

1 Title: Molecular evidence for a recent founder event in the UK populations of the
2 adonis blue butterfly (*Polyommatus bellargus*)

3

4 Running title: Molecular analysis shows a founder event in the adonis blue

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21 Keywords:

22 Lepidoptera, calcareous grasslands, mtDNA, founder event, metapopulation

23 Summary

24

25 Contrary to accepted theories of post-glacial colonisation of the UK approximately
26 10,000 ybp, historical population data for *Polyommatus bellargus* suggests the
27 butterfly was either extremely rare or not present before 1775. We examined the
28 phylogeography of the species by sequencing the ‘hypervariable’ mitochondrial
29 control region of UK and French butterflies. Overall, twenty-two polymorphic
30 nucleotide sites were identified within the control region. French specimens were
31 highly variable, with seventeen polymorphic sites, whereas most UK specimens were
32 monomorphic. Average nucleotide diversity was 0.026 (S.D. 0.016, n = 8) in France,
33 whilst the UK values ranged from 0.00 (n = 6) (for every UK population outside
34 Dorset, n = 43) to 0.01 (S.D. 0.008, n = 7) (Dorset). The mean number of pairwise
35 differences among the French samples was 7.42, whilst the UK values ranged from
36 0.00 (all populations except Dorset) to 0.295 (Dorset). One French haplotype differed
37 from the predominant UK version by just a single nucleotide substitution. It seems
38 implausible that the species can have been resident in the UK for 10,000 years without
39 accumulating variation at this mitochondrial region. Thus, the results suggest that
40 either a severe genetic bottleneck or founder event has occurred recently in the UK.

41 Introduction

42 A combination of both historical and contemporary demographic processes will
43 determine the genetic structure and geographical distribution of genetic diversity
44 among populations (Templeton *et al*, 1995). The phylogeography of many European
45 species reflects the location of their glacial refugia, as well as the nature of the post-
46 glacial colonisation. Specifically, where colonisation has been rapid, genetic diversity
47 is often reduced, particularly in northern areas of Europe such as the UK (Hewitt
48 1996; Hewitt, 1999). Genetic diversity is also likely to be low for species that have
49 colonised more recently because they are often at the edge of their range, where the
50 cycles of expansion and subsequent bottlenecks will result in impoverished diversity.
51 Conserving genetic diversity is vital since it provides adaptive capacity and
52 evolutionary potential (Frankham ref?).

53

54 Phylogeographic studies can allow inferences to be made about the history of
55 population divergence based on associations between the geographical distribution of
56 mitochondrial DNA haplotypes and their genealogical relationships (Avise, 2000).
57 This approach has been used to elucidate colonisation patterns for many species
58 (Hewitt, 1999), including butterflies such as the marsh fritillary (Joyce and Pullin,
59 2001). In this study, we use MtDNA haplotype diversity to shed light on patterns of
60 colonisation in the Adonis blue butterfly (*Polyommatus bellargus* (Rottemburg)
61 (Lepidoptera: family Lycaenidae; subfamily Polyommatinae: tribe Polyommatini).

62

63 *P. bellargus* is a local species in the UK, where it exists in a metapopulation structure,
64 at the north-western edge of its European range (Harper et al. 2000; 2003; 2006).

65 Although the species is widespread in Europe, it is confined to the warmer southern

66 counties of the UK, specifically on areas of south facing calcareous grassland
67 (Thomas, 1983; Emmet and Heath, 1990; Bourn and Warren, 1998; Bourn *et al*, 1999;
68 Stewart *et al*, 2000; Asher *et al*, 2001).

69

70 It has been suggested that many butterfly species, including *P. bellargus*, colonised
71 the UK during the first half of the Flandrian period, around 9,500 to 10,000 years BP
72 (Dennis, 1977). This was because much of the UK was glaciated 18,000 years BP, at
73 which time most of its present flora and fauna were confined to refugia in southern
74 parts of Europe. They remained there until the ice, which covered most of northern
75 Europe, retreated around 10,000 years BP, and they then began to expand northwards,
76 recolonising areas such as Britain (Dennis, 1977; Hewitt, 1999).

