Genetic Analysis of Founder Bottlenecks in the Rare British Butterfly *Plebejus argus*

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Abstract: *Fragmented populations of the silver-studded blue butterfly (*Plebejus argus*: Lycaenidae) provide a model system for metapopulations in which extinctions, colonizations, and artificial introductions have all been documented. The genetic consequences of population turnover were investigated using allozyme and mtDNA/RFLP markers and via studies of fluctuating asymmetry. Evidence for changes in genetic diversity was studied in samples of 40–90 individuals from one source and eight descendent populations in North Wales. The genetic data were analyzed using a novel permutation method, which is potentially applicable to analyses of other levels of biodiversity, such as species diversity or species counts. Significant reductions in the numbers of rare allozyme alleles were found in descendent populations, presumably as a result of drift during colonization bottlenecks. Similar trends were evident in the mtDNA data, but were largely non-significant. The reduced statistical power of mtDNA was probably due more to low overall numbers of alleles detected (19) compared with allozymes (40 alleles at 12 loci) than to any conflict between marker loci. Expected heterozygosity (H_e) also generally declined during colonization, but the changes were rarely significant, probably because drift-induced changes in the frequencies of common alleles may actually increase H_e in a bottlenecked population. Finally, overall gene frequency changes, measured by F_{st}, were often significant. The values of the effective population size (N_e) estimated for two introductions (N_e = 71 and 29) suggested bottleneck population sizes of approximately half the number of diploid genomes (180 and 60 respectively) actually introduced. In contrast to evidence for loss of genetic diversity, there was no evidence for any increase in fluctuating asymmetry among descendent populations.* Plebejus argus *maintains large enough populations locally and colonizes in sufficient numbers that the deleterious effects of bottlenecking are temporary, affect only the rarest alleles, and cause no observed disturbances to developmental stability. As a result a single introduction of 90 mated females to Rhŷd-y-foel in 1942 has led to the successful establishment of a healthy metapopulation now numbering about 100,000.*

Análisis Genético de Cuellos de Botella en la Mariposa Británica *Plebejus argus*

Resumen: *Poblaciones aisladas de la mariposa* Plebejus argus *(Lycaenidae) proporcionan un sistema modelo de metapoblaciones, en las cuales; extinciones, colonizaciones y liberaciones artificiales han sido documentadas. Utilizando muestras grandes de 40–90 individuos, las consecuencias genéticas producidos por disturbios poblacionales fueron estudiados mediante el uso de alozimas y marcadores ADNmt/RFLP y con estudios de asimetría fluctuante. Las evidencias de cambios en la diversidad genética fue estudiada en una población original y ocho poblaciones descendientes en North Wales. Los datos genéticos fueron analizados mediante un nuevo metodo de permutaciones, el cual se puede utilizar en análisis de otros niveles de biodiversidad, así como en diversidad de especies. Encontramos reducciones significativas en el número de alelos*

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raros de alozimas en las poblaciones descendientes, probablemente como resultado de la deriva génica durante colonizaciones cuellos de botella. Tendencias similares fueron evidentes en los datos de ADNmt, pero fueron mayormante no significativas. El reducido poder estadístico del ADNmt probablemente se debe al número bajo de alelos (19) comparado con el número de alozimas (40 alelos en 12 loci), mas que a un conflicto entre loci marcadores. La heterozigocidad esperada (H_e), generalmente también es reducida durante una colonización, pero los cambios son raramente significativos, probablemente debido a que cambios en las frecuencias de alelos comunes inducidos por la deriva génica puede, a veces, causar un incremento en H_e en una población en situación de cuello de botella. Finalmente, cambios en frecuencias de alelos, medidos por F_{st}, fueron frecuentemente significativos. El tamaño efectivo de la población (N_e) estimado para dos introducciones (N_e = 71 y 29) sugiere tamaños poblacionales cuello de botella de aproximadamente la mitad del número actual de genomas diploides liberados (180 y 60 respectivamente). En contraste con la evidencia de pérdida de diversidad genética, no hay evidencia de incrementos en la asimetría fluctuante de las poblaciones descendientes. Plebejus argus *mantiene poblaciones locales lo suficientemente grandes y coloniza en números tales que los efectos negativos de los cuellos de botella son temporales, afectando únicamente los alelos mas raros sin causar disturbios en la estabilidad ontogenética. Como resultado, una sola liberación de 90 hembras inseminadas en Rhyd-y-foel en 1942 logró el establecimiento de una metapoblación nueva, la cual cuenta hoy con aproximadamente 100,000 habitantes.*

Introduction

Habitat destruction caused by intensive agriculture and urban development is fragmenting the distributions of formerly widespread species. Population fragmentation has become particularly acute in the British Isles, where many threatened species are now confined to small pockets of suitable habitat. The ecology of subdivided populations, especially metapopulations—groups of populations characterized by recurrent extinction and colonization—has been explored in theory and in its effects on conservation (Gilpin & Hanski 1991; Hanski & Gilpin 1996). These demographic changes will also cause repeated, small population "bottlenecks," or "founder effects," which can strongly influence the genetics of remnant populations, but the amount of genetic change and its importance in terms of fitness and conservation has become controversial. Simple genetic arguments generated serious concerns about the loss of genetic variation in small, isolated populations and the consequent long-term viability of endangered species (Frankel & Soulé 1981; Soulé 1987; Gilpin 1991). Although the mood in conservation has swung away from the importance of genetics and more toward demographic considerations (Lande & Barrowclough 1987; Lande 1988; Nunney & Campbell 1993), recent theory shows that rather large populations are required to prevent populations suffering from random fixation of mildly deleterious mutations (Lynch et al. 1995; Lande 1995). Meanwhile, more and more populations are going extinct or becoming isolated, and conservation managers are proceeding with introductions, reintroductions, and management of existing metapopulations. We must be sure that we do not underestimate the importance of population genetics in conservation.

An increased frequency of bottlenecks and inbreeding may be the most important genetic consequence of population fragmentation. The genetic effects of small population size and population bottlenecks has also been studied extensively in theory (Wright 1931, 1978; Gilpin 1991; Lande 1995; Lynch et al. 1995) and in the laboratory (e.g., Bryant et al. 1990; Leberg 1992; Brakefield & Saccheri 1994; Saccheri 1995). There are, however, relatively few studies of the genetics of natural bottlenecks relevant to conservation (Mallet 1996), and the fitness consequences of reduced genetic variation may only be expressed in some environments. For example, it has long been known that inbreeding depression in plants will be more strongly expressed under competitive than under optimal conditions (e.g., for plants, see Darwin 1875), and recent experiments on animals have provided clear demonstrations of this effect (Jiménez et al. 1994; Keller et al. 1994). Introductions into natural, low-diversity populations of house mice (*Mus*) and desert topminnows (*Poeciliopsis*) have shown enormous selective advantages for genes from genetically more varied populations (Berry et al. 1991; Scriven & Bauchau 1992; Vrijenhoek 1994), again suggesting problems of a narrow genetic base in the wild.

