302 were not significant for any group, not supporting any population expansion.

303 The heterozygosity excess test performed on microsatellite data did not reveal any evidence
304 for a historical or recent bottleneck in any group (one-tailed Wilcoxon test: all groups $p > 0,59$).
305 Likewise, the distribution of allele frequencies was L-shaped supporting the idea of long-term
306 stable population sizes. Also the M ratio of allelic richness to allelic size range did not indicate
307 bottleneck for the genetic populations analysed (all value ≤ 0.36) (see Online Resource
308 Appendix 10 for Table).

309

310 **Recent migration rate**

311 In total 21 individuals (14.2% of the sampled population) ($p < 0.05$) were identified as first
312 generation migrants by GENECLASS. The major proportion of migrants were found in the
313 Greek Is., Sardinia, Corsica and Israel. Fourteen individuals emigrated from Northern-central
314 Italy populations, in particular, seven from the Taro Park, six from Tuscany and one from Veneto
315 (see details in Online Resource Appendix 11).

316

317 **Discussion**

318 The molecular analysis of both mitochondrial and nuclear markers confirmed the genetic

319 divergence between Canary and Mediterranean populations of the Stone-curlew first found by
320 Mori et al. (2014a). Furthermore, the addition of specific microsatellites (Mori et al 2014b) and of
321 new sampled populations illuminated the intraspecific diversity of the taxon in both the Canary
322 Islands and the Mediterranean region. In the range of the previously described four subspecies
323 (Mediterranean: *B. o. oedichnemus*, *B. o. saharae*; Canary Islands: *B. o. insularum*, *B. o.*
324 *distinctus*), microsatellites identified seven different groups: two (Gran Canaria+Tenerife,
325 Fuerteventura) in the Canary Islands and five (Balearic Islands, Corsica, Sardinia+Sicily,
326 North-central Italy) in the Mediterranean basin.

327 As expected considering the large geographic distance, the Canary Island populations were
328 well-differentiated from the Mediterranean ones. In spite of the increased number of samples
329 analysed in Mori et al. 2014a, mitochondrial markers showed no genetic variation within the
330 Canary Islands, confirming previous results (Mori et al. 2014a). As already reported for several
331 avian species in the Macaronesian region (eg. Agudo et al. 2011; Spurgin et al. 2014), these
332 results could indicate a recent bottleneck or a founder effect, although the statistical test results
333 were not significant. In contrast to the findings of Mori et al. (2014a), nuclear markers identified
334 a clear divergence between the Eastern (Fuerteventura) and Western (Gran Canaria, Tenerife)
335 Canary Islands, supporting the presence of two distinct taxonomic units, identifiable with the two
336 previously described subspecies (*B. o. insularum* and *B. o. distinctus*) (Fig. 3c).

337 As for Macaronesia, mtDNA failed to identify the two subspecies reported for the
338 Mediterranean basin, *saharae* and *oedichnemus*. However, specific microsatellite markers
339 revealed cryptic genetic structure. The primarily-mainland populations clustered into two groups,
340 northern (Taro Park, Veneto, Tuscany) and southern (Tunisia, Israel, Greek Islands, Cyprus).
341 Interestingly, the populations of islands such as Sicily, Corsica and Balearics (Fig 3b) showed
342 differentiation from the other Mediterranean samples, with the exception of the Sardinian
343 population, which was genetically close to Sicily. This result supports the role of Western
344 Mediterranean islands as epicentres of incipient differentiation for taxa including those with high
345 over-sea dispersal propensities, such as birds (Blondel et al. 2010; Pons et al. 2015a,b).

346 Among Western insular populations, Corsica was particularly interesting. Consistently with
347 the high level of bird endemism reported for this island (see Pons et al. 2015a,b and references
348 therein), Corsican Stone-curlews showed an exclusive nuclear pattern not shared with the other
349 nearest analysed populations (Sardinia and North-central Italy). Little information about the
350 status of Stone-curlews in Corsica exists, but new investigations (Jolin 2004; Seguin 2015)
351 found a recent, marked increase of the breeding population (now estimated as ~170 pairs). As
352 the mtDNA analyses did not provide any evidence of a founder effect, the genetic
353 distinctiveness of Corsican birds might be due to a recent and independent colonization wave

354 possibly from the nearby (but no sampled) southern France populations.

