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Microsatellite analysis reveals the spatial dynamics of *Bombus humilis* and *Bombus sylvarum*

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Abstract

1. Substantial reductions in the distributional ranges of several species of bumblebee have been recorded in the UK. Loss and fragmentation of suitable foraging and nesting habitat to agricultural intensification is thought to be the main driving force behind declines.

2. Limited knowledge of species ecology means that effective conservation management prescriptions cannot be put into place.

3. Here we investigated the spatial dynamics of two UK Biodiversity Action Plan bumblebee species, *Bombus sylvarum* and *Bombus humilis*. For the first time, microsatellite DNA analysis was used to estimate foraging distances in rare bumblebees.

4. Sisterhoods were identified between bees sampled at discrete forage patches within a fragmented landscape. Using these sisterhoods, minimum estimates of maximum and mean foraging distances were calculated from distances separating sister bees.

5. Minimum mean foraging distances were calculated as 475 ± 97m for *B. humilis* and 231 ± 58m for *B. sylvarum*. Mean distances were significantly greater for *B. humilis* than *B. sylvarum* ($P<0.001$).

6. The differences between the spatial scales that the species were recorded over highlighted a need for further research into the spatial dynamics of rare and more ubiquitous foraging bumblebees.

Introduction

Human-driven habitat loss and fragmentation is a major concern in conservation biology and landscape ecology (Gilpin, 1987; Opdam, 1990; Hanski & Gilpin, 1991; Reed, 2004). Agricultural intensification has resulted in the fragmentation, degradation and loss of semi-natural habitats (Robinson & Sutherland, 2002). It has been hypothesised that these impacts are driving declines in the abundance and distribution of a number of British bumblebees (*Bombus* species) (Williams, 1986; Rasmont & Mersch, 1988; Osborne *et al.*, 1991; Osborne & Corbet, 1994; Goulson, 2003; Williams & Osborne, 2009). If this is the case, lost foraging and nesting habitat must be recreated and the spatial scales for such habitat creation must be appropriate to target species thereby increasing habitat connectivity and countering the effects of fragmentation (Steffan-Dewenter *et al*., 2002; Steffan-Dewenter, 2003).

Whilst some species of bumblebee remain ubiquitous throughout the British Isles, many others have been recorded as suffering national declines in their distribution and abundance (Williams, 1982; Rasmont & Mersch, 1988; Goulson *et al*., 2008; Williams & Osborne, 2009). Due to their key role as pollinators in ecosystems, these declining bees are increasingly being recognised as conservation priority species (UKBAP, 1995; UKBAP, 1999). If habitat management plans are to effectively conserve declining species, knowledge of individual species' habitat requirements is needed (Williams, 1995; Williams & Osborne, 2009). Previous studies of colony development times, foraging and nesting behaviour have demonstrated inter-species variation and it is this variation that has been linked to differential declines (summarised in Williams & Osborne 2009). Significant differences in the way bumblebee species exploit spatially variable resources have also been recorded (summarised in Goulson & Osborne, 2010). As yet, however, such studies have only been

carried out on more ubiquitous species. With common bumblebee species appearing to be relatively unaffected by habitat fragmentation, it is possible that rarer bees may have more restricted foraging ranges than more common bumblebees, driven perhaps by a greater need to maximise energy efficiency on foraging trips. Some anecdotal evidence has in fact been provided for this in the form of a study of mortality of bumblebees by road kill (Williams, 1985). If this were the case, restricted foraging range could make the bees more vulnerable to the effects of habitat fragmentation partly explaining differential declines recorded between species. Such differences in foraging range would also have implications for conservation habitat management planning.

This study focused on the spatial dynamics of the two UK Biodiversity Action Plan species, *Bombus humilis* Illiger and *Bombus sylvarum* (Linnaeus), and investigated the foraging distances of workers travelling from nest to forage patch. Traditional mark-recapture techniques for studying animal movements have been used in bumblebee studies (Kwak, 1987; Kearns & Inouye, 1993; Walther-Hellwig & Frankl, 2000; Kreyer *et al*., 2003; Wolf & Moritz, 2008) but, due to the tendency of bumblebees to be forage patch constant (Plowright & Laverty, 1984; Osborne & Williams, 2001), unless bees are marked leaving the nest, mark-recapture is an ineffective technique for investigating flight distances within the landscape. Locating bumblebee nests is a notoriously difficult task and has yet to be done with any reliability for rarer species (Fussell & Corbet, 1992; Harvey, 2000a; Carvell, 2002; Edwards, 2002; Kells & Goulson, 2003; Osborne *et al*., 2007). Additionally, attempts at attracting *B. humilis* and *B. sylvarum* queens into artificial nest boxes for controlled experimental study have been unsuccessful (Harvey, 2000a; Edwards, 2002). With mark-recapture methods being impractical, alternative methods to investigate the localised spatial patterns of bumblebees are required.

Harmonic radar has been used with great success to track bumblebees in an experimental environment (summarised in Goulson & Osborne, 2010). The technique would however be unsuitable for use in the rough grassland and scrub covered landscapes within which *B. sylvarum* and *B. humilis* are both associated (Harvey, 1999; Carvell, 2002).

