Molecular Ecology (2006) **15**, 4375–4386 doi: 10.1111/j.1365-294X.2006.03121.x

Extremely low effective population sizes, genetic structuring and reduced genetic diversity in a threatened bumblebee species, *Bombus sylvarum* (Hymenoptera: Apidae)

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Abstract

Habitat fragmentation may severely affect survival of social insect populations as the number of nests per population, not the number of individuals, represents population size, hence they may be particularly prone to loss of genetic diversity. Erosion of genetic diversity may be particularly significant among social Hymenoptera such as bumblebees (*Bombus* spp.), as this group may be susceptible to diploid male production, a suggested direct cost of inbreeding. Here, for the first time, we assess genetic diversity and population structuring of a threatened bumblebee species (*Bombus sylvarum*) which exists in highly fragmented habitat (rather than oceanic) islands. Effective population sizes, estimated from identified sisterhoods, were very low (range 21–72) suggesting that isolated populations will be vulnerable to loss of genetic variation through drift. Evidence of significant genetic structuring between populations ($\theta = 0.084$) was found, but evidence of a bottleneck was detected in only one population. Comparison across highly fragmented UK populations and a continental population (where this species is more widespread) revealed significant differences in allelic richness attributable to a high degree of genetic diversity in the continental population. While not directly related to population size, this is perhaps explained by the high degree of isolation between UK populations relative to continental populations. We suggest that populations now existing on isolated habitat islands were probably linked by stepping-stone populations prior to recent habitat loss.

Keywords: *Bombus*, conservation, microsatellites, nest-density, population genetics

Received 4 April 2006; revision received 26 July 2006; accepted 7 August 2006

Introduction

The importance of reductions in genetic diversity on survival of animal populations following population subdivision has been the subject of debate. Some authors suggest that in fragmented populations environmental factors are of greater importance to survival than genetic factors (Caro & Laurenson 1994). Others argue that processes such as long-range dispersal, kin recognition and polyandry maintain diversity in wild populations (Pusey & Wolf 1996). Recent reviews indicate an emerging consensus that genetic factors are important (Keller & Waller 2002; Frankham 2005) and may impact on threatened species (Spielman *et al*. 2004) although most studies to date focus on vertebrates. The importance of these factors in invertebrates is less well documented, with a few notable exceptions. Recent studies of butterfly species, for example, have revealed a genetic pattern to population decline (Saccheri *et al*. 1998; Schmitt & Hewitt 2004).

Social insects may be particularly susceptible to losses of genetic diversity following population subdivision since sociality leads to decreased effective population sizes: most individuals in a population are sterile workers (Chapman & Bourke 2001; Packer & Owen 2001). Studies of social insects so far have largely concentrated on ants

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(Hymenoptera: Formicidae), e.g. increased queen-male relatedness and/or positive inbreeding coefficients among fragmented populations have been observed (Hasegawa & Yamaguchi 1995; Cole & Wiernasz 1997; Sundstrom *et al*. 2003). Other studies of social insects find no evidence of a reduction in genetic diversity in fragmented populations (e.g. Gyllenstrand & Seppa 2003).

Should erosion of genetic diversity occur, haplodiploid organisms might be expected to suffer less from inbreeding depression than diploids because deleterious recessive alleles may be purged through selection on the haploid sex (see Sorati *et al*. 1996; Henter 2003). In haplodiploid Hymenoptera, however, an additional cost of reduced genetic diversity owing to population subdivision is the production of diploid males as a consequence of single-locus complementary sex determination (sl-CSD) (Duchateau *et al*. 1994; Cook & Crozier 1995). Usually males develop from unfertilized eggs and are haploid, females develop from fertilized eggs and are diploid. If the complementary sex determining-locus (CSD-locus) is diploid but homozygous, however, a diploid male will result. When such a matched mating occurs, half the workers (on average) will develop into males and do not contribute any resources to the colony. Matched matings are more probable in populations with reduced allele number at the CSD-locus. In bees, some studies report low levels of diploid male production despite high levels of inbreeding (e.g. Paxton *et al*. 2000) while others report high levels of diploid male production (e.g. Zayed & Packer 2001) and propose diploid male production as a measure of sustainability of fragmented bee populations (Zayed *et al*. 2004). Recently, modelling has suggested that this phenomenon can lead to 'an extinction-vortex' (Zayed & Packer 2005).

Bumblebees are ecologically and economically important as pollinators (Corbet *et al*. 1991; Fussell & Corbet 1992; Fussell & Corbet 1993). Some species have suffered declines in range and abundance across the Holarctic in the last 60 years (Williams 1982, 1986; Batra 1995; Koisor 1995; Rasmont 1995), a trend evident in the UK (e.g. extinction of *Bombus subterraneus*). Despite this, studies of population genetics have focused on ubiquitous species (Estoup *et al*. 1996; Pirounakis *et al*. 1998; Widmer *et al*. 1998; Widmer & Schmid-Hempel 1999). Little is known of the population structure of rare and declining species that typically exist in highly fragmented populations. Even basic aspects of population ecology such as effective population sizes within habitat fragments are unknown.

