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Population structure and inbreeding in a rare and declining bumblebee, *Bombus muscorum* (Hymenoptera: Apidae)

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Abstract

Owing to habitat loss populations of many organisms have declined and become fragmented. Vertebrate conservation strategies routinely consider genetic factors, but their importance in invertebrate populations is poorly understood. Bumblebees are important pollinators, and many species have undergone dramatic declines. As monoandrous social hymenopterans they may be particularly susceptible to inbreeding due to low effective population sizes. We study fragmented populations of a bumblebee species, on a model island system, and on mainland Great Britain where it is rare and declining. We use microsatellites to study: population genetic structuring and gene flow; the relationships between genetic diversity, population size and isolation; and frequencies of (sterile) diploid males — an indicator of inbreeding. We find significant genetic structuring ($\theta = 0.12$) and isolation by distance. Populations > 10 km apart are all significantly differentiated, both on oceanic islands and on the mainland. Genetic diversity is reduced relative to closely related common species, and isolated populations exhibit further reductions. Of 16 populations, 10 show recent bottlenecking, and 3 show diploid male production. These results suggest that surviving populations of this rare insect suffer from inbreeding as a result of geographical isolation. Implications for the conservation of social hymenopterans are discussed.

Keywords: *Bombus*, diploid males, Hymenoptera, inbreeding, microsatellites, population genetics

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Introduction

The destruction of natural habitats as a result of human activity is one of the most serious threats to survival faced by many species. Formerly widespread species become restricted to remaining fragments of suitable habitat, and the resulting populations are frequently small and isolated. Without frequent immigration these populations are prone to the loss of genetic diversity through bottlenecks and drift (Frankham *et al*. 2002; Keller & Waller 2002). The maintenance of genetic diversity is crucial for the long-term survival of many populations and species (Frankham *et al*. 2002; Hansson & Westerberg 2002; Keller & Waller 2002; Reed & Frankham 2003). Reduced genetic diversity lowers the capacity of a population to respond to environmental change, and may lead to inbreeding depression caused by the expression of deleterious alleles.

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It has been argued that in wild populations, the impact of inbreeding will be negligible relative to demographic and environmental stochasticity (Caro & Laurenson 1994; Caughley 1994), and avoided due to processes such as long-range dispersal, kin recognition and polyandry (Pusey & Wolf 1996). There is, however, now a wealth of evidence suggesting that inbreeding may reduce both individual and population performance. Numerous effects have been documented, including a reduction in larval survival, adult longevity, egg-hatching rates and resistance to disease and environmental stress (reviewed in Keller & Waller 2002). However, the vast majority of such studies have focused on vertebrate and plant species. The relatively large population sizes of invertebrates compared to vertebrates may explain why few signs of inbreeding have been detected (Van Dongen *et al*. 1998; Gyllenstrand & Seppa 2003; Henshaw & Crozier 2004; Keller *et al*. 2004; Molbo *et al*. 2004). In contrast, Saccheri *et al*. (1998) found reduced heterozygosity in small isolated populations of the butterfly *Melitaea cinxia* and a resultant increase in extinction risk. Given the vast number of invertebrate species, and the

diversity of their life histories, it is not yet possible to generalize as to the relative importance of genetic processes in the decline of endangered populations.

As a result of their sociality, social insects exhibit characteristics which may increase their susceptibility to inbreeding. Effective population sizes may be small, despite an apparent abundance of (sterile) workers, and haplo-diploidy may reduce genetic variation (Pamilo & Crozier 1997; Chapman & Bourke 2001). It has been argued that population fitness will not be reduced by inbreeding in haplo-diploid Hymenoptera, as haploid males provide a mechanism for the purging of recessive deleterious alleles (Sorati *et al*. 1996). However, the single-locus complementary sex determination (sl-CSD) system found in social hymenopterans has an important consequence for the fitness of populations that begin to lose genetic diversity. Individuals homozygous at the sex locus develop into sterile diploid males, which do not benefit the colony, and therefore represent a cost (Cook & Crozier 1995). The overall fitness of the population is thus directly related to the number of different alleles at the sex locus, which may in turn be related to the size and isolation of the population.

