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Analysis of museum specimens suggests extreme genetic drift in the adonis blue butterfly (*Polyommatus bellargus*)

G. L. HARPER[1]*, N. MACLEAN[2] and D. GOULSON[2]

[1]*School of Applied Sciences, University of Glamorgan, Llantwit Road, Trefforest, Mid Glamorgan CF37 1DL, UK*
[2]*Biodiversity and Ecology Division, School of Biological Sciences, University of Southampton, Bassett Crescent East, Southampton SO16 7PX, UK*

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We amplified microsatellite DNA from museum specimens over 100 years old of the adonis blue butterfly, *Polyommatus bellargus*. These results were compared with butterfly samples taken from the same site near Folkestone in southern UK in 1998/9, 200 generations later, and with samples from other extant UK populations. Dramatic changes in allele frequencies have occurred over time, which is indicative of substantial genetic drift or extinction/recolonization. Patterns of heterozygosity in the 1998/9 sample are indicative of a past bottleneck, and one was known to have occurred in the late 1970s in this and many other UK populations. One allele present at high frequency in 1896 was not detected in any extant UK population, suggesting that it may have been lost from the UK (a 'ghost' allele), although the allele may well persist elsewhere within the range of the species. Although the present study is relatively small in scale (20 museum specimens from one site), it serves to reinforce the enormous potential of museum specimens in well represented taxa such as butterflies for examining the effects of demographic events spanning many years. © 2006 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2006, **88**, 447–452.

ADDITIONAL KEYWORDS: bottleneck – genetic drift – Lepidoptera – Lycaenidae – microsatellites – Museum specimens.

INTRODUCTION

Neutral theory predicts that if a population undergoes a significant demographic contraction, there will be considerable losses of genetic diversity (Wright, 1969; Nei, Maruyama & Chakraborty, 1975; Chakraborty and Nei, 1977; Lacy, 1987). On this basis, low levels of genetic diversity have frequently been used to infer past population bottlenecks (Bonnell and Selander, 1974; O'Brien *et al*., 1983; Ellegren *et al*., 1996). However, this apparently obvious conclusion may be erroneous and ignores other alternative explanations, such as selective sweeps (Bouzat, Lewin & Page, 1998b). In attempts to further elucidate causal factors for observed allele frequency distributions, a number of statistical methods have been devised based on theoretical effects of population declines. For example, it has been proposed that an excess of heterozygosity relative to allele diversity at individual loci can be used to infer a recent population decline (Cornuet and Luikart, 1996; Luikart, Cornuet & Allendorf *et al.*, 1999). To date, many studies concerning changes in population structure have used this type of indirect methodology to infer demographic changes that populations may have undergone (Mallet *et al*., 1993; Le Page *et al*., 2000; Spencer, Neigel, and Leberg, 2000; Waits *et al*., 2000).

Recent advances in molecular biology allow the extraction of DNA from historic and even ancient tissue specimens (Thomas *et al*., 1989; Hausworth, 1994; Roy *et al*., 1994; Bouzat *et al.*, 1998a). Where suitable museum samples exist, we can now reconstruct population structures, and measure changes in allele frequency and gene coalescence that resulted from founder events and bottlenecks. Two previous studies that have directly compared allele frequencies in

*Corresponding author. E-mail: glharper@glam.ac.uk

museum specimens with that of individuals from extant populations both provide compelling evidence for a link between the occurrence of a bottleneck event and a subsequent reduction in genetic diversity (Bouzat *et al*., 1998a; Groombridge *et al*., 2000). This effect is exemplified by the presence of what are known as 'ghost' alleles, alleles that occurred in historical populations but can no longer be found in the modern day populations (Bouzat *et al*., 1998a; Groombridge *et al*., 2000).

There are obvious limitations to using DNA from preserved specimens, the most important of which is the quality of the DNA that can be obtained. DNA in old specimens is often severely degraded, frequently to fragments of just a few hundred base pairs (Paabo, 1989; Cockburn and Fritz, 1996). In this situation, a polymerase chain reaction (PCR)-based methodology using short DNA markers such as microsatellites is most suitable (Thomas *et al*., 1989; Ellegren, 1991; Cano and Poinar, 1993; Mitton, 1994; Hammond, Spanswick & Mawn, 1996).

