

1 **Running Head: Genetic variability in the Eurasian Stone-curlew**

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3 **A first assessment of the genetic variability in the Eurasian Stone-curlew**  
4 ***Burhinus oedicephalus***

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23 The Eurasian Stone-curlew is a species of conservation concern in Europe. We investigate for the  
24 first time the extent of population structure among populations sampled from six geographic areas,  
25 representing four subspecies inhabiting the western part of the species' distribution range. Both  
26 mitochondrial and nuclear markers did not fully support current subspecies boundaries. However,  
27 both markers support significant differentiation of the Canary Island populations from those  
28 sampled from the Mediterranean. Further work is needed to establish the taxonomic status of this  
29 putative distinct Macaronesian taxon. More broadly, further research is required in order to design  
30 and implement an effective conservation plan for this species.

31

32 **Keywords:** avian subspecies, microsatellite, NADH2, shorebirds

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34 The Eurasian Stone-curlew *Burhinus oedicephalus* is a species of European conservation concern  
35 (SPEC3, BirdLife International 2012). It is considered an intra-Palaeartic migrant, but several  
36 populations are probably facultative migrants or resident (Vaughan & Vaughan-Jennings 2005).

37 With the exception of populations in the United Kingdom (Green *et al.* 1997), wintering ranges and  
38 migration routes are not well understood due to the lack of focal studies and the scarcity of ringed  
39 bird recoveries (Cramp & Simmons 1983). Very little data exists on dispersal (see Vaughan &  
40 Vaughan-Jennings 2005), as detailed information on the ecology and behaviour of this species are  
41 difficult to collect, given its secretive and crepuscular habits (Cramp & Simmons 1983). Hence, the  
42 utilization of molecular markers are an essential tool with which to investigate populations  
43 movements.

44 Genetic structure among Eurasian Stone-curlew populations has to date not been studied but  
45 this is important in the context of the six recognized subspecies (five, according to Rasmussen &  
46 Anderton 2005) described only on the basis of phenotypic characters (size and plumage coloration)  
47 (Vaughan & Vaughan-Jennings 2005), sometimes from only a few specimens (e.g. for Canary  
48 Islands). Geographic variation is complex and mainly clinal, making the assignment of individuals,  
49 particularly with intermediate characters, to subspecies difficult (Cramp & Simmons 1983). Cramp  
50 and Simmons (1983) suggest that the Canary Islands host two subspecies: *B. o. distinctus* (generally  
51 brown like the nominate, but with upperparts more heavily streaked and a paler ground-colour)  
52 situated on the western islands of the archipelago (Gran Canaria, Tenerife, La Gomera, El Hierro  
53 and La Palma ), and *B. o. insularum* (smaller, with more rufescent underparts and less heavily  
54 streaked upperparts than *B. o. distinctus*) situated on the eastern islands (Lanzarote, Fuerteventura,  
55 Lobos and other small eastern islands), which represent the smallest distinctive breeding  
56 populations of this species (Delany *et al.* 2009). Northern European populations are attributed to the  
57 nominate *B. o. oedicnemus*, the largest, darkest and most heavily streaked Palearctic subspecies,  
58 whereas the taxonomic position of the Mediterranean populations are not yet well defined. Birds  
59 from France, Spain and northern Italy are attributed to *B. o. oedicnemus*, but those from Greece,  
60 some Mediterranean Islands (e.g. Sardinia, Sicily, Balearic Is., Cyprus), the eastern Balkans and  
61 western Turkey tend towards *B. o. saharae*, the subspecies occurring in the Maghreb area, which is

62 paler than the nominate with a sandy-pink background colour (Cramp & Simmons 1983). The  
63 situation is further complicated by the pattern of movement exhibited by some populations  
64 attributed to both *B. o. oediconemus* and *B. o. saharae*, that seem to share their wintering grounds in  
65 Africa (Vaughan & Vaughan-Jennings 2005).

66 Many species of conservation concern are polytypic and quite often subspecies have varying  
67 conservation status (Haig *et al.* 2006). Although a correct subspecies designation may entail  
68 important implications for wildlife management, subspecies descriptions often rely on phenotypic  
69 characters with little or no support from molecular data (Zink 2004, Phillimore & Owens 2006,  
70 Haig & D'Elia 2010, Hull *et al.* 2010).