The only previous estimates of genetic structuring of threatened bumblebee species are of *Bombus muscorum* (Darvill *et al*. 2006). Almost all of the populations studied were on oceanic islands, as few mainland populations remain. Here for the first time we examine population genetic structure in fragmented bumblebee populations (*Bombus sylvarum*) persisting in habitat (rather than oceanic) islands. This species has experienced major range contraction in recent years. We compare genetic diversity between highly fragmented populations in the UK and a continental population, where populations of this species are less fragmented (see below). At present there are few estimates available of the nest density of common bumblebee species (Darvill *et al*. 2004; Knight *et al*. 2005), and none for species of threatened status, largely due to difficulties in reliably locating nests. Yet effective population size is a fundamental attribute of populations: with no knowledge of population size, it is impossible to develop sensible conservation strategies. Genotypic data is used to establish sisterhoods among workers to estimate the number of nests per population, and effective population size is calculated from these estimates.

Materials and methods

Study species and sampling

We assess the population genetic structure of *Bombus sylvarum*, with reference to that of *Bombus pascuorum*, both belonging to the subgenus, *Thoracobombus*. In the UK *B. sylvarum* occurs in discrete and isolated populations, is currently known from only seven localities, is in danger of extinction (Edwards & Telfer 2001) and is designated on the Biodiversity Action Plan (UKBAP). Across Europe it has a wide distribution from Scandinavia to the Mediterranean and eastwards to the Urals (Edwards & Telfer 2001). In France, *B. sylvarum* is more widespread, particularly in the south and east [Rasmont, Atlas Hymenoptera (http://zoologie.umh.ac.be/hymenoptera/)] and can be found even in agricultural areas in central regions (Goulson, personal observation). Both species have an annual colony cycle. Queens are monoandrous (Estoup *et al*. 1995a).

Localities with recent records of *B. sylvarum* in the UK (Edwards & Telfer 2001) were visited during the summers of 2003 and 2004 (Fig. 1). Sampling began in early July and queens were observed on the wing in this period, although most sampling was carried out in mid-August when nests are firmly established (Table 1). All known UK populations of *B. sylvarum* were visited. A collection of *B. sylvarum* was additionally obtained from Epénède in the Charente Department of France (summer 2004). Areas roughly 100 m^2 were searched within sites and where possible were separated by a distance of 500 m to avoid over-sampling of sisters. Bees were netted on the wing or over flowers. Samples were largely nonlethal [tarsal clips following Holehouse *et al*. (2003), tarsi stored immediately in absolute ethanol]. Microsatellite data from previous studies of *B. pascuorum* at two sites in the UK (Darvill *et al*. 2004; Knight *et al*. 2005) were used for comparison. An additional sample of *B. pascuorum* was obtained from around Foix in the Pyrenees, France.

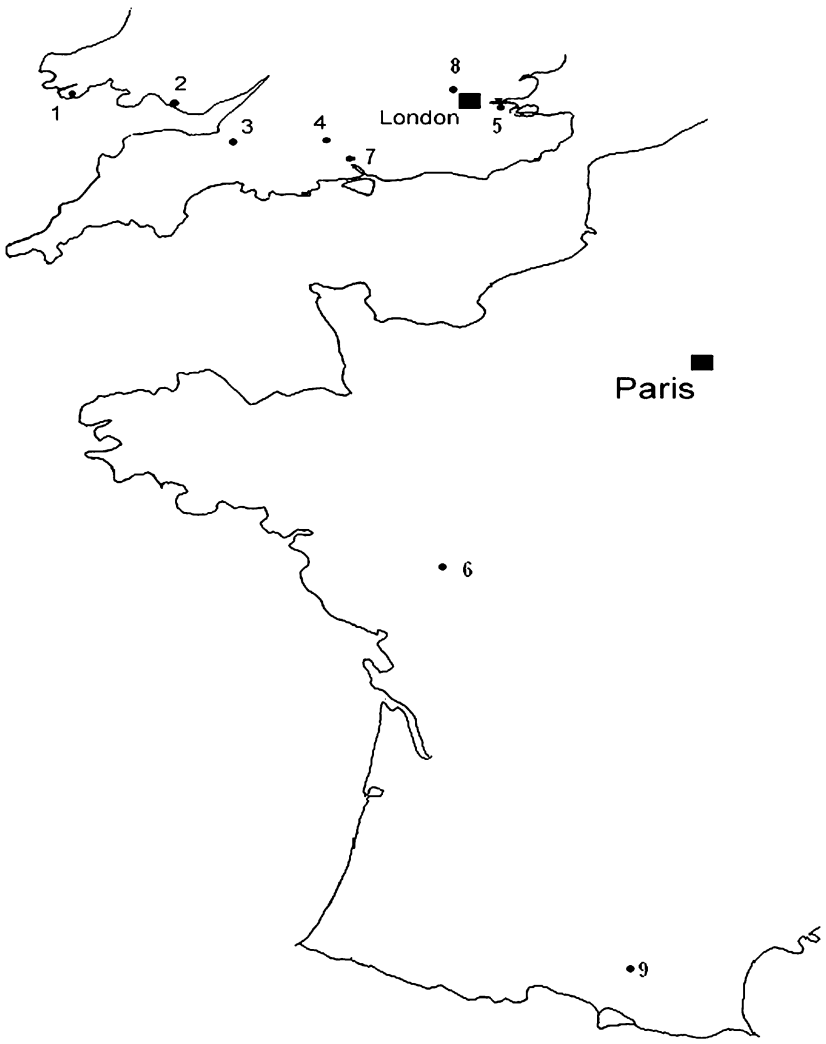

Fig. 1 Map showing approximate location of sample sites. (1) Castlemartin (SR 9295) (2) Margam moors (SS 7784) (3) Ham Wall (ST 4539) (4) SPTA (SU 0747) (5) Cliffe Pools (TQ7177) (6) Epénède (Lat: 42°57′50″N, Long. 1°36′25″E) (7) Landford (SU2519) (8) Rothamsted (TL 1213) (9) Foix (Lat: 46°03′48″N, Long. 0°32′12″). Figures in parenthesis after the locality name indicate the ordnance survey grid reference of the centre-point (UK sites) or latitude and longitude (French sites).