355 Mitochondrial and nuclear markers showed different power in describing the genetic structure
356 of *B. oedicnemus*, as found in Mori et al (2014a). We found a congruence pattern at a broader
357 geographic level (distinction between Canary Islands and Mediterranean populations), while
358 discordance was found at a fine scale level, with a more structured pattern explained by nuDNA
359 markers. Discordant patterns between mitochondrial and nuclear genetic markers (i.e. mito-
360 nuclear discordance), frequently identified, also in previous studies on birds (Toews and
361 Brelsford 2012 and references therein), can be explained by differences in the mutation rate and
362 effective population size between the two markers, selective sweeps, introgression or
363 demographic disparities and sex-biased asymmetries (Zink and Barrowclough 2008). In the
364 Stone-curlew, the female dispersal could be an important factor influencing the incongruence
365 between the two markers. Unfortunately, the data did not allow to make reliable assumptions on
366 this discordance, even though new sex-linked markers are under development (Baratti et al. in
367 progress).

368 On the basis of the first-generation migrants test, the Northern-central Italy populations seem
369 to be the main external source of individuals for all the other studied areas, with the exception of
370 Sicily. Although data regarding the dispersal movements of Stone-curlews are extremely scarce
371 (Vaughan & Vaughan-Jannings 2005), our results suggests that Northern Italy birds could
372 disperse longer distances than do the remaining populations. Unlike the other populations,
373 which are mainly resident or partially migratory, most of the Northern Italy populations are
374 characterized by full migration and also by a remarkably positive population trend (Biondi et al.
375 2015). At the species level, migration and population trend are positively correlated with
376 dispersal distance in birds (Paradis et al. 1998). Our findings suggest that this pattern could also
377 be true at the population level. It is interesting to observe that contact between Stone-curlews
378 from Northern Italy and Tunisia, Corsica and Sardinia probably occurs during the wintering
379 period (Giunchi et al. 2015; Giunchi et al., unpublished data). Contrary to what has been
380 observed in other bird species (e.g. *Ciconia ciconia*; Itonaga et al. 2010), however, the dispersal
381 pattern seems not to be influenced by the preferred migration routes, as we did not observe any
382 relevant bias in the gene flow among Northern Italy, Tunisia, Corsica and Sardinia populations.

383 In this study, we detected a level of population differentiation not detected in a previous paper
384 by the authors (Mori et al. 2014a). Most part of the genetic Mediterranean diversity is located in
385 islands, confirming their importance for the conservation of Stone-curlews in the Mediterranean
386 biodiversity hotspot. We suggest considering the Mediterranean and Canary Islands populations
387 as two different Evolutionary Significant Units (ESUs) (Moritz 1994) for their significant
388 mitochondrial and nuclear distinctiveness.

389 Within the Canary Islands the two groups observed correspond to the two previously
390 described subspecies (*B. o. distinctus* and *B. o. insularum*). The genetic identification of the
391 Canary subspecies is of particular concern given their small population size (especially for
392 *distinctus* populations) and their generally decreasing trend (with the exception of Gran Canaria
393 population) (Delany et al. 2009), encouraging their conservation. In the current Spanish
394 Catalogue of Threatened Species (Real Decreto 139/2011) both subspecies have different
395 categories of threat (“Vulnerable” for *distinctus*, and “Especial Protection Regime” - equivalent to
396 Least Concern in IUCN categories - for *insularum*). However, given the high risk of habitat loss
397 and fragmentation (Madroño et al. 2004), the negative impact of power lines (García del Rey
398 and Rodríguez-Lorenzo 2011) and the genetic distinctiveness observed in this paper, the
399 Canarian populations should be considered of a high conservation concern (see also Seoane et
400 al. 2011).