An alternative technique for measuring foraging range and foraging patterns in bumblebees is the use of microsatellite genetic analysis to infer relationships between individuals sampled across a landscape (Chapman *et al*., 2003; Darvill *et al*., 2004; Knight *et al*., 2005; Herrmann *et al*., 2007). Chapman *et al*. (2003) sampled bees from habitat 'islands' within an urban landscape whereas Darvill *et al*. (2004) and Knight *et al*. (2005) sampled bumblebees at known distances within a continuous habitat. The studies used the proportion of sister pairs (bees from the same colony) at different distances to estimate foraging distances. Whilst the studies were able to demonstrate significant differences between *Bombus* species, this was only possible for those that were nationally ubiquitous species as rare species were too sparsely distributed for estimations to be derived. Thus, there is currently no evidence available on the movements of *B. humilis* and *B. sylvarum* within the landscape when foraging.

Molecular techniques have been used with *B. humilis* and *B. sylvarum* to study migratory distances and genetic isolation of populations (Ellis *et al*., 2006). This present study aimed to further develop the use of microsatellite analyses to assess the ecology of these species by investigating movements within a landscape of fragmented forage patches. In previous studies of foraging distances, bees have been surveyed in designated patches within larger diffuse areas of suitable habitat (Darvill *et al*., 2004; Knight *et al*., 2005). By contrast, this study surveyed discrete forage patches surrounded by areas of scrub and semi-natural grassland not supporting favoured forage species (Connop,

2008). Discrete forage patches were identified prior to this study during bee surveys across a range of sites in South Essex supporting *B. humilis* and *B. sylvarum* populations (Connop, 2008). These patches provided ideal locations for sampling both a large number and proportion of foraging workers to obtain an estimate of the foraging distances of *B. humilis* and *B. sylvarum* within the landscape.

Materials and Methods

DNA sample collection and processing

Sample collection

Key forage patches visited by *B. humilis* and *B. sylvarum* workers at Wat Tyler Country Park (51:32:59N, 0:30:08E), Hadleigh Castle Country Park SSSI (51:32:43N, 0:35:37E), and Canvey Northwick SSSI (51:31:23N, 0:32:20E), Essex, UK, were identified during extensive bee walk surveys in 2003, 2004 and 2005 (Connop, 2008). Patches comprised areas of suitable forage surrounded by areas of scrub and semi-natural grassland not supporting favoured forage species. Patches supported the highest numbers of each of the *Bombus* species throughout the South Essex region (Connop, 2008). Site selection for surveys was based on advice from Peter Harvey (personal communications) and habitat suitability. The forage patches identified during these surveys were mapped (Figure 1). The patches were then surveyed twice weekly during August and early September 2005. Surveys were timed to correspond with the main foraging period of the bees

(Connop, 2008). Any *B. humilis* and *B. sylvarum* workers observed foraging on these areas were caught in queen bee marking plunger cages (Kwak, 1987). Identification of the bees followed Prŷs-Jones and Corbet's (1987) Naturalist Handbook key.

Due to the conservation status of the two species, non-lethal DNA mid-tarsal clip sampling (sampling the terminal portion of the tarsus of a mid-leg) was carried out and the bees were released immediately afterwards. This DNA sampling technique was used as it was considered to have no significant impact on worker foraging ability (Holehouse *et al*., 2003). The tarsal clip methodology also served to mark sampled workers against re-sampling. Samples were immediately preserved in 100% ethanol.

In total 150 *B. humilis* and 150 *B. sylvarum* workers were sampled. This comprised thirty workers of each species from each of the following forage patches: Wat Tyler Country Park (centre patch), Canvey Wick (west and east patches) and Hadleigh Castle Country Park (benfleet and marsh patches). Thirty workers from each patch was considered to be a sufficiently large sample size to demonstrate enough genetic variability for microsatellite analysis whilst being a realistic sample size from rare bumblebee populations (personal correspondence with Jon Ellis and Ben Darvill).

Microsatellite genotyping

Prior to DNA extraction, tarsal segments were cut into smaller segments in order to improve quality of template DNA (Ellis *et al*., 2006). DNA was extracted using the HotSHOT protocol (Truett *et al*., 2000). The samples were genotyped at 11 of the microsatellite loci developed by Estoup *et al*. (1995; 1996): B10, B96, B101, B116, B118, B119, B121, B124, B126, B131 and B132. PCR

reactions were performed in 25 µl reactions containing; 15 ng DNA, 0.25 µl Thermostart DNA polymerase (Abgene Ltd, Epsom UK), 2.5 µl Thermo-Start buffer (providing 1.5 mM Mg), 0.5 µM of each primer, 0.2 mM of each d'NTP and made up with de-ionised H_2O. Primers were fluorescently labelled with FAM or HEX (Eurogentec, Southampton, UK) with multiplexes of up to three loci. The reaction cycle was: 95°C for 15 min; then 35 cycles of 95°C for 30 seconds, the annealing temperature for 45 s, and 72°C for 40 seconds, followed by an extension period of 72°C for 10 minutes. Genotypes were assessed by comparison with internal size standards (ROX400, Applied Biosystems) using a BaseStation 51 DNA fragment analyser. Allele sizes were assigned using Cartographer^{TM} (version 1.0) software (MJ Research Inc., San Francisco, CA). Identical sample controls were used throughout for each species.

Any cases of scoring ambiguity or non-amplification were reprocessed until allele sizes could be confirmed. Across both species, 270 of 4050 genotypes were retyped following the same PCR conditions to assess an error rate for allele scoring.