Habitat fragmentation further increases the susceptibility of populations to a loss of genetic diversity, through drift and bottlenecks (Frankham *et al*. 2002; Keller & Waller 2002). Previous studies of social Hymenoptera have largely focused on ant species and report mixed findings, generally related to the colony structure present. For example, fragmented populations of the wood ant, *Formica lugubris*, showed no detectable inbreeding and had high genetic variability, perhaps as a consequence of polygyny (more than one queen per nest) inflating the effective population size (Gyllenstrand & Seppa 2003). Similarly, Maki-Petays *et al*. (2005) found little evidence for reduced genetic diversity in two polygynous ant species in response to habitat fragmentation. A significant amount of genetic structuring was found between subpopulations, although a closer analysis revealed that social structure played a key role in restricting gene flow. Conversely, five studies of monogyne ants (only one queen per nest) provide some evidence of inbreeding in social insects. Sundstrom *et al*. (2003) report a high degree of population structure at a local scale in *Formica exsecta* along with sex-biased gene flow, significant inbreeding coefficients (F_{IS}) and a high degree of queen–male relatedness. Two further studies of the same population of the same species also report evidence for inbreeding (Pamilo & Rosengren 1984; Pamilo 1991). Similarly, inbreeding has been shown in ant species where very few colonies participate in nuptial flights at any one time (Hasegawa & Yamaguchi 1995). Finally, there is some evidence of inbreeding in the lek-mating species *Pogonomyrmex occidentalis* (Cole & Wiernasz 1997).

Bumblebees are social hymenopterans which live in annual colonies founded by a single queen. The majority of species are monoandrous (Estoup *et al*. 1995b; Schmid-Hempel & Schmid-Hempel 2000; Sauter *et al*. 2001; Payne *et al*. 2003), which decreases the amount of genetic variation present in each colony, relative to that of polygynous or polyandrous species, and therefore increases their susceptibility to inbreeding (Chapman & Bourke 2001). In addition, the effective population size is determined by the number of successful nests in a given area, rather than the number of (sterile) workers. Many bumblebee populations have declined dramatically in recent decades, both in Europe and North America, primarily as a result of agricultural intensification and associated habitat loss (reviewed in Goulson 2003). Of the UK's 25 native species, three are now extinct, and several remain only in small isolated populations (Goulson 2003). As bumblebees are important crop and wildflower pollinators (Corbet *et al*. 1991), their declines may have serious consequences for agriculture and for wildflower populations.

Previous studies of bumblebee population genetics have focused on common and widespread species: *Bombus terrestris* (Estoup *et al*. 1996; Widmer *et al*. 1998), *Bombus pascuorum* (Pirounakis *et al*. 1998; Widmer & Schmid-Hempel 1999), and *Bombus ignitus* (Shao *et al*. 2004). Genetic differentiation between mainland sites separated by several hundreds of kilometres was low, and genetic variability was high. Comparisons between mainland sites and distant offshore islands found significant genetic differentiation, and reduced genetic diversity on some islands, possibly as a consequence of founder effect and drift (Estoup *et al*. 1996; Widmer *et al*. 1998; Shao *et al*. 2004).

Here, we study a rare and declining bumblebee species which exists in a series of small fragmented populations, in order to

1 determine whether the populations exhibit genetic structuring, and estimate dispersal range;
2 attempt to establish the relationship between genetic diversity, population size and isolation; and
3 detect the presence of diploid males as an indicator of inbreeding.

Materials and methods

Study species

Once widespread on the mainland, *Bombus muscorum* (L.) now survives only in a series of small fragmented populations, although it is still relatively abundant on some Scottish islands (Edwards & Broad 2005). Within the UK, a number of different subspecies are recognized, differentiated on the basis of coat colour. Three races occur within the study areas: (i) *Bombus muscorum sladeni* (Vogt) is found in south and central England and Wales; (ii) *Bombus muscorum pallidus* (Evans) in northern England, mainland Scotland and some Inner

Fig. 1 Map of the study areas, showing the locations of the populations in (A) the Hebrides, NW Scotland, and (B) Southern UK. *Bombus muscorum sladeni* occur at Dungeness and Elmley, *Bombus muscorum pallidus* on Staffa, Lunga and Colonsay, and *Bombus muscorum smithianus* at all other sites.

Hebridean islands; and (iii) *Bombus muscorum smithianus* (auctt. nec White) on the Shetland Isles, the Outer Hebrides and some of the Inner Hebrides. Of these three races, *B. muscorum sladeni* has declined the most, primarily as a result of agricultural intensification, and is now very rare. *B. muscorum* occurs in a range of habitats (Goulson *et al*. in press). In the south, it occurs on coastal marshes, shingle and calcareous grasslands, and is strongly associated with Fabaceae (Goulson & Darvill 2004). In the north, it is also frequently found on moorland and machair (Goulson *et al*. 2005).

