

1 **Historical and current patterns of gene flow in the butterfly *Pararge aegeria***

2

3 **Short running title: phylogeography of *Pararge aegeria***

4

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### 39 **Acknowledgements**

40 Support for this research was provided by; a Nigel Groome PhD studentship awarded to

41 Breuker for Livraghi, a Santander Student Research Grant to Livraghi, a Santander Research

42 Scholarship to Breuker, a Marie Curie International Outgoing Fellowship within the 7th

43 European Community Framework Programme to Dincă (project no. 625997), an European

44 Union’s Seventh Framework programme for research and innovation under the Marie Curie  
45 grant agreement No 609402 - 2020 researchers: Train to Move (T2M) to Vodã, the projects  
46 “Barcoding Italian Butterflies” and “Barcoding Butterflies of the Tuscan Archipelago  
47 National Park”, and the Spanish Plan Nacional I+D+I CGL2016-76322-P (AEI/FEDER, UE).

48

49

## 50 **Abstract**

51

### 52 **Aim**

53 We have investigated the phylogeography and genetic structure of the Speckled Wood  
54 butterfly (*Pararge aegeria*) across its entire distribution range and studied its dispersal both  
55 on mainland and across sea straits. The apparent lack of gene flow between Sardinia and  
56 Corsica was further investigated by means of mating experiments.

57

### 58 **Location**

59 Europe and North Africa

60

### 61 **Methods**

62 We sampled 345 individuals and sequenced one mitochondrial gene (*Cytochrome c Oxidase*  
63 *subunit I, COI*) for all samples and two nuclear genes (*wingless* and *zerknüllt*) for a subset of  
64 the specimens. A total of 22 females from Corsica and Sardinia were used to establish a  
65 series of crosses to investigate reproductive compatibility and were screened for the presence  
66 of *Wolbachia*. Bayesian inference (BI) and haplotype networks were employed to infer  
67 phylogenetic relationships and a Principal Coordinate Analysis (PCoA) was used to represent

68 geographical patterns of genetic diversity. Mating and courtship data were analysed using  
69 linear mixed effect models.

70

## 71 **Results**

72 We detected two main *COI* lineages separated by the Mediterranean Sea and maintained over  
73 relatively short sea straits. While nuclear gene variation was generally in agreement with that  
74 of *COI*, this was not the case in all areas (e.g. Iberian Peninsula and Corsica/Sardinia).

75 Mating experiments revealed no evidence of reproductive isolation between the lineages, nor  
76 clear relation to *Wolbachia* infection status.

77

## 78 **Main conclusions**

79 We propose that following the post-glacial recolonisation of Europe, the ancestral *COI*  
80 lineage of *P. aegeria* was maintained in North Africa and Mediterranean islands, while a new  
81 lineage colonised from Eastern Europe, replacing and apparently outcompeting the ancestral  
82 variant. Several hypotheses are discussed that may explain the local discordance between the  
83 nuclear genes and *COI*, including sex-specific dispersal, selection and differential rates of  
84 gene evolution.

85

86 **Editor** Simone Fattorini

87 **Keywords:** *Pararge aegeria*, Speckled Wood butterfly, phylogeography, barcoding, pre- and  
88 postzygotic barriers, *wingless*, *zerknüllt*, life-history variation, selection, and gene flow

## 89 **Introduction**

90 Climatic fluctuations during the Pleistocene period in Europe had a tremendous impact on the  
91 emergence of different lineages for many temperate species (Cooper *et al.*, 1995; Taberlet *et*  
92 *al.*, 1998; Seddon *et al.*, 2001). During cold periods most European species were presumably  
93 restricted to Mediterranean areas. Due to the geographic configuration of the Mediterranean  
94 region, a series of areas separated by mountain chains and sea channels have been hypothesised  
95 to function as differentiation centres for many organisms (e.g. Hewitt, 1999; Hewitt, 2000;  
96 Schmitt, 2007). In Europe, such areas have been typically identified in the Iberian and Italian  
97 Peninsulas and the Balkans, which were isolated from each other to various degrees during the  
98 long cold periods that characterized the Pleistocene. The large Mediterranean islands, Maghreb  
99 and Asia Minor represented further important refugia and centres of differentiation for species  
100 living in the Mediterranean area (Habel *et al.*, 2009; Habel *et al.*, 2011; Dapporto *et al.*, 2011,  
101 2012).

102 Following isolation, populations of many species in glacial refugia evolved into distinct  
103 lineages and (sub-)species (Ribera and Volger, 2004). During the relatively short warm periods,  
104 thermophilic species that were constrained to these areas began northwards expansions and  
105 recolonised previously glaciated regions. It has been inferred on a number of occasions that  
106 although lineages and sister species can (post-glacially) expand over thousands of kilometres  
107 in Europe, they tend to form only very limited areas of overlap or they establish contact zones  
108 along even narrow sea straits when they meet in re-colonized areas (Waters, 2011 for a review,  
109 Dapporto *et al.*, 2011, 2012, 2017; Vodă *et al.*, 2015a,b; Habel *et al.*, 2017 for Mediterranean  
110 butterflies). Several explanations for this have been proposed including density dependent  
111 phenomena, climatic and environmental preferences, reproductive interference, dispersal  
112 limitation and/or competitive exclusion (Waters, 2011; Vodă *et al.*, 2015b; 2016). Due to the  
113 large number of potential mechanisms that can determine patterns of mutual exclusion,

114 understanding the processes responsible for the observed distributions requires a  
115 multidisciplinary approach (Vodã *et al.*, 2015b for Mediterranean butterflies). Studying the  
116 spatial distribution of highly diverging genetic lineages and their tendency to form extended  
117 parapatric areas of contact has fundamental implications in understanding the onset of the  
118 speciation process (e.g. Arias *et al.*, 2008, Habel *et al.*, 2017 for butterflies in particular).

119 The Speckled Wood butterfly, *Pararge aegeria* (Linnaeus, 1758) has been widely used as a  
120 model system to study the distribution of genetic lineages and their spatial segregation; it is  
121 an ubiquitous species with a widespread distribution (ranging from the Maghreb, throughout  
122 Europe and reaching western Asia), experiencing various environmental settings from cold  
123 and wet conditions in northern Europe to hot and dry conditions in southern Europe and north  
124 Africa (Weingartner *et al.*, 2006; Habel *et al.*, 2013; Tison *et al.*, 2014). Moreover, this  
125 species occurs in many Mediterranean islands and the Atlantic island of Madeira, thus also  
126 allowing the study of dispersal both on mainland and across sea straits (Dapporto *et al.*,  
127 2017). These attributes mean the species is highly suitable for studying the distribution of  
128 genetic lineages and their spatial segregation and giving insight into broad biogeographical  
129 patterns associated with responses to both biotic and abiotic factors and into the evolution of  
130 different lineages. The model species also has potential to provide valuable insights on how  
131 species react in time and space to environmental pressures across large geographic ranges  
132 (Parmesan, 1999; Oliver *et al.*, 2015).

