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

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

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Summary

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

Introduction

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

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

P. bellargus is a local species in the UK, where it exists in a metapopulation structure, at the north-western edge of its European range (Harper et al. 2000; 2003; 2006). Although the species is widespread in Europe, it is confined to the warmer southern

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

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

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

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

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

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

Materials and Methods

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

PCR amplification and sequencing of a 722 bp fragment encompassing the entire mitochondrial control region and a section of the 12SrRNA gene was achieved using invertebrate specific oligonucleotide primers TM-N-193 (Met-20) (5'-TGG GGT ATG AAC CCA GTA GC) (Simon *et al*., 1994) and 12s-332 (5'-TAG GGT ATC TAA TCC TAG TT) (Taylor *et al*, 1993). Each 25µl PCR reaction contained 50-100ng of template DNA; 2U *Taq* DNA polymerase (ABgene, UK); 0.2µmoles of each primer; 20mM $(NH_4)SO_4$; 75mM Tris-HCl, pH 8.8; 0.01%(v/v) Tween® 20; 1.5mM $MgCl_2$; 0.25mM dNTPs (ABgene, UK). Amplifications were carried out under the following conditions: 1x 94°C, 4 min; 30x 94°C, 1min, 45°C, 1min, 72°C, 2.5min; 1x 72°C, 7 min. Negative controls for each batch of PCRs showed no contamination.

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

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

Statistical Analysis

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

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

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

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

Results

All variation was found to be within the initial 193bp of control region, the remaining 529bp of the amplicon was found to be monomorphic among all 58 *P. bellargus* individuals sequenced. The UK populations of *P. bellargus* were particularly impoverished of variation in the control region. The only divergence between the sequences was by either one or two indels of a (TA) repeat unit in the latter part of a short microsatellite repeat ($(TA)_3C(AT)_n$) (See figure 1). The addition of a single repeat occurred in three individuals and the addition of two repeat units was present in a single individual, all four originating from a Dorset population (variation at this microsatellite was not found to exclusively relate to the geographic origin of the haplotype; it is present in both the French and UK butterflies). All other UK samples were monomorphic; represented by a single mtDNA haplotype (represented by 46 sequences) (shown in figure 2). However, the French specimens showed a much higher level of haplotypic variation than that observed in the UK, and there was significant divergence between the UK and French specimens. The eight French specimens were represented by six haplotypes (figure 2).

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

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

Discussion

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

The analysis of the UK and French haplotype variation reveals a separation of the UK haplotypes from the majority of those found in France (figures 2 and 3). With the exception of "Fr2", a minimum of 11 base pair differences can be found between any two UK and French sequences. The maximum likelihood tree echoes this pattern, with 99.9% of the bootstraps separating the two groups. The only exception is haplotype "Fr2", which groups strongly with UK haplotypes. The six French haplotypes were obtained from just eight specimens, so it is likely that with more 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

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

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

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

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

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Figure legends

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

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

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

Figures:

Figure 1

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UK              CTTTATTTAGCTTATTTTTAAAAAATAATTTTTTATTTTTATTATAAAAATTATTAAAAATG
Fr1             :::::::::::::::::::::::::-:::::::::T::::::::::G:::::::::::::::-:
Fr2             ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Fr4             ::::::::::::::::::::::::--::::::::::T::::-:::::G::::::::::::::::
Fr6             ::::::::::::::::::::::::--::::::::::T:::--:::::G::::::::::::::::
Fr3             :::::::::::::::::::::::::-:::::::::T::::-:::::G::::::::::::::::
Fr5             :::::::::::::::::::::::::-:::::::::T::::-:::::G::::::::::::::::

UK              G-TTTAAGAATATAATTATTTTTACCGTTGATTGGGTTTTTCTTTTATTATTTACCGTGCAC
Fr1             :G::::::::::::::::::::::-:::A::::::A:-::::::::C::::::::::::::::::
Fr2             :-:::::::::::::::::::::::::A::::::::::::::::::::::::::::::::::::
Fr4             :G::::::::::::::::::::::-:::A::::::A:-::::::::C::::::::::::::::::
Fr6             :G::::::::::::::::::::::-:::A::::::A:-::::::::C::::::::::::::::::
Fr3             :G::::::::::::::::::::::-:::A::::::A:-::::::::C::::::::::::::::::
Fr5             :-:::::::::::::::::::::::-:::A::::::A:-::::::::C::::::::::::::::::