It is not clear which genetic techniques should be used in conservation. Allozymes were often used as genetic markers in earlier work (Avise 1994; Bruford & Wayne 1994; Brookes et al. 1996); however, it is possible that strong selection controls protein polymorphisms so that allozymes reflect current selection rather than past population bottlenecks. Some recent studies have shown differences between the evolution of DNA and allozymes (reviewed by Avise, 1994; Mallet, 1996). Fluctuating asymmetry (FA), the unsigned difference between the right and left sides of a bilaterally symmetrical character, may reflect environmental or genetic stresses that an individual experiences during its development

(Palmer & Strobeck 1986). Accordingly, individuals with the highest fitness, or "developmental stability" should be the most symmetrical. Several authors have suggested that measures of FA may be a useful tool in conservation biology, providing a surrogate for estimating the fitness effects of inbreeding (Leary & Allendorf 1989; Clarke 1995).

We examine the genetic effects of artificial introductions and natural colonizations of the silver studded blue butterfly *Plebejus argus* in North Wales. This butterfly is now confined to highly restricted populations in Britain and represents an ideal model system in which to study these effects because it is the subject of conservation measures and because its documented demographic history includes information on introductions and natural colonizations. We ask whether these bottlenecks have resulted in a detectable loss of genetic diversity in initial colonizations and introductions and during spread within a new metapopulation in the Dulas Valley. We also use fluctuating asymmetry as a preliminary surrogate method to test whether the bottlenecks have had any effect on fitness. In the course of this work, we developed methods to test for subtle changes in genetic diversity, and we compared mtDNA and allozyme markers.

Methods

Plebejus argus in North Wales

Over the last 100 years, the British range of *P. argus* has declined precipitously because of its habitat destruction and changes in land use (Thomas 1985*a*, 1993). Owing to its weak dispersal capacity, *P. argus* is incapable of recolonizing naturally, except over short distances (Thomas 1985*a,b*; Lewis et al. 1997). In North Wales *P. argus* occurs on species-rich calcareous grasslands that are localized because of the distribution of limestone outcrops and because the grasslands are fragmented on each outcrop (Thomas & Harrison 1992). In this area *P. argus* is restricted to limestone grasslands that provide high densities of both host plants (various Leguminosae and Cistaceae) and mutualist ants (*Lasius* spp., Jordano & Thomas 1992; Jordano et al. 1992). The larvae of *P. argus* secrete droplets containing sugars and amino acids (Glen 1996). These are collected by *Lasius* ants, which are presumed to protect the larvae from natural enemies. The butterfly depends on high densities of both host plants and *Lasius*. We concentrated on nine populations derived from a single and distinctive source area, The Great Orme's Head near Llandudno in N. Wales (Fig. 1), where there is a population of about 10^6 adults. It has been suggested that *P. argus* has probably been present on this limestone headland for 1,000–10,000 years (Dennis 1977). Two successful introductions and a series of well-documented natural colonizations have occurred in the last 50 years (Thomas & Harrison 1992). In 1942, 90 mated females were taken from the Great Orme's Head and introduced to suitable habitat at Rhŷd-y-foel within the Dulas Valley, 15 km away (Marchant 1956). There were no prior historical records of *P. argus* in the Dulas Valley, but the introduced population colonized a number of suitable patches nearby and now forms a large metapopulation, distributed patchily over 7 km^2, with a total population size estimated at about 10^5 individuals by 1983 (Dennis 1972, 1977; Thomas & Harrison 1992). Metapopulation model simulations predicted successful establishment and long term persistence in the Dulas Valley (Hanski & Thomas 1994). A second introduction of 30 mated females was made in 1983 from the Great Orme to Graig Fawr, 29 km away (Thomas 1983). Several thousand individuals were present at Graig Fawr in 1990, representing an approximate doubling of population size per generation (*P. argus* is univoltine) since 1983. Finally, a natural colonization from the Great Orme to a nearby site, Happy Valley, also occurred soon after 1983 (Thomas & Harrison 1992), and this population currently numbers about 10^3 adults. This well-documented history of *P. argus'* demographic success provides an excellent model system in which to study intermediate term ($\sim$ 50 generations) consequences of artificial introductions and successive natural colonizations.

Collecting Sites

All samples were collected in 1992 and 1994. The sampling sites, mapped in Fig. 1, were the source population, Great Orme's Head (abbreviated to "GO," grid reference SH 768 824); one naturally colonized population, Happy Valley ("HV," SH 780 832); the introduced populations, Graig Fawr ("GF," SJ 059 803) and Rhŷd-y-foel ("RYF," SH 913 765); and populations naturally colonized from RYF, Borth-ŵryd ("BW," SH 913 763), Garth Gogo ("GG," SH 917 762), Mynydd Marian ("MM," SH 891 773), Plâs-newydd ("PN," SH 905 766), and Terfyn ("T," SH 913 776). We collected only males to avoid excessive damage to the populations.

Allozymes

Individuals were immediately frozen in liquid nitrogen for transport and then stored at −80°C for analysis of genetic variability at 12 polymorphic allozyme loci: phosphoglucomutase, *Pgm*; glucose-6-phosphate isomerase, *Gpi*; glutamate oxaloacetate transaminase, *Got-f*; mannose-6-phosphate isomerase, *Mpi*; malic enzyme, *Me*; sorbitol dehydrogenase, *Sdh*; isocitrate dehydrogenase, *Idh*; fumarate hydratase, *Fum*; peptidase (phe-pro), *Pp*; peptidase (leu-gly-gly), *Lgg-f* and *Lgg-s*; and adenylate kinase, *Ak-f*. Standard cellulose acetate plates were used to separate alleles using methods given by Emelianov et al.