133 Using variation in the mitochondrial cytochrome *c* oxidase subunit I (*COI*) gene, the nuclear  
134 *wingless* gene and in microsatellites, two main lineages of *P. aegeria* have been identified  
135 (Weingartner *et al.*, 2006; Habel *et al.*, 2013; Dapporto *et al.*, 2017), in agreement with the  
136 subdivision of this species into two subspecies (ssp. *aegeria* and ssp. *tircis*). The *aegeria*  
137 lineage occurs in Maghreb, the Balearic Islands and in Sardinia, and the *tircis* lineage in  
138 mainland Europe and Asia. Accordingly, the two lineages are separated by three sea channels

139 – the Gibraltar strait between Morocco and Spain, the strait of Sicily between Sicily and  
140 Tunisia, and the strait of Bonifacio between Sardinia and Corsica (Vodá *et al.*, 2016;  
141 Dapporto *et al.*, 2017). The differentiation between Corsican and Sardinian populations of *P.*  
142 *aegeria* is also evident at the morphological level, with a divergence in male genital shape  
143 between the two lineages (Dapporto *et al.*, 2012). A recent study by Longdon *et al.* (2017)  
144 examining the modes of transmission in a range of different Rhabdoviruses and their  
145 population genetics, which often reflect those of their hosts (Wilfert & Jiggins, 2014;  
146 Longdon *et al.*, 2017), highlighted discrete Sardinian and Corsican populations of the *P.*  
147 *aegeria* specific Rhabdovirus PAegRV (for a detailed description of this recently discovered  
148 virus see Longdon *et al.*, 2015). This strongly suggests limited dispersal between the islands.  
149 The variation between populations on Corsica and Sardinia thus represents a particularly  
150 intriguing case, which is a focus of this study. These islands are separated by less than 12 km  
151 of sea straits in which several small adjacent islands could potentially act as stepping stones.  
152 Moreover, in contrast to the areas separated by the Gibraltar and Sicilian channels, these  
153 islands were connected during the Last Glacial Maximum (LGM) suggesting that the two  
154 different populations were established from different source populations following relatively  
155 recent post glacial dynamics (Dapporto, 2010) and thereafter there has been little or no  
156 dispersal over the Bonifacio strait.

157 Several explanations can be provided for the observed distributions of island populations.  
158 Corsica and Sardinia have different environmental settings, with considerable variation in  
159 temperature and rainfall (reflected in the vegetation) (Hijmans *et al.*, 2005). It is highly  
160 unlikely that climatic differences alone prevent the European lineage from establishing  
161 populations on Sardinia and *vice versa*, but local adaptation may reduce the likelihood of  
162 colonization (cf. Richter-Boix *et al.*, 2013). Climatic factors and their effects on host plants  
163 have indeed imposed strong selection pressures in *P. aegeria* that influence egg-laying

164 strategies (Hill *et al.*, 1999; Gibbs & Van Dyck, 2010; Gibbs *et al.*, 2012). Furthermore, it  
165 may be possible that reproductive isolation is emerging between the two lineages; female  
166 mate choice, in particular, has been recorded as a factor in maintaining reproductive isolation  
167 in several butterfly species (e.g. Dincă *et al.*, 2013; Pinzari & Sbordoni, 2013).

168 Even in the absence of reproductive barriers, hybrid fitness could be reduced, thus explaining  
169 the mutual exclusion pattern. Although very little is understood about the reduction in hybrid  
170 fitness at the molecular level (Presgraves *et al.*, 2003; Rogers & Bernatchez, 2006), three  
171 specific forms of post-zygotic isolation have been described: sterility of F1 hybrids, lethality  
172 of F1 hybrids and degeneracy of F2 hybrids (Dobzhansky, 1970; Dumas *et al.*, 2015). Thus, it  
173 may be possible that no strict pre- or postzygotic barriers exist, but that immigrants and their  
174 (hybrid) offspring find themselves at a selective disadvantage compared to the endemics.

175 To address these issues, we sampled numerous populations of *P. aegeria* to cover as much as  
176 possible of their European and North African range. We specifically focused on Corsica and  
177 Sardinia, the closest areas where the two lineages can be found with apparent lack of gene  
178 flow and sequenced *COI* as well as two nuclear developmental genes for a subset of  
179 individuals. The transcription factor-encoding *zerknüllt* (*zen*)(for a description of this gene in  
180 *P. aegeria* see Ferguson *et al.*, 2014), was added to data on the traditionally used gene  
181 *wingless* (*wg*), encoding a signalling protein to increase the nuclear sequence depth (see also  
182 Wahlberg and Wheat, 2008). This allowed us to investigate, with high spatial resolution, the  
183 distribution of the two genetic lineages and their intra-lineage genetic diversity over the study  
184 area. Moreover, to test the hypothesis that pre- or postzygotic barriers affect gene flow  
185 between the two lineages over Sardinia and Corsica the reproductive strategies of Sardinian  
186 and Corsican *P. aegeria* were examined using courtship and mating experiments.

## 187 **Material and Methods**



## 188 **DNA extraction, amplification, sequencing, and phylogenetic analyses**

189 Using PCR and direct Sanger sequencing (see Appendix S1 in Supporting Information,  
190 including details on primers and cycling conditions), we obtained sequences of; the 658 bp  
191 barcoding region of *COI* for 345 individuals spanning from North Africa to northern Europe,  
192 of a 411 bp region of the gene *wingless* (*wg*) for a subset of 87 individuals, and of the entire  
193 coding region of *zen* (1599 bp) for 79 individuals. We further obtained two outgroup  
194 sequences of *wg* and *zen* belonging to the closely related species *Pararge xiphioides*  
195 (Staudinger, 1871) (cf. Weingartner *et al.*, 2006). Bayesian inference (BI) for the three genes  
196 was employed to infer phylogenetic relationships with BEAST 1.8.0 (Drummond *et al.*,  
197 2012). Patterns of genetic variation were inferred by maximum parsimony haplotype  
198 networks using the program TCS 1.21, with a 95% connection limit (Clement *et al.*, 2000).  
199 Representations of genetic diversity were created for the three genetic markers by calculating  
200 matrices of p-distances for each of them, and subsequently analysing and plotting these using  
201 R and QGIS 2.0.1. (QGIS Development Team 2009). Further details on sequencing and the  
202 phylogenetic, haplotype network and genetic distance analyses can be found in Appendix S1.