77 There is no fossil evidence for butterflies to test this theory, but data for Coleoptera
78 are broadly consistent with it (Osborne, 1976). However, since the Flandrian period,
79 there have been several smaller scale temperature variations, including the “little ice
80 age”, dated to the late medieval period (Grove, 1988), and it is possible that this
81 climatic cooling resulted in a contraction of the range of *P. bellargus*, culminating
82 once again in its exclusion from Britain.

83

84 Evidence already exists for the butterfly’s susceptibility to climatic change; for
85 example, it is known that a drought in 1976 severely affected the host plant,
86 *Hippocrepis comosa*, causing UK populations of *P. bellargus* to crash. Many of the
87 more isolated northerly populations have not recovered from this event (Thomas,
88 1983; Emmet and Heath 1990; Pearman *et al*, 1998; Asher, 2001; Harper *et al*, 2003)

89

90 It is notable that the species appears to have been extremely rare or not present in the
91 UK prior to 1775 CE, when the first confirmed record of the species was made
92 (Harris, 1775; as cited by Emmet & Heath, 1990). In Wiltshire the species was not
93 identified until 1883, yet there are now over 90 confirmed populations in the county
94 (Fuller, 1995). The collecting of butterflies as a hobby began during the last quarter
95 of the 17th century, and one of its chief proponents, James Pettiver, “the father of
96 British entomology”, named and described the majority of the British butterfly species
97 (Emmet & Heath, 1990). Notably he made no description that fits *P. bellargus*,
98 although this species is a conspicuous butterfly and most of his collecting trips were
99 in the south east of the UK, where the species currently occurs. Despite a period of
100 intense entomological activity, no description of this species appears in any work on
101 the British fauna prior to 1775(summarised in Emmet & Heath, 1990). Emmet and
102 Heath (1990) conclude that *P. bellargus* “must have been an extremely rare species”.
103 This historical population data for *P. bellargus* implies that the present day
104 populations of the butterfly in the UK may have a more recent origin.

105
106

107 In order to test this theory, we use the mitochondrial control region to characterise
108 contemporary populations of *P. bellargus* from the UK and France. These data can
109 infer the likely source population and time at which the UK was colonised. In many
110 insect species, the control region is one of the most variable regions in the
111 mitochondrial genome, and has been described as “hypervariable” (Simon *et al.*,
112 1994; Taylor, 1993; Brookes *et al.* 1997). It has been applied to lepidopteran species
113 to deduce both phylogenetic relationships (Taylor, 1993) and population demography
114 (Brookes, 1997).

115

116 Materials and Methods

117

118 Fifty adult male specimens of *P. bellargus* were collected from throughout the UK
119 range (the Isle of Portland, the Isle of Wight, Kent, South Downs, Sussex, Salisbury
120 Plain, Dorset) during June and August in 1998 and 1999. These localities represent
121 the geographic spread of the UK populations. Eight adult butterflies from southern
122 France were also collected during September 1999 (5°22'E 44°45'N). For details of
123 DNA extraction method refer to Harper *et al.*, 2001.

124

125 PCR amplification and sequencing of a 722 bp fragment encompassing the entire
126 mitochondrial control region and a section of the 12SrRNA gene was achieved using
127 invertebrate specific oligonucleotide primers TM-N-193 (Met-20) (5'-TGG GGT
128 ATG AAC CCA GTA GC) (Simon *et al.*, 1994) and 12s-332 (5'-TAG GGT ATC
129 TAA TCC TAG TT) (Taylor *et al.*, 1993). Each 25µl PCR reaction contained 50-
130 100ng of template DNA; 2U *Taq* DNA polymerase (ABgene, UK); 0.2µmoles of each
131 primer; 20mM (NH₄)SO₄; 75mM Tris-HCl, pH 8.8; 0.01%(v/v) Tween[®] 20; 1.5mM
132 MgCl₂; 0.25mM dNTPs (ABgene, UK). Amplifications were carried out under the
133 following conditions: 1x 94°C, 4 min; 30x 94°C, 1min, 45°C, 1min, 72°C, 2.5min; 1x
134 72°C, 7 min. Negative controls for each batch of PCRs showed no contamination.