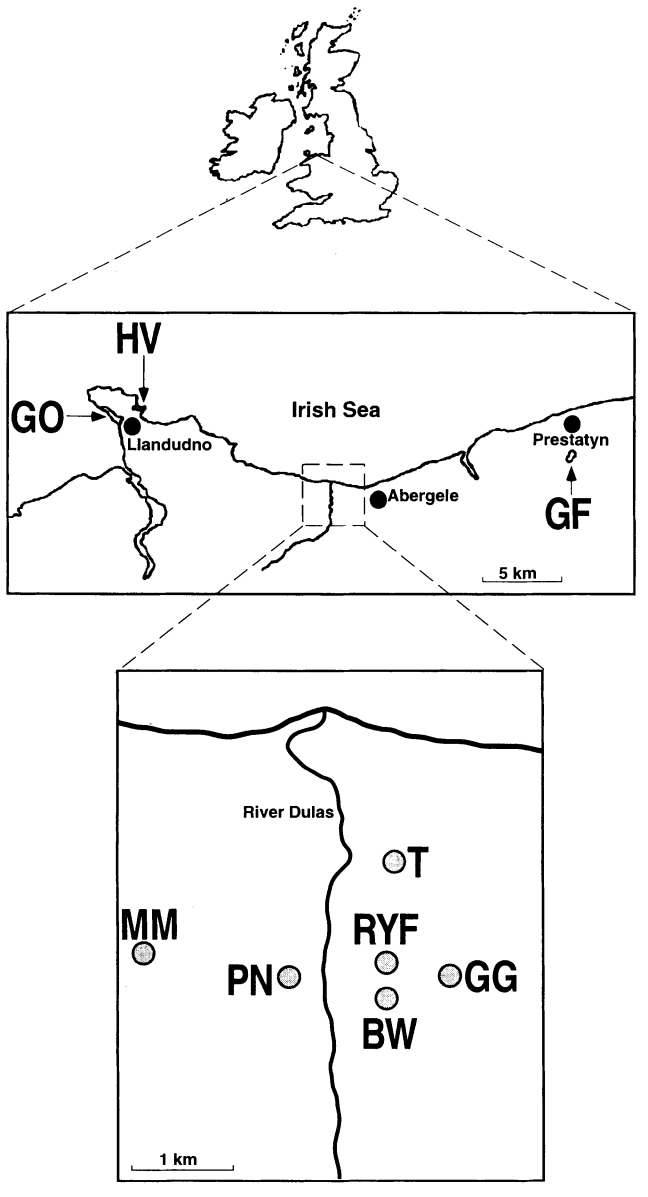

Figure 1. Map of study sites in North Wales. Populations sampled shown on this map were source population, Great Orme's Head (GO); a naturally colonized population, Happy Valley (HV); the introduced populations, Graig Fawr (GF) and Rhŷd-y-foel (RYF); and the Dulas Valley populations naturally colonized from RYF, Borth-ŵryd (BW), Garth Gogo (GG), Mynydd Marian (MM), Plâs-newydd (PN), and Terfyn (T). The Dulas Valley metapopulation is shown enlarged.

(1995) as modified from other sources (Richardson et al. 1986; Pasteur et al. 1988; Mallet et al. 1993).

DNA Extraction

Each butterfly thorax was homogenized in 250 μL TNES buffer (0.01 M Tris, 0.1 M NaCl, 1 mM EDTA, 0.1% SDS) and incubated overnight at 55°C with 250 μg Proteinase K. An equal volume of 2.6 M NaCl was added, and the mixture was centrifuged for 5 minutes. The supernatant was mixed with an equal volume of cold absolute ethanol and centrifuged for 20 minutes at 4°C. The DNA precipitate was washed in 70% ethanol and air-dried before dissolving in 100 μL H_2O. In the course of this study we also showed that PCR amplification of DNA could be achieved on a few wing scales scraped from live or dead butterflies (Rose et al. 1994); although less reliable than standard DNA extraction, the method will be of use especially with more highly endangered species.

Mitochondrial DNA

Approximately 780 bp of the "AT-rich" (= control) region of the mtDNA genome was amplified using the primer pair met39- (5′-TGGGGTATGAACCCAGTAGC-3′) and 12s348+ (5′-TAGGGTATCTAATCCTAGTT-3′; Taylor et al. 1993). The PCR was carried out in 50 μL total reaction volume, containing 0.5 μL DNA extract (approx. 100 ng DNA), 200 μM each dNTP, 0.3 μM each primer, 1.5 mM Mg^{2+}, 1 unit of *Taq* polymerase and 5 μL Advanced Biotechnologies Buffer IV, under the following conditions: 95°C for 10 minutes, 51°C for 40 seconds, 72°C for 1 minute; followed by 29 cycles of 94°C for 1 minute, 51°C for 40 seconds, and 72°C for 1 minute. Separate digests of this region with a total of 24 restriction enzymes were disappointing because no polymorphic restriction sites were found. However, high resolution gels showed that fragments varied in length. To reveal this polymorphism, 16 μL of each 50 μL PCR reaction were digested with 10 units each of *Ssp*I and *Rsa*I and 2 μL of reaction buffer, for three hours. 1 μL of each sample was then run on a 32 cm non-denaturing polyacrylamide gel (8% polyacrylamide, 10% glycerol, 0.5 × TBE) for 16 hours at 50 mA constant current and then silver-stained. These conditions produced six restriction fragments, of which five displayed discrete length variation between individuals (Fig. 2). Each fragment had 2–7 length variants, giving a total of 336 potential haplotypes, of which 19 were actually found in North Wales. (Only 3 of 1150 *P. argus* scored from Britain and Europe digested with *Ssp*I and *Rsa*I gave variation in fragment number as opposed to fragment length.) This length variation was carefully tested and calibrated via repeat PCR of the same samples and by running the same samples repeatedly alongside haplotypes that were putatively the same and different. Although length differences were slight, the variants could in practice be scored unambiguously.

Statistical Analysis of Genetic Data

We examined changes in heterozygosity, in allelic number, and also in gene frequency as indicators of genetic change wrought by genetic drift during introductions

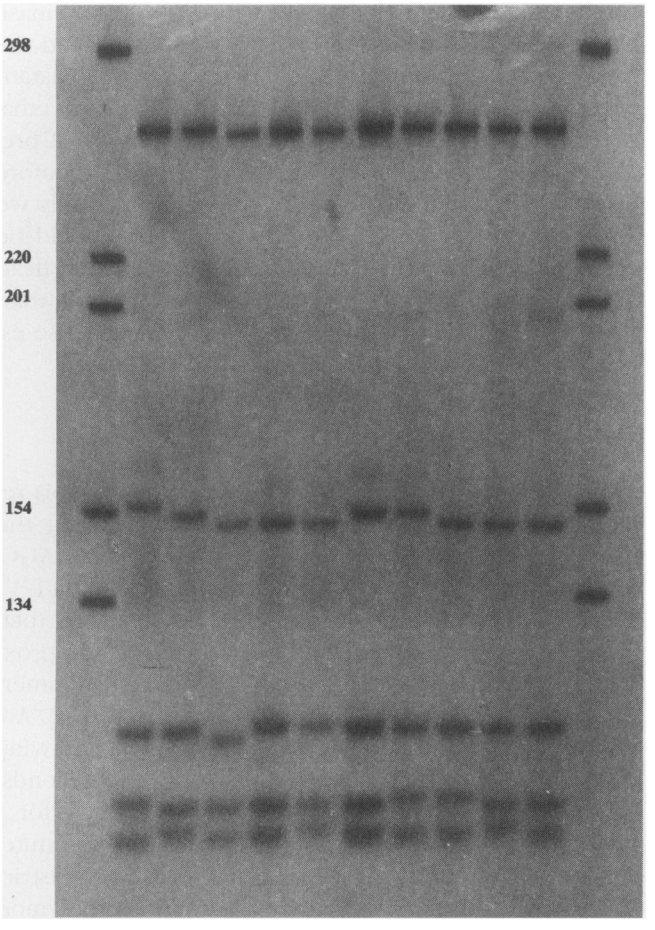

Figure 2. Length variation in mitochondrial restriction fragments of Plebejus argus. *Five restriction fragments of the PCR-amplified AT rich region are shown for 10* P. argus *individuals (a sixth ~ 60 bp restriction fragment was run off the end of the gel). Lanes (from left to right) 1 and 12 molecular weight markers (sizes in bp); lanes 2-11 a sample of 10 of the alleles detected in the* P. argus *populations. Length variation can be seen in each of the five restriction products. Allelic scores for each lane are as follows reading from the top to the bottom of the gel (S = slow, M = medium, F = fast): 2 SSSSF, 3 SMSFS, 4 FFFFF, 5 FFSFF, 6 FFSFS, 7 SMSFF, 8 SMSSF, 9 SFSSF, 10 SFSFS, 11 SFSFF.*