203

## 204 **Sardinian and Corsican samples**

205 Between the 6<sup>th</sup> and the 12<sup>th</sup> of May 2014 *P. aegeria* females were collected in the field from  
206 11 different localities in: Sardinia (Aritzo, Desulo and Tempio Pausania), Corsica (Asco,  
207 Zonza, Bavella, Bonifacio, Solenzara, Cavallo Morto and Pietralba) and La Maddalena  
208 (Sualeddu), a smaller island off the north coast of Sardinia. In total 32 females were caught:  
209 18 from Corsica, 13 from Sardinia and 1 from La Maddalena. Eggs from collected females  
210 were obtained *in-situ* and brought to the laboratory in Oxford, UK. Upon hatching these eggs  
211 were put on host plants for this species in Europe (a mix of *Poa trivialis* and *Dactylis*

212 *glomerata*) and reared at  $21 \pm 2^\circ\text{C}$  (60% RH, 16L:8D) (cf. Gibbs *et al.*, 2010b). Rearing and  
213 mating conditions in this study included full daylight spectrum lamps, including UV-light  
214 (Osram Biolux). The females collected in the field laid readily on these plant species, as did  
215 all the females used in this experiment. Pupae were sexed and kept individually, to ensure  
216 virgin adults were available for setting up crosses. The offspring of a total of 22 of the field-  
217 collected females provided the adults to perform the crosses detailed below (Appendix S2 in  
218 Supporting Information for details on collection).

219

## 220 **Pre-zygotic reproductive barriers: courtship behaviour in Sardinian and Corsican**

### 221 *Pararge aegeria*

222 Crosses were performed with the offspring of the wild-caught females. Four types of crosses  
223 were set up: Corsican male/Corsican female (CC), Corsican male/Sardinian female (CS),  
224 Sardinian male/Sardinian female (SS) and Sardinian male/Corsican female (SC). In total 72  
225 crosses generated data to be used in the analyses (CC=27, CS=20, SC=11, SS=14). To  
226 perform the crosses, newly eclosed virgin females were placed in cages along with an  
227 artificial flower containing 10% honey solution (Gibbs *et al.*, 2012). A newly eclosed virgin  
228 male was then introduced into a female's cage and the total courtship duration (seconds) was  
229 timed using stopwatches. The primary aim of these crosses was to establish how willing  
230 males and females of the two islands were to mate with each other, and having done so, what  
231 the reproductive output was. Thus, no mate choice experiments *per se* were conducted (i.e.  
232 where by a female needed to choose between a male from her own island or the other one).  
233 *Pararge aegeria* have a courtship very similar to that described for the grayling where  
234 courtship is initiated by a wing flick used by males, to the front and side of the female  
235 (Davies, 1978 and references therein). We used this male wing flick as our cue for courtship

236 initiation. If the male was unsuccessful at initiating mating after numerous bouts of courtship  
237 between 8am and 6pm, it was removed and replaced with a new virgin male the following  
238 morning (8am). After mating had finished an egg laying plant was placed in the cage and the  
239 male was removed.

240

## 241 **Reproductive barriers**

242 After mating the female was left to oviposit for six days and all eggs laid in that period were  
243 collected. All females were allowed to oviposit on the exact same host plant species (a mix of  
244 *P. trivialis* and *D. glomerata*). Six days represent the period of peak egg laying in *P. aegeria*,  
245 usually followed by a rapid increase in mortality of both eggs and females (Gibbs *et al.*,  
246 2010b). Female age throughout the experiments was recorded as it affects willingness to  
247 mate, and reproductive output (Gibbs *et al.*, 2010a,b). After six days females were removed  
248 and used for wing measurements. From the collected eggs, the first eight larvae to hatch from  
249 a particular cross were each reared through on a mix of *P. trivialis*, *D. glomerata*,  
250 *Brachypodium sylvaticum* and *Festuca rubra*. The hatching success of the remaining eggs  
251 was noted and the remaining larvae sacrificed. Larvae placed on food plants were monitored  
252 to eclosion and the proportion of individuals surviving to adulthood and the sex ratio of the  
253 adults was recorded. Pupae were sexed and kept individually, to ensure that virgin adults  
254 were available to set-up mating pairs in backcrosses.

255 After the individuals used in the crosses had been sacrificed their forewings were removed  
256 and the dorsal side of the forewing was placed between glass slides and photographed using a  
257 Leica MZ6 dissection microscope with integrated camera (Leica IC80 HD camera with Las  
258 EZ software suite) under controlled light conditions. Wing area (mm<sup>2</sup>) of both forewings was  
259 measured using ImageJ software (Abramoff *et al.*, 2004; Breuker *et al.*, 2010), and the

260 average forewing area was used as a proximate measure of individuals' size (cf. Merckx &  
261 Van Dyck, 2006), and included as a covariate in the models.

262

### 263 **Backcrosses**

264 The offspring resulting from the aforementioned crosses (both hybrids and pure-bred  
265 Sardinian and Corsican individuals; F1), were crossed amongst each other (see below; i.e.  
266 backcrossed) to generate an F2 (see also Longdon *et al.*, 2017) under similar conditions. For  
267 the backcrosses hatching success of a sample was assessed only for a minimum of ten and a  
268 maximum of 20 eggs, as this was considered a representative sample size. The aim of the  
269 backcrosses was to test for fertility issues of the hybrids versus pure-breds. Thus, only those  
270 crosses for which a successful mating was observed were included in the analyses, and no  
271 behavioural data was collected. A hybrid male or female, was backcrossed to either a  
272 purebred Sardinian or Corsican specimen (Appendix S2, Table S2.2; a total of 54  
273 successfully mated backcrosses were obtained). After the male had been removed females  
274 were provided with an egg laying plant and allowed to oviposit for six days (see also original  
275 crosses).

276

### 277 ***Wolbachia* presence**

278 The wild-collected Sardinian and Corsican females whose offspring were used as parents in  
279 the crosses (with the exception of females 3 and 14; Appendix S2) were screened for the  
280 presence of *Wolbachia*, as this has been shown to sometimes affect reproductive output and  
281 fertility in insects, and the presence of this endosymbiont has been reported in *P. aegeria*  
282 ovaries (reviewed in Carter *et al.*, 2013). In order to screen for *Wolbachia*, we PCR amplified  
283 *Wolbachia* specific sequences (*Wolbachia* surface protein – *wsp*) using previously described

284 primers (Dobson *et al.*, 1999). The PCR products were run on a gel and screened for the  
285 presence of amplification. The gene *caudal* was used as a positive control and absence of  
286 *Wolbachia* had been verified for a number of samples in a separate study using RNA  
287 sequencing (Quah *et al.* 2015). Individuals used in the crosses presented in this study were not  
288 tested for *Wolbachia* prior to mating, as that was not feasible given the design of the  
289 experiments, nor postmating. Whether or not *Wolbachia* infection was detected in the  
290 mothers of the animals used to establish the crosses was used as a fixed factor in the models  
291 described below.