Data analysis

Hardy-Weinberg and Linkage Disequilibrium

Tests for linkage disequilibrium (non-random association of alleles) and departure from Hardy-Weinberg equilibrium were carried out on subsamples of workers from each of the sites using GENEPOP version 3.1 (Raymond & Rousset, 1995). Following the methodology of Chapman *et al*. (2003), twenty workers were randomly selected ten times from each site using a random number generator. This subsampling method reduced the comparison of non-independent genotypes due to

the presence of sisters in samples prior to the determination of sibships. This method was used in preference to the analysis of a single sister from each identified sister pair as it required no assumption of kinship within the dataset and was therefore not affected by the high level of falsely rejected sister pairs which would be frequent within the dataset due to the use of the most stringent resolving power for Kinship analysis. Sequential Bonferroni corrections (Rice, 1989) were applied to minimise type I errors for multiple tests.

Estimating foraging range

Microsatellite Analyser (MSA) was used to check datasets for typographical errors (Dieringer & Schlötterer, 2003). Polymorphic allele scores for each individual bee and allelic frequencies within a population can be used to calculate kinship (relationships between individual bees from the population). In this study the program KINSHIP (Goodnight & Queller, 1999) within the program KINGROUP v2 (Konovalov *et al*., 2004) was used for kinship calculations. In studies of monoandry and polyandry in European bumblebees only *Bombus hypnorum* was shown to exhibit polyandry (Estoup *et al*., 1995; Schmid-Hempel & Schmid-Hempel, 2000). For this study it was therefore assumed that *B. humilis* and *B. sylvarum* queens were once-mated and that, due to their haplodiploidy, the expected coefficient of relationship between sister bees was 0.75. KINSHIP used this correlation coefficient, with individual worker microsatellite scores and population allelic frequencies to calculate the likelihood of sibships, the null hypothesis being zero relatedness (Goodnight & Queller, 1999). Likelihood tests were performed between all individuals of the population. The accuracy with which sister-pairs were identified was maximised empirically by performing 2,000,000 simulations. The occurrence of sisters in each population was accepted at the $P \leq 0.001$ level to minimise type I errors.

Foraging ranges were estimated for each species by identifying sister pairs foraging at separate forage patches. All sister pairs identified against a *P*-value of $P \leq 0.001$ were used for the foraging distance estimates. The distance between forage patches was known from mapping data, so a theoretical mid-point nest could be calculated. This nest distance represented a minimum distance that one of the sister bees must have travelled for both bees to be present at the different forage patches. From this, a minimum estimate of maximum foraging distance and mean minimum foraging distance for each species could be calculated and compared. As the data represented discrete values, a non-parametric Mann-Whitney U exact test was used to compare the distances separating the sister pairs identified for each *Bombus* species.

Relative frequencies of sister pairs within the landscape were also compared at the 'patch' and 'site' spatial scales. Sister pairs were assigned scores dependent upon whether they were recorded at the same patch or at different patches (scores 1 or 2 respectively). As the scores represented discrete values, Mann-Whitney U exact tests were used to rank the scores and to assess whether there was a significant difference between the spatial distributions of *B. sylvarum* and *B. humilis* sister pairs. This methodology was repeated at the site spatial scale.

Estimating colony number

Total numbers of *B. humilis* and *B. sylvarum* colonies were determined by grouping all sisters identified by Kinship analysis at the $P \leq 0.001$ level across the whole study site into colony groupings. Noncircular nests (i.e. incidences when bee X was found to be related to bee Y, bee Y

was found to be related to bee Z, but bee Z was not found to be related to bee X) were found at a low frequency for both species as an inevitability of using the most rigorous significance level. In such cases, data was re-examined and bees accepted as sisters at the less stringent level of $P \leq 0.01$ (as described in Knight *et al*. 2005). When no such relationship was found between the individuals, the sisters were divided on the basis of omitting the bee collected furthest away from the other sisters. Whilst this method reduced the resolving power of the Kinship analysis, this was a rare occurrence (3.6% of *B. humilis* and 2.1% of *B. sylvarum* sister pairs) and would therefore have had little effect on the results of the colony groupings. An alternative method for establishing nest estimates, Colony v1.2 (Wang, 2004), has been demonstrated as giving more accurate nest reconstruction for haplodiploid populations (Lepais *et al*., 2010). However it is the experience of the authors that the resolving power of this programme is reduced when dealing with rare bumblebee populations with low genetic variability. Thus Kinship reconstruction was deemed to be the most appropriate method for resolving colonies within the present study.

In previous studies, attempts have been made to estimate the total number of nests at each sample site by calculating the number of nests from which no worker was sampled by fitting the colony data to a mathematical distribution model (Chapman *et al*., 2003, Darvill *et al*., 2004, Knight *et al*., 2005). However, this requires assumptions of the spatial distribution and frequency distributions of the bees to be made. The choice of distribution model is hugely influential in determining the predicted colony abundance per site and is largely based on derived conjecture rather than real evidence. For this study, therefore, total number of resolved colonies was used as a relative measure of population size rather than attempting to estimate an effective population size.

Measures of genetic variation were also calculated for each site: allelic richness and heterozygosity. Allelic richness was calculated using Fstat 2.9.3.2 (Goudet, 2001) and heterozygosity was calculated using MSA (Dieringer and Schlötterer, 2003).