and natural colonizations. Arguably, a measure of allelic diversity which includes both numbers of alleles (n_a) and their relative frequency could be most informative. The obvious choice as a measure of allelic diversity is expected heterozygosity (Nei's unbiased measure—Nei 1978),

$$H_e = \frac{2N}{(2N-1)} \sum_{i=1}^{n} (1 - p_i^2),$$

where n is the number of alleles at a locus, N is the number of genotypes in the sample, and p_i is the frequency of the ith allele. However, H_e gives rise to a number of problems (Leberg 1992). The H_e declines predictably *on average* under bottleneck-induced genetic drift as alleles are lost (Wright 1931), but changes in the frequency of common alleles in a limited sample of loci can sometimes cause a strong increase in H_e. In the short term, this can cause bottlenecked populations to become more "heterozygous" than their parent populations, although this problem should disappear as more loci are sampled. Secondly, in conservation we are more interested in irretrievable loss of alleles than in changes in frequency; selection can restore perturbed gene frequencies, but lost alleles can be replaced only by immigration or mutation. When some alleles are very rare, it is impossible to count alleles accurately using samples from populations. Consequently, neither numbers of alleles (n_a) nor heterozygosity (H_e) at limited samples of loci give clear results.

Unless one can sample every individual in each population, all of these measures cause difficulties in statistical tests. For example, to use parametric statistical tests on changes in H_e or n_a requires estimates of the underlying allele frequency distributions. During population bottlenecks, rare alleles are the most likely to be lost. Rare alleles will also be missed in most samples, so we are unable to make reasonable predictions about their frequency distributions. Instead, we have used a pairwise permutation method to test the null hypothesis that two samples could have been drawn from the same population with respect to both H_e and n_a. To do this, we wrote a computer program which mixes all the individual genotypes from the pair of population samples to be tested and withdraws at random and without replacement, the number of genotypes obtained in the actual sample sizes from each of the two populations. Permutations were performed over complete genotypes as opposed to over loci in order to avoid assumptions about the interdependence of loci and without replacement to avoid assumptions about the frequency distributions of alleles in the sampled populations. The H_e and n_a of each of the two artificially generated populations were then determined. Allozyme H_e values were based on Nei's unbiased estimate (see above) averaged over loci; mtDNA genetic diversity (equivalent to heterozygosity) was estimated in the same way:

$$H_e = \frac{N}{(N-1)} \sum_{i=1}^{n} (1 - p_i^2).$$

Allelic and heterozygosity differences in the simulated populations were then compared with the observed differences. This test is used to estimate the probability that the observed change in n_a (total over loci sampled) or H_e (averaged across loci) was greater than that expected under randomization. For two populations of sizes N_1 and N_2, there are in fact $(N_1 + N_2)!/N_1!N_2!$ possi-

ble permutations; if $N_1 = N_2 = 40$, there are 1.08×10^{23} such possibilities, so simulation is necessary to sample the potential space. Each pairwise probability was based on 1000 trials.

Differences in gene frequency between pairs of populations were measured using estimates of F_{st} (Wright 1978). To test for significant differences in gene frequency between pairs of populations, we performed contingency table tests using the statistic

$$X^2 = \sum \frac{(O-E)^2}{E}.$$

This method has been criticized (Raymond & Rousset 1995). However, the alternative suggested by Raymond and Rousset, an "exact" probability test, has its own flaws; the method assumes, as do most other tests, that, if columns are populations and rows are alleles, the row totals are correct relative estimates of the gene frequencies in the overall population. We have extensively tested the X^2 method under simulations of the null hypothesis (unpublished; again, we made the assumption of actual row and column totals being at their true values); X^2 was found to be somewhat conservative but similar to the asymptotic result where X^2 is distributed approximately as a χ^2.

Fluctuating Asymmetry

Butterfly forewings from the individuals used in the genetic analyses were dissected and dry mounted between two microscope slides. Wings were viewed and measured using NIH Image v.1.55 computer software, connected to a binocular microscope via a video camera fitted with a macro lens. Nine wing spot characters were measured (see Fig. 3) for 34–40 individuals from each of the nine North Wales populations. The imaging software allows contrast to be accentuated via a "thresholding" algorithm to make the butterfly wingspots appear completely black on a white background. After standardized thresholding, the Cartesian coordinates of the "center" of each spot (≡ the center of the ellipse that best fits the shape of the spot) were determined. Each of the nine characters was measured as a straight line distance between spot centers (see Fig. 3).

For each character, and for each population, FA was calculated as a dimensionless variance:

$$FA = var\left[\frac{R-L}{(R+L)/2}\right],$$

where R and L are the measured distances for the right and left side of the character, respectively. This is the most powerful measure for detecting FA differences between populations when, as here, FA is proportional to character size (Palmer & Strobeck 1986). Two repeated measurements of each character were made for each individual so that the measurement error variance could be partioned out of the estimate of FA. In all cases, the variance due to measurement error never exceeded 1% of the total variance due to differences between sides. For each character, FA differences between all nine North Wales populations were assessed with an F_{max} test, which is based on the ratio of the maximum sample variance to the minimum sample variance.

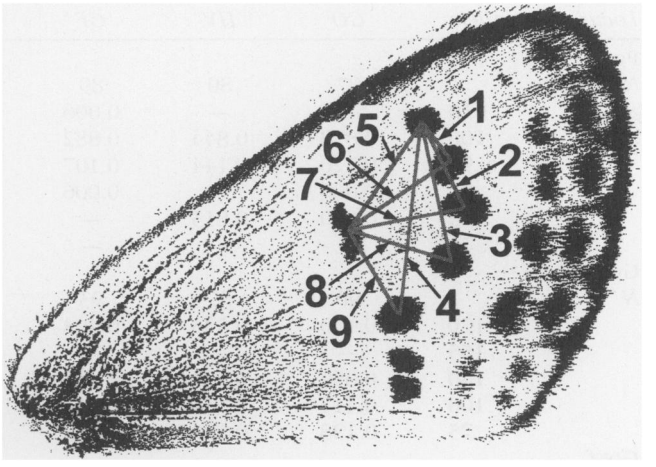

Figure 3. Wing measurements taken in studies of fluctuating asymmetry (FA) in Plebejus argus. *Enhanced photograph of the wing spots on the underside of the left forewing and the nine characters (distances between spots) used in the measurement of FA.*

Results

Genetic Data

Allele frequencies at allozyme and mtDNA loci are shown in Tables 1 and 2. The first question of interest is whether there has been a loss of genetic diversity in descendent populations. More alleles were found at the source population (GO), both for mtDNA and for allozymes, than in any descendent population. We recorded 36 allozyme alleles at GO and 23–29 alleles in descendent populations. Similarly, we found 10 mtDNA alleles at GO, whereas descendent populations had 4–9 alleles. Reduction in numbers of alleles is an expected result of a bottleneck induced by colonization. However, only four allozyme alleles (Got-f/130, Idh/200, Fum/150, Lgg-f/80) did not appear in any descendent population in the Dulas Valley, where we sampled six populations; so presumably at least 32 of the GO alleles were introduced in 1942. These losses were balanced by the appearance of four rare alleles in the Dulas Valley not detected in the GO source population (Pgm/110, Pgm/70, Gpi/155, and

Table 1. Frequencies, heterozygosity, and number of allozyme alleles for *Plebejus argus* at various sites in North Wales.