292

### 293 **Statistical analyses of the courtship and mating experiments**

294 Linear mixed effect models (fitted by maximum likelihood t-tests use Satterthwaite  
295 approximations to degrees of freedom) were constructed to investigate variability amongst  
296 the crosses in reproductive output (both number of laid eggs and egg hatching success), larval  
297 survival and courtship duration. The latter can be considered largely the net result of the  
298 choosiness of the female, and the willingness and effectiveness of the male (e.g. Holveck *et*  
299 *al.*, 2015). Fixed effects tested for inclusion were age of both male and female at the time of  
300 mating, wing size (measured as wing size), *Wolbachia* infection and type of cross. Both male  
301 and female maternal origin were included as random factors. Model selection has been  
302 carried out based on Minimum models were constructed using Akaike Information Criterion  
303 (AIC) value as a guideline, and these are the models presented in this study. This means that  
304 non-significant fixed covariates and interactions were removed. Once model selection had  
305 been completed, significance of the remaining fixed effects was provided through use of the  
306 lmerTest package (Kuznetsova *et al.*, 2016) providing. All residuals for included effects were  
307 tested for normality and log and square root transformations were used where appropriate

308 (e.g. courtship duration). Both male and female maternal origin were kept as random factors  
309 in all the models, and as the models tested the significance of differences between the various  
310 cross types, cross type was always included as a fixed effect. Analyses were performed using  
311 R (3.4.0) (R Development Core Team 2016) with packages ‘lme4’ (Bates *et al.*, 2015)  
312 ‘lmerTest’ (Kuznetsova *et al.*, 2016)). Chi-square tests were used to test for cross type and  
313 fertility associations; while for the backcrosses the Fisher's Exact Test for Count Data was  
314 used, as some counts were low (Appendix S2).

315

## 316 **Results**

### 317 ***COI* variation reveals the presence of two distinct lineages**

318 We obtained 345 sequences with 27 haplotypes characterized by 28 variable nucleotide sites  
319 for the *COI* gene. Haplotype networks based on *COI* sequences show a discrete boundary  
320 between North African and European populations, forming two distinct lineages separated by  
321 a minimum of seven mutations (1.1%), with a single divergent specimen from Cyprus in  
322 evidence (Figure 1, Appendix S4). North African haplotypes show significant population  
323 structure, with several highly frequent haplotypes occurring throughout the areas analysed. In  
324 contrast, populations in continental Europe are characterised by one main haplotype,  
325 separated from several low frequency ones by a maximum of two mutations (Figure 1). The  
326 two haplogroups are also supported in our phylogenetic analyses (Appendix S4).

327 Interestingly, the islands of Sardinia, Mallorca, Menorca and Ponza are all populated  
328 exclusively by North African haplotypes, even though they are in closer proximity to  
329 continental Europe (Figure 1, 2a,b). Furthermore, we found evidence of only one individual  
330 carrying the Sardinian haplotype in Corsica (Bonifacio), suggesting very limited gene flow  
331 (of matriline) between the two islands.

332 When splitting the populations based on the *COI* lineages, we observed a marginally  
333 significant negative Tajima's D for the European lineage (Tajima's D = -2.10,  $p=0.05$ ),  
334 signifying expansion and/or recent selective sweep, but not for the North African one  
335 (Tajima's D = -0.91,  $p> 0.10$ ). Overall genetic diversity was also higher for the North African  
336 lineage compared to the European populations (average nucleotide diversity,  $\pi$  was 0.0025  
337 and 0.0013 respectively). This was also evident when the genetic differences among the  
338 nearest four specimens to each 0.2x0.2 square of latitude and longitude is plotted on a map  
339 (Appendix S1; Figure 3). Average genetic divergence between the lineages is 0.30% for *wg*  
340 and 0.50% for *zen* (based on mutations in aligned sequences). Geographical locations  
341 corresponding to the North African lineage were shown to harbour more genetic  
342 heterogeneity. Interestingly, the populations in Romania and Alps are also more variable,  
343 suggesting increased genetic diversity for the European clade in central and Eastern Europe.

344

#### 345 **Nuclear genes versus *COI* lineages**

346 Sequence variation in the developmental genes *wg* and *zen* was generally in agreement, but  
347 locally discordant with the mtDNA, since the pattern of genetic clustering showed a south-  
348 western genotype mainly distributed across north Africa, Iberia, southern France, Sardinia  
349 and Sicily and a north-eastern genotype in the Italian Peninsula, north Europe and Eastern  
350 Europe (Figure 2b,c, Appendix S5 and S6). The Iberian Peninsula and Sicily were inhabited  
351 solely by *COI* haplotypes belonging to the European lineage, while nuclear sequences also  
352 belong to the south-western lineage (Figure 2b,c).

353

#### 354 **Courtship behaviour**

355 Females in pure-bred Corsican crosses were significantly slower in mating than any of the  
356 other crosses (full minimum mixed model AIC=142.1, BIC=156.6, df resid = 52). Not only  
357 did they take longer to mate compared to Sardinian females in pure-bred Sardinian crosses  
358 (CC versus SS;  $t=-2.80$ ,  $df=59$ ,  $p=0.0068$ ), but Sardinian females also mated more readily  
359 with Corsican males, than Corsican females did (CC versus CS  $t=-3.61$ ,  $df=59$ ,  $p<<0.001$ ).  
360 Sardinian males also mated more readily with Corsican females than Corsican males did (CC  
361 versus SC  $t=-2.18$ ,  $df=59$ ,  $p=0.033$ ). Female age and size, male size or temperature did not  
362 improve the model.

363

#### 364 **Reproductive barriers**

365 *Female fecundity*: Reproductive output (i.e. number of eggs laid) was significantly affected  
366 by female age and size, as well as cross type (AIC=605.8, BIC=626.3, df resid = 63).

367 Females that were older at the time of mating laid more eggs in the six days following mating  
368 than those that mated young, having presumably stored mature eggs for fertilisation ( $t=3.25$ ,  
369  $df=71.90$ ,  $p=0.0018$ ). Larger females laid significantly more eggs ( $t=2.88$ ,  $df=67.48$ ,  
370  $p=0.0053$ ). Sardinian females (i.e. SS ( $9.57\pm 4.83$ ) and CS ( $16.69\pm 4.25$ )) laid significantly  
371 less eggs than Corsican females (i.e. CC ( $36.72\pm 3.96$ ) and SC ( $24.45\pm 3.55$ )), regardless  
372 whom they mated with (SS versus CC  $t=-3.31$ ,  $df=20.53$ ,  $p=0.0034$ ; CS versus CC  $t=-3.87$ ,  
373  $df=15.23$ ,  $p=0.0015$ ). There was no significant difference between CC and SC ( $t=-1.75$ ,  
374  $df=71.82$ ,  $p=0.085$ ).