Results

Amplification of loci

Of a total of 11 microsatellite loci, 8 provided reliable genotypic information and were sufficiently polymorphic on both species. B10 could not be amplified reliably and locus B119 was monomorphic for both *Bombus* species so both loci were removed from further analysis. Locus B101 was found to be monomorphic for *B. sylvarum*: it was therefore removed from further analysis but only for this species. Error rates for other loci were calculated as 2.5% for homozygotes and 3.1% for heterozygotes for *B. humilis*, compared with 2.2% and 4.4% respectively for *B. sylvarum*.

Hardy-Weinberg and Linkage Disequilibrium

After correcting for multiple tests there was no evidence of significant linkage disequilibrium between loci in *B. humilis* or *B. sylvarum*. For *B. humilis* no locus deviated significantly from Hardy-Weinberg equilibrium. However, one locus of *B. sylvarum* (B118) was found not to meet Hardy-Weinberg assumptions. Analysis with MICROCHECKER (van Oosterhout *et al*., 2004) revealed a lack of heterozygosity and evidence of null alleles for *B. sylvarum* at locus B118.

KINSHIP analysis of sibship assumes no linkage disequilibrium, no inbreeding and no mutation (Queller & Goodnight, 1989), so this locus was removed from the *B. sylvarum* dataset prior to analysis.

Foraging range

The forage patches sampled in this survey were located at a range of distances apart: from approximately 500m to 5km (Figure 1). For these analyses, type II error rates generated from the KINSHIP program were 0.38 for *B. humilis* sisters (n=150, 9 loci, type II error when $P \leq 0.001$) and 0.31 for *B. sylvarum* sisters (n=150, 8 loci, type II error when $P \leq 0.001$). In total, 55 *B. humilis* and 94 *B. sylvarum* sister pairs were identified. At these error rates it would be expected that 20 *B. humilis* sister pairs and 29 *B. sylvarum* sister pairs would be falsely rejected. Whilst this error rate is substantial and would have been increased further by genotyping errors, the error would result in the rejection of sister pairs rather than the false acceptance of sister pairs. These errors would be randomly distributed and, whilst reducing the number of sister pairs on which subsequent analyses were based, they would not be expected to bias the overall patterns of forage visitation.

To avoid sampling effort bias and enable direct comparisons of the two species, analyses were based on equivalent sample sizes of *B. humilis* and *B. sylvarum* workers sampled from the same forage patches. Results were calculated based on all sibships identified at the $P<0.001$ probability level. The maximum and mean distances apart that the sisters of each *Bombus* species were found were 5060 m and 950 ± 194 m for *B. humilis* and 4560 m and 461 ± 116 m for *B. sylvarum*. Based on these data, using a theoretical mid-point nest, a minimum estimate of the maximum distance that one of the sisters must have travelled was calculated as 2530 m for *B. humilis* workers and 2280 m

for *B. sylvarum* workers. Using the same method, minimum estimates of mean foraging distance were calculated as 475 ± 97 m for *B. humilis* and 231 ± 58 m for *B. sylvarum*. A Mann-Whitney U exact test for independent samples revealed a significant difference ($P<0.001$) between the mean distances apart that sisters of the two species were recorded. This indicated that *B. humilis* sisters were more likely to be found foraging at greater distances apart than *B. sylvarum* sisters.

The relative spatial frequency of sibships of each *Bombus* species was analysed at three spatial scales. In the first instance, patches where DNA was sampled were grouped by their relative distances apart (Figure 2). These categories were selected to correspond with sampling distances and forage distance results of other microsatellite studies (Chapman *et al*., 2003; Darvill *et al*., 2004; Knight *et al*., 2005; Darvill *et al.*, 2006). A comparison of the frequencies of *B. humilis* and *B. sylvarum* sister pairs identified at each relative distance is shown in Figure 2. *B. sylvarum* sisters demonstrated a bimodal distribution, with the majority of *B. sylvarum* sister pairs being recorded at the same forage patch or between 0-1 km apart, none were recorded 2-3 km apart, but 12 of 94 pairs were recorded at patches 3-5 km apart. In contrast, *B. humilis* sister pairs were more evenly distributed across the relative distances, demonstrating divergence between the spatial distributions of the two species.

Relative distributions were also compared at the ‘patch’ and ‘site’ spatial scales. Mann-Whitney U exact tests for independent samples revealed no significant difference ($P=0.11$) between the distribution of *B. humilis* and *B. sylvarum* sisters at a site scale, with both species much more frequently recorded at the same site than at different sites. For both species however, a proportion of sisters pairs were recorded foraging across different sites. In contrast, Mann-Whitney U exact tests revealed a significant difference ($P<0.001$) between the distributions of the two species at a patch

scale. *B. sylvarum* sisters were more frequently recorded at the same patch than at different patches whereas the frequency of *B. humilis* sisters at different patches was approximately equal to those distributed at the same patch.