401 In Mediterranean populations, microsatellite markers supported a genetic substructure,
402 suggesting an interesting level of genetic differentiation, meaningful for the conservation status
403 of the species and, generally, for the Mediterranean biodiversity hotspot. However, the
404 taxonomic assignment of the five groups identified in the Mediterranean basin in the range of *B.*
405 *o. oediconemus* and *B. o. saharae* is difficult using genetic data only, given the lack of systematic
406 phenotypic and ecological studies on these populations.

407 On the basis of the first-generation migrants detection, Mediterranean islands resulted a
408 recipient population respect to the mainland populations, but the mechanisms of dispersal of
409 this species are almost unknown. The mainland Northern-central Italy populations seem to play
410 an important and unexpected role as a source of immigrants for most of the other populations.
411 This long-range dispersal may inhibit further diversification within the Mediterranean basin (see
412 also Weeks and Claramunt 2014), but the mechanisms underlying this phenomenon are not
413 known. Further investigations regarding both genetic and behavior are thus needed to
414 understand dispersal in this species, especially the role of sex in this behavior and whether
415 long-range movements indicate a substantial saturation of suitable breeding habitats within the
416 Italian peninsula.

417 Molecular results obtained in this study show that in spite of habitat fragmentation and
418 negative population trend, the Stone-curlew exhibited a significant level of diversity and
419 connectivity that has several practical implications for its conservation. The data supported both
420 the existence of all subspecies described for this area and the Macaronesian and
421 Mediterranean islands as significant reservoirs of intraspecific diversity. These aspects are
422 particularly relevant for the conservation of the taxon and the identification of nuclear genetic
423 groups should be considered for planning networks among diversity resource centers. As

pointed out by Illera et al. (2016), the taxonomic status of all endemic subspecies recognized on the Canary Islands should be revised using an integrative taxonomic approach, as it is likely that several of them could be raised to full species status (see e.g. Sangster et al. 2016). Given the genetic distinctiveness of Canary Stone-curlews recorded in our analysis, it seems necessary to plan future investigation on morphological and behavioral characters and to sample population from nearby areas (e.g. continental Spain and Morocco). A multidisciplinary approach will have significant implications for the conservation of the Stone-curlew in the Mediterranean biodiversity hotspot and it will provide also useful information for better understanding the evolutionary history of Canarian taxa.

Acknowledgements

We are grateful to all the people who helped us conduct field work. We thank for the Canary Islands: Wildlife Rehabilitation Centres, Á.C. Moreno, R. Barone and V.R. Cerdeña; Balearic Islands: L. Parpal, J. Solà and G. Tavecchia; Italy: A. Barbon, N. d'Apolito, S. Franci, M. Dragonetti, C. Fiesoli, P. Giovacchini, F. Corsi, C. Caccamo, E. Pollonara, M.T. Spena, G. Insacco, D. Pisu and all staff of the Taro River Regional Park (Parma District); Corsica Island: J.F. Seguin; Greece and Cyprus: C. Barboutis; Tunisia: S. Nouira, R. Ouni; Israel: R. Nathan, N. Aljadeff. We thank also all the students who helped us in the field and the laboratory. We are also grateful to Lorenzo Zane for support for statistical analyses. We appreciate the improvements in English usage made by Caitlin Stern through the Association of Field Ornithologists' program of editorial assistance.

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FIGURE CAPTIONS

Figure 1. Distribution of the five *Burhinus oedicnemus* subspecies and sampling locations in the Mediterranean area and Canary Islands: TEN: Tenerife; GC: Gran Canaria; FV: Fuerteventura; BAL: Balearic Islands; SAR: Sardinia; COR: Corsica; TAR: Taro River Regional Park; VEN: Veneto; TUS: Tuscany; SIC: Sicily; TUN: Tunisia; GRE: Greek Islands; CYP: Cyprus; ISR: Israel.

Figure 2. A median-joining network of Stone-curlew NADH2 haplotypes. Circle size is proportional to haplotype frequency. Haplotypes are colour-coded according to the cluster subdivisions that resulted from Bayesian analysis in STRUCTURE.