375 *Offspring fitness and the effect of temperature on egg hatching success*: All four types of  
376 crosses were similar in terms of infertile (i.e. egg hatching success =0%, or no eggs laid,  
377 despite having been observed to mate successfully) versus fertile (i.e. egg hatching success >  
378 0%) crosses *per se* (chi-square 1.58,  $df=3$ , and  $p=0.66$ ). Egg hatching success was very high,



379 with no significant differences in hatching success between the different cross types (CC  
380  $94.38 \pm 2.62\%$ , CS  $92.53 \pm 2.83\%$ , SC  $92.85 \pm 4.23\%$ , and SS  $99.1 \pm 0.59\%$ ). Hatching success  
381 was only affected by temperature, but not female age at mating or female size (AIC=506.6,  
382 BIC=523.9, df resid=57). Within the temperature range used (range: 22.1 – 25.4°C), more  
383 eggs hatched successfully at higher temperatures ( $t=2.43$ ,  $df=60.82$ ,  $p=0.018$ ).

384 There were no significant differences (i.e.  $P \gg 0.05$ ) in survival of the offspring (i.e. from  
385 larval hatching to eclosion as an adult) between the crosses (full model with only cross type  
386 AIC=32.7, BIC=48.0, df resid=58).

387 *Wolbachia infection status*: The majority of the field-collected females tested for *Wolbachia*  
388 were found to be infected, with the exception of five females: three from Aritzo (Sardinia),  
389 one from Desulo (Sardinia), and one from Bonifacio (Corsica). However, Aritzo is not a  
390 location free from *Wolbachia*, as other females collected there were infected (Appendix S3).  
391 We cannot rule out *Wolbachia* presence in populations from Desulo and Bonifacio as only a  
392 single specimen was collected in each of these localities. The *Wolbachia* infection status of  
393 the mothers of the specimens used to establish the crosses was not a factor that significantly  
394 improved the statistical models reported earlier, and therefore not included in the reported  
395 final models. Finally, for each of the four cross types Chi-squared tests were used to evaluate  
396 the presence of sex ratio distortion in the surviving offspring. No significant sex ratio  
397 distortion was found in any of cross types: CC (chi-square=0.12,  $df=1$ ,  $p=0.73$ ), CS (chi-  
398 square = 0.017,  $df=1$ ,  $p=0.90$ ), SC (chi-square = 0.059,  $df=1$ ,  $p=0.81$ ) or SS (chi-square =  
399 0.76,  $df=1$ ,  $p=0.78$ ). The lack of sex ratio distortion and the absence of fertility problems  
400 suggests that cytoplasmic incompatibility does not explain the lack of gene flow between  
401 Sardinia and Corsica.

402 *Sterility of F<sub>1</sub> hybrids*: F1 hybrids were backcrossed to either pure-bred Sardinians or  
403 Corsicans (Appendix S2). There were no differences between the 10 types of crosses in terms  
404 of fertility (Fisher's Exact Test for Count Data, p=0.13).

405

## 406 **Discussion**

407

408 Corsica and Sardinia are characterised by the occurrence of a variety of endemic populations  
409 for various butterfly species (Aubert *et al.*, 1997, Grill *et al.*, 2002; Dapporto, 2010 ). This is  
410 likely to be the result of the long-term isolation of these islands since the early or late  
411 Miocene (Ketmaier *et al.*, 2006). Mutually exclusive pairs of cryptic butterfly species such as  
412 *Aricia agestis* and *A. cramera* or *Polyommatus icarus* and *P. celina* have been shown to  
413 occur on Corsica and Sardinia (Dincă *et al.*, 2011, Vodă *et al.*, 2015a,b). Such divergence  
414 between Corsican and Sardinian populations is in many ways unexpected as the islands are  
415 separated by a narrow sea strait (approximately 12 km wide, while the shortest distance  
416 between Corsica and Sardinia, represented by the small islands in between is about six km),  
417 and were connected during the last glaciation period (Dapporto, 2010 and references therein).  
418 Similarly, in Sweden, *P. aegeria* revealed little to no gene flow between the populations of  
419 the island Öland and the near-by mainland (separated by five km) (Tison *et al.*, 2014). Even  
420 though the data presented in this study confirm that even short sea straits can provide a strong  
421 barrier to the dispersal of *P. aegeria*, we observed some markedly discordant patterns  
422 between the nuclear and mitochondrial genes. For instance, the Iberian Peninsula is inhabited  
423 solely by *COI* haplotypes belonging to the European lineage, but the nuclear markers at the  
424 same locations clustered together with North Africa and Sicily. This pattern is reinforced by  
425 the geometric morphometric split observed for male genitalia shape between populations of  
426 *P. aegeria* where the same east-west differentiation pattern is observed (Dapporto *et al.*,

427 2012). The conservative nature of nuclear markers (Wahlberg and Wheat, 2008) was most  
428 notably exemplified between Corsica and Sardinia, given the similarity in nuclear sequences  
429 despite the occurrence of different lineages.

430 The presence of the North African *COI* lineage on several Mediterranean islands is intriguing  
431 (Vodă *et al.* 2015b, 2016; Dapporto *et al.*, 2017), as they are in closer proximity to the  
432 European mainland and in this region wind generally blows from west-northwest (Dapporto  
433 *et al.*, 2012). Thus, one would expect them to be more easily colonised from either the Italian  
434 Peninsula (in the case of Ponza and Sardinia) or the Iberian Peninsula (in the case of Mallorca  
435 and Menorca). The higher genetic heterogeneity observed in the Maghreb lineage (Figure 2),  
436 suggests the presence of ancestral populations not only in North Africa, as suggested by  
437 Weingartner *et al.* (2006), but also in other Mediterranean islands. This is in stark contrast to  
438 the reduced genetic variation observed in the European clade in the circum-Mediterranean  
439 populations, suggestive of a recent colonisation and population expansion from Eastern  
440 continental areas. The significant negative Tajima's D for European populations also supports  
441 this hypothesis, because low frequency variants segregating at high frequencies can indicate  
442 population expansion by founder effect and gene surfing (Waters, 2011). Given the higher  
443 genetic variation found in the Alps and Romania (Figure 3) one could propose a putative  
444 centre of origin for the European populations further east, and then, as found in other  
445 Lepidopteran species (Mende and Hundsdoerfer, 2013), the contact zone among genetic  
446 variants has likely shifted to the west (Figure 4). This could have occurred as a phalanx-like  
447 colonisation over the mainland, which was impeded at sea straits, resulting in the island  
448 lineages being unexpectedly similar to the North African populations (Dapporto *et al.*, 2012).  
449 The populations in Sardinia, Mallorca, Menorca and Ponza might thus represent "relict"  
450 populations harbouring the ancestral *COI* haplotypes, which have not been replaced due to  
451 the physical barriers imposed by the sea straits.

