Apidologie 38 (2007) 518–524
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DOI: 10.1051/