Figure 3. Bar plots estimated by STRUCTURE showing the assignment probability of each sampled individual to the genetic clusters. Each bar represents a single individual with populations separated by black lines. a) STRUCTURE plot for $K = 2$, calculated for all samples ($N = 198$); b) STRUCTURE plot for $K = 6$ calculated for the Mediterranean cluster ($N = 150$) with LOCPRIOR option; c) STRUCTURE plot for $K = 2$ calculated for the Canary Island cluster ($N = 48$)

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Table 1. Details of Stone-curlew samples used for genetic analyses.

Sampling areas (acronym)	Sampling localities	Movements ^a	Putative subspecies ^a	Type of sample	N (micro)	N (mtDNA)
Taro River Regional Park (TAR)	Parma (44.76 N, 10.18 E)	mainly migrant	<i>oedicnemus</i>	Blood or plucked feathers	24	41
Veneto (VEN)	Veneto (45.72 N, 12.40 E)	mainly migrant	<i>oedicnemus</i>	Plucked feathers	17	15
Tuscany (TUS)	Maremma (42.65 N, 11.26 E)	mainly resident	<i>oedicnemus</i>	Plucked feathers	19	14
Corsica (COS)	Aghione (42.11 N, 9.40 E) ? Luccione (42.55 N, 9.42 E) Ghisonaccia (42.02 N, 9.41 E)		<i>oedicnemus</i>	Shed feathers	10	8
Sardinia (SAR)	Asinara Island (40.99 N, 8.23 E)	mainly resident	<i>oedicnemus</i>	Blood or plucked feathers	7	6
Sicily (SIC)	Siracusa (37.15 N, 15.23 E) Ragusa (36.96 N, 14.56 E)	mainly resident	<i>oedicnemus</i> *	Blood or plucked feathers Recovery centre (part)	20	46
Balearic Islands (BAL)	Menorca (39.96 N, 4.07 E)	mainly resident	<i>oedicnemus</i>	Recovery centre	21	18
Tunisia (TUN)	Sidi-Thabet (36.52 N, 10.01 E) Kairouan (35.59 N, 10.01 E) Kerkenna Is. (34.45 N, 11.14 E) Gabes (33.46 N, 10.11 E) Bouhedma (34.48 N, 9.45 E)	resident/ partial migrant?	<i>saharae</i>	Blood or plucked feathers	17	17
Greek Islands (GRE)	Crete (35.37 N, 24.81 E) Rhodes (36.23 N, 27.94 E)	resident	<i>saharae</i>	Dead birds	6	5
Israel (IS)	31.74 N, 34.99 E	resident	<i>saharae</i>	Plucked feathers	7	7
Cyprus (CYP)	34.89 N, 32.97 E	resident	<i>saharae</i>	Dead birds	2	2
Eastern Canary Islands (FV)	Fuerteventura (28.42 N, 14.01 W)	resident	<i>insularum</i>	Recovery centre	9	7
Western Canary Islands (GC, TEN)	Gran Canaria 27.95 N, 15.57 W Tenerife 28.31 N, 16.49W	resident	<i>distinctus</i>	Blood (GC) Shed feathers (TEN)	23 16	22 6

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^a According to Cramp and Simmons (1983) and Vaughan and Vaughan-Jennings (2005).