Table 1 Dates of visits to each locality and number of bees collected (workers and males). Figures in parenthesis indicate the locality number (Fig. 1)

	Year 1		Year 2	
Site	Dates sampled	Sample size	Dates sampled	Sample size
Castlemartin (1)	15–17/8/03	16	10–12/8/03	27
Cliffe Pools (5)	22–23/7/03, 1/8/03	3	20/7/04, 20/8/04	28
Epénède (6)	n/a	0	September 04	28
Ham Wall (3)	27/8/03	13	17/8/04, 25/8/04	38
Margam (2)	18–19/8/03	16	10/8/03	21
Salisbury Plain (4)	8–12/7/03, 13/8/03, 22/8/03	8	2–5/8/04	31

DNA extraction, PCR and genotyping

DNA was extracted from tarsi using the HotSHOT protocol (Truett *et al*. 2000). To improve template DNA quality, tarsi were cut into segments before extraction. Nine loci were amplified: B10, B11, B96, B118, B132, B131, B116, B124 and B126 (Estoup *et al*. 1995a; Estoup *et al*. 1996). Polymerase chain reactions (PCRs) were performed in 10-μL reactions using QIAGEN multiplex kits [10 ng template DNA, 5 μL master mix, 1 μL Q-solution, 1 μL

primer mix (each primer 0.2 μM), 2 μL H_2O]. Multiplexes contained up to four loci per reaction. Primers were fluorescently labelled with FAM, HEX (Sigma-Genosys) or NED (Applied Biosystems). With multiplexes of four loci, two were size-plexed with the same dye. The reaction cycle was: 95 °C for 15 m; 35 cycles of three steps: 94 °C for 30 s, 51 °C for 90 s and 72 °C for 90 s; followed by a 10 m extension period at 72 °C. Genotypes were assessed by comparison with internal size standards (ROX350, Applied Biosystems) using an ABI PRISM 377 automated sequencer. Allele sizes were scored using GENOTYPER 2.1 (GENESCAN software, Applied Biosystems, PerkinElmer).

Statistical methods

Sisters were identified and removed from the data set so as to leave only one representative per nest. Using polymorphic genetic markers, kinship can be inferred from estimates of relatedness. In haplodiploid social insects, the expected coefficient of relatedness between sisters (workers from the same nest) is 0.75. The presence of sisters in each population was calculated using KINSHIP 1.3.1 (Goodnight & Queller 1999) applying a significance level of 0.01; the null hypothesis being that worker pairs are unrelated (maternal and paternal coefficients of relatedness set at 0), the primary hypothesis being that worker pairs are sisters (i.e. maternal and paternal coefficients of relatedness set at 0.5 and 1, respectively). The number of nests per population was estimated from the genotypic identification of sisters: by estimating the number of nests from which one worker was caught, the number from which two workers were caught and so on, it is possible to estimate the number of nests from which no individuals were caught, by extrapolation after fitting the data to a Poisson distribution (see Darvill *et al*. 2004). Estimates were made per site. The value of the zero category (number of nests with no individuals caught) was estimated using the program FITTING (Abramson & Gahlinger 2001). Effective population size was estimated using Wright's (1933) formula which is:

$$N_e \text{ (haplodiploids)} = \frac{9NfNm}{2Nf + 4Nm}$$

where *Nf* is the number of females and *Nm* is the number of males. In this case, this is effectively the number of nests multiplied by 1.5, assuming each queen mates with a single male and each male mates with a single queen. At sites with large enough sample sizes over 2 years, effective population size was also estimated from changes in allele frequency using the program CONE (Anderson 2005).

After removal of sisters, typographical error-checking of data sets was made in MSA (Dieringer & Schlötterer 2002). None of the programs described below can handle both diploid and haploid data simultaneously, so males were removed from the data set prior to subsequent statistical analyses.

Probability tests for departure from Hardy–Weinberg equilibrium (HWE) and genotypic disequilibrium were then performed in GENEPOP On The Web (Raymond & Rousset 1995). Sequential Bonferroni corrections were applied to minimize the occurrence of type I errors (Rice 1989). Regarding departure from HWE, individual Bonferroni tables were made pertaining to populations across all loci pooled and to loci across all populations pooled. Additionally, evidence for the presence of null alleles at each locus within each population was assessed using MICROCHECKER (van Oosterhout *et al*. 2004).