71 Here we investigate for the first time the population genetic structure of the Eurasian Stone-  
72 curlew and the degree of differentiation among populations using mitochondrial and nuclear mark-  
73 ers. We sample populations from six geographic areas, located across the western part of the  
74 species' distribution range. We also determine whether our sampled populations can be reliable as-  
75 signed using molecular data to the four subspecies reported for the region.

76

## 77 **METHODS**

78 Blood and feather samples of adult Eurasian Stone-curlews were collected from different areas of  
79 the Mediterranean basin and Canary Islands primarily during the breeding season (April – August;  
80 Fig. 1, Table 1). A portion of the mitochondrial NADH2 gene was sequenced from 115 individuals  
81 using standard laboratory procedures (Appendix S1). Thirteen microsatellite markers designed for a  
82 wide range of birds (Küpper *et al.* 2008; Dawson *et al.* 2010), were tested on *Burhinus* (Table S1).

83 Standard summary statistics were estimated using MEGA 5 (Tamura *et al.* 2011) and neutrality  
84 tests conducted with DNAsp 4.10 (Rozas *et al.* 2003). Pairwise  $\Phi_{ST}$ -values and analyses of  
85 molecular variance (AMOVA) were calculated using ARLEQUIN 3.5.1 (Excoffier *et al.* 2010) and  
86 corrections were made for multiple comparisons (Appendix S1). A medium-joining network was

87 constructed using the R-package *pegas* (Paradis 2010). For the microsatellite analyses, deviations  
88 from Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium (LD) was determined using  
89 GENEPOP 4.0 (Rousset 2008), and corrections were made for multiple comparisons (Appendix  
90 S1). Expected ( $H_E$ ) and observed heterozygosity ( $H_O$ ) as well as the inbreeding coefficient ( $F_{IS}$ )  
91 were calculated using GENETIX 4.05 (Belkhir *et al.* 2004), and values of allelic richness were  
92 determined using FSTAT 2.9.3 (Goudet 2001).

93

## 94 **RESULTS**

95 A total of 26 haplotypes were obtained for 653 bp of NADH2 (Table 2). Significant mtDNA  
96 differentiation was detected only for the Canary Island populations relative to the combined  
97 Mediterranean samples ( $\Phi_{ST} = 0.46$  on average; Table S2). The most common NADH2 haplotype  
98 H1 was shared by 44 samples from SIC, NIT and TUN that encompass the range of both the  
99 subspecies *B. o. oedignemus* and *B. o. saharae*. The second most common haplotype H2 (20  
100 samples) was detected in all the Canary Island samples (ECI, WCI). Tunisian samples were  
101 represented by three different haplotypes (H1, H3, H4) shared with NIT and SIC (Figs. 1 & 2).

102 Only five of the 13 microsatellite loci tested were polymorphic (Table S1; Appendix S2), these  
103 loci were used for all subsequent analyses. Deviation from Hardy-Weinberg equilibrium (HWE)  
104 was detected for the loci TG01040 (in all populations except GRE) and TG05053 (in NIT, SIC and  
105 TUN). The use of MICROCHECKER (Van Oosterhout *et al.* 2004) indicated that the lack of HWE  
106 at these loci may be a consequence of the presence of null alleles (see Appendix S1 for details).  
107 Null alleles were revealed in all populations at the locus TG01040, but only for SIC and NIT at  
108 locus TG05053. No evidence of linkage disequilibrium was found among all pairs of loci across all  
109 sampled populations.

110 All populations, with the exception of ECI and NIT, deviated from the HWE with values of  $F_{IS}$   
111 being significantly positive (Table 2). Pairwise  $F_{ST}^{(ENA)}$  values corrected for null alleles, were very

112 similar to those produced with the original data with differences  $< 0.0098$ . This suggests that the  
113 influence of null alleles is likely small and thus, does not influence the biological interpretation of  
114 the results. For this reason, all further analyses were performed using the original genotypic  
115 frequencies. Pairwise  $F_{ST}$ -values supported the divergence between the Canary Islands (ECI, WCI)  
116 and the other populations (0.12 on average), but significant divergences were also obtained between  
117 TUN and SIC (0.025), and between WCI and ECI (0.054) (Table S2).