135

136 PCR products were purified from the agarose gel by excision of the band, then a
137 Qiaquick gel purification kit (Qiagen, USA) was used to isolate the DNA. Each
138 sequencing reaction was carried out via the manufacturers instructions using Big-Dye
139 Terminators (PE-Applied Biosystems, USA) and run on 5% denaturing
140 polyacrylamide gel by vertical electrophoresis at 20-60mA for 2 hours using a Perkin-

141 Elmer ABI 377 automated sequencer. The region was sequenced using both Met20
142 and 12Sr348, so that both the forward and reverse sequences were obtained.

143

144 Statistical Analysis

145

146 Sequence data were subjected to alignments using the computer programme Clustal-X
147 (Thompson *et al* 1997), highlighting any sequence variation between the control
148 regions of the individuals studied. The *P. bellargus* control region was also compared
149 with other species: *Jalmenus evagoras* (from Ebor, Australia) (Lepidoptera: family
150 Lycaenidae; subfamily Theclinae: tribe Zeziini) (GenBank L16849) and *Strymon*
151 *melinus* (from North America) from the same subfamily (Theclinae: tribe Eumaeini;
152 classification follows Eliot, 1973) (GenBank L16850).

153

154 Basic statistics (haplotype number, transition:transversion ratio (TS:TV), nucleotide
155 composition and mean number of pair-wise differences between haplotypes (Tajima
156 1983; Nei 1987)) were calculated using Arlequin (Schneider *et al.*, 2000). The
157 relationships between populations were calculated in Arlequin, using Tamura's (1992)
158 genetic distance. This distance measure was considered most appropriate because of
159 the high A+T content of the sequences, and also on the basis of the transition:
160 transversion ratio, which was higher than the expected ratio of 1:2 (Tamura, 1992;
161 Oyler-McCance *et al.*, 1999).

162

163 A Tree representing the relationship between the haplotypes was constructed in *Phylip*
164 *3.57c* (Felsenstein, 1993) using a maximum likelihood method, without the
165 assumption of a molecular clock. Published control region sequences for *J. evagoras*

166 and *S. melinus* were used in the analysis as outgroups. The data were bootstrapped in
167 the subroutine *SEQBOOT*, with 1000 iterations, and then 1000 distance matrices were
168 created from the bootstrapped data using the subroutine *DNADIST*. These matrices
169 were used to create 1000 Neighbour Joining trees, using the subroutine *NEIGHBOUR*,
170 invoking option J to randomise the input order. Finally, a maximum likelihood
171 consensus tree was created using the subroutine *CONSENSE*. A minimum spanning
172 network between haplotypes was also created using *MINSNET* (Excoffier, 1993).

173

174 Results

175 All variation was found to be within the initial 193bp of control region, the remaining
176 529bp of the amplicon was found to be monomorphic among all 58 *P. bellargus*
177 individuals sequenced. The UK populations of *P. bellargus* were particularly
178 impoverished of variation in the control region. The only divergence between the
179 sequences was by either one or two indels of a (TA) repeat unit in the latter part of a
180 short microsatellite repeat ((TA)₃C(AT)_n) (See figure 1). The addition of a single
181 repeat occurred in three individuals and the addition of two repeat units was present in
182 a single individual, all four originating from a Dorset population (variation at this
183 microsatellite was not found to exclusively relate to the geographic origin of the
184 haplotype; it is present in both the French and UK butterflies). All other UK samples
185 were monomorphic; represented by a single mtDNA haplotype (represented by 46
186 sequences) (shown in figure 2). However, the French specimens showed a much
187 higher level of haplotypic variation than that observed in the UK, and there was
188 significant divergence between the UK and French specimens. The eight French
189 specimens were represented by six haplotypes (figure 2).