Population genetic structure was assessed using Weir & Cockerham's (1984) estimators, implemented in FSTAT 2.9.3.2 (Goudet 2001). Global and pairwise values of θ [equivalent to Wright's (1951) F_{ST}] were generated. Standard errors were calculated by jackknifing over loci. Significance levels of population differentiation were calculated by randomizing alleles over all samples, with tests based on 1000 permutations; for pairwise estimates multilocus genotypes were randomized between populations and a strict Bonferroni correction was applied (implemented as standard in FSTAT). Allelic richness was also calculated in FSTAT, using a resampling procedure to avoid bias of sample size on number of alleles detected in a population (El Mousadik & Petit 1996). Nei's unbiased expected heterozygosity was calculated in MSA (Dieringer & Schlotterer 2003).

To assess whether there were any differences in genetic diversity (expected heterozygosity and allelic richness) between continental and UK populations of *B. sylvarum*, a Friedman's test was performed with genetic diversity in each population examined by locus. In this approach each population is ranked according to its diversity at a particular locus, the average rank of each population across all loci is then calculated and the null hypothesis that the ranks do not differ from the expected value is tested using chi-square. Additionally, gene diversity was calculated in MSA under the heterozygosity range option. Bootstrapping was performed (10 000 permutations) to estimate the maximum and minimum estimates of gene diversity. Differences in gene diversity between populations were then assessed by examination of these confidence intervals (by locus). To examine whether there was any effect of population size (effective population size based on nest density estimates) and degree of isolation (shortest geographical distance between population pairs) on either measure of genetic diversity, a univariate general linear model was applied (variables first tested for normality using the Kolmogorov-Smirnov statistic). All statistics were performed in SPSS version 12.

Genetic isolation by distance (IBD) was examined in each species with Mantel tests calculated in IBD (Bohonak 2002). Rousset's (1997) distance $F_{ST}/(1 - F_{ST})$ was used [in this case $\theta/(1-\theta)$] and regressed against log (geographical distance).

The software BOTTLENECK (Cornuet & Luikart 1996) was used to test for evidence of recent reductions of effective population size. Gene diversity excess was estimated using the Wilcoxon signed rank statistic and the mode-shift of allele frequency distribution was examined. Both the infinite allele model (IAM) and two-phase model (TPM) of microsatellite mutation were implemented (authors have argued that bumblebee microsatellites do not follow the stepwise mutation model (SMM), Estoup *et al*. 1995b; Shao *et al*. 2004). For TPM tests, number of multistep changes and variance were set at 10%. Statistics were calculated using 10 000 iterations.

Diploid male detection

Individuals were sexed from their morphology before genotyping. Diploid males were detected by the presence of heterozygous loci. To guarantee accuracy of all genotypes any individuals with the possibility of scoring error (e.g. weak strength of initial amplicons, excessive stuttering, etc.) where re-amplified and re-typed.

Results

In total, 240 *Bombus sylvarum* were genotyped. At two sites, sample sizes were very low (Elmley RSPB reserve, Kent and Canvey Island, Essex, 10 bees in total); these were not included in further analysis. Of the remaining 230 bees, 191 were workers, giving a mean sample size per population of 31.8 (range 18–40). The additional sample of *Bombus pascuorum* from Foix consisted of 32 workers. Previously published data for this species were also included in analysis of population structure (Darvill *et al*. 2004; Knight *et al*. 2005; two populations, sample size 125 and 237 workers, respectively).

Effective population size

At $P < 0.01$, some of the identified nests were noncircular (8 of 38), i.e. individual A was a sister of individuals B and C, but individual B was not a sister of individual C. To resolve noncircularities when encountered, the significance of all potential sister pairs was examined. If all relationships were significant at $P < 0.05$ then nests were completed, but if the relationship of potential sisters was not significant then original sister pairs were deemed to be false, or nests were divided in the most parsimonious way (Darvill *et al*. 2004; Knight *et al*. 2005). The total number of sister pairs detected per site ranged from 4 (Margam, year 1) to 13 (Salisbury Plain), and the number of nests from which only one individual was caught from 5 (Epenede) to 16 (Cliffe Pools) (average type II error for detection of sister pairs 0.29 ± 0.03). The number of nests detected per site varied from 14 (Epénède) to 48 (Margam) giving estimates of effective population sizes in the range 21–72 (Table 2). Where sample sizes allowed the number of nests to be calculated over 2 years, effective population size remained similar at one site (Castlemartin) with an estimate of 49.5 in year 1 and 48 in year 2, but increased at the second (Margam: year 1 $N_e = 39$, year 2 $N_e = 72$). Estimates of Ne from changes in allele frequency could also only be estimated from these two sites: at Castlemartin the

Table 2 Measures of genetic diversity (second generation only), and effective population size for populations of *Bombus sylvarum*, and *Bombus pascuorum*. *n*, sample size of genotyped workers (both years); n_s, sample size of workers after sisters deleted (both years); H_E, expected heterozygosity; Population size, effective population size of the second generation, from estimates of nests detected. Figures after the locality name indicate the site number on the map (Fig. 1), see also supplementary data