Sample collection

During the summers (June–September) of 2003 and 2004, individuals of *B. muscorum* were collected from 14 islands in the Inner and Outer Hebrides (Scotland, UK), and from two southern UK sites (Fig. 1). All known southern UK populations were visited, although at some, workers were insufficiently abundant for adequate samples to be collected. Previously recorded populations on the Scottish mainland were also visited, but here too workers were very scarce or absent, and samples were not collected. Nonlethal

tarsal samples were taken following Holehouse *et al.* (2003). Workers were caught from numerous locations within each population (where possible > 200 m from one another) to minimize the probability of sampling individuals from the same colony. When encountered, male bumblebees were also caught in order to assess the frequency of diploid males, and were destructively sampled to conclusively assess their sex. Samples were preserved in pure ethanol and stored at ambient temperature.

Molecular methods

DNA was extracted using the HotShot protocol (Truett *et al.* 2000). Workers were genotyped at up to nine microsatellite loci: B132, B131, B118, B100, B96, B10, B11, B124, B126 (Estoup *et al.* 1995b, 1996). B100 was found to be monomorphic, as was B11, in all but one population. Microsatellites were amplified by polymerase chain reaction (PCR) in 10-μL volumes using QIAGEN Multiplex PCR kits. Each reaction contained approximately 10 ng template DNA, 1 μL Q-solution, 5 μL PCR Master Mix and 0.2 μM of each primer. Samples were initially denatured at 95 °C for 15 min, followed by 35 cycles of denaturing at 94 °C for 30 s, annealing at 51 °C for 90 s and extension at 72 °C for 90 s. A final extension step at 72 °C for 10 min then followed. PCR products were visualized on an ABI PRISM™ 377 semi-automated sequencer using an internal size standard (GeneScan ROX 350, Applied Biosystems).

Fragment sizes were scored using Genotyper (Applied Biosystems). Repeat PCRs were carried out on individuals believed to be diploid males, and on any samples that had failed to amplify or were uncertainly scored.

Statistical methods

The data set was first checked for unexpected mutation steps, large gaps in the data or unusually sized alleles using MSA (Dieringer & Schlotterer 2003). A number of different software applications were used for subsequent analyses, some of which do not deal well with missing data. Where a full data set was not available (for Elmley at B10, and several populations at B131) the locus or populations were excluded from the analysis. Tests for genotypic linkage disequilibrium and departure from Hardy–Weinberg equilibrium (HWE) were performed using GENEPOP version 3.4 (Raymond & Rousset 1995). Sequential Bonferroni corrections (Rice 1989) were applied to minimize type I errors.

The genetic population structure was assessed with *F*-statistics (Wright 1951), using Weir & Cockerham (1984) estimators (F, f and θ), as implemented in FSTAT version 2.9.3 (Goudet 2001). Global *F*-statistics were calculated for all populations, and pairwise θ for all pairs of populations. Means and standard errors of the estimates were obtained by jackknifing over samples and loci. Significance levels were determined by permuting alleles (100 000 permutations) using MSA, applying Bonferroni corrections (Rice 1989).

An analysis of molecular variance (AMOVA) was performed using ARLEQUIN version 2.001 (Schneider *et al.* 2000), with the populations divided into three groups, corresponding to the three recognized subspecies, and also to their geographical locations. The significance of the results was estimated by performing 16 000 permutations.

Genetic isolation by distance is expected to increase linearly with the logarithm of physical separation in two-dimensional space (Rousset 1997; Hardy & Vekemans 1999). Isolation by distance between the Hebridean populations was therefore examined by regression of pairwise estimates of genetic distance, as defined by $\theta/(1-\theta)$, against the corresponding logarithms of the geographical separation between populations (Rousset 1997). A Mantel test (Mantel 1967) was used to assess the significance of any correlation, performing 50 000 permutations in ISOLATION BY DISTANCE (Bohonak 2002).

A neighbour-joining (NJ) tree (Saitou & Nei 1987) relating the populations was constructed using Nei's chord distance (Nei *et al.* 1983). To assess the stability of the tree nodes, 1000 bootstrap replications were performed using the PHYLIP package of programs (Felsenstein 2004). SEQBOOT, GENDIST and NEIGHBOUR were used to create replica data sets, calculate Nei's chord distance, and construct NJ trees, respectively. The final condensed consensus tree was produced using MEGA version 3 (Kumar *et al.* 2004), showing only nodes supported by more than 50% of bootstraps.