118 The analysis of molecular variance (AMOVA) carried out on mitochondrial and nuclear data  
119 recovered significant genetic structure for both datasets (global  $\Phi_{ST} = 0.17$  for mtDNA and  $F_{ST} =$   
120 0.11 for microsatellites;  $P < 0.001$ ).

121 The major partition of variance was consistent with the separation of the Mediterranean from the  
122 Canary Island populations (NIT, SIC, TUN, GRE vs. WCI, ECI;  $\Phi_{CT} = 0.32$ ,  $P = 0.07$  and  $F_{CT} =$   
123 0.11,  $P = 0.001$ ; Table S3).

124

## 125 **DISCUSSION**

126 Our data only partially agree with the current subspecies delimitation across the southwestern part  
127 of the Eurasian Stone-curlew's distribution range. Indeed, mitochondrial analyses do not support  
128 any divergence between eastern and western Canary Island populations nor within the  
129 Mediterranean basin, but the Canary Island populations appear genetically isolated relative to  
130 Mediterranean populations. The microsatellite data only partially supported the mitochondrial  
131 results as these data indicate significant nuclear genetic divergence between SIC and TUN, and  
132 between WCI and ECI, results not recovered in the mtDNA analyses. The different pattern of  
133 variability of mitochondrial and nuclear markers recorded for Canary Island populations makes it  
134 difficult to reach a firm conclusion with respect to the distinction between western and eastern  
135 subspecies.

136 There was a general lack of genetic variability in mtDNA haplotype diversity for Canary Island  
137 populations, a result that is consistent with possible founder events or bottlenecks, and which has  
138 been reported for several avian species in the Macaronesian region (Illera *et al.* 2012). However,  
139 bottlenecks or any demographic events were not detected by analytical software or neutrality tests  
140 (Tajima's *D* and Fu and Li's *D*; data not reported) possibly due to our relatively small sample size.

141 The pattern of genetic diversity and structure observed among the Mediterranean populations is  
142 much more complicated. Both mitochondrial and nuclear markers did not support substantial  
143 distinction between *B. o. saharae* and *B. o. oediacnemus*, thus supporting the need for caution  
144 recommended by Cramp and Simmons (1983) when assigning names to geographically or  
145 phenotypically intermediate populations of Eurasian Stone-curlew (e.g. Italy). The microsatellite  
146 data, however, revealed unexpected differences between SIC and TUN, notwithstanding the  
147 proximity of these two geographic areas. While we cannot exclude the possibility of our results  
148 being unduly influenced as a consequence of small sample size, this result would suggest  
149 unexpected patterns of movement within the Mediterranean area, which at the moment are difficult  
150 to explain given our limited knowledge of these populations (Vaughan & Vaughan-Jennings 2005).

151 The different results obtained using mitochondrial and nuclear markers are frequently reported  
152 for avian species, and could be a consequence of several demographic events, such as sex-biased  
153 dispersal (see Zink & Barrowclough 2008), already documented in other waders (Colwell 2010)  
154 and more generally in birds (Greenwood 1980). Even though we are aware that this discrepancy  
155 could be due to the different in effective population size ( $N_e$ ) and mutation rates between maternal  
156 and bi-parentally inherited markers (Brito 2007), this aspect deserves further investigation as the  
157 characteristics of the dispersal behaviour of the Eurasian Stone-curlew are at present unknown  
158 (Vaughan & Vaughan-Jennings 2005). In particular, future analyses that take into account the sex of  
159 individuals could be extremely useful in order to clarify the mechanisms shaping the present genetic

160 structure, which could be mainly driven by the most philopatric sex (see e.g. Ortego *et al.* 2011,  
161 Küpper *et al.* 2012).