Population	n	n_s	H_E	Allelic richness	Nests detected year 1	Nests detected year 2	Population size
B. sylvarum							
Castlemartin (1)	40	27	0.41 ± 0.11	2.85 ± 0.70	33	32	48
Cliffe Pools (5)	30	25	0.33 ± 0.12	2.94 ± 0.78	n/a	40	60
Epenede (6)	18	10	0.53 ± 0.09	4.00 ± 0.85	n/a	14	21
Ham Wall (3)	30	21	0.37 ± 0.11	3.25 ± 0.97	n/a	26	39
Margam (2)	37	29	0.44 ± 0.07	3.14 ± 0.75	26	48	72
SPTA (4)	36	26	0.40 ± 0.11	3.41 ± 0.83	n/a	38	57
Average (all)	**31.8 ± 3.2**	**23.0 ± 2.8**	**0.41 ± 0.03**	**3.27 ± 0.17**	n/a	**33.0 ± 4.86**	**49.5 ± 7.30**
UK average	**34.6 ± 2.0**	**25.6 ± 1.3**	**0.39 ± 0.02**	**3.12 ± 0.10**	n/a	**36.8 ± 3.72**	**55.2 ± 5.58**
B. pascuorum							
Rothamsted (8)	125	n/a	0.52 ± 0.11	5.71 ± 1.01	n/a	n/a	n/a
Lanford (7)	237	183	0.52 ± 0.15	6.22 ± 1.19	n/a	n/a	n/a
Foix (9)	32	26	0.52 ± 0.11	7.07 ± 1.24	n/a	n/a	n/a
Average (all)	**131.3 ± 59.3**	**n/a**	**0.52 ± 0.002**	**6.33 ± 0.40**	n/a	n/a	n/a
UK average	**181.0 ± 56.0**	**n/a**	**0.52 ± 0.02**	**5.96 ± 0.26**	n/a	n/a	n/a

maximum likelihood estimate of Ne was 16.4, but the upper confidence interval could not be estimated by the program (the lower confidence interval was 6.2 ± 0.08), similarly the maximum likelihood estimate of Ne could not be calculated at all for the Margam population. This happens most often when there is so little genetic differentiation between the first and second generation that the difference in allele frequency could easily be accounted for by the fact that these generations are sampled randomly (Anderson 2005).

Departure from Hardy–Weinberg equilibrium

Of the nine loci, B10 did not amplify reliably so was not scored. *B. pascuorum* was not scored at B131 or B116. For *B. sylvarum*, probability tests by locus across all populations pooled revealed significant departure from HWE at B116; tests by population across loci pooled revealed no significant departure in any population. MICROCHECKER (van Oosterhout *et al*. 2004) revealed evidence of null alleles at B116 in the Cliffe population, at B126 in the Ham Wall and Salisbury Plain (SPTA) populations and at B11 in the Ham Wall population. No locus pairs showed significant linkage disequilibrium. Consequently, analyses were made excluding B116 and were repeated excluding B116, B126 and B11 (those loci that showed evidence of null alleles in at least one population). As data were collected over two generations, (Table 1) all parameters were estimated a final time using data from one generation only (the second year of sampling) without data from B116. The results of this final analysis are presented below. Unless otherwise stated all parameter estimates between data sets remained qualitatively similar with no difference in the statistical significance of results. Results across all data sets are summarized in Table 3.

Genetic structuring of populations

Global values of θ were significant in *B. sylvarum* (0.084 ± 0.017 SE, $P < 0.001$) even when the French population was removed (0.084 ± 0.021 SE, $P < 0.001$). Global values were significant but much lower in *B. pascuorum* (all populations θ = 0.015 ± 0.012 SE, $P < 0.001$) but nonsignificant when the French population was removed (UK populations, θ = 0.001 ± 0.002 SE). Pairwise values of θ also indicated significant genetic structuring in *B. sylvarum*: all population pairs are significantly differentiated from one another (Table 4).

Analysis revealed that genetic diversity differed significantly across populations in terms of allelic richness considered by locus with the French population showing the highest mean rank diversity (Table 5; $\chi^2 = 11.1$, d.f. = 5, $n = 7$, $P < 0.05$). Genetic diversity across populations did not differ significantly in terms of expected heterozygosity, although the French population exhibited the greatest mean rank diversity (Table 5; $\chi^2 = 6.84$, d.f. = 5, $n = 7$, $P = 0.23$). Additionally, by locus examination of

Table 3 Comparison of population genetic parameter estimates across datasets. Figures in parenthesis indicate the total number of loci from which parameters were calculated. Global θ Weir & Cockerham's (1984) F_{ST}; H_E, expected heterozgosity; N_a, allelic richness; IBD, isolation by distance

		Both generations, minus B116 (7)	Both generations, minus B116, B124 and B11 (5)	Single generation, minus B116 (7)
Global θ		0.088 ± 0.012, *P < 0.001*	0.084 ± 0.017, *P < 0.001*	0.087 ± 0.016, *P < 0.001*
Mean H_E	UK only	0.38 ± 0.01	0.39 ± 0.02	0.42 ± 0.03
	Epénède	0.53 ± 0.08	0.53 ± 0.09	0.55 ± 0.12
Mean *Na*	UK only	3.15 ± 0.10	3.11 ± 0.10	3.57 ± 0.15
	Epénède	4 00 ± 0.85	4.00 ± 0.85	4.40 ± 1.12
IBD		No evidence	No evidence	No evidence
Bottlenecks		In no populations	In no populations	One population

Table 4 Pairwise θ and significance between all population pairs of *Bombus sylvarum*, **P* < 0.05 (below the diagonal) and pairwise distance between populations (kilometres, above the diagonal)