Butterflies are particularly well represented in museum collections. Historical population data are available for most butterflies in Britain as a result of a nationwide network of amateur lepidopterists. This wealth of data allows specific populations with known histories to be targeted to reveal genetic information. The present study examines changes in microsatellite allele frequencies over more than 100 years in the Adonis blue butterfly, *Polyommatus bellargus* Rottemburg, using modern and preserved specimens. *Polyommatus bellargus* is a rare and southern species in the UK confined to south-facing calcareous grassland, a habitat that was extensively lost to agricultural 'improvement' during the 20th century. The population reached an all-time low in the late 1970s following the drought of 1976, which caused the larval foodplant, *Hippocrepis comosa* to wilt (Thomas, 1983). Subsequently, the species has recovered slightly, but remains very locally distributed. The present study focuses on a population known to have undergone a severe bottleneck at this time. We test whether analyses of current heterozygosity can reveal known past bottlenecks, and we compare past genetic diversity with that remaining in the present day.

MATERIAL AND METHODS

Butterflies were collected in 1998 (32 specimens) and 1999 (42 specimens) from a well-known population at Cheriton Hill, near Folkestone, Kent, UK. Between 1978, when *P. bellargus* underwent a severe decline, up until 1992, the population remained extremely small (Kent Division of English Nature, unpublished data). The population has since expanded to become one of the largest in the UK. Twenty preserved specimens of *P. bellargus* specimens were obtained from the Hope Entomological Collections at the Oxford Natural History Museum. All were captured at the same site at Folkestone during 1896. Both modern and museum specimens were screened at four polymorphic microsatellite loci, identified by Harper *et al*. (2000). These markers have also been used to screen 1173 individuals from 26 modern-day populations; a study spanning the entire modern day geographical distribution of the species within the UK (Harper, Maclean and Goulson, 2003).

DNA was extracted by salting out (Sunnucks and Hales, 1996; Aljanabi and Martinez, 1997). From the modern specimens, the head was used for extraction, whereas a single leg was used for the 1896 specimens. The extraction method followed that previously described by Harper *et al*. (2003). To avoid contamination, the DNA from the museum specimens was extracted in a separate laboratory from the modern-day specimens.

Four microsatellites were employed in the analysis (Lb1/57, LbG2, Lb4/18 and Lb 4/19). Primer sequences and PCR conditions for the modern specimens were as previously described (Harper *et al*., 2000). For the museum specimens, the amount of DNA template was increased to 50–100 ng and the primer concentration was increased to 0.4 μM per reaction. The microsatellites were scored using a 5% denaturing polyacrylamide gel by vertical electrophoresis at 20–60 mA for 2 h on a Perkin Elmer ABI 377 automated sequencer running GENESCAN and GENOTYPER 2.5 software (Perkin Elmer Applied Biosystems).

STATISTICAL ANALYSIS

To test for temporal subdivision of allele frequencies between 1998 and 1999, they were examined using an exact *G*-test. No significant differences were found ($P > 0.05$), and hence these samples were pooled. Departures from Hardy–Weinberg equilibrium for both modern and 1896 populations at each locus were tested using ARLEQUIN (Schneider, Roessli, and Excoffier, 2000). A previous study demonstrated that at least four out of the five microsatellites available for *P. bellargus* have a high incidence of null alleles (Lb1/57, Lb4/18 and Lb4/19) (Harper *et al*., 2000). Where a significant heterozygote deficit was found, estimations were made of the frequency of null alleles (n) using the method of Brookfield (1996).

To test for evidence of a recent reduction in effective population size (N_e), the microsatellite data were analysed using the programme BOTTLENECK 1.2.02 (Cornuet and Luikart, 1996; Piry, Luikart & Cornuet, 1999). Statistics for all three models of mutation were calculated [stepwise (SMM), two phase (TPM) and the infinite allele (IAM)]. A Wilcoxon signed rank test was

performed to test the hypothesis that the average standardized difference across loci for each population was not significantly different from zero (Spencer *et al*., 2000). A second indication of a bottleneck is a mode-shift away from an 'L-shaped' distribution of allele frequencies, to one with fewer alleles in the low frequency categories. These were all assessed using BOTTLENECK (Cornuet and Luikart, 1996; Piry *et al*., 1999).