162 In summary, our preliminary results suggest a complex and partially unexpected genetic  
163 structure that deserves further in-depth molecular analyses. This seems particularly critical in order  
164 to better assess the taxonomic status of the populations of Eurasian Stone-curlew from the Canary  
165 Islands, given that divergence from Mediterranean populations is supported by both mitochondrial  
166 and nuclear loci. We suggest that additional markers and samples are needed in order to fully  
167 support the recognition of a separate Macaronesian species, but it is important to stress the  
168 conservation values of these populations, which our analysis confirm as being the smallest  
169 distinctive breeding populations of the Eurasian Stone-curlew. A better understanding of the pattern  
170 of genetic diversity and structure seems crucial if effective conservation measures are to be  
171 developed and implemented for this species.

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174 We are grateful to all the people who helped us conduct field work; in particular Ángel C. Moreno,  
175 Denise Grassi and to the staff of the Taro River Regional Park (Parma, Italy). Samples were also  
176 provided by the Wildlife Rehabilitation Centres of Gran Canaria and Fuerteventura, by Gianni  
177 Insacco (Regional Rescue Centre of Comiso, Sicily) and by Christos Barboutis (Greek Is.). We  
178 appreciate the improvements in English usage made by Peter Lowther through the Association of  
179 Field Ornithologists' program of editorial assistance. The comment of an anonymous reviewer  
180 greatly improved an earlier draft of the manuscript.

181

182 **DATA ACCESSIBILITY:** GenBank accession numbers for haplotype sequences of NADH2 are  
183 (JX270972–JX270995) and (KJ689457–KJ689458). Individual mitochondrial haplotypes and  
184 microsatellite allelic scores are available in Appendix S2.

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## 258 **ONLINE SUPPORTING INFORMATION**

259 Additional Supporting Information may be found in the online version of this article:

260

261 **Appendix S1.** Details of molecular data collection and genetic analyses.

262 **Appendix S2:** Mitochondrial haplotypes and microsatellite genotypes for six Eurasian Stone-curlew  
263 populations

264 **Table S1.** Details of the 13 microsatellite loci tested in *B. oediacnemus*.

265 **Table S2.** Pairwise comparisons of population  $F_{ST}$ -values for microsatellite DNA (below the  
266 diagonal) and  $\Phi_{ST}$  values for mtDNA (above the diagonal)

267 **Table S3.** Summary of hierarchical AMOVAs for mtDNA and the microsatellite loci under two  
268 alternate population structures.