		*Population**								
Locus	*Allele*	*GO*	*HV*	*GF*	*RYF*	*T*	*BW*	*GG*	*PN*	*MM*
Pgm										
N		87	80	89	41	84	48	46	83	89
	110	—	—	0.006	—	—	—	—	0.018	—
	100	0.828	0.844	0.882	0.890	0.810	0.802	0.815	0.892	0.787
	90	0.126	0.144	0.107	0.098	0.161	0.198	0.185	0.090	0.208
	80	0.006	—	0.006	—	0.030	—	—	—	—
	75	0.040	0.013	—	0.012	—	—	—	—	—
	70	—	—	—	—	—	—	—	—	0.006
Gpi										
N		90	87	93	42	88	49	47	83	89
	170	0.400	0.523	0.403	0.381	0.392	0.449	0.543	0.404	0.494
	155	—	—	—	—	0.006	—	—	—	—
	140	0.056	0.023	—	—	—	—	—	—	0.011
	100	0.417	0.333	0.414	0.524	0.591	0.439	0.394	0.560	0.466
	75	0.128	0.121	0.183	0.095	0.011	0.112	0.064	0.036	0.028
Got-f										
N		90	87	93	42	86	49	47	83	89
	130	0.017	—	0.011	—	—	—	—	—	—
	100	0.983	1.000	0.989	1.000	1.000	1.000	1.000	1.000	1.000
Mpi										
N		73	67	84	38	76	42	37	67	72
	105	0.007	0.007	—	0.026	0.007	0.024	0.014	0.015	0.014
	100	0.719	0.739	0.810	0.776	0.592	0.702	0.757	0.627	0.694
	90	0.253	0.246	0.190	0.197	0.401	0.262	0.176	0.336	0.264
	80	0.021	0.007	—	—	—	0.012	0.054	0.022	0.028
Me										
N		85	72	91	40	86	42	40	83	87
	115	0.324	0.396	0.626	0.475	0.355	0.369	0.175	0.452	0.397
	100	0.676	0.604	0.374	0.525	0.645	0.631	0.825	0.548	0.603
Sdh										
N		79	72	88	39	80	38	39	79	85
	100	0.968	1.000	0.903	0.897	0.962	0.961	0.936	0.930	0.971
	65	0.032	—	0.097	0.103	0.038	0.039	0.064	0.070	0.029
Idh										
N		72	67	81	31	74	35	25	71	80
	200	0.007	—	—	—	—	—	—	—	—
	100	0.993	1.000	1.000	0.984	1.000	1.000	1.000	1.000	0.994
	60	—	—	—	0.016	—	—	—	—	0.006
Fum										
N		77	71	85	42	84	45	42	83	87
	−20	0.435	0.444	0.388	0.250	0.292	0.622	0.369	0.633	0.471
	−100	0.558	0.549	0.594	0.750	0.708	0.378	0.631	0.367	0.529
	−150	0.006	0.007	0.018	—	—	—	—	—	—
Pp										
N		81	72	91	39	84	41	37	83	87
	110	0.006	—	—	—	—	—	0.014	—	—
	100	0.975	1.000	1.000	1.000	1.000	0.988	0.986	1.000	1.000
	90	0.019	—	—	—	—	0.012	—	—	—
Lgg-f										
N		88	78	89	41	85	43	41	83	89
	105	0.006	0.019	0.129	—	0.006	—	—	0.012	—
	100	0.949	0.897	0.871	1.000	0.871	0.930	0.963	0.904	0.904
	95	0.028	0.064	—	—	0.124	0.070	0.037	0.078	0.090
	90	0.011	0.013	—	—	—	—	—	0.006	0.006
	80	0.006	0.006	—	—	—	—	—	—	—
Lgg-s										
N		85	72	91	41	86	42	39	83	89
	115	0.012	0.021	—	—	0.017	0.024	0.026	0.012	0.028
	100	0.988	0.979	1.000	1.000	0.983	0.976	0.974	0.988	0.972
Ak-f										
N		73	73	85	36	75	37	31	74	77
	115	0.233	0.151	0.259	0.194	0.127	0.176	0.177	0.236	0.175
	100	0.740	0.849	0.741	0.806	0.820	0.811	0.790	0.723	0.799
	80	0.027	—	—	—	0.053	0.014	0.032	0.041	0.026
Average H_e		0.249	0.229	0.245	0.214	0.236	0.242	0.220	0.245	0.245
Total n_a		36	29	25	23	27	26	26	28	29

**Population abbreviations as in Figure 1.*

Table 2. Frequencies, allelic diversity, and numbers of mtDNA alleles for *Plebejus argus* at various sites in North Wales.

		*Population**								
	Allele	*GO*	*HV*	*GF*	*RYF*	*T*	*BW*	*GG*	*PN*	*MM*
n		81	85	89	42	84	50	47	83	90
	A	—	0.082	0.022	—	—	—	—	—	—
	B	0.123	0.118	—	0.071	0.036	0.140	0.170	0.060	0.056
	C	0.420	0.365	0.652	0.595	0.786	0.620	0.574	0.663	0.433
	D	0.160	0.188	0.056	0.095	0.119	0.160	0.170	0.157	0.100
	E	0.173	0.176	0.213	0.048	0.060	0.020	0.043	0.048	0.378
	F	—	0.024	0.022	—	—	0.020	0.021	0.036	—
	G	0.025	0.012	—	—	—	—	0.021	0.012	0.033
	H	0.012	—	0.034	—	—	—	—	—	—
	I	0.037	—	—	—	—	—	—	—	—
	J	0.012	0.024	—	—	—	—	—	—	—
	M	0.012	—	—	—	—	—	—	0.012	—
	S	—	—	—	—	—	—	—	0.012	—
	T	—	—	—	0.024	—	—	—	—	—
	U	0.025	—	—	—	—	—	—	—	—
	V	—	—	—	—	—	0.020	—	—	—
	A6	—	—	—	0.095	—	0.020	—	—	—
	A7	—	—	—	0.048	—	—	—	—	—
	A8	—	—	—	0.024	—	—	—	—	—
	A9	—	0.012	—	—	—	—	—	—	—
H_e		0.759	0.788	0.530	0.632	0.368	0.580	0.623	0.534	0.663
n_a		10	9	6	8	4	7	6	8	5

**Definitions of population abbreviations provided in Figure 1.*

Idh/60). Similarly, although four mtDNA alleles were apparently lost during colonization of the Dulas Valley, seven alleles not known to be present at GO appeared in the Dulas Valley metapopulation. The sampling effort was greater in the Dulas Valley overall (398 individuals) than in GO (90 individuals), so it is not surprising that rare alleles were found in descendent populations, but were absent from the GO sample. However, this result underlines the difficulties of making inferences from numbers of alleles in samples.

Alleles that were lost were almost always the rarer ones, as expected under drift. There were only two cases where alleles at a frequency, $p_i > 0.05$ in the GO sample were apparently lost (Table 1): Gpi/140 (p_i = 0.056), absent from most of the Dulas Valley populations (though present at MM), and mtDNA/B (p_i = 0.123) absent from Graig Fawr. Once again, it is not clear what this result means. Existing rare alleles are expected to be absent from samples as well as to be lost if the base population is subjected to the sampling effect of a bottleneck. Investigating allelic loss risks confusing sampling variation due to genetic drift with sampling variation due to sample size.