Colony number

Colony numbers were fairly consistent between patches, with Canvey and Wat Tyler having slightly higher colony numbers per thirty workers than Hadleigh Castle for both *Bombus* species (Table 1). This supported the assumption that forage patches had a similar relative attractiveness to both *B. humilis* and *B. sylvarum* workers. Despite following a similar pattern between patches however, the number of colonies identified at each patch, site and over the entire study area were consistently higher for *B. humilis* than *B. sylvarum* (Table 1). In contrast to this, allelic richness and heterozygosity were greater for *B. sylvarum* (Table 1). The mean number of workers per colony was low for both *B. humilis* and *B. sylvarum*. The low occurrence of sibships within the dataset supported the use of the subsampling procedure to assess linkage disequilibrium and departure from Hardy-Weinberg.

Discussion

Microsatellite DNA analysis proved to be an effective technique for identifying relationships between *B. humilis* and *B. sylvarum* workers. Despite both species being nationally rare and the South Essex population being genetically isolated from other UK populations (Ellis *et al*., 2006) there was sufficient genetic variability within the populations to identify sibships between the bees.

Estimating foraging range

Foraging range estimates compared well with those for more ubiquitous species (summarised in Goulson & Osborne, 2010). The *B. sylvarum* mean foraging range of 231m was the lowest of any species in all studies (Goulson & Osborne, 2010), whilst 475m for *B. humilis* was lower than the lowest median foraging distance estimated for *B. pascuorum* and *B. terrestris* in urban/suburban park habitats (Chapman *et al*., 2003). Chapman *et al*. (2003) studied bumblebee foraging behaviour in a continuous patchwork of urban/suburban gardens and parks. Darvill *et al*. (2004) and Knight *et al*. (2005) investigated bumblebee movements at fixed points within areas of continuous suitable habitat. Interestingly, minimum estimates of maximum foraging distances in these studies were similar to the minimum mean foraging distances for *B. humilis* in the present study and those in Chapman *et al*. (2003), but minimum estimates of maximum foraging distances in the present study were considerably greater than those recorded by Darvill *et al*. (2004), Knight *et al*. (2005) and Wolf and Moritz (2008). This could indicate a necessity for bees to forage over greater distances in fragmented or patchy landscapes, or could represent a greater likelihood of identifying sister bees on islands of suitable habitat within landscapes featuring large areas of unsuitable foraging habitat. However, differences between the methods used for assessing foraging dynamics in this and previous studies (Chapman *et al*., 2003; Darvill *et al*., 2004; Knight *et al*., 2005; Wolf and Moritz, 2008), made it difficult to draw direct comparisons. A further study using the same sampling and microsatellite techniques used here but with *rare and more ubiquitous species* foraging on the same forage sites would need to be carried out for more definitive conclusions to be drawn.

The aim of the present study was to compare the minimum estimates of maximum foraging distance and mean foraging range of *B. sylvarum* and *B. humilis* workers within the same landscape. In a field-based experiment such as this, it was impossible to standardise the value of each patch in terms of its 'attractiveness' to foraging workers. However, the experiment was designed in a way to minimise the effects of these variables: by selecting discrete forage patches with the highest densities of floral groups on which both *Bombus* species have been most frequently recorded foraging in the region (Harvey, 1999; 2000a; Connop, 2008); and by selecting forage patches with the highest densities of both *B. humilis* and *B. sylvarum* within the study region. These design principles ensured that each forage patch represented the most attractive areas of the landscape to both *Bombus* species and that the level of attraction to each patch was relatively consistent between the *Bombus* species. It is also possible that differences in foraging distance could be affected by variation in colony number and size (Herrmann *et al*., 2007) leading to bias in the probability of detecting sister pairs within equally large samples. Nevertheless, *B. humilis* and *B. sylvarum* are considered to have similar sized colonies (Goulson, 2003) and colony development follows a similar temporal pattern, peaking in late summer (Goulson *et al*., 2005) , thus colonies would be expected to be of similar size. Moreover, if the probability of finding sister pairs was more biased towards one of the study species, it would be expected that sister pairs of one species would have been found more frequently throughout the sampling distances. This was not the case however as, of the two study species, *B. sylvarum* sisters were more frequently found within the same patch and *B. humilis* sisters were more frequently found at different patches and different sites (Figure 2). Thus, the data generated should provide an accurate representation of comparative spatial distributions of *B. humilis* and *B. sylvarum* within and between these patches.

Minimum estimates of mean foraging distance were substantially different between the two species (475 ± 97 m and 231 ± 58 m for *B. humilis* and *B. sylvarum*), with *B. humilis* sisters operating over significantly greater spatial scales than those of *B. sylvarum* ($P<0.001$). Maximum distance they were recorded apart was also greater for *B. humilis* than *B. sylvarum* (2530 m and 2280 m respectively). In addition to differences between mean and maximum foraging distances there were differences between the relative distances over which *B. humilis* and *B. sylvarum* sisters foraged (Figure 2). *B. sylvarum* sisters were significantly more frequently recorded foraging at the same forage patch, whereas *B. humilis* sisters were more evenly distributed across forage patches. For the purpose of this study, all identified sister pairs were treated independently (Figure 2). Previous studies have indicated that sister bees may forage away from their nest to avoid competition with nest mates and to reduce predation and parasitism (Dramstad, 1996). However, the proximity of nest mates whilst foraging may be dependent on worker number, forage availability and predation/parasitism pressure. Thus, if a number of sister pairs are compared from a single colony, the distances separating them may not be independent. However, in light of the ecological similarity of the two *Bombus* in terms of their colony size (Goulson, 2003), the selection of the most suitable foraging patches within the study area, the substantial number of sisters bees identified, and the randomised nature of sampling, comparative distances separating sisters in the study should be representative of foraging distributions.