Possible loss of genetic variation through bottlenecking — a recent reduction in population size without substantial subsequent immigration — was tested for using BOTTLENECK version 1.2.02 (Piry *et al.* 1999). Three mutation models have been proposed for microsatellites: the stepwise-mutation model (SMM); the infinite allele model (IAM); and the two-phase mutation model (TPM). Shao *et al.* (2004) followed the IAM, arguing that bumblebee microsatellites, which are interrupted-repeats, have been shown to not fit the SMM (Estoup *et al.* 1995a,c). Indeed, as dinucleotide repeats, multistep changes may be frequent in these microsatellites (Huang *et al.* 2002). However, in support of the TPM, others have argued that mutation patterns involve a majority of one-step changes, with a smaller proportion of multistep changes (Ellegren 2000; Schlotterer 2000). Since this debate is ongoing, tests were conducted using both IAM and TPM allowing for varying multistep changes between 5% and 10%. In each case, 100 000 iterations were performed.

In order to ascertain whether the genetic diversity of the populations was related to either their isolation or to their area, a two-way analysis of variance (ANOVA) was performed using SPSS version 12. Genetic diversity was assessed by calculating Nei's unbiased heterozygosity (Nei 1987) and allelic richness using FSTAT. Allelic richness was calculated because the average number of alleles per locus

is sensitive to sample size (El Mousadik & Petit 1996). The habitat area utilized by each population was defined as the number of kilometre squares in which *B. muscorum* was found foraging. Isolation was measured as the distance to the nearest population, or (in the case of the Hebridean populations) the distance to the mainland, whichever was smaller. The area of land within 5, 10, 15 and 20 km of each Hebridean island was estimated using 1:50 000 Ordnance Survey maps, in order to take into account the size as well as the proximity of potential source populations. The allelic richness and H_E of Hebridean populations were compared to southern UK populations using Mann–Whitney *U*-tests in SPSS version 12. In addition, the H_E of both Hebridean and southern UK populations of *B. muscorum* was compared to that of the closely related species, *Bombus pascuorum* (data from Widmer & Schmid-Hempel 1999). Data from a previous study of *Bombus terrestris* and *B. lucorum* are also included in Table 1 for comparison (from Estoup *et al*. 1996).

Results

Hardy–Weinberg and linkage disequilibrium

In total, 854 females and 64 males were genotyped (Table 1). Neither global tests by population nor by locus detected any significant deviation from HWE. Significant linkage disequilibrium ($P < 0.05$) was found between two pairs of loci, B132-B131 and B96-B126, when testing each locus pair across all populations. Tests within each population found significant linkage disequilibrium in only one population (Staffa) for these locus pairs. A global test across all populations excluding Staffa found no significant linkage disequilibrium, so subsequent analyses were carried out with and without this population.

Population structure

Overall genetic structuring was high, with $\theta = 0.119 \pm 0.023$ SE ($P < 0.00001$) (excluding Staffa, $\theta = 0.120 \pm 0.025$ SE $P < 0.00001$). Estimates of F_{IS} were small (mean $F_{IS} = 0.01 \pm 0.01$ SE). Pairwise θ values were highly significant ($P < 0.01$) for 109 of 120 comparisons, and were significant ($P < 0.05$) for three pairs of populations (Mingulay-Muldoanich, Mingulay-Sandray and Muldoanich-Sandray). The remaining seven comparisons between the cluster of islands comprising Mingulay, Muldoanich, Pabbay, Sandray and Barra were not significant. Coll and Tiree also showed no significant differentiation from one another.

Table 1 The sample size, average (unbiased) heterozygosity (H_E) and allelic richness of each of the 16 populations (± SE), along with data for mainland continental populations of *Bombus pascuorum* (from Widmer & Schmid-Hempel 1999), *Bombus terrestris* and *Bombus lucorum* (from Estoup *et al*. 1996). Allelic richness and H_E were calculated using all loci except B131