It might be imagined that a measure of genetic diversity, expected heterozygosity, H_e, would give a better idea of the loss of genetic variation during bottlenecks. At mtDNA, H_e did in general decrease, from 0.759 at GO, but one descendent population, HV, actually had a higher H_e (0.788); the rest had H_e between 0.368 and 0.663 (Table 2). At allozymes, the source population, GO, had the highest H_e (0.249), but descendent populations (0.214–0.245) differed little (Table 1). These results for H_e and ν_α are consistent with mild levels of genetic drift induced by founder effects, but it is difficult to make clear inferences on the basis of either.

Clearly, statistical analysis is needed to test whether the colonizations and extinctions have resulted in a loss of genetic diversity. The raw results of pairwise permutation and X^2 tests for change in H_e, n_a and gene frequency between samples are given in Table 3 and Fig. 4. We would like to find out whether the initial natural colonizations or introductions from the Great Orme resulted in a loss of genetic diversity. There are three tests here of the null hypothesis, consisting of comparisons between GO and immediate daughter populations HV, GF, and RYF. Because these three comparisons can be considered tests of the same hypothesis, a simple Bonferroni correction can be made, giving p = 0.05/3 = 0.017, equivalent to a 5% significance level in a single test (Rice 1989). Table 3 shows that the numbers of allozyme alleles are significantly reduced in all three daughter populations, whereas there is little effect on heterozygosity; in contrast, at mtDNA there was a strong effect on H_e for the introduction to GF, but no effect on allelic number during any colonization. Table 3 also demonstrates that allele frequencies at allozymes and mtDNA have significantly changed after both introductions, but not after the natural colonization from GO to HV.

Table 3. Pairwise probabilities of obtaining the data under the null hypothesis of no differentiation between Great Orme (GO) and descendent populations of *Plebejus argus*, together with F_{st} values for the pairwise differentiation.

*Population**		*Probability of change in* n_a	*Probability of change in* H_e	*Probability of change in gene frequency*	*Pairwise* F_{st} *between GO and descendant population*
Daughter populations of GO					
HV	allozymes	**0.003**	**0.043**	***0.042***	**0.002**
	mtDNA	0.822	0.526	*0.121*	0.002
GF	allozymes	**0.000**	**0.634**	***0.000***	**0.017**
	mtDNA	0.089	0.000	*0.000*	0.024
RYF	allozymes	**0.001**	**0.018**	***0.006***	**0.009**
	mtDNA	0.785	0.077	*0.005*	0.010
Average of daughter populations	allozymes	**0.001**	**0.232**	***0.016***	**0.009**
	mtDNA	0.565	0.201	*0.042*	0.012
Daughter and grand-daughter populations of RYF					
GG	allozymes	**0.007**	**0.027**	***0.061***	**0.004**
	mtDNA	0.220	0.037	*0.245*	0.007
MM	allozymes	**0.027**	**0.685**	***0.001***	**0.002**
	mtDNA	0.007	0.034	*0.023*	0.012
PN	allozymes	**0.008**	**0.710**	***0.000***	**0.010**
	mtDNA	0.511	0.000	*0.007*	0.015
BW	allozymes	**0.003**	**0.455**	***0.126***	**0.005**
	mtDNA	0.478	0.003	*0.040*	0.016
T	allozymes	**0.000**	**0.193**	***0.000***	**0.010**
	mtDNA	0.004	0.000	*0.000*	0.057
Average of all desendent populations of GO	allozymes	**0.006**	**0.346**	***0.030***	**0.007**
	mtDNA	0.365	0.085	*0.055*	0.018

**Definitions of population abbreviations provided in Figure 1. Typographic conventions (underlining, bold face, and italicization) as in Figure 4.*

Another null hypothesis of conservation interest from Table 3 is that there are no changes between GO and any descendent population. Because, for each genetic parameter/marker combination, there are eight tests, $p = 0.05/8 = 0.006$ is equivalent to a 5% significance level in a single test. Here, the null hypothesis for n_a is rejected for allozymes in a number of populations, but rejected convincingly for mtDNA only at Terfyn. In contrast, H_e at mtDNA is strongly reduced at four sites, whereas results at allozymes provide little evidence for a reduction in H_e.

Another way of testing these hypotheses is to estimate the average probability of obtaining these results under the null hypothesis, rather than to worry about specific populations (Table 3). The test for n_a for allozymes in the three immediate daughter populations gives $p = 0.001$. For all eight descendent populations, the same test has average $p = 0.006$. The null hypothesis is rejected. The results for gene frequency change also show significant changes in descendent population gene frequencies on average, both at allozymes, and, at least in the three daughter populations, at mtDNA. In contrast, there is little evidence from Table 3 that there are changes in n_a at mtDNA or in H_e at either marker type.

In Fig. 4 we give probabilities of obtaining results as extreme as ours if there are no differences in H_e, n_a, and gene frequency along the routes (or most likely routes) of introduction or colonization for both allozymes and mtDNA. Because there are nine different comparisons for each type of change (H_e, n_a, or gene frequency) for each marker type (mtDNA or allozymes), the simple Bonferroni-corrected probability level equivalent to nominal "significance" of 0.05 should be 0.05/9 or 0.006. Apart for the changes in n_a at the initial colonization events, and a number of allele frequency changes, few of the other pairwise comparisons are significant under this test. Apparently then, subsequent colonizations within the Dulas Valley from Rhŷd-y-foel did not result in detectable loss of alleles or their diversity.

In these populations changes in heterozygosity of allozymes are somewhat unpredictable (Fig. 4, Table 3). After Bonferroni correction, essentially no changes of heterozygosity are significant except at mtDNA data; these are GO→GF, GO→PN, GO→BW, and GO→T (Table 3). This may be because heterozygosity is affected by changes in the frequencies of more abundant alleles as well as by losses of rare alleles and because the mtDNA fragment samples more rare alleles than do allozymes. Slight reductions in heterozygosity due to loss of rare alleles can be counteracted by frequency changes at commoner alleles (Tables 1, 2). In contrast, contingency table X^2 tests demonstrate that changes in allozyme and mtDNA allele frequency have in fact occurred in many cases of natural colonization and introduction, even after Bonferroni correction.