190

191

192 Of the 58 sequences analysed, seven substitutions and 15 indels characterised a total
193 of nine haplotypes, of which six were transitions, and two were transversions. As
194 expected for insect MtDNA, there was an extremely low G+C content in the sequence
195 data (Clary & Wolstenholme, 1985) and whilst this varied slightly between
196 individuals, the nucleotide ratios were on average found to be: A 36.4%; C 5.74%; G
197 6.63%; T 51.5%. Nucleotide diversity (average number of nucleotide differences per
198 site between two sequences) was 0.021 (S.D. = 0.012, n = 58) overall, with the French
199 population at 0.026 (S.D. = 0.016, n = 8) and the UK values ranging from 0.00 (n = 6)
200 (All UK populations except Dorset, n = 43) to 0.01 (S.D. 0.008, n = 7) (Dorset). The
201 mean number of pairwise differences among the French samples was 7.42, whilst the
202 UK values ranged from 0.00 (all populations except Dorset) to 0.295 for Dorset, the
203 overall number of pairwise differences between all haplotypes being 3.25. Gene
204 diversity (the probability that two randomly chosen haplotypes are different) ranged
205 from 0.153 (n = 50) for the UK, to 0.929 (n = 8) for France.

206

207 The French haplotypes showed much higher levels of variation than in the UK, with
208 seventeen polymorphic nucleotide sites. Haplotypes can be characterised by between
209 one and sixteen nucleotide changes between them, although the majority appear to be
210 from one to three mutational steps (see figure 2). Most have at least eleven nucleotide
211 differences compared with the predominant UK haplotype, but a single French
212 haplotype ("Fr2") is almost identical to the predominant UK haplotype ("UK1"), with
213 only one nucleotide change separating them (figure 1). The maximum likelihood tree
214 (figure 3) shows that all but one ("Fr2") of the French haplotypes group away from
215 the UK haplotypes (and "Fr2") very robustly, supported by 100% of the bootstraps.

216

217

218 Discussion

219

220 The lack of haplotype diversity and polymorphism within the UK populations of *P.*
221 *bellargus* is most unusual when compared to the results of similar surveys in other
222 taxa. MtDNA studies of vertebrates (e.g. Avise, 1986; Moritz *et al.*, 1987) and
223 invertebrates (Smith & Brown 1990; Brookes *et al.* 1997, Joyce & Pullin 2001)
224 generally reveal a much higher degree of differentiation at the population level than
225 observed within *P. bellargus*. The A+T-rich mitochondrial control region sequenced
226 for this study is considered to be hypervariable in many insect species (Zhang &
227 Hewitt, 1997), and has been found to contain sufficient variation for demographic
228 analyses in several lepidopteran species (Taylor *et al.*, 1993; Brookes *et al.*, 1997).
229 This is in sharp contrast to the three haplotypes, varying by just one or two TA repeats
230 at a short microsatellite, found for *P. bellargus* across its entire UK range. However, it
231 is notable that in France, *P. bellargus* had far higher levels of mitochondrial diversity.

232

233 The analysis of the UK and French haplotype variation reveals a separation of the UK
234 haplotypes from the majority of those found in France (figures 2 and 3). With the
235 exception of “Fr2”, a minimum of 11 base pair differences can be found between any
236 two UK and French sequences. The maximum likelihood tree echoes this pattern,
237 with 99.9% of the bootstraps separating the two groups. The only exception is
238 haplotype “Fr2”, which groups strongly with UK haplotypes. The six French
239 haplotypes were obtained from just eight specimens, so it is likely that with more
240 comprehensive screening, additional haplotypes would be identified, a proportion of

241 which would probably bridge this 11bp difference. The minimum spanning network
242 tree provides a putative pattern of descent for the UK haplotypes: with “UK2” and
243 “UK3” both stemming from “UK1”, via the sequential insertion of (TA) repeats (or a
244 single insertion of a (TA)₂ repeat in “UK3”).