The more limited distribution of *B. sylvarum* sibships when compared to *B. humilis* married well with previous studies which demonstrated inter-species differences in foraging range (Walther-Hellwig & Frankl, 2000; Darvill *et al*., 2004; Knight *et al*., 2005). If this is the case, the requirement of greater concentrations of suitable forage over smaller spatial scales in relation to *B. sylvarum* nest sites may make the species more prone to the impacts of habitat fragmentation than other

bumblebee species. This, in part, might explain why this *Bombus* species in particular has become so rare in the UK. Such findings are consistent with anecdotal evidence from Bumblebee Working Group surveys which indicated that *B. humilis* was better able to survive in smaller fragmented patches of habitat than *B. sylvarum* which seemed to have a requirement for 10km^2 of continuous suitable habitat (Edwards, 2002).

The results of the present study support the theory that *Bombus* species operate on different spatial scales with *B. sylvarum* being more restricted in its landscape movements, tending to operate within patches and particularly within sites, whereas *B. humilis* appeared to operate less within patches and more on a site scale. Nevertheless, sister workers of both *B. humilis* and *B. sylvarum* were recorded operating between sites. Indeed *B. sylvarum* appeared to have a bimodal distribution (Figure 2), with the majority of sister pairs being recorded at the same patch and at patches between 0-1 km apart, none were recorded at patches 2-3 km apart, but numbers rose again at patches 3-4 km apart. Whilst the study can provide no definitive evidence for why this might be, almost all of the sister pairs located 3-4 km apart were recorded between Wat Tyler Country Park Centre and Canvey Wick West patches (Figure 1). It is possible that the lack of suitable forage, presence of a leading-line in terms of a sea-wall, and lack of roads between these sites may have increased their landscape connectivity (Williams, 1985; Bhattacharya *et al*., 2003). Whilst this study could provide no evidence for such habitat connectivity, this occurrence of sister bees between different sites demonstrated the significance of the landscape in supporting these rare bumblebee populations, and supports previous studies which have argued the importance of a network of sites on a landscape scale for supporting populations of these bees (Hanski & Gilpin, 1991; Edwards, 2002; Steffan-Dewenter & Tscharntke, 1999; Ellis *et al*., 2006).

This study provided no evidence for the reasons behind foraging range differences. Further research is required to assess whether inter-species differences could be due to variation in worker size limiting forage ability (Steffan-Dewenter *et al*., 2002; Ings *et al*., 2005; Knight *et al*., 2005; Peat *et al*., 2005), differences in nest density and/or nest size (Knight *et al*., 2005) requiring further foraging trips for nest mate avoidance to reduce intra-colony competition, or increased need for energy efficiency caused by edge of climatic niche effects (Williams, 1988; Williams *et al*., 2007; 2009).

Colony number

The number of colonies rather than number of individuals are a measure of the effective population size of eusocial insects (Chapman and Bourke, 2001). Therefore, by calculating an estimate of the number of colonies at each patch, site and for the whole study area it was possible to identify the relative population size of *B. humilis* and *B. sylvarum* and the relative importance of each South Essex site in supporting the metapopulations of each species. Results indicated that *B. humilis* was the more common of the two species within the study area. This was the case for all sites surveyed. This corresponded to patterns observed during timed bee counts carried out across the sites in 2003-2005 (Connop, 2008) and with national patterns of decline which have recorded the distribution of *B. sylvarum* as being more restricted than that of *B. humilis* (Williams, 1982; Edwards, 2002).

Colony numbers were relatively high for both species over the entire study area when compared to national average effective population sizes for these two species calculated by Ellis *et al*. (2006). However, with sibships resolved at the $P \leq 0.001$ level leading to high type II error rates, colony numbers are likely to be inflated for both species. With colony numbers likely to be over-

estimations, the combination of national patterns of decline in the two study species (Edwards, 2002), the landscape-scale loss to development of key bumblebee habitat in the South Essex region (Harvey, 2000b), and the isolated nature of the South Essex populations when compared to estimated threshold migratory distances in bumblebee queens (Darvill *et al*. 2006; Ellis *et al*., 2006; Lepais *et al*. 2010), these populations must be considered at risk. This concern is supported by the general lack of genetic variability in terms of allelic richness recorded for both species within the study (Table 1). It is hoped that data generated within this study will act as baseline on which future population monitoring can be based.

Implications for habitat management

This study represents a snap-shot of foraging behaviour in a specific habitat type. The overarching conclusion that must be drawn from the data is the need for further research into the comparative spatial dynamics of foraging bumblebee workers, with particular focus on comparing declining species with more ubiquitous ones. Nevertheless, the results of this study represent the only current data on the spatial dynamics of foraging *B. humilis* and *B. sylvarum* workers. Until further evidence is generated therefore, the results of this study must be adopted as a precautionary principle in the design of habitat management for the conservation of these two *Bombus* species.