452 However, it must be noted that *COI* is maternally inherited and it can only trace the dynamics  
453 of females. Nuclear genes show a general correspondence into two main southern and  
454 northern groups but also areas of discrepancy where the northern *COI* lineage is associated to  
455 southern *wg/zen* genes. Our data suggest that hybrid sterility and hybrid-purebred  
456 incompatibilities do not limit introgression between these islands, and there appear to be no  
457 obvious pre- or postzygotic barriers. Moreover, we observed that the two *COI* lineages are  
458 highly inter-fertile and also that there are temperature-related differences across types in both  
459 female fecundity and offspring fitness during the egg stage, indicating possible effects of  
460 local adaptation to temperature during oviposition and embryogenesis. Other Speckled wood  
461 populations across Europe show significant and distinct population structuring, evidenced by  
462 sequence analyses of the *P. aegeria* specific Rhabdovirus PAegRV (Longdon *et al.*, 2017)  
463 and population genetic analyses (Tison *et al.*, 2014). For the UK in particular, this is  
464 remarkable, given the relatively recent contraction and subsequent expansion of *P. aegeria* in  
465 the UK (Hill *et al.*, 1999; Longdon *et al.*, 2017). A nuclear gene such a *zen* evolves relatively  
466 slowly, not least as it has an important developmental role in the specification and  
467 functioning of the serosa, an extra-embryonic tissue involved in drought resistance (Ferguson  
468 *et al.*, 2014), and has been shown to be under negative selection in *P. aegeria* (Livraghi *et al.*,  
469 unpubl). Viral genes evolve much faster, showing a higher propensity to population  
470 structuring (of their hosts; see Longdon *et al.*, 2015). The differences in the spatial patterning  
471 of nuclear and *COI* as well as PAegRV variation might thus reflect complex patterns of past  
472 and current selection, past isolation and recolonisation events, in theory including sex-biased  
473 dispersal (Toews & Brelsford, 2012).

474 Although dispersal may be more costly to female *P. aegeria*, often lowering reproductive  
475 output (Hughes *et al.*, 2003), females have been shown to be the most dispersive sex in  
476 typical metapopulation dynamics in for example the UK and Belgium (Hughes *et al.*, 2003;

477 Bergerot *et al.*, 2012). Male-biased dispersal would not satisfactorily explain the Sardinia-  
478 Corsica results since PAegRV is transmitted to offspring by both males and females. Thus, in  
479 the case of male-biased dispersal, genetic variation observed for PAegRV genetic variation  
480 would reflect nuclear variation; instead it reflects the observed *COI* pattern, arguing against  
481 male-biased dispersal. Consequently, the similarity between the islands in terms of variation  
482 of slowly evolving nuclear genes between the islands is likely to be historical, rather than the  
483 result of an ongoing process of male-specific dispersal.

484 At present we do not know enough about the differences between populations across the  
485 whole of the geographical range of *P. aegeria* in terms of the selection pressures operating on  
486 dispersal propensity, reproductive strategies and the trade-offs made between reproduction  
487 and dispersal. Strong differences between *P. aegeria* populations are not only evident on the  
488 basis of sequence variation, but also in terms of expression patterns of specific miRNA genes  
489 (Quah *et al.*, 2015). This has been shown for egg production in Corsican (specifically Zonza)  
490 and Belgian populations (Quah *et al.* 2015). This leads one to hypothesise that female  
491 reproductive strategies, and the genes involved therein, are very likely to be under selection  
492 in response to habitat variation (e.g. temperature and oviposition plants) with significant  
493 population differences, as observed in other *P. aegeria* populations across Europe (Gibbs &  
494 Van Dyck 2009; Gibbs *et al.*, 2010b). Such differences may possibly also exist in our study  
495 area since Sardinian and Corsican females significantly differed in reproductive output.

496 *Pararge aegeria* is confirmed as a highly suitable model to study the distribution of genetic  
497 lineages and their spatial segregation in order to reveal broad biogeographical patterns  
498 associated with responses to both biotic and abiotic factors and to the evolution of different  
499 lineages. Open questions to pursue are whether the historical polymorphisms of nuclear genes  
500 are: actively maintained by selection in the areas of discordance, simply the result of different  
501 evolutionary rates of nuclear genes versus *COI per se* (i.e. neutral variation; when genes

502 likely to be under different selection pressures show similar patterns) and/or whether sex-  
503 biased dispersal underpins observed patterns of discordance between nuclear genes and *COI*.  
504 The wider availability of other molecular techniques such as RAD-seq and genome-wide  
505 association study (GWAS) for non-model organisms now provides the opportunity for more  
506 in-depth analyses of population genetics and the adaptive nature of particular SNPs across  
507 different selective environments. Studies on gene flow and local adaptation in a life-history  
508 context are now more pertinent than ever given that most species are facing rapid  
509 environmental changes (e.g. climate and land use), and our data suggests that *P. aegeria*  
510 would be an excellent model for these kinds of studies.

511

#### 512 **Conflict of Interest**

513

514 The authors declare no conflict of interests

515

#### 516 **Data availability**

517 Sequence data are publicly available via GenBank (MH090747-MH090823; dedicated  
518 databases are publicly available for *COI* and *wg* sequences through the Barcode of Life Data  
519 (BOLD) system ([dx.doi.org/10.5883/DS-PARARGE](https://dx.doi.org/10.5883/DS-PARARGE)), and for *zen* sequences through a *P.*  
520 *aegeria hox3* sequence database (DOI [10.24384/000476](https://doi.org/10.24384/000476)).

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725 **Biosketch**

726 Members of the research team are actively engaged in studying: 1) life history evolution and  
727 maternal effects in response to environmental variation, aiming to synthesise life history  
728 models with developmental genetic models of evolution, and 2) insect biogeography,  
729 systematics and conservation, with a specific interest in unravelling the historical and  
730 present-day factors responsible for species distributions across mainland Europe and  
731 Mediterranean islands.

732

733 **Tables and Figures - Legends**

734 **Figure legends**

735

736 **Figure 1**

737 Haplotype network based on *COI* sequences of *Pararge aegeria* from the study area. Each  
738 colour indicates the geographic location of the haplotypes, as indicated in the legend, and the  
739 size of the circle corresponds to the frequency of a haplotype. The number of nucleotide  
740 changes at each node is shown as white circles (putative ancestral haplotypes).