Population	Subspecies	Sample size	Allelic richness*	H_E
Barra	*smithianus*	50 ♀ 3 ♂	3.10 ± 0.66	0.393 ± 0.113
Mingulay	*smithianus*	49 ♀ 1 ♂	2.99 ± 0.64	0.374 ± 0.115
Muldoanich	*smithianus*	25 ♀ 6 ♂	3.63 ± 0.90	0.421 ± 0.103
Pabbay	*smithianus*	37 ♀ 16 ♂	3.33 ± 0.68	0.399 ± 0.118
Sandray	*smithianus*	58 ♀ 1 ♂	3.05 ± 0.63	0.367 ± 0.111
Outer Hebrides average			3.22 ± 0.12	0.391 ± 0.010
Colonsay	*pallidus*	67 ♀ 0 ♂	3.21 ± 0.50	0.416 ± 0.086
Lunga	*pallidus*	36 ♀ 6 ♂	3.43 ± 0.56	0.507 ± 0.108
Staffa	*pallidus*	52 ♀ 0 ♂	3.33 ± 0.51	0.484 ± 0.091
Canna	*smithianus*	62 ♀ 3 ♂	3.11 ± 0.57	0.433 ± 0.086
Coll	*smithianus*	70 ♀ 0 ♂	3.46 ± 0.69	0.499 ± 0.091
Eigg	*smithianus*	64 ♀ 2 ♂	3.30 ± 0.51	0.533 ± 0.094
Muck	*smithianus*	52 ♀ 0 ♂	2.91 ± 0.42	0.425 ± 0.088
Rum	*smithianus*	42 ♀ 1 ♂	2.91 ± 0.48	0.451 ± 0.077
Tiree	*smithianus*	119 ♀ 2 ♂	3.27 ± 0.55	0.499 ± 0.086
Inner Hebrides average			3.21 ± 0.07	0.472 ± 0.014
Overall Hebrides average			3.22 ± 0.06	0.443 ± 0.014
Dungeness	*sladeni*	23 ♀ 6 ♂	3.95 ± 0.62	0.522 ± 0.088
Elmley†	*sladeni*	48 ♀ 17 ♂	4.06 ± 0.92	0.496 ± 0.125
Southern UK average			4.01 ± 0.06	0.509 ± 0.013
B. pascuorum		an average of 22.7 per site	5.49 ± 0.16	0.563 ± 0.009
B. terrestris		an average of 37.5 per site	5.96 ± 0.12	0.610 ± 0.009
B. lucorum		40	7.00 ± 2.00	0.598 ± 0.115

*For *B. pascuorum*, *B. terrestris* and *B. lucorum* the average number of alleles per locus is given. However, when sample sizes are similar, the two measures are comparable.
†For Elmley, data for B10 was incomplete.

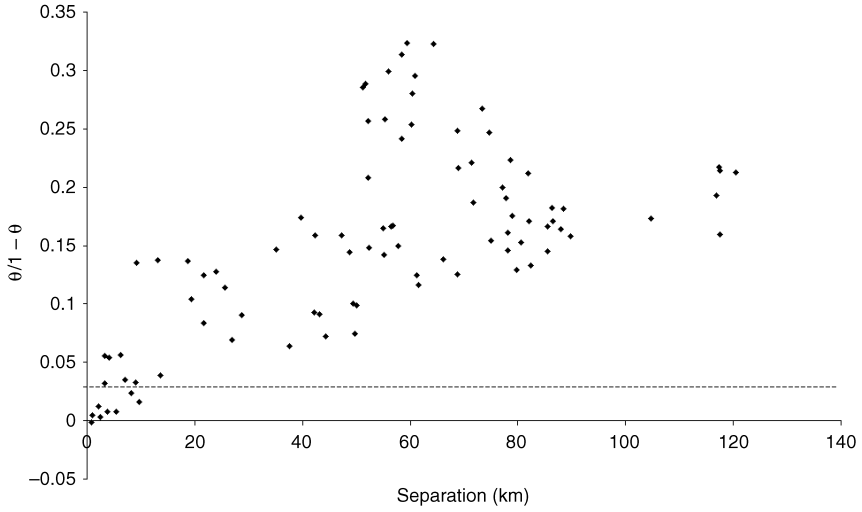

Fig. 2 The (unlogged) physical separation of populations and the genetic differentiation between them. Mantel test, $P < 0.0001$. Population pairs above the dotted line are significantly differentiated from one another ($P < 0.05$).

Table 2 Analysis of molecular variance (AMOVA) examining the partitioning of genetic variation*

Source of variation	d.f.	Sum of squares	Variance	% total	*P*
Within populations	1595	2503.443	1.56956	87.00	< 0.000001
Among populations within subspecies groups	12	340.418	0.24297	13.47	< 0.000001
Among subspecies groups	2	37.336	−0.00841	−0.47	0.58
Total	1609	2881.197	1.80411		

*Prior to performing this AMOVA, population Elmley and locus B131 were removed as ARLEQUIN does not perform well when some populations are missing data for one or more loci.