Genetic data generated in the present study allowed the following inferences about ecological variables of the two rare bumblebee species that would deserve to be considered in habitat management:

First, the seemingly more limited foraging dynamics of *B. sylvarum* supports the need for targeted habitat management for nesting sites in the proximity of foraging sources. Second, the occurrence

of nestmates within single forage patches highlights the need to avoid cutting or grazing management of forage patches during times of bumblebee foraging activity. This is particularly the case in *B. sylvarum* conservation areas where loss of single forage patches could have a greater impact due to the greater proportion of workers from a single colony expected to be foraging on a single patch. If habitat management must be carried out during the foraging season, it should be carried out in a mosaic pattern to ensure that not all forage from a single patch, or from within a colony's foraging range, is removed. Third, *B. sylvarum* and *B. humilis* utilise a network of forage sources over site- and landscape-scales therefore conservation of a single site might not be sufficient to support populations. A network of forage and nesting habitat at a site- and landscape-scale is required to support viable metapopulations and to buffer colonies against the effects of forage patch losses.

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Figure Legends

Figure 1. The South Essex DNA sampling sites. Sites are those from which thirty *B. sylvarum* and *B. humilis* mid-tarsal clips were taken during the 2005 DNA sampling. [Canvey = the Canvey Wick site; HCCP = Hadleigh Castle Country Park; Wat Tyler = Wat Tyler Country Park]

Figure 2. Frequency of *B. humilis* and *B. sylvarum* sister pairs across all patch distances. Sister bees are all of those determined from kinship likelihood analysis at the $P<0.001$ probability level.

Table 1. *B. humilis* and *B. sylvarum* colony structure and measures of genetic diversity within the South Essex study area. Allelic richness and H_E were calculated using all loci for *B. humilis* and all loci except B101 and B118 for *B. sylvarum*. n = sample size, H_E = expected heterozygosity.

	B. humilis	*B. sylvarum*
H_E (± S.E.)	0.46 ± 0.07	0.52 ± 0.11
Allelic Richness (± S.E.)	4.00 ± 0.34	5.57 ± 1.59
Mean number of workers/colony	1.27	1.54
Reconstructed colonies		
HCCP Benfleet (n=30)	23	21
HCCP Marsh (n=30)	23	16
HCCP All (n=60)	43	33
Canvey East (n=30)	29	26
Canvey West (n=30)	29	21
Canvey All (n=60)	56	46
Wat Tyler All (n=30)	29	23
All study area (n=150)	118	97

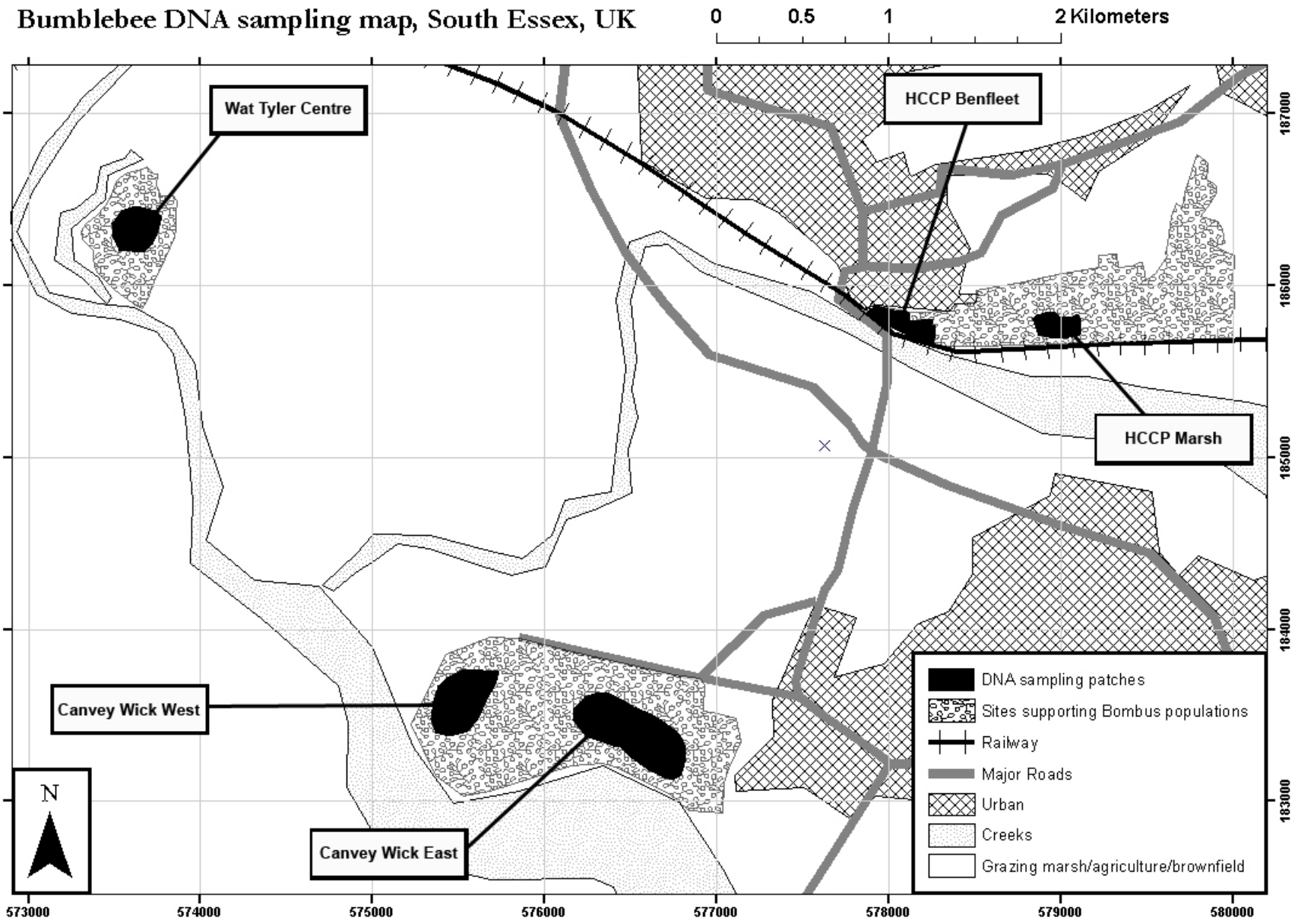
Bumblebee DNA sampling map, South Essex, UK
0
0.5
1
2 Kilometers
Wat Tyler Centre
HCCP Benfleet
HCCP Marsh
Canvey Wick West
Canvey Wick East
N
DNA sampling patches
Sites supporting Bombus populations
Railway
Major Roads
Urban
Creeks
Grazing marsh/agriculture/brownfield
187000
186000
185000
184000
183000
573000
574000
575000
576000
577000
578000
579000
580000