741

742 **Figure 2**

743 A Principal Coordinates Analysis projection of the p-distances genetic variation in *COI*,  
744 among the *Pararge aegeria* specimens (dots), in the bidimensional Red, Green, Blue (RGB)  
745 space (a), spatial distribution of genetic variants of *COI* (b), RGB PCoA projection of p-  
746 distances genetic variation in concatenated nuclear dataset (c), and spatial distribution of  
747 nuclear genes (d).

748



749 Figure 3

750 Distribution of the genetic richness of *Pararge aegeria* in the study area based on 0.25x0.25  
751 degree squares for which at least 4 specimens were sequenced in a 100km radius. Genetic  
752 richness was calculated separately for the two lineages identified in this study for each of  
753 these squares. The method involves calculating matrices of p-distances (proportions of  
754 nucleotide differences), taking geographic distances into account. At the end, a single value,  
755 indicating the genetic differentiation of four specimens closest to each other weighted for  
756 their distance from the centre of their locations, is then plotted onto a map. This has been  
757 represented here as a heat map of sequence variation across a wide geographical range (full  
758 range 0% (green) to 1.6% (red); values indicated in figure)(for full details on the genetic  
759 richness method see Supplementary File 1).

760 Figure 4

761

762 Proposed hypothesis for the historical biogeography of *Pararge aegeria*. The ancestral  
763 lineage (blue circles) was present throughout the range of *P. aegeria* in Europe (A), without  
764 substantial differentiation of the nDNA markers due to unrestricted dispersal between  
765 populations. During the last glacial period (possibly also including previous series of glacial  
766 events) (B) the range retracted southwards (red arrows), and gene flow was restricted  
767 between the refugia due to the Alps and Pyrenees acting as barriers, which allowed for  
768 periods of differentiation (yellow circles in C). Following the warming of the climate, the  
769 eastern lineage spread northwards and westwards (red arrows in D), where it could have  
770 introgressed with the nuclear genome of warm adapted populations in the Iberian peninsula  
771 as well as the islands of Ibiza, Corsica and Sardinia resulting in the discordance between the  
772 markers (indicated by blue and yellow circles in D). This introgression was presumably  
773 hindered by sea straits, giving rise to the sharp boundary observed for the *COI* data.

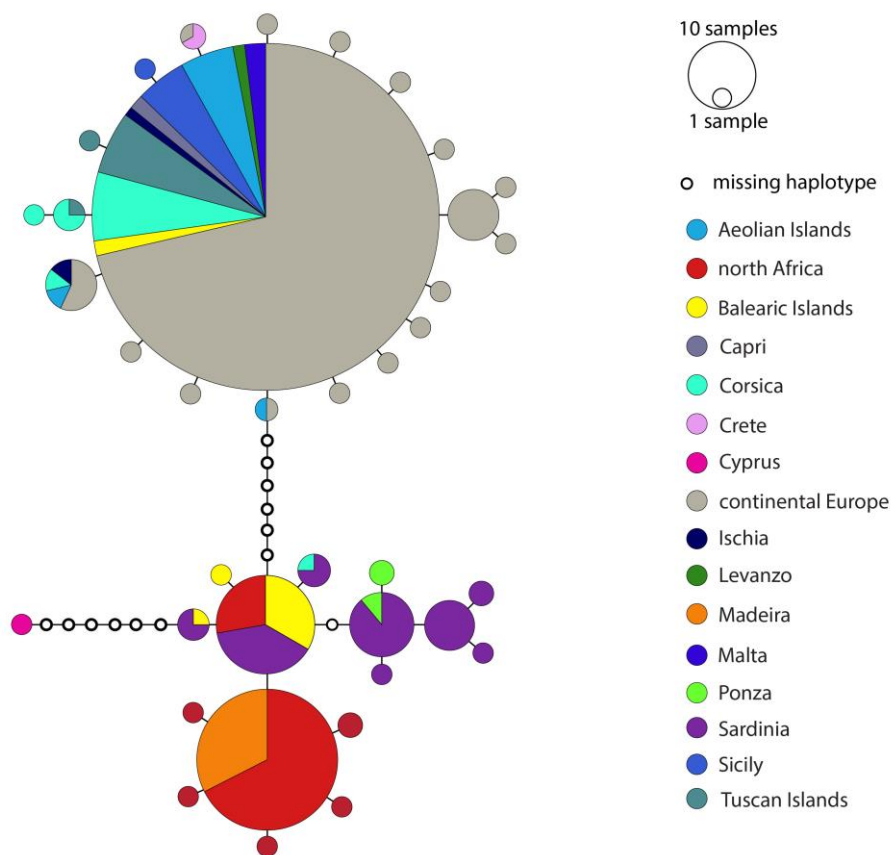
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777 **Figures**

778 Figure 1



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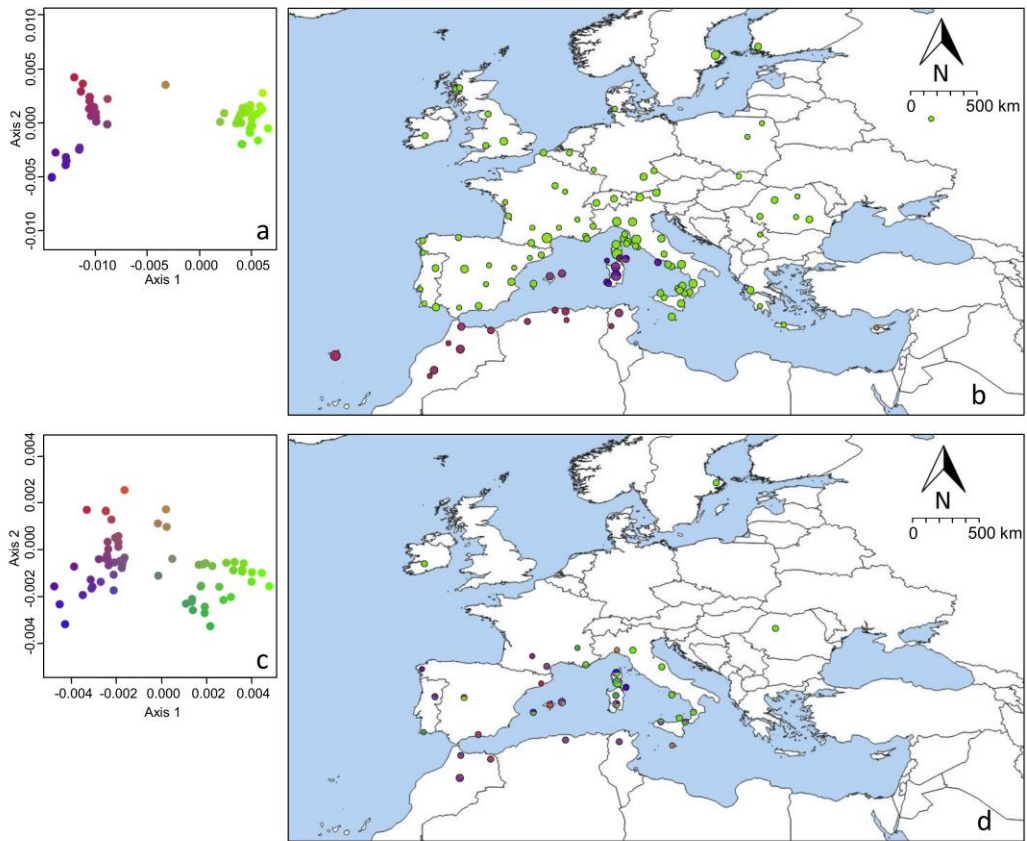
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790 Figure 2