* According to Mori et al. 2014a

664 **Table 2.** Mitochondrial and nuclear diversities for the global population, each sampling
 665 locations, and each of the two principal clusters identified by the STRUCTURE analysis
 666 (Mediterranean cluster and Canary Island cluster). N, number of samples; K_{AR} , allelic richness;
 667 K_{PAR} , private allele richness; H_O , average observed heterozygosity; H_E , average expected
 668 heterozygosity; F_{IS} , inbreeding coefficient; N_h , number of haplotypes; h haplotype diversity; π ,
 669 nucleotide diversity. In bold significant value ($p < 0.05$).
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Sampling areas	Microsatellites						mtDNA			
	N	K_{AR}	K_{PAR}	H_E	H_O	F_{IS}	N	N_h	h	π
Taro Park	24	1.68	0.19	0.66	0.66	0.02	41	11	0.710	0.00378
Tuscany	19	1.66	0.17	0.64	0.66	0.01	14	2	0.264	0.00040
Sardinia	7	1.68	0.19	0.63	0.66	0.03	6	3	0.060	0.00184
Corsica	10	1.61	0.14	0.57	0.61	0.01	8	2	0.250	0.00192
Veneto	17	1.66	0.17	0.64	0.64	0.04	15	6	0.571	0.00120
Sicily	20	1.67	0.18	0.65	0.61	0.09	46	17	0.807	0.00652
Greek Is.	6	1.66	0.19	0.60	0.61	0.07	5	5	1.000	0.00552
Tunisia	17	1.70	0.21	0.67	0.63	0.09	17	4	0.551	0.00329
Balearic Is.	21	1.67	0.18	0.66	0.65	0.04	18	4	0.399	0.00066
Israel	7	1.71	0.24	0.66	0.70	0.02	7	1	0.000	0.00000
Cyprus	2	1.65	0.21	0.49	0.60	0.13	2	2	1.000	0.00153
Fuerteventura	9	1.65	0.20	0.61	0.64	0.01	7	1	0.000	0.00000
Gran Canaria	23	1.64	0.23	0.62	0.60	0.06	22	1	0.000	0.00000
Tenerife	16	1.61	0.23	0.59	0.60	0.02	6	1	0.000	0.00000
Mediterranean cluster	150	8.07	3.03	0.69	0.64	0.08	179	40	0.624	0.00393
Canary Is. cluster	48	0.30	1.63	0.64	0.61	0.07	35	1	0.000	0.00000
ALL	198			0.66	0.64		214	41	0.710	0.00378

671 **Table 3.** Estimates of population pairwise genetic distance values calculated for microsatellite
672 loci below the diagonal (F_{ST}) and for mtDNA above the diagonal (Φ_{ST}). In bold are values that
673 were significant after the Benjamini-Yekutieli correction. Populations with $N \leq 7$ were excluded
674 from the analysis.
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	Taro Park	Veneto	Tuscany	Corsica	Sicily	Tunisia	Balearic Is.	Fuertev entura	Gran Canaria	Tenerife
Taro Park	-	0.144	0.161	0.043	-0.010	-0.006	0.143	0.318	0.410	0.307
Veneto	0.020	-	0.035	0.008	0.104	0.073	0.006	0.642	0.766	0.628
Tuscany	0.021	0.024	-	0.060	0.114	0.109	0.043	0.848	0.910	0.840
Corsica	0.045	0.048	0.039	-	0.009	-0.034	0.013	0.595	0.771	0.571
Sicily	0.020	0.034	0.017	0.044	-	-0.007	0.104	0.239	0.324	0.227
Tunisia	0.028	0.032	0.030	0.037	0.034	-	0.069	0.406	0.559	0.389
Balearic Is.	0.026	0.041	0.019	0.040	0.028	0.032	-	0.756	0.840	0.747
Fuerteventura	0.063	0.106	0.080	0.123	0.066	0.066	0.074	-	0.000	0.000
Gran Canaria	0.094	0.074	0.110	0.163	0.093	0.113	0.114	0.055	-	0.000
Tenerife	0.094	0.122	0.111	0.183	0.095	0.126	0.119	0.077	0.025	-