To test for changes in allele frequency over time, modern-day and museum samples were compared using an exact *G*-test with 5000 permutations in FSTAT 2.9.1 (Goudet, 2000). Estimates were made for allele number, observed (H_O) and expected heterozygosity (H_E) and frequency data were calculated for both populations using FSTAT 2.9.1. Differences in mean expected heterozygosity (H_E) and mean number of alleles per locus between the 1896 and modern *P. bellargus* populations were tested using a one-way analysis of variance (ANOVA). Heterozygosity data were normalized by arcsine transformation. Sample size affects parameters such as allelic diversity, confounding the ANOVA analysis. To overcome this, a permutation approach was taken, whereby a random subset of 20 individuals was repeatedly sampled, without replacement, from the 74 individuals from the modern population. Permutations were performed over complete genotypes, as opposed to over loci, to avoid assumptions about the interdependence of loci and, without replacement, to avoid assumptions about the frequency distributions of alleles in the sampled population. The average number of alleles per locus was then calculated from all the artificially generated sample datasets to allow an unbiased ANOVA comparison.

In addition to the exact test of population, two other estimates of genetic distance were calculated for the two temporally defined populations. The first of these was θ, Weir and Cockerham's F_{ST} analogue (Weir and Cockerham, 1984), calculated using FSTAT version 2.9.1. This estimator is particularly relevant because it is independent of the effects of sample size (Weir and Cockerham, 1984). The second distance measure was Slatkin's R_{ST} (Slatkin, 1995), calculated using RST Calc. Version 2.2 (Goodman, 1997). Before calculation of R_{ST}, data were standardized for variance between sample sizes using a standardization procedure within the RST Calc 2.2 package.

RESULTS

DNA from modern specimens could be visualized as a single high molecular weight band on an ethidium bromide-stained agarose gel. By contrast, the DNA from the 1896 specimens was seen as a faint smear at the base of the gel, approximately 100–300 base pairs in size. Four of the five microsatellites consistently amplified from both butterfly time periods. All attempts to optimize Lb1/41 from the museum specimens failed; therefore, this marker was not included in the analysis.

As expected, three loci (Lb1/57, Lb4/18 and Lb4/19) all displayed heterozygote deficits under the Hardy–Weinberg equilibrium, possibly indicative of the presence of null alleles. The fourth locus, LbG2, showed no significant deviations between the calculated H_O and H_E. When the null allele frequencies were calculated for the 1896 sample (Table 1), all three loci affected displayed values between 0.2 and 0.35; this is comparable with other published studies (Neumann and Wetton, 1996; Van Treuren, 1998) and also with those calculated for the modern-day populations (Harper *et al*., 2003).

The program BOTTLENECK revealed evidence for a past bottleneck in the modern population, but not

Table 1. Calculated values for the Hardy–Weinberg equlibrium showing observed heterozygosity (H_O), expected heterozygosity (H_E), calculated probability rating, and also the number of alleles (n), effective number of alleles (N_E) and null allele frequencies for all microsatellite loci in each population

Population	Locus	H_O	H_E	P	n	N_E*	Null allele frequency
1896	Lb1/57	0.333	0.953	< 0.001	10	–	0.34
	LbG2	0.588	0.685	0.6	7	–	0
	Lb4/18	0.529	0.756	0.02	5	–	0.23
	Lb4/19	0.467	0.864	< 0.001	8	–	0.21
	Mean	0.479	0.816	–	7.5	–	0.20
Modern	Lb1/57	0.508	0.836	< 0.001	13	8.6*	0.25
	LbG2	0.731	0.764	0.07	7	4.4*	0
	Lb4/18	0.667	0.828	< 0.001	9	6.9*	0.27
	Lb4/19	0.600	0.717	0.037	8	7.1*	0.07
	Mean	0.627	0.786	–	9.25	6.8*	0.15

*These values are calculated using the permutation approach, thus removing the effects of the larger sample size of the modern day population.

in the 1896 sample. Although neither population exhibited a shift in allele frequencies away from an L-shaped distribution, the Wilcoxon signed rank test uncovered an excess of heterozygosity relative to allele number in the modern population, but only under the IAM (assigned $P = 0.031$). However, both TPM and SMM showed no significant excess (0.92 and 0.90, respectively). The population from 1896 showed no evidence for a recent contraction in size under any of the mutation models tested (Wilcoxon signed rank test: IAM $P = 0.16$, TPM = 0.91, SMM = 0.94).