An AMOVA found significant structuring within populations ($P < 0.000001$) and among populations within subspecies groups ($P < 0.000001$), but not between subspecies groups ($P = 0.58$), confirming that the observed structuring was due to genetic differentiation between populations rather than a subspecies effect (Table 2).

Gene flow between populations

There was a highly significant correlation between genetic distance ($\theta/1 - \theta$) and the natural logarithm of physical separation ($P < 0.0001$, $R^2 = 0.514$), for the 14 Hebridean populations (Fig. 2). Some populations separated by distances of between 3 and 10 km were significantly genetically differentiated from one another, and all populations more than 10 km apart were all significantly differentiated. The two southern UK populations were 49 km apart, and were separated by a significant genetic distance of 0.040 ($P = 0.0072$).

The NJ tree grouped together populations which were geographically close to one another (Fig. 3). This corresponds well with the results of the isolation by distance analysis. This tree is largely in accordance with the three

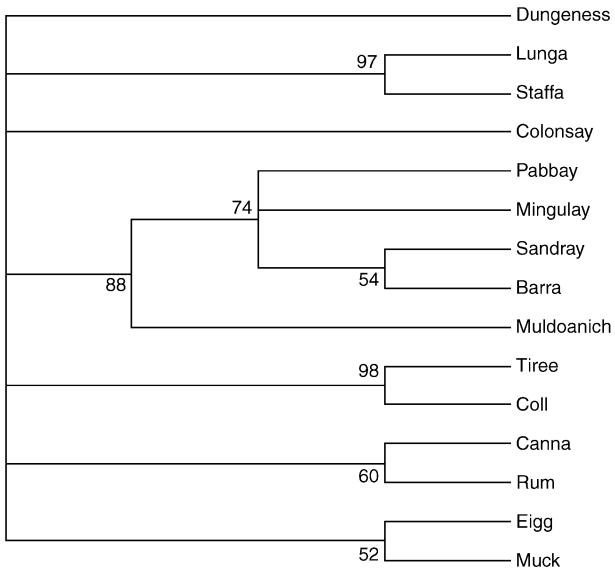

Fig. 3 Condensed neighbour-joining tree (unrooted) relating 15 of the 16 *Bombus muscorum* populations (Elmley was excluded due to missing data at the locus B10). Numbers represent boot strap support. Only nodes supported by more than 50% of bootstraps are shown.

subspecies of *Bombus muscorum* previously designated on morphological grounds. However, microsatellite data alone are not ideal for phylogenetic analyses (Frankham *et al*. 2002), and further studies using mitochondrial DNA are needed to clarify the status of these phenotypes.

Population bottlenecks

Under IAM, 10 of the 14 Hebridean populations (Barra, Coll, Canna, Eigg, Colonsay, Lunga, Muck, Rum, Staffa, Tiree) showed significant signs of recent bottlenecking (Wilcoxon test, one-tailed for heterozygote excess, $P < 0.05$). The remaining islands (Mingulay, Muldoanich, Pabbay, Sandray) along with the two southern UK populations (Dungeness and Elmley) showed no sign of recent bottlenecking. Under both TPM and SMM, only Eigg showed significant signs of recent bottlenecking ($P = 0.004$).

Genetic diversity

The ANOVA found no relationship between expected heterozygosity (H_E) and either isolation or habitat area ($F_{1,12} = 0.389$, $P = 0.545$ and $F_{1,12} = 0.720$, $P = 0.414$, respectively). The observed variation in H_E was, however, well explained by the location of the islands, with Outer Hebridean islands having significantly lower values on average ($F_{1,13} = 16.43$, $P = 0.002$). No relationship was found between allelic richness and either isolation or habitat area ($F_{1,12} = 0.128$, $P = 0.727$ and $F_{1,12} = 0.158$, $P = 0.699$, respectively). In contrast to H_E, variation in allelic richness was not well explained by location, with the Inner and Outer Hebrides having a similar number of alleles per locus ($F_{1,13} = 0.006$, $P = 0.941$). The more complex measures of isolation which took into account the size as well as the proximity of potential source populations did not better explain the observed variation in H_E or allelic richness.

Overall, allelic richness was significantly lower in the Hebridean populations than in southern UK sites (one-tailed Mann–Whitney; $P < 0.01$). H_E was also lower in the Hebridean populations, although the difference was not significant (one-tailed Mann–Whitney; $P = 0.1$). Both Hebridean and southern UK populations of *B. muscorum* showed significantly lower H_E than populations of the closely related species, *Bombus pascuorum* (one-tailed Mann–Whitney; $P < 0.001$ and $P = 0.021$, respectively).