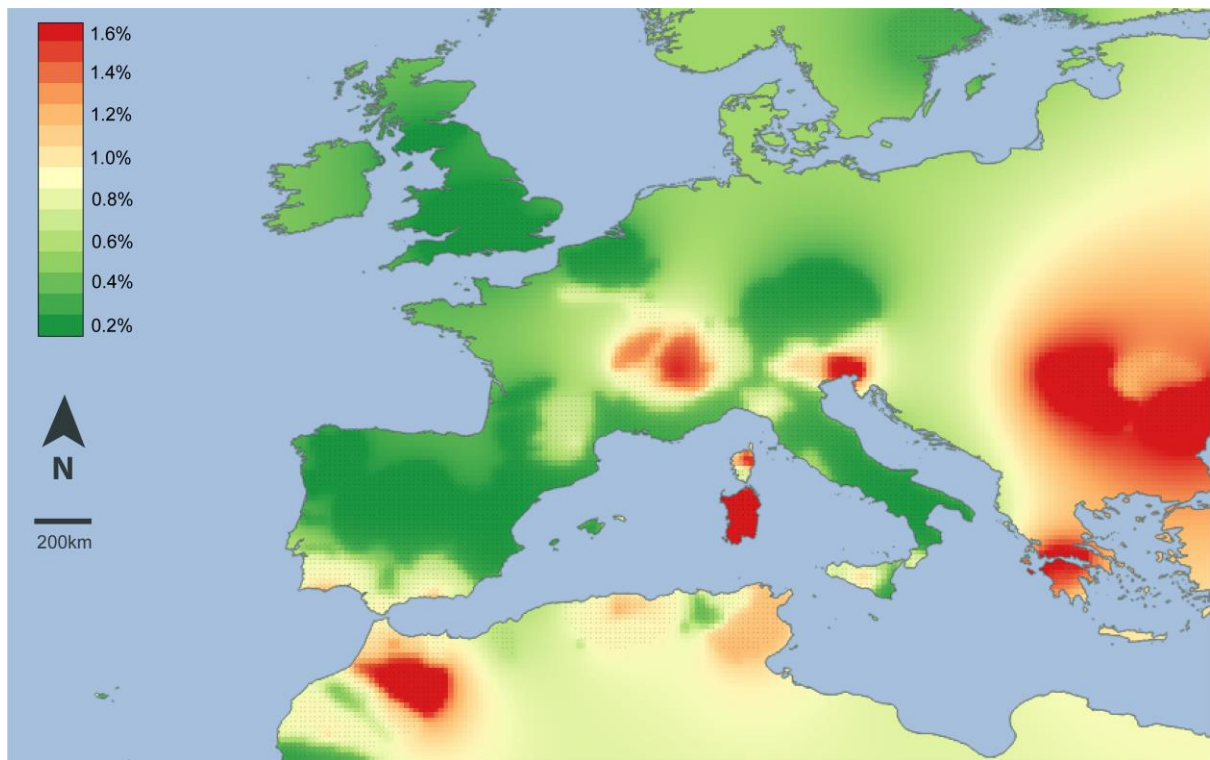
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795 Figure 3



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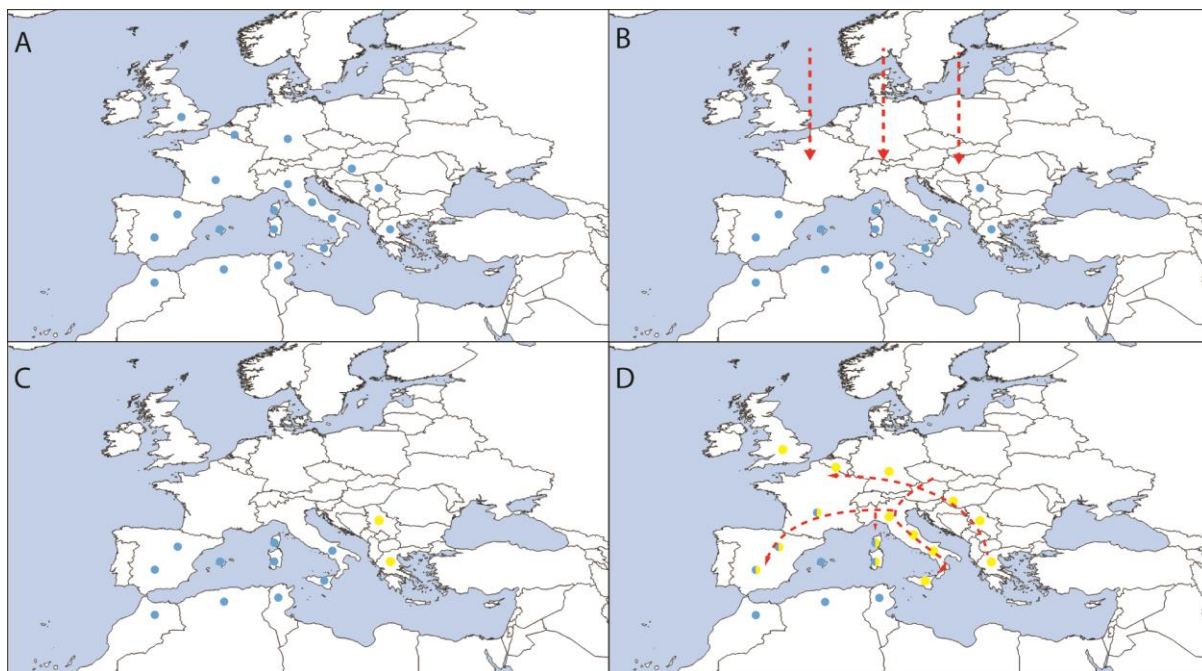
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801 Figure 4



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