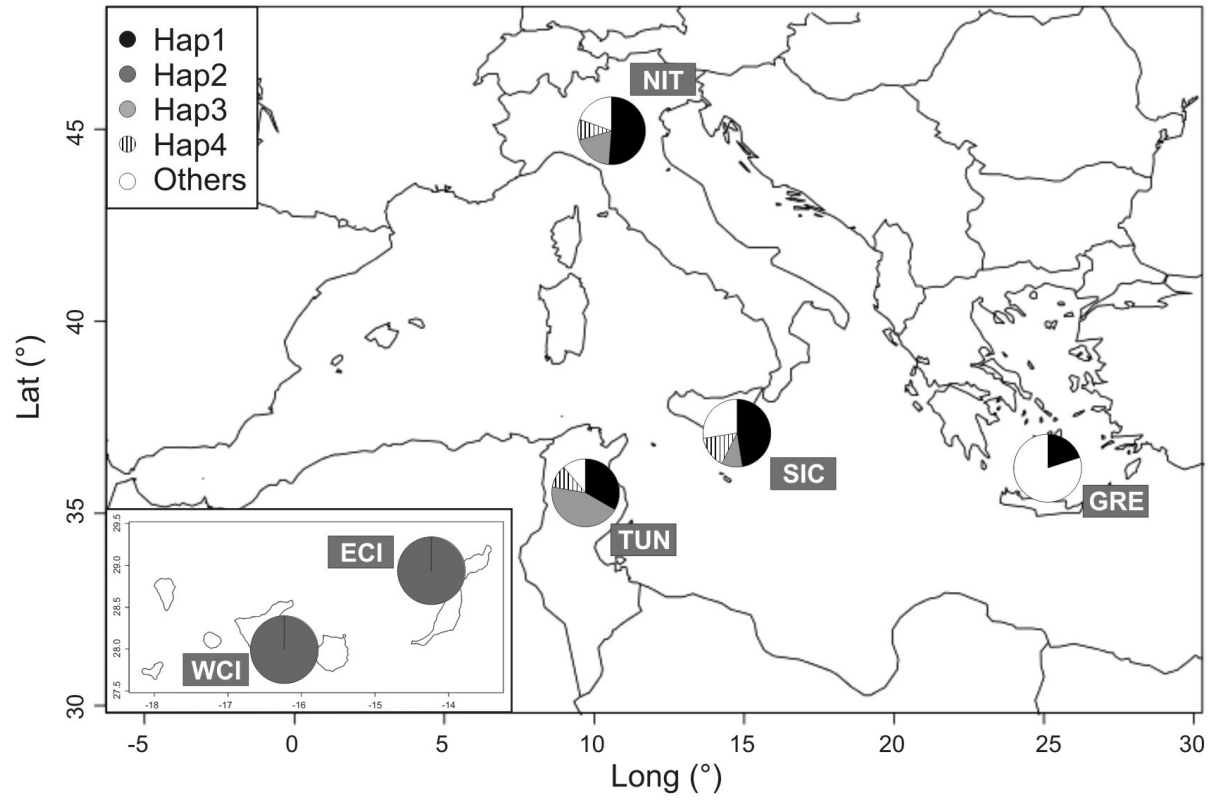
269 **FIGURE CAPTIONS**

270

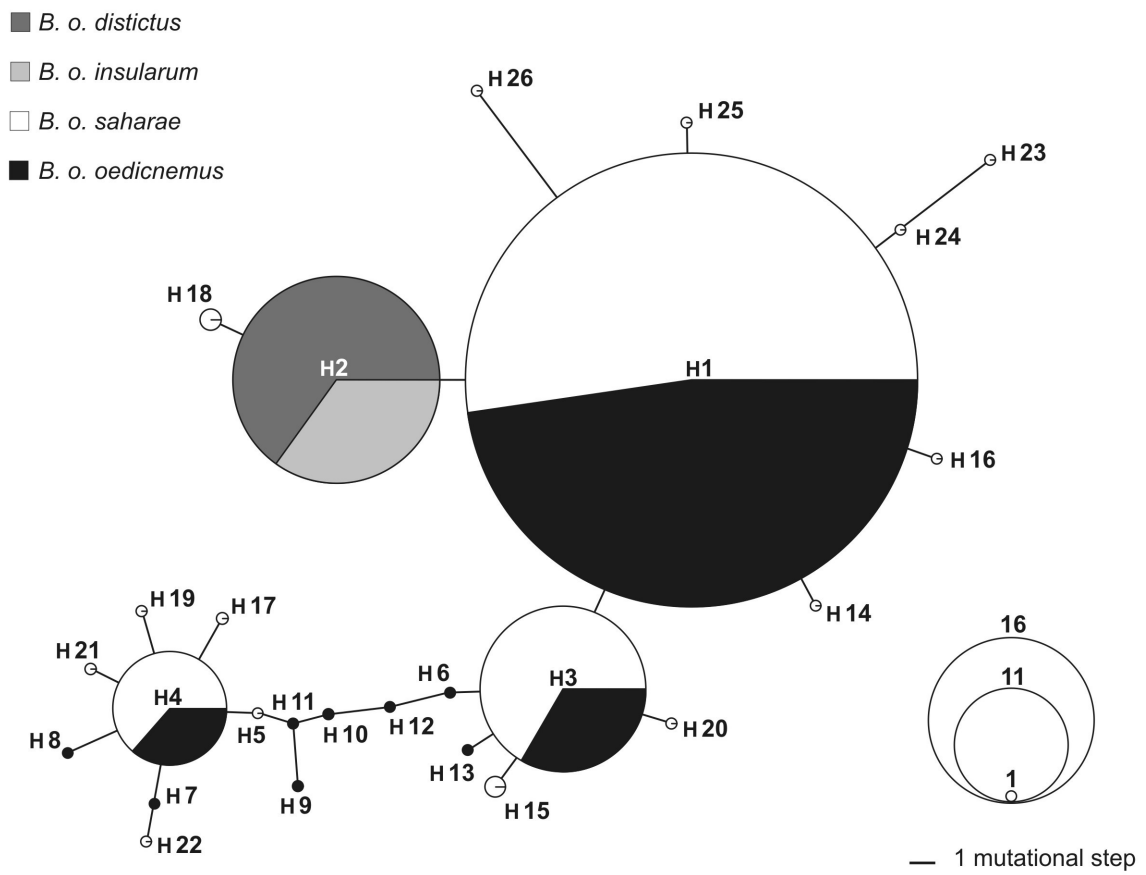
271 **Figure 1.** Map reporting the haplotype (Hap) frequencies recorded in each of the six sampling  
272 areas: NIT - northern Italy; SIC - Sicily; TUN - Tunisia; GRE - Greek Islands; ECI - Eastern Canary  
273 Islands; WCI - Western Canary Islands.