Diploid males

In total 64 males were caught and genotyped, 41 from the Hebrides and 23 from southern UK sites. Of these, three were diploid (at three or more loci), representing an overall frequency of 5% with respect to haploid males. Two of these were caught in the Hebrides (on Pabbay and Tiree) and one in the south (Elmley), representing frequencies of 5% and 4%, respectively. Few males were caught from most populations and the diploid males detected represent a considerable proportion of the males collected (50% of males caught on Tiree, 6.3% of Pabbay males, and 5.9% of Elmley males).

Discussion

Hardy–Weinberg and linkage disequilibrium

Global tests by population and by locus found no deviation from HWE suggesting that null alleles are absent, or at very low frequencies, and that mating is random. Linkage disequilibrium was found between two pairs of loci in one population (Staffa). Physical linkage of these loci in just 1 of 16 populations is unlikely. Four other hypotheses could explain this result: (i) selection acting on certain genotypic combinations; (ii) recent immigration from a genetically differentiated population; (iii) a recent population bottleneck; and (iv) sampling bias due to the collection of groups of workers from the same colonies. Of these explanations, the last two seem the most likely: under the TPM, a recent bottleneck was detected on Staffa; Staffa is the smallest of all of the populations, with an area of just 0.5 km^2, so it is possible that a small number of workers from the same colony were collected in the course of sampling.

Population structure and gene flow

Genetic structuring over all populations was high ($\theta = 0.119$), and an AMOVA confirmed that the observed structuring was largely due to differences between individual populations, and not due to differences between the three subspecies that were sampled. Significant genetic differentiation was evident between populations as little as 3 km apart, and all populations separated by 10 km or more were significantly differentiated from one another. A clear pattern of isolation by distance was evident (Fig. 2), with the populations demonstrating a regional equilibrium between gene flow and drift (see Hutchison & Templeton 1999). In support of this, the NJ tree grouped together populations which were geographically close (Fig. 3). It is clear that dispersal in this species is limited, and that gene flow over distances greater than 10 km is uncommon. The two southern UK populations, separated by a distance of 49 km, were significantly differentiated from one another, but the genetic distance between them was lower than might be expected from comparison with populations in the Hebrides (0.040), possibly suggesting that long-distance dispersal is slightly more common over land. It could be argued that large bodies of water represent a greater obstacle than large areas of unsuitable habitat, since the latter would contain some suitable forage, enabling dispersing bees to top-up energy reserves en route. Overland gene flow might therefore occur over much greater distances.

However, it is perhaps more likely that this similarity occurs because in the recent past these populations were linked by stepping-stone populations that have since disappeared (Edwards & Broad 2005). Until recently, these populations were probably part of a much larger metapopulation extending around the coast of southern England and Wales.

Given that the bumblebee species previously studied are common and ubiquitous, it is perhaps unsurprising that little genetic variation was detected over large distances (Estoup *et al*. 1996; Pirounakis *et al*. 1998; Widmer & Schmid-Hempel 1999). Even with limited dispersal, if all populations are contiguous, genetic cohesion will remain relatively high. However, these previous studies reveal that susceptibility to genetic differentiation is not unique to *Bombus muscorum*. The population of *Bombus terrestris* on Samos Island, just 3 km away from the mainland, was found to be significantly differentiated from continental populations (Estoup *et al*. 1996). Furthermore, the island of Elba, less than 10 km from the mainland, was highly differentiated from continental populations ($\theta > 0.11$). Differentiation between island populations and the mainland was attributed to founder effect and genetic drift, suggesting that dispersal over these distances is infrequent.

Genetic diversity and bottlenecks

The majority of the Hebridean populations (10 of the 14) showed signs of recent bottlenecking under the IAM mutation model. Under the more conservative TPM, a bottleneck was detectable in one population (Eigg). No clear relationship was evident between population size, isolation and genetic diversity, possibly as a result of the obscuring effect of these frequent bottlenecks. It was, however, clear that the more isolated Outer Hebridean islands had significantly reduced genetic diversity compared to the Inner Hebrides. The southern UK populations were more heterozygous than those in the Hebrides, and allelic richness was also significantly higher in the south. It is possible that the recent bottlenecks experienced by many Hebridean populations may account for these differences. Alternatively, this may reflect the relatively recent contraction in the range and effective population size of southern sites. In the absence of severe bottlenecks, reduced effective population size results in a gradual loss of genetic diversity, rather than a sudden drop (Frankham *et al*. 2002). Southern populations may therefore not yet have reached mutation–drift equilibrium. Nevertheless, all populations of *B. muscorum* showed significantly reduced genetic variation compared to other *Bombus* species previously studied (Table 1). In particular, the closely related species *Bombus pascuorum* shows significantly higher genetic variation than *B. muscorum*. It seems probable that the low genetic diversity in remaining isolated populations is due to drift acting on small effective population sizes, coupled with periodic bottlenecks.