Figure 1.

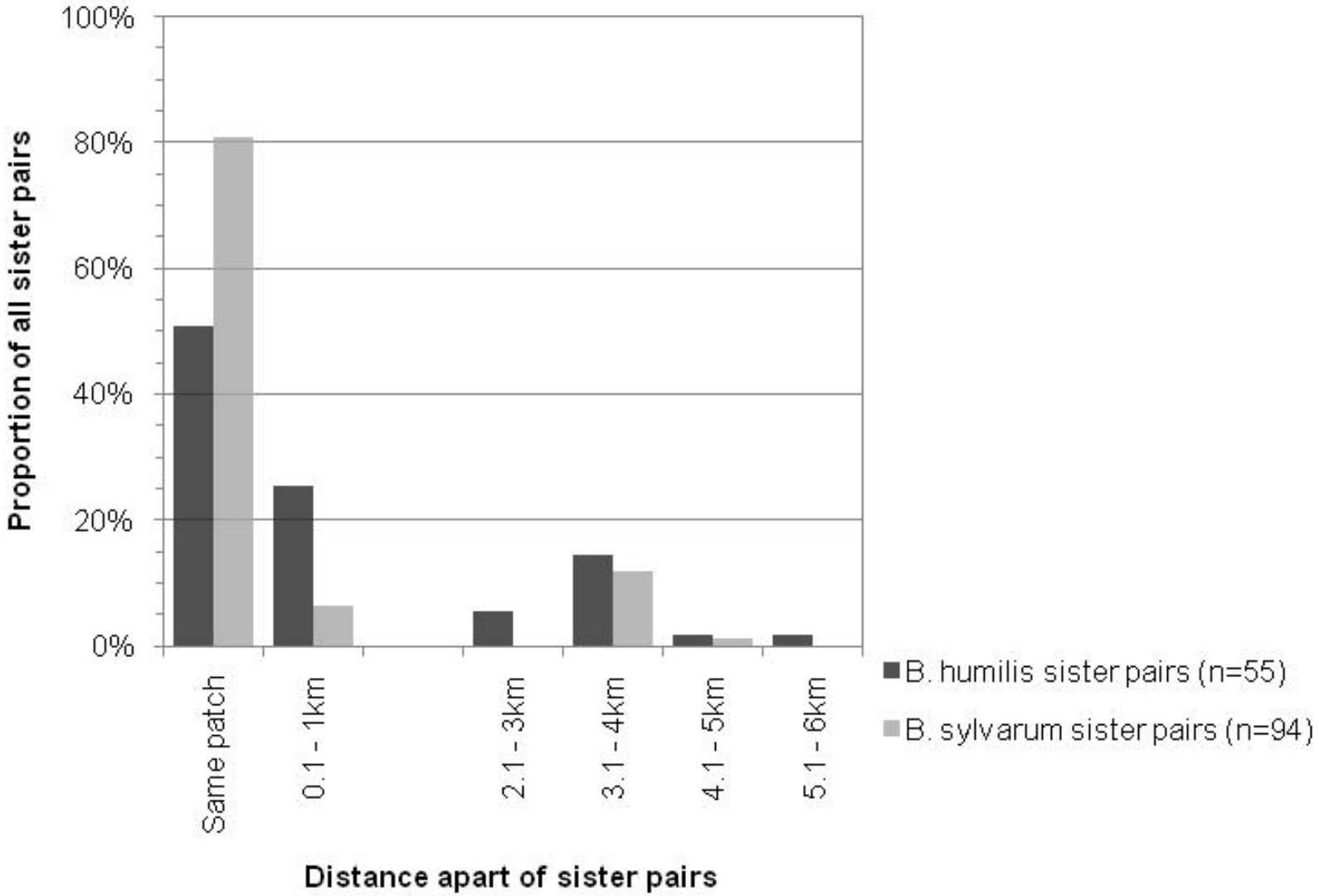
100%
80%
60%
40%
20%
0%
Proportion of all sister pairs
Same patch
0.1 - 1km
2.1 - 3km
3.1 - 4km
4.1 - 5km
5.1 - 6km
Distance apart of sister pairs
B. humilis sister pairs (n=55)
B. sylvarum sister pairs (n=94)

Figure 2.