Seven alleles were found in the 1896 population that were no longer detected in modern samples at Folkestone (LbG2: 182, 184; Lb4/18: 147; Lb4/19: 254; Lb1/57: 203, 215, 223; Fig. 1). One allele at LbG2 (184) was unique to the 1896 museum specimens and, at that time, comprised the dominant allele with a frequency of 0.56. This allele was absent in an extensive population survey of 1173 butterflies from 26 current UK populations of *P. bellargus* (Harper *et al*., 2003). Three out of the four loci (Lb1/57, LbG2 and Lb4/18) showed significant shifts in allele frequencies between the 1896 and modern samples (exact *G*-test for each loci: $P = 0.001$) (Fig. 1). Lb4/19 showed no significant differences (exact *G*-test, $P = 0.2$). Overall loci differentiation was significant (exact *G*-test, $P = 0.001$). Similarly, F_{ST} and R_{ST} values indicated marked differentiation over time ($F_{ST} = 0.0942$, $P = 0.001$; $R_{ST} = 0.216$, $P < 0.0001$).

Expected heterozygosity (H_E) in the 1896 population at Folkestone (0.816) was higher than that of the modern population (0.786), although this difference was not significant ($F_{3,12} = 2.01$, $P = 0.16$) (Table 1). Similarly, allele number was higher in the 1896 population (mean of 7.5 per locus) compared to the modern sample (mean of 6.75 after accounting for the greater sample size), but this difference was not significant ($F_{3,12} = 0.63$, $P = 0.61$) (Table 1).

DISCUSSION

We successfully extracted DNA from more than 100-year-old museum specimens, and compared genetic diversity at microsatellite loci in two samples of butterflies collected 102 years (approximately 200 generations) apart from the same site near Folkestone in southern UK. Significant changes had occurred in allele frequency over time. In 1896, the population contained more alleles and higher heterozygosity than it does at present. Heterozygosity was higher than that found in any of 26 extant UK populations surveyed by Harper *et al*. (2003). Reassuringly, analysis of the modern samples provides evidence for a past genetic bottleneck, and this site is known to have undergone a