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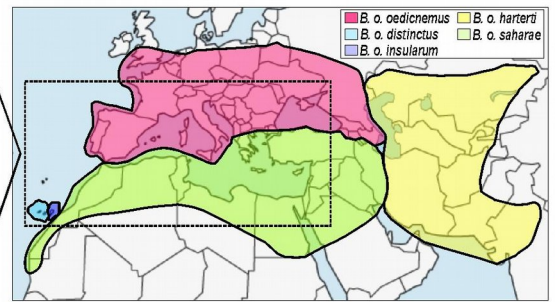
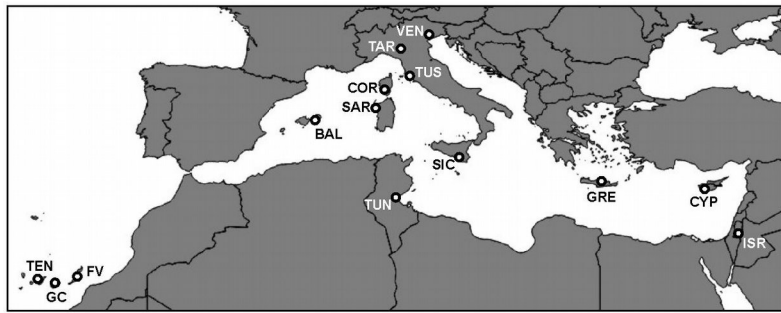