	Castlemartin	Cliffe Pools	Epenede	Ham Wall	Margam	SPTA
Castlemartin	—	378	748	163	85	219
Cliffe Pools	0.083*	—	612	228	294	166
Epenede	0.010*	0.113*	—	672	704	616
Ham Wall	0.114*	0.083*	0.075*	—	82	62
Margam	0.123*	0.111*	0.065*	0.063*	—	134
SPTA	0.086*	0.065*	0.087*	0.092*	0.046*	—

Table 5 Mean rank diversity of populations (expected heterozygosity and allelic richness) averaged across loci as calculated in Friedman's test [n(loci) = 7]

	Mean rank	
Population	Allelic richness	Expected heterozygosity
Castlemartin	2.43	3.43
Cliffe Pools	2.57	2.29
Margam	3.86	3.71
Ham Wall	3.29	3.29
SPTA	3.57	3.43
Epenede	5.29	4.86

maximum and minimum gene diversity estimates between UK populations and the French population did not reveal significantly lower gene diversity in any pairwise comparison. There was no significant effect of population size and degree of isolation on either measure of genetic diversity.

There was no evidence for a pattern of genetic isolation by distance among *B. sylvarum* population pairs [UK only ($Z = 2.09$, $r^2 = 0.00001$, $P = 0.47$); all ($Z = 3.45$, $r^2 = 0.00002$, $P = 0.45$); Tables 4 and Fig. 2].

Under the IAM, one population of *B. sylvarum* (Castlemartin) showed signs of having passed through a recent bottleneck, but this was not evident under the TPM or SMM (data sets from both years combined showed no evidence of any bottlenecks).

Diploid males

A total of 39 *B. sylvarum* males were caught, one of which was diploid (one of three males sampled at Castlemartin). Of all 191 *B. sylvarum* workers, none was homozygous at all loci, therefore the frequency of undetected diploid males is likely to be very low in this species. Regarding accuracy of genotypes, the average number of individuals (males and workers) per locus that needed to be re-amplified and re-scored (due to potential scoring errors) was 22.75 ± 3.09, equating to 9.5% of the total.

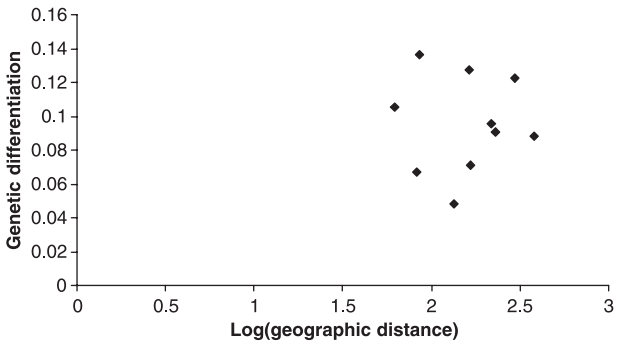

Fig. 2 Pairwise genetic differentiation ($\theta/(1 - \theta)$) vs. pairwise log(geographical distance) between all UK population pairs of *B. sylvarum*.

Discussion

Effective population size

Estimates of nest density in declining bumblebee species were previously anecdotal and in the order of 1–2 nests km^{-2} (Edwards 1999, 2000, 2001, 2002, 2003). Here the first empirical estimates of number of nests per population (directly related to population size) in a threatened bumblebee species are provided. Estimates of effective population size range from 21 to 72 (the figure of 1–2 nests km^{-2} provides estimates of number of nests per population in our sampled areas of 1–20). Although type II errors in detection of sister pairs are unavoidably large due to the low degree of genetic polymorphism and a consequent reduction in statistical power, they are similar across all populations, so we reason that interpopulation comparisons remain valid. Additionally, although foraging range is an important aspect of nest density estimates and has not been quantified in *Bombus sylvarum*, the fragmented UK sites studied are mostly small and all are surrounded by extensive areas of agricultural land with few recent records of this species, hence it is unlikely that individuals sampled originate from a much greater area. As *B. sylvarum* is more widespread in France, however, the population size estimate in this case may more accurately simply reflect the number of colonies utilizing the sample site. Estimates of effective population size are extremely low for this species and are likely to be overestimates in most populations: firstly due to the degree of type II error observed, significant sister pairs are likely to have been under-detected. Under-detection of sister pairs then leads to an overestimate of the number of nests in a population. Secondly, the use of Wright's formula to estimate effective population size assumes each female mates with a single male, if a male mates with several females the population size will be somewhat lower. Finally not all nests of bumblebees survive to produce reproductive individuals (Muller & Schmid-Hempel 1992). At the only site where Ne could be estimated from changes in allele frequency between generations (Castlemartin), the estimate was extremely low (16.4), approximately three times less than the estimates based on nest density at this site, although this may also be inaccurate owing to little genetic differentiation between generations. There is some debate as to what constitutes the minimum viable population size (see Frankham *et al*. 2002), but in general populations of less than 50 are expected to swiftly become inviable through inbreeding, and even in populations of up to 500 loss of

genetic diversity through drift will generally exceed the rate at which new alleles arise through mutation. Thus we would expect these bumblebee populations to be suffering from inbreeding in terms of population subdivision and any associated fitness costs (discussed below) and also to be more prone to stochastic extinctions.