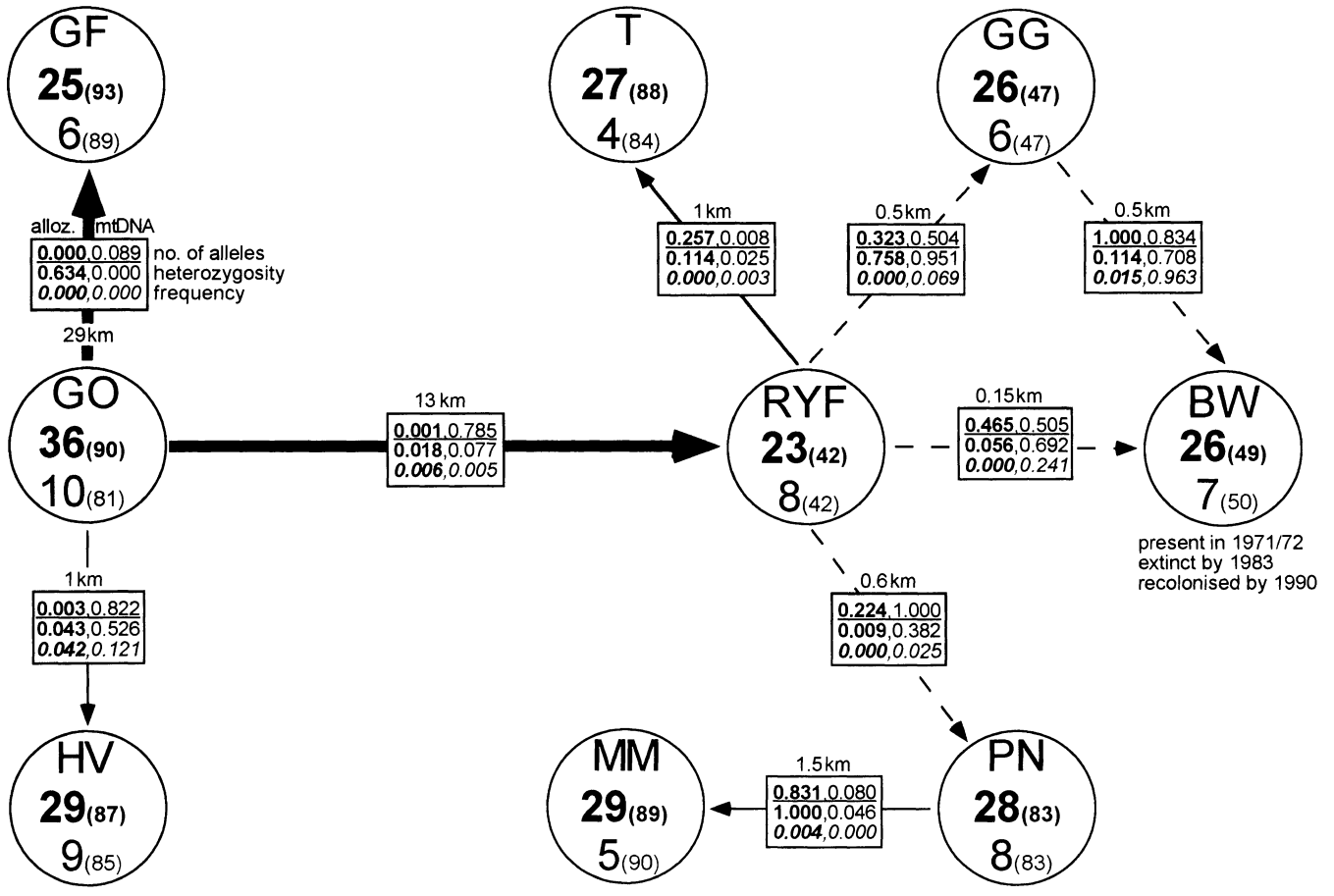

Figure 4. Routes between populations, numbers of alleles, sample sizes, and estimates of probability for no genetic differences in Plebejus argus. *Schematic representation of the routes of artificial introduction and natural colonization connecting the sampled populations of* P. argus *within North Wales. Populations connected by bold arrows indicate artificial introductions, thin arrows represent routes of natural colonization, and dashed arrows have been used where the exact route of colonization is not known and the population could have been colonized from alternative sources. The boxes superimposed on the arrows contain the two-tailed permutation probabilities of the null hypothesis of no difference in number of alleles (n_a, underlined), heterozygosity (H_e, normal typeface), and X^2 probabilities for lack of differentiation in gene frequencies (italicized) for the allozyme (bold) and mtDNA (normal typeface) data. The numbers of alleles are given within the circles representing each population for allozymes (bold) and mtDNA (normal typeface). Because the numbers of alleles in a sample depends strongly on the number of individuals sampled, sample sizes are also given in parentheses.*

Fluctuating Asymmetry

We test the null hypothesis that FA does not differ between source and colonized populations. The FA values for each character and for each population are shown in Table 4. Only character 1 shows a significant difference in FA between populations. Interestingly, the population with the lowest FA for this character is GO, the source population, which also has the largest allozyme H_e and n_a of all nine populations. The population with the highest FA for this character is GG, which has one of the lowest allozyme heterozygosities. However, this pattern of FA variation is not repeated across other characters. In fact, one significant difference out of nine can be put down to chance (i.e., 1/9 is not significantly different from 5%). When all characters are considered together, there was no overall evidence for FA variation across populations (Kendall's coefficient of concordance, $W = 0.18$, $\chi^2 = 12.74$, $p > 0.05$, Sokal & Rohlf 1981).

Discussion

Evidence for Genetic Changes Accompanying Colonization and Introduction

In *Plebejus argus*, bottlenecks appear to have produced significant reductions of genetic diversity, with about

Table 4. Fluctuating asymmetry values for nine *Plebejus argus* wing characters ($\times 10^5$).

		Character								
*Population**	N	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*
RYF	34	3.279	1.872	1.697	1.287	1.570	1.843	1.727	1.839	3.588
GF	40	2.977	1.610	1.416	1.099	1.468	1.727	2.329	3.430	2.253
GG	40	10.71	3.950	1.870	0.975	1.413	2.458	2.193	3.054	3.956
GO	40	2.641	1.435	1.237	1.019	1.839	2.182	1.881	3.198	3.222
HV	40	4.917	1.902	0.978	0.817	1.647	2.977	1.678	2.787	5.174
MM	40	4.042	1.610	1.161	0.679	1.442	1.232	1.933	2.539	2.007
PN	40	4.625	1.497	1.299	0.915	1.649	2.147	2.268	3.312	3.117
BW	40	2.933	1.519	1.543	0.793	1.901	1.809	1.816	3.269	3.457
T	40	4.376	1.552	1.012	0.817	1.870	1.667	1.354	2.130	2.476
Test (p) of F_{max}		<0.01	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

**Definitions of population abbreviations provided in Figure 1.*

23% of enzyme alleles and about 36% of mitochondrial alleles having been lost on average from samples of daughter populations compared with a sample of the source. Because the losses were of rare alleles, the effects were relatively mild and difficult to detect using standard statistical tests. After introduction, there were no significant changes in n_a between any adjacent pair of Dulas Valley populations (Fig. 4). This may be because most of the alleles lost were rare. Once lost during the initial introduction, rare alleles would be unavailable for loss during subsequent colonizations. However, the lack of strong reductions of allelic number between the initial introduction site in the Dulas Valley, RYF, and its own daughter populations may be in part due to the low sample size at RYF ($n = 42$).

In our tests, n_a was a sensitive indicator of bottlenecks, but it should be borne in mind that few alleles missing from samples may actually have been lost from the populations. Tables 1 and 2 reveal that losses during colonization of the Dulas Valley were more than balanced by apparent gains of alleles that were not found on the Great Orme (although they probably occur there at low frequency). Permutation analysis looks for losses of alleles between pairs of *samples*, not for actual losses between pairs of *populations*. There may, of course, be many rare alleles in the extremely large GO population which were neither collected in our GO sample nor were introduced to, or colonized, any of the daughter populations. The permutations strictly test only whether the underlying allele frequencies have been changed in a way which reduces n_a in the daughter samples.