The cost of inbreeding

Theory predicts that, as monoandrous social hymenopterans, fragmented populations of bumblebees are susceptible to a loss of genetic diversity (Chapman & Bourke 2001). Here, for the first time, we provide evidence from wild populations suggesting that this is indeed the case. A recent meta-analysis concluded that the relationship between genetic diversity and population fitness was highly significant, and that reduced heterozygosity has a deleterious effect (Reed & Frankham 2003), but there nevertheless remains a clear distinction between inbreeding and inbreeding depression. It has been suggested that haploid males in Hymenoptera provide a mechanism for the purging of recessive deleterious alleles, reducing their susceptibility to inbreeding depression (Sorati *et al*. 1996). Indeed, the only study of bumblebees to date found no evidence for reduced immune response or body size following inbreeding (Gerloff *et al*. 2003). However, males express genes relevant to only a fraction of the bumblebee life cycle. Deleterious mutations affecting the development of female morphology, or traits such as hibernation survival and nest-foundation success would not be exposed to purging. Although it is true to say that many deleterious mutations will be purged by haploid males, in our view it is not true that bumblebees are invulnerable to inbreeding depression. Indeed, a recent meta-analysis concluded that, although haplo-diploid insects suffer less from inbreeding than diploid insects, substantial inbreeding depression does occur, in one case resulting in a 38% decrease in longevity and a 32% reduction in fecundity (Henter 2003). The reduced genetic diversity of remaining populations of *B. muscorum* may therefore reduce their fitness directly, and will reduce their capacity to respond to environmental change.

The single-locus complementary sex determination (sl-CSD) system found in bumblebees presents an additional cost of inbreeding through the production of sterile diploid males (Cook & Crozier 1995). Their production in the wild has been proposed as an indicator of the vulnerability of bee populations (Zayed *et al*. 2004). For the first time in naturally occurring populations of bumblebees, we find diploid males at detectable frequencies. Although few individuals were detected, males were infrequently encountered while sampling workers, and therefore the diploid males detected represent a considerable proportion of the males collected from within those populations. It is likely that continued erosions of effective population size and genetic diversity will result in higher levels of diploid male production, with concomitant consequences for population fitness (Zayed & Packer 2001).

Conservation

In the wake of agricultural intensification, populations of many rare hymenopterans have declined and fragmented.

The distributions of bumblebees are reasonably well known, and demonstrate that remaining populations frequently survive only in small, isolated pockets of suitable habitat. Despite the habitat within these areas remaining suitable, populations within them often become extinct. For example, *Bombus subterraneus* was widespread in England during the early 1900s, occurring in a range of flower-rich habitats (Alford 1975). By the 1960s it was restricted to a few isolated coastal sites in Essex and Kent, but these populations rapidly became extinct, despite the continued suitability of the habitat. The species was last recorded in Britain in 1988 at Dungeness. Seven once widespread UK species have undergone similarly dramatic declines in recent decades, trends which are mirrored throughout Europe and North America (reviewed in Goulson 2003). The distributions of other hymenopterans are less well known, but many are considered scarce or threatened, and exist only in small isolated populations (Edwards & Broad 2005). To date, it was not known whether genetic factors might be contributing to their continued declines. Here, for the first time, we demonstrate that populations of a rare and threatened hymenopteran exhibit reduced genetic diversity, and we show that long-range dispersal is uncommon. Additionally, we detect diploid males, an indicator of inbreeding. If, as seems likely, genetic factors are accelerating the declines of social hymenopterans, many of which are important crop and wildflower pollinators (Corbet *et al*. 1991), ecosystems engineers (Jones *et al*. 1994), and natural pest-predators (Van Mele & Cuc 2000), then steps must be taken to conserve whatever genetic diversity remains.

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BD, JSE and GCL are all students of Dr Goulson and share his interest in the ecology and conservation of bumblebees. Areas of research include: the comparative population structure and conservation genetics of rare versus common bee species; foraging behaviour; nesting ecology; scent marking and dietary specialization; and the use of molecular tools to delineate species boundaries and detect cryptic species.