                                            ▼
UK              CGTAT-ATATACATATATATA--TATATTAAATTTTTAATTAATTATTAATTTTAATAATT
Fr1             :::::T:::::T::::::::::--C::::::::::::::::::::::::::::::::::::::
Fr2             :::::-:::::::::::::::::--:::::::::::::::::::::::::::::::::::::::
Fr4             :::::T:::::T::::::::::--C::::::::::::::::::::::::::::::::::::::
Fr6             :::::T:::::T::::::::::TAC::::::::::::::::::::::::::::::::::::::
Fr3             :::::T:::::T::::::::::--C::::::::::::::::::::::::::::::::::::::
Fr5             :::::T:::::T::::::::::--C::::::::::::::::::::::::::::::::::::::
```

Indels:-

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

Figure 2

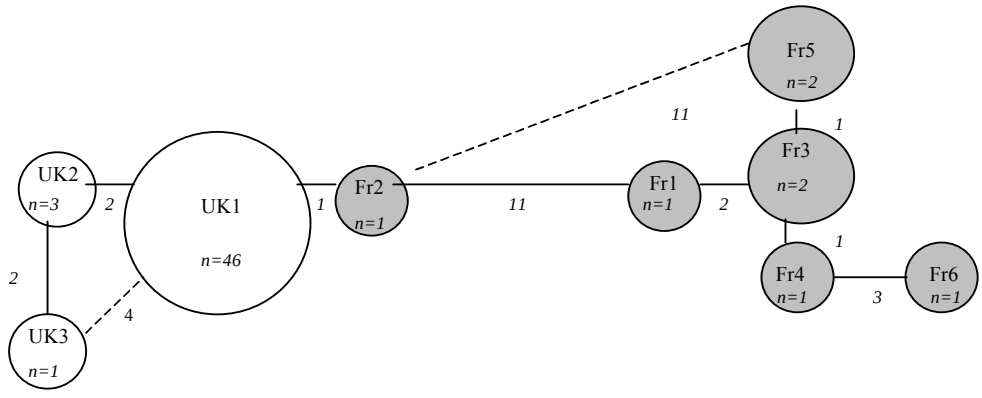
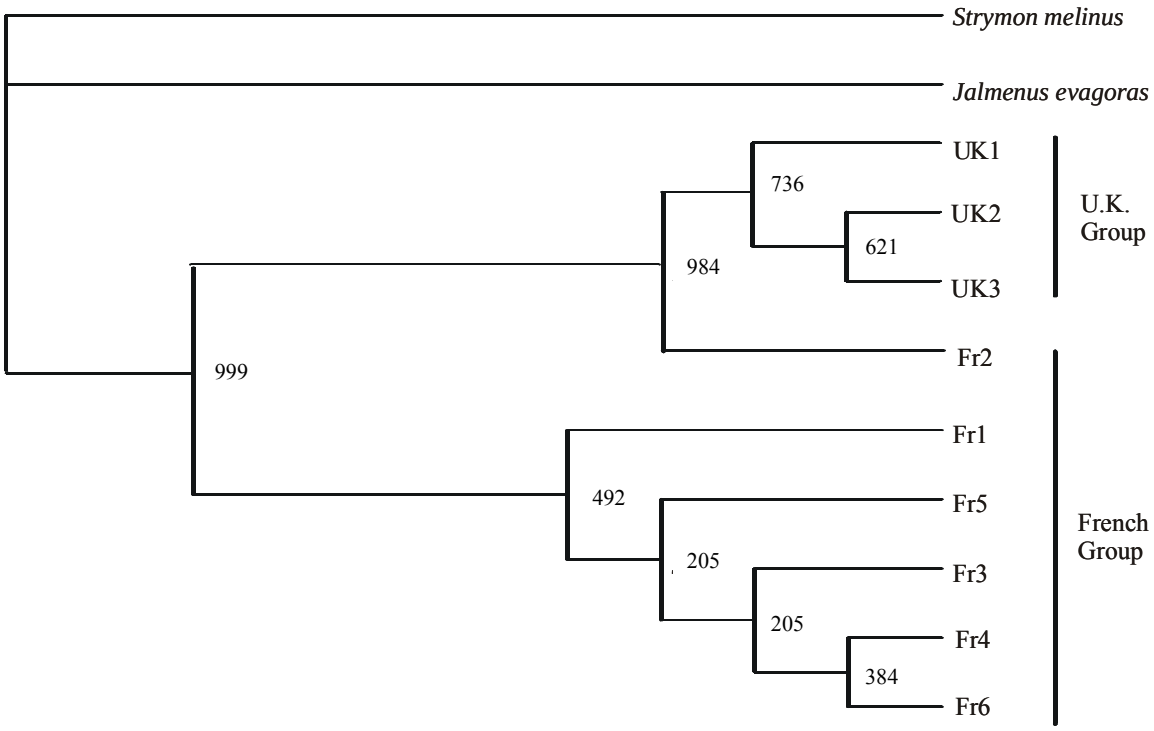


Figure 3.


Strymon melinus
Jalmenus evagoras
UK1
736
UK2
621
UK3
984
U.K.
Group
999
Fr2
Fr1
492
Fr5
205
Fr3
205
Fr4
384
Fr6
French
Group