In contrast to the results for n_a, there is very little evidence for a reduction of genetic diversity as measured by H_e, at least for allozymes. Our results therefore concur with Leberg (1992), in that n_a is more sensitive than H_e as a measure of reduction in genetic diversity. In conservation the complete loss of variation (i.e., n_a is equivalent to allelic "richness," to use the ecological term applied to species diversity) is more important than the changes in frequency ("evenness") that are also incorporated into diversity measured by H_e. It does seem here that statistical convenience coincides with conservation interest.

Significant differences in allozyme frequencies provide further evidence for founder effects during or after introductions and colonizations (Table 3, Fig. 4). Although the exact dates of natural colonization within the Dulas Valley are unknown, all populations were established at least 20 years ago, except Mynydd Marian, where a population was first recorded in 1983 (Dennis 1972, 1977; Thomas & Harrison 1992). The Borth-ŵryd population was extinct in 1983, but was recolonized by 1990, presumably from Rhŷd-y-foel and/or Garth Gogo (Thomas 1985*a*,*b*; Thomas & Harrison 1992; Fig. 4). Direct estimates of dispersal suggest that allozyme frequency differences will persist because of extremely low rates of gene flow between established local populations (Thomas 1985*a*,*b*; Lewis et al. 1997). In terms of conservation biology, significant differences in gene frequency provide a clue that subpopulations could easily diverge under selection (as well as drift). This potential for spatial differentiation means that local populations may have divergent characteristics of interest to conservation managers.

F_{st} is the standardized variance in gene frequency and can be used to estimate the approximate single generation bottleneck size required to produce the observed change in gene frequencies between source and daughter populations,

$$F_{st} \cong \frac{1}{2N_e},$$

where N_e is the effective population size causing the changes in allele frequency. Many of these changes in allele frequency are shown to be of low probability under the null hypothesis of allele frequency identity (Fig. 4, Table 3). For the Dulas Valley (using the average of the F_{st} values for all daughter populations) and Graig Fawr

introductions, the estimated $N_e \cong 71$ and 29 individuals, respectively (based on allozyme data). These estimates of N_e are approximately half the actual number of genomes introduced. Inoculum sizes of 90 and 30 mated females were introduced to Rhŷd-y-foel and Graig Fawr, giving approximately 180 and 60 introduced diploid genomes, respectively. Under a wide variety of conditions, effective population sizes are expected to be about half the actual population sizes (Nunney 1993), as found here.

Comparisons Between Markers

Mitochondrial DNA should be more sensitive to population bottlenecks than allozymes for two reasons (Birky et al. 1983; Moritz et al. 1987; Harrison 1989). Firstly, maternal inheritance ensures that the effective population size of mtDNA is approximately one quarter that of nuclear genes. Secondly, the mtDNA region sampled here was far more variable than any of the allozyme loci. At mtDNA, a higher proportion of alleles were lost between source and daughter populations than at any allozyme locus, and there was a higher F_{st} between populations, as expected; however, more allozyme alleles were lost overall because of the greater number of loci and total numbers of alleles sampled. This may explain why few daughter populations had significant reductions in n_a at mtDNA (Table 3; Fig. 4); those that lost most alleles were either at the ends of chains of colonizations (MM), or were at relatively long distances from their source population (T), and may represent the effects of multiple or particularly narrow bottlenecks. Mitochondrial H_e always declined in comparison to Great Orme source (except at Happy Valley), in most cases significantly (Table 3). However, for reasons already mentioned, these changes are due to random gene frequency changes as well as losses of alleles, and so are less convincing evidence for conservation-relevant effects of bottlenecks. In conclusion, the most informative genetic markers would be loci with many alleles at a locus, but with many independent loci; short-sequence repeats, or microsatellite loci would be ideal in this respect, and are currently under development for *P. argus*.

Analysis of Genetic Diversity

Genetic diversity is analogous to species diversity, in that we are interested both in allelic numbers (similar to species richness) and frequencies (evenness). As with species diversity, genetic diversity measures that incorporate both number and frequency are problematic; here heterozygosity (mathematically equivalent to Simpson's species diversity index, Simpson 1949), H_e, may actually increase after a bottleneck when limited numbers of loci are sampled. But the numbers of alleles, n_a, are not particularly easy to estimate. In studies of species counts one often attempts to estimate the asymptomatic species richness given infinite sampling effort. This procedure likely causes problems in estimating numbers of species, but would be virtually impossible to use for numbers of alleles, many of which may be unique (unlike members of a sexual species). To circumvent these problems, we have used a binary permutation method that simply asks whether two samples of genotypes could have been drawn from the same underlying population. This method may be generally applicable in studies of biological diversity, as well as in studies of its genetic components.

Fluctuating Asymmetry

The artificial introductions and natural colonizations of *P. argus* have not been associated with any significant changes in FA. There is no evidence from our data for reduced developmental stability as a result of loss of genetic variation. However, although many studies have shown an inverse relationship between FA and isozyme heterozygosity, both within individuals and populations, controlled experiments using inbred and outbred lines have found no association between FA and genetic variability (see Fowler & Whitlock 1994 for a recent review). Our results could be interpreted in two ways. One possibility is that the allelic losses we observed were too small to affect FA very much. Alternatively, FA may be an unreliable correlate of heterozygosity and fitness (Fowler & Whitlock 1994) and may therefore be useless as a tool in conservation biology. Although we cannot distinguish between these possibilities, the FA data give no evidence for fitness changes as a result of the significant genetic changes we have observed. Only the rarer marker alleles, presumably those less likely to be maintained in balanced polymorphisms, were lost. Hence a lack of reduction in fitness is, perhaps, not unexpected.

Genetic Success of Introductions and Colonizations

Genetics and conservation have had an unhappy marriage (Lande 1988; Soulé & Mills 1992; Nunney & Campbell 1993; Avise 1994; Caro & Laurenson 1994). Some conservation geneticists stress that maintenance of genetic variability should form an integral part of management strategies; others argue that genetic problems are trivial in the face of more pressing demographic concerns. Despite the significant loss of rare alleles measured here, introduced populations of *P. argus* in North Wales now represent some of the largest in the UK, with approximately 10^5 adults present across the entire metapopulation (Thomas 1993). The population at Borth-ŵryd within the Dulas Valley was extinct in 1983 (Thomas 1983), but recovered rapidly to about 2000 individuals by 1994 (Lewis et al. 1997). Yet Borth-ŵryd today has a genetic diversity similar to that of other Dulas Valley popula-

tions. The Dulas Valley metapopulation has grown to a size at which genetic drift is unlikely to cause further loss of genetic variability, and occasional immigration will usually replace alleles lost in individual populations. Non-significant changes of fluctuating asymmetry between source and daughter populations may also suggest that there has been little or no change in relative fitness associated with the introductions or subsequent colonizations. Lost rare alleles may be important for future adaptability, but by definition they cannot contribute to current fitness in most individuals in the source population. *Plebejus argus* from North Wales is a good example of where the success of artificial introductions may depend more on the existence of suitable habitat than on genetic factors. Despite statistically significant loss of genetic variability, artificially introduced and naturally colonized populations of *P. argus* are thriving.

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