Departure from Hardy–Weinberg equilibrium

After removal of sisters, probability tests revealed deviation from HWE at the locus B116. This is most likely due to the presence of null alleles as primers were developed for *Bombus terrestris* (Estoup *et al*. 1995a; Estoup *et al*. 1996). This would seem most likely as MICROCHECKER (van Oosterhout *et al*. 2004) indicated the presence of null alleles at this locus as well as two others (B126 in two populations and B11 in one population). Other possible causes of HWE-departure are selection (unlikely in microsatellites); linkage (unlikely as there was no evidence of linkage between any loci here); nonrandom mating; and the Wahlund effect. The latter two may be expected to show a pattern across all loci within a population, hence we suggest null alleles as the most likely explanation for the observed departure.

Genetic structuring of fragmented populations

In the last 50 years or more *B. sylvarum* has suffered declines in range and abundance (Williams 1982; Edwards & Telfer 2001) and given the very low estimates of effective population size described above and the large geographical distances between populations (Fig. 1), patterns of genetic structuring are unsurprising (global $\theta = 0.084$, all pairwise comparisons significant). In contrast, the ubiquitous *Bombus pascuorum* showed much lower levels of structuring even over large scales (1000 km), with none between the UK populations sampled. The observed value of θ for *B. sylvarum* is lower than the estimate for the closely related and also declining *Bombus muscorum* ($\theta = 0.119$, Darvill *et al*. 2006). Although interspecific comparisons based on microsatellite data must be made with caution, most likely this is because most current-day populations of this latter species are oceanic island populations where gene flow has presumably always been limited owing to the necessity of crossing open water to reach other populations. Populations of the study species of interest here, on the other hand, have most probably become isolated from one another more recently and are not separated by sea. Indeed, the degree of structuring between mainland populations of *B. muscorum* is also lower than that of the island populations supporting this explanation ($\theta = 0.038$, two populations, Kent UK, Darvill *et al*. 2006). Alternatively, there may be undetected interconnecting populations on the mainland. The possibility that until recently the study populations may have been connected by stepping stone populations, or that there are undetected connecting populations may explain the relatively low levels of genetic differentiation observed, despite the very low estimates of population size.

No isolation by distance (IBD) was observed across our UK study populations. There are several potential explanations for this. First, we may have sampled too few populations (only seven populations are known to exist in the UK): Peterson & Denno (1998) found it is necessary to sample at least 15 populations to detect IBD [although Darvill *et al*. (2006) detected a significant trend of IBD in just 14 populations of *B. muscorum*]. Second, following moderately recent fragmentation our study populations may not be in mutation-drift equilibrium and thus may break a basic assumption of stepping-stone isolation-by-distance models, i.e. any pattern of IBD may be obfuscated by the occurrence of random genetic drift. The relative importance of genetic drift vs. gene flow can be tested for (Hutchison & Templeton 1999; Johansson *et al*. 2006) by correlating the residuals of an initial linear regression of genetic differentiation with the logarithm of geographical distance against the logarithm of geographical distance. When genetic drift is relatively more important than gene flow, a high degree of scatter in the distribution of residuals is expected. We have not performed this analysis due to low statistical power. Alternatively, a lack of isolation by distance could simply indicate long separated populations between which there is an absence of gene flow. In this case, random factors (drift) may influence the degree of relatedness between populations, although these individual populations may be in mutation-drift equilibrium. Given the very small effective population sizes estimated in these populations of *B. sylvarum* and that the current-day populations are (in general) so far apart from one another that gene flow between them seems highly unlikely (mean distance between populations = 181.1 km ± 31.8, only three known remaining populations are closer than 100 km to one another) significant genetic drift and an absence of gene flow between populations seems likely. Indeed, the only estimate for fragmented populations of bumblebees to date suggests that gene flow over 10 km is uncommon (Darvill *et al*. 2006). If a similar figure applies to the *B. sylvarum* then gene flow is likely between only two populations [Canvey Island, Essex where numbers were too low for meaningful analysis and Cliffe Pools RSPB reserve, Kent (site 5, Fig. 1)]. Between the two UK samples of *B. pascuorum*, which are 125 km apart, there is no significant genetic differentiation, reflecting the connectivity between populations in this ubiquitous species.

Social Hymenoptera have been hypothesized to be particularly susceptible to losses of genetic diversity owing to decreased effective population size due to sociality (Chapman & Bourke 2001; Packer & Owen 2001). We provide evidence of low effective population sizes in

B. sylvarum but find no association between population size and genetic diversity. The UK populations, however, do show significantly reduced genetic diversity relative to the French population at least in terms of allelic richness, although this was not evident when examining measures of heterozygosity. We speculate that observed reductions in allelic richness in the highly fragmented UK populations are attributable to their increased isolation from one another and small size so that the only possible source of variation in these populations is mutation alone (and not migration). Conversely, the French population may be more genetically diverse because of gene flow. Alternatively, given that there is no evidence of bottlenecking in most populations and that in some instances the value of θ between UK and continental sites is as low as 1%, reductions in genetic diversity may indicate a phylogeographical signal in that UK populations are of more recent origin and may be derived from fewer foundresses hence genetic diversity is lower. In summary, habitat fragmentation may have led to the reduction of genetic diversity in UK populations of *B. sylvarum* through metapopulation structure breakdown. More data are necessary to substantiate this, including better knowledge of current continental distributions and a wider analysis of continental populations, as well as analysis of historical UK populations from museum samples (if possible). Evidence to suggest a loss of genetic diversity with increasing fragmentation is a concern for the future sustainability of these populations if there are any associated fitness costs with reductions of genetic diversity.