274

275 **Figure 2.** A median-joining network of Eurasian Stone-curlew NADH2 haplotypes. Circle size is  
276 proportional to haplotype frequency. Haplotypes are colour coded by subspecies following Cramp  
277 and Simmons (1983) and Vaughan and Vaughan-Jennings (2005).



**Figure 1**



**Figure 2**

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 279  
 280

**Table 1.** Details of Eurasian Stone-curlew samples used for genetic analyses. All samples were collected during the breeding season (April-August) with the exception of Gran Canaria, where 6 out of 23 individuals were collected during the post-breeding period (November).

Sampling areas (acronym)	Sampling localities	Movements <sup>a</sup>	Putative subspecies <sup>a</sup>	<i>n</i>
Northern Italy (NIT)	Parma (44.76 N, 10.18 E)	mainly migrant	<i>oediconemus</i>	42
Sicily (SIC)	Siracusa (37.15 N, 15.23 E) Ragusa (36.96 N, 14.56 E)	mainly resident	<i>saharae?</i>	52
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>	28
Greek Islands (GRE)	Crete (35.37 N, 24.81 E) Rhodes (36.23 N, 27.94 E)	resident	<i>saharae</i>	6
Western Canary Islands (WCI)	Gran Canaria (27.97 N, 15.61 W) La Gomera (28.14 N, 17.20 W)	resident	<i>distinctus</i>	24
Eastern Canary Islands (ECI)	Fuerteventura (28.42 N, 14.01 W)	resident	<i>insularum</i>	9

<sup>a</sup> According to Cramp & Simmons (1983) and Vaughan & Vaughan-Jennings (2005).

**Table 2.** Estimates of genetic variation for six Eurasian Stone-curlew populations:  $n$ , number of samples;  $N_A$ , average number of alleles;  $A$ , allelic richness;  $P$ , number of private alleles;  $H_O$ , average observed heterozygosity;  $H_E$ , average expected heterozygosity;  $F_{IS}$ , inbreeding coefficient;  $N_H$ , number of haplotypes;  $h$ , haplotype diversity;  $\pi$ , nucleotide diversity. Significant values ( $P < 0.05$ ) are indicated in bold.

Population	Microsatellites (five loci)							NADH2			
	$n$	$N_A$	$A^*$	$P$	$H_O$	$H_E$	$F_{IS}$	$n$	$N_H$	$h$	$\pi$
NIT	15	4.4	3.177	0	0.587	0.633	0.108	41	11	0.702	0.005
SIC	20	4.4	3.267	0	0.500	0.615	<b>0.212</b>	40	12	0.751	0.006
TUN	25	4.6	3.254	2	0.576	0.635	<b>0.113</b>	9	4	0.750	0.005
GRE	6	3.2	2.777	0	0.367	0.481	<b>0.321</b>	5	5	1.000	0.006
WCI	23	3.6	2.598	1	0.409	0.484	<b>0.177</b>	13	1	0.000	0.000
ECI	9	3.0	2.576	0	0.533	0.535	0.061	7	1	0.000	0.000

\*Allelic richness is based on minimum sample size of four diploid individuals and calculated over the five loci analysed for all populations.