245

246 The similarity between “Fr2” and the UK haplotypes suggests that the UK population
247 may have originated via a recent and rapid colonisation event from France. The
248 maternal inheritance pattern of the mitochondrial genome provides a powerful
249 indicator of such colonisation events (Moritz, 1991; Harrison, 1989), and additionally
250 can be used to estimate the numbers of individuals mediating them. In this study,
251 where all of the observed UK haplotypes appear to stem from a single predominant
252 version (which is closely related to a haplotype found in France where widespread
253 variation is present), the most plausible explanation is that the colonisation of the UK
254 by *P. bellargus* was mediated by very few female butterflies. Furthermore, the severe
255 lack of sequence variation observed in the control region among UK butterflies tends
256 to indicate that this colonisation was a recent event, because variation at this non
257 coding site would have accrued among the UK haplotypes by mutation over longer
258 time periods. It has been proposed that in fast colonising events, pioneers rapidly
259 expand to fill new areas, and that the genes of these individuals will subsequently
260 dominate the new population genome (Hewitt, 1999). It is plausible that following the
261 initial colonisation of the UK, the butterfly spread rapidly across suitable habitats to
262 occupy its present range.

263

264 An alternative possibility is that a range wide bottleneck reduced the UK variability to
265 just a few closely related haplotypes. This rationale is improbable, because the UK

266 population would need to have been reduced to one or a few females (and an
267 unknown number of males) in order to eradicate virtually all variation. The more
268 probable outcome of this scenario would be the loss of most, but not all UK
269 populations, leaving a few butterflies in core areas. This would inevitably result in
270 the fixation of different haplotypes in separate geographic regions, a pattern that is not
271 found.

272

273 If this genetic evidence for a recent UK founder event is combined with the historical
274 population data for *P. bellargus*, and geological evidence for climatic fluctuations,
275 then the evidence for a recent colonisation becomes more convincing. The failure of
276 entomologists to describe *P. bellargus* anywhere in the UK until 1775 (Emmet and
277 Heath, 1990) suggests that *P. bellargus* must have either been extremely rare before
278 this date, or was not present in the UK. The latter explanation would infer that the
279 colonisation of the UK may have occurred as recently as within the last 250 years,
280 perhaps as global temperatures increased after the “little ice age”. This cannot rule
281 out the possibility that the butterfly may have previously been native to the UK prior
282 to this date, and subsequently became extinct, but it does provide compelling evidence
283 that contemporary populations of *P. bellargus* in Britain are the descendants of recent
284 colonists, almost certainly from France. This type of biogeographic event is generally
285 accepted as a route of colonisation, but whether this happened through a chance
286 natural event (perhaps a mated female was blown from France during a storm) or at
287 the hands of man will remain unknown. If the colonisation was anthropogenic, this
288 inevitably raises the issue of whether the species should be considered to be native to
289 Britain, leading to questions about its conservation. There is often debate as to the
290 natural range of species, and those that are deemed not to be native generally receive

291 much lower conservation efforts. Even species which are natural recent colonists are
292 generally given low priority of importance to conservation. The comparative lack of
293 genetic diversity within the UK also implies that these populations may be less
294 important in the conservation of the species, in comparison to the French populations
295 that appear to be much richer in diversity and hence in adaptive potential.

296

297 One important caveat of this work is that because of the inheritance pattern of
298 mtDNA, no inferences can be made towards male mediated gene flow. Although
299 there is disagreement about whether dispersal is male or female mediated in
300 butterflies (Goulson 1993; Kuussaari *et al.*, 1996; Barascud, 1999; Mouson *et al.*,
301 1999), mark-release-recapture studies of *P. bellargus* have indicated that the male is
302 the main proponent of gene flow (Thomas, 1983; Emmet & Heath, 1990; Rusterholz,
303 & Erhardt, 2000). Thus although the UK was probably colonised by a very small
304 number of females it is possible that a larger number of males have made the crossing,
305 so that levels of variation in nuclear DNA may be higher.

306

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481

482 Figure legends

483

484 Figure 1. Sequences of the Met20 amplified control region of six French and one UK
485 *P. bellargus* individuals. A colon (:) indicates identity with the predominant UK
486 haplotype; and a dash (-) indicates a deduced indel. The haplotypic variation within
487 the UK is indicated at the bottom of the figure.

488

489 Figure 2. . A minimum spanning network (Excoffier, 1993) showing the number of
490 base changes between haplotypes. Each haplotype is represented as a circle with its
491 relationship to the most similar haplotypes (defined by the number of base changes)
492 represented as a line. A dotted line indicates an alternative relationship. The numbers
493 of base pair differences between haplotypes is only indicated where values are >1.
494 Shaded circles are French and non-shaded are UK haplotypes.