Only one population showed evidence of a genetic bottleneck (under the IAM, but not under the TPM nor the SMM). Unless severe and recent, bottlenecks may remain undetected (Luikart & Cornuet 1998). In the UK, bumblebees have probably been declining in range gradually since at least 1947 when the postwar drive for increased agricultural production and self-sufficiency manifested itself in the 1947 Agriculture Act (Goulson 2003). Until relatively recently, these species were more widespread and probably existed as much larger populations which would not be prone to bottlenecking.

Diploid male production as a cost of reductions in genetic diversity

Evidence of reduced genetic diversity in fragmented populations does not mean that *B. sylvarum* will suffer from fitness costs. Although declines in heterozygosity have been shown to be detrimental to survival of wild butterfly populations (Saccheri *et al*. 1998), inbreeding may not have such fitness effects in haplodiploid species in particular, because deleterious alleles may be purged from the population through expression in the haploid sex (Sorati *et al*. 1996), although this will not apply to deleterious alleles for sex-limited traits in females. To our knowledge, only four studies have examined fitness implications of inbreeding in bumblebees, all using *B. terrestris*. Duchateau *et al*. (1994) reported only slight effects on colony growth in inbred colonies with diploid males. Gerloff *et al*. (2003) found that inbred workers did not suffer from a reduced encapsulation response and Gerloff & Schmid-Hempel's (2005) study suggested no effect of inbreeding on reproductive output or cumulative fitness. Alternatively, Beekman *et al*. (1999) reported that inbred queens exhibited reduced egg laying. Clearly, the picture is far from clear.

The production of diploid males is one acknowledged potential cost of reduced genetic diversity at the sex-locus, in Hymenoptera with sl-CSD (Duchateau *et al*. 1994; Cook & Crozier 1995). Recently suggested as a measure of sustainability of fragmented bee populations (Zayed *et al*. 2004) this cost may outweigh any potential benefits of haplodiploidy and potentially lead to a so-called 'extinction-vortex' (Zayed & Packer 2005). Here, very few diploid males were observed (1 of 39 males sampled). There are a number of possible reasons for a low frequency of diploid male production (suggested by Paxton *et al*. 2000): first, the sex-mechanism may not be single-locus CSD, but multilocus. This is perhaps an unlikely explanation here, as *B. terrestris* is known to exhibit sl-csd (Duchateau *et al*. 1994) and sl-csd is ancestral to Hymenoptera. Second, sperm selection may occur where a sperm has a higher probability of fertilizing an egg if the sex alleles of the egg and sperm are not matched. This would also seem unlikely as mating trials between sibs in *B. terrestris* produce diploid males in the expected frequencies (Duchateau *et al*. 1994). Third, there may be recognition of mates with a shared sex allele. In this study, such interpretations are premature. If a queen founds a colony following a matched mating, the greatest effect of diploid male production is presumably likely to be early on, when there are only a small number of workers to forage and perform nest duties. Such an inbred colony may not survive much past the initial founding stages, and thus we would not expect to detect many diploid males later into the season (higher mortality rates have been observed in colonies producing inviable or viable diploid males in bumblebees (Plowright & Pallett 1979), ants (Ross & Fletcher 1986) and stingless bees (Carvalho 2001). Although the diploid male observed was collected on 16 August, a time likely to be after the early stages of nest foundation, it must be stressed that in this study very few sites were sampled very early in the season and relatively few males were encountered while collecting. Therefore, due to very low sample sizes and timing of collection, it is not possible for us to estimate or comment on a potential cost of reduced genetic diversity in terms of diploid male production. Such a study would be most useful and highly welcomed, providing a great opportunity to assess fitness impacts in

wild populations of an invertebrate species. It would necessitate sampling of large quantities of males early on in the season and assumes that diploid males are mostly viable. Although diploid males are mostly viable in the related species *B. terrestris* and one diploid male was observed here, viability of diploid males may be low (e.g. only ~5% of diploid males of the Hymenopteran *Bracon hebetor* are viable while the majority of *B.* nr. *hebetor* are (Holloway *et al*. 1999). If this is the case, there may be unobserved fitness effects due to diploid male production in *B. sylvarum* as diploid males may only rarely survive to adulthood. Hence, even in a study where large numbers of adult males are sampled from wild populations the cost of diploid male production could be under-estimated.

In summary, we report very low population sizes in fragmented populations of *B. sylvarum*. Results demonstrate that these populations are sufficiently isolated to lose genetic diversity through drift, with perhaps no gene flow between most remaining populations. Our data indicate that previous hypotheses that social Hymenoptera may be susceptible to genetic differentiation and losses of diversity following population subdivision may be true, but it remains to be established whether this results in a loss of fitness.

Acknowledgements

Thanks must be given to the RSPB; the MoD and Defence Estates; the Countryside Council for Wales; and English Nature for access to land and permission to collect; to Mike Edwards for advice; to Dr M. Dixon and Prof N. Maclean for laboratory use. Funding was provided by NERC as part of J. Ellis's PhD thesis.

Supplementary material

The supplementary material is available from
http://www.blackwellpublishing.com/products/journals/suppmat/MEC/MEC3121/MEC3121sm.htm

Table S1 Allelic richness (N_a), observed heterozygosity (H_O) and expected heterozygosity (H_E) by population and locus in (a) *Bombus sylvarum* and (b) *Bombus pascuorum*

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