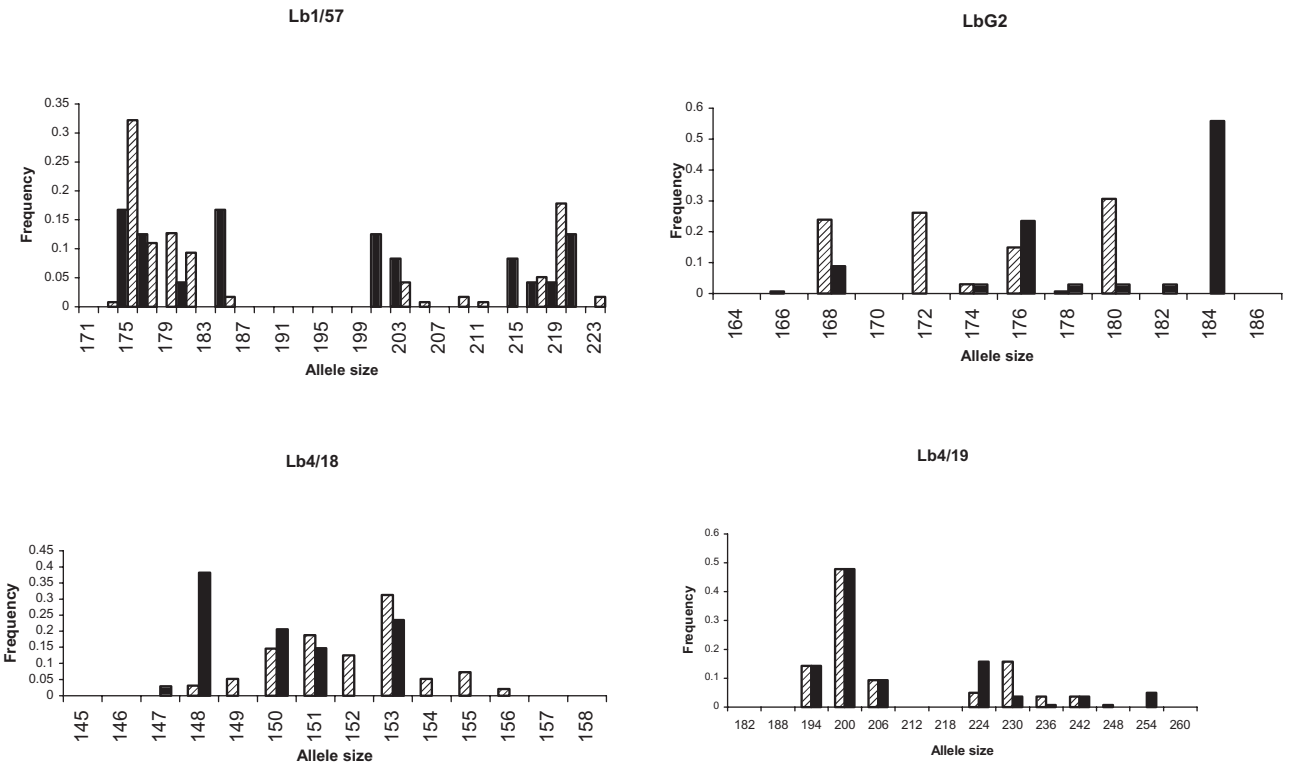

Figure 1. Combined frequency histograms of allele sizes (in bp) for all four loci in the modern (grey) and 1896 (black) populations of *Polyommatus bellargus* at Cheriton Hill.

severe bottleneck in the last 1970s. This bottleneck probably explains the reduced genetic diversity in the extant population. Seven alleles present in 1896 were not detected in 1998. One of these alleles was not detected in any extant UK population, suggesting that it may have been lost from the UK.

It must be noted that the analyses provide only weak evidence for a past bottleneck, although we know that it did occur. When the data were analysed for an excess of heterozygosity relative to allele number, a proposed characteristic of a population bottleneck (Hedrick *et al*., 1986; Cornuet and Luikart, 1996), significant excess was only found under the IAM (assigned $P = 0.031$) rather than the TPM and SMM. This may indicate constraints on the analytical power of the tests, such as: (1) The presence of null alleles in the population may have a confounding effect on the analysis (Cornuet and Luikart, 1996; Le Page *et al*., 2000); (2) the disjunctive patterns of allele size distributions present for most loci (Fig. 1) may indicate a departure from the SMM towards the IAM; and (3) four loci may be too few to allow the test to achieve sufficient analytical power (Cornuet and Luikart, 1996). This lack of concordance between the IAM, TPM and SMM was also observed during the screening of 26 modern day populations of *L. bellargus* using the same markers (Harper *et al*., 2003). Here, the IAM was consistently shown to be the most effective measure for demographic change within these data.

Significant differences were found between the allele distributions in 1896 and 1998/9 as determined by the *G*-test, and temporal change was also indicated by significant pairwise F_{ST} and R_{ST} values. One allele (184 bp) present at LbG2 in 1896 was not detected in 1173 butterflies collected in 1998/9 from 26 sites spanning the entire UK range (Harper *et al*., 2003). Similar 'ghost' alleles have been described in other studies (Bouzat *et al*., 1998a; Groombridge *et al*., 2000). In 1896, this allele was the most common at this locus, with a frequency of 0.56. This dramatic shift in allelic distribution is unlikely to have occurred in a population at equilibrium, and can only be attributed to rapid drift via one or more major demographic changes, such as bottlenecks or cycles of population extinction and recolonization (Wright, 1940; Slatkin, 1977; Whitlock and McCauley, 1990; Wade and McCauley, 1988). Anything other than a severe bottleneck or extinction event would be unlikely to remove such a common allele because theoretical predictions suggest that declines will generally only remove rare alleles (Nei *et al*., 1975; Leberg, 1992).

To date, few studies have attempted to directly measure temporal changes in allele frequencies over long time periods in wild populations. Museum specimens provide an enormous and largely untapped resource in this respect, particularly for taxa that are well represented, such as butterflies (by contrast, studies of vertebrates are likely to be hampered by limited availability of specimens; Bouzat *et al*., 1998a). There are many thousands of documented *P. bellargus* specimens available in entomological collections, which could enable the elucidation of detailed information concerning the origin and route of temporal changes in allele frequencies. Not only could this address questions of ecological/conservation importance particularly relevant to species existing in closed populations, but also it would enhance the current understanding of mutational patterns and evolution in microsatellite DNA.

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