495

496 Figure 3. An unrooted maximum likelihood consensus tree of control region
497 haplotypes, from UK and French populations of *P. bellargus*. Equivalent published
498 sequences for *Jalmenus evagoras* and *Strymon melinus* have been included as
499 outgroups. Tree created using the DNADIST programme in PHYLIP3.57c. Figures
500 in italics indicate bootstrap values after 1000 replications

501

502

503

504

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506 Figures:

507 Figure 1

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508 UK          CTTTATTTAGCTTATTTTTTAAAAATAATTTTTTATTTTATTATATAAAAAATTATTA AAAATG
509 Fr1          ::::::::::::::::::::::::::::::-::::::::::T::::::::::G::::::::::-:
510 Fr2          ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
511 Fr4          ::::::::::::::::::::::::::::::-:-----:T::::-:-----:G::::::::::
512 Fr6          ::::::::::::::::::::::::::::::-:-----:T::::-:-----:G::::::::::
513 Fr3          ::::::::::::::::::::::::::::::-:-----:T::::-:-----:G::::::::::
514 Fr5          ::::::::::::::::::::::::::::::-::::::::::T::::-:-----:G::::::::::
515
516 UK          G-TTTAAGAATATAAATTATTTTTACCGTTGATTGGGTTTTTCTTTTATTATTTACCGTGCAC
517 Fr1          :G::::::::::-:::::A:::::A:-::::::::::C::::::::::
518 Fr2          :-::::::::::-:::::A::::::::::-::::::::::-::::::::::
519 Fr4          :G::::::::::-:::::A:::::A:-::::::::::C::::::::::
520 Fr6          :G::::::::::-:::::A:::::A:-::::::::::C::::::::::
521 Fr3          :G::::::::::-:::::A:::::A:-::::::::::C::::::::::
522 Fr5          :-::::::::::-:::::A:::::A:-::::::::::C::::::::::
523
524
525
526 UK          CGTAT-ATATACATATATATA--TATATTAATTTTTAATTAATTATTAATTTTAATAATT
527 Fr1          ::::T:::::T::::::::::--C::::::::::
528 Fr2          ::::-::::::::::-::::::::::
529 Fr4          ::::T:::::T::::::::::--C::::::::::
530 Fr6          ::::T:::::T::::::::::TAC::::::::::
531 Fr3          ::::T:::::T::::::::::--C::::::::::
532 Fr5          ::::T:::::T::::::::::--C::::::::::
533

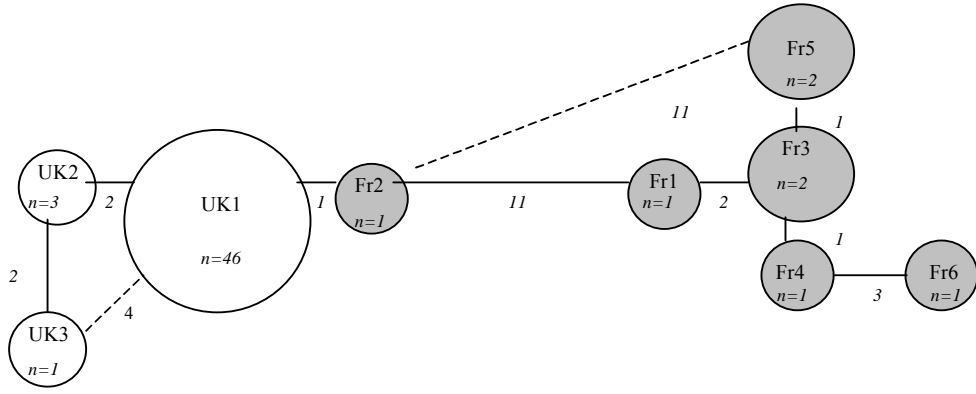
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534 Indels:-

535 ▼ Insertion of either TA or TATA here (UK only).

536

537 Figure 2



538

539

540 Figure 3.

541

542