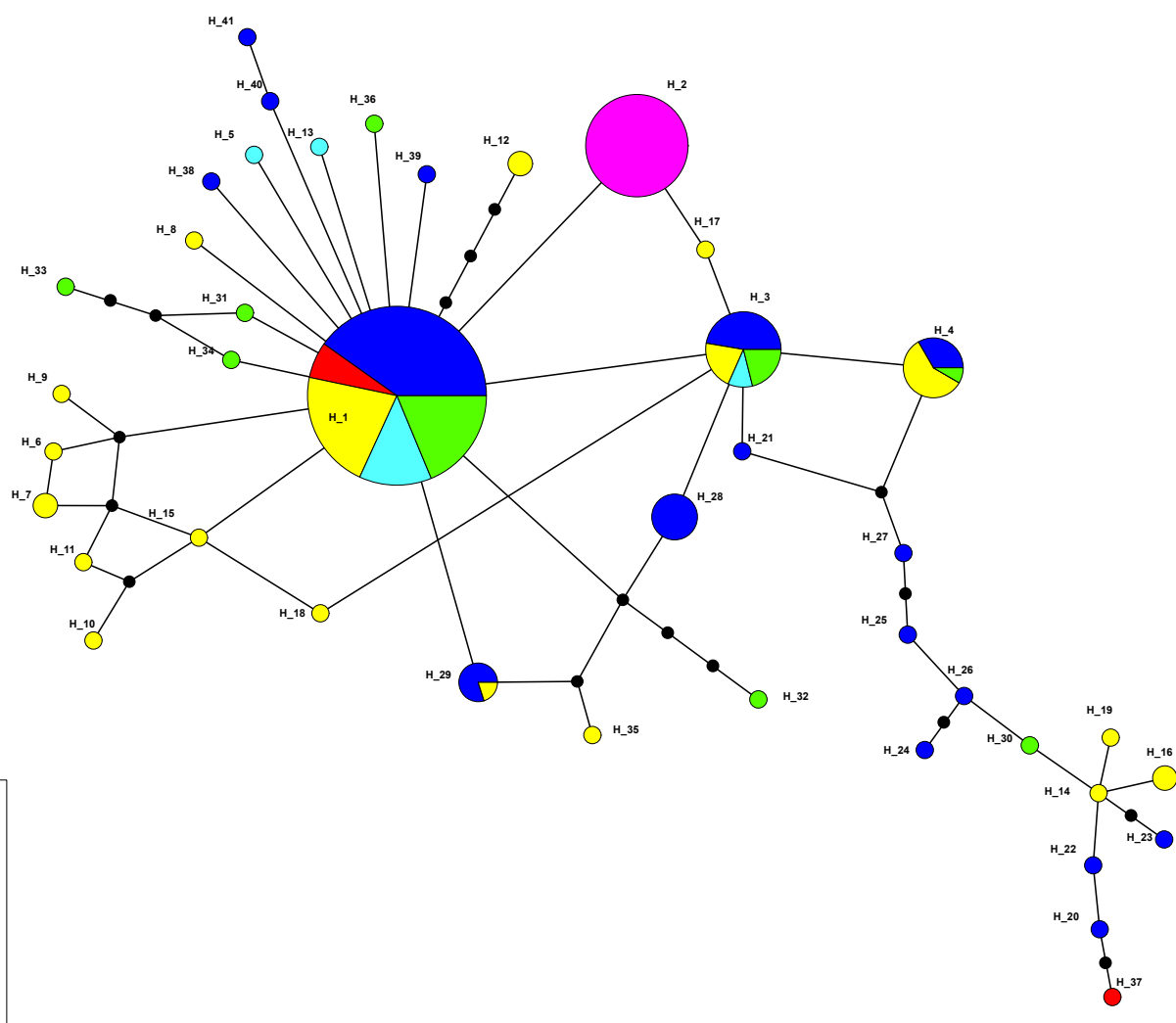
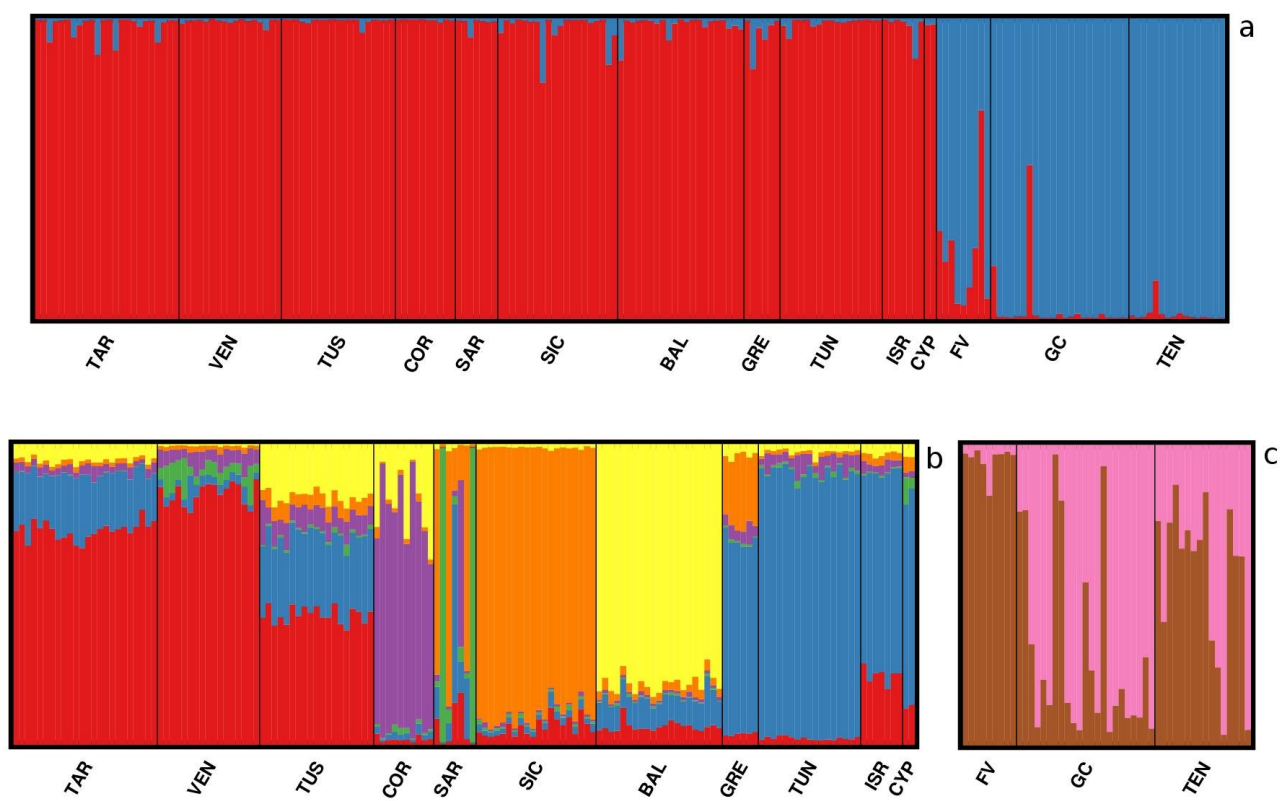


Fig. 1



680 Fig. 2

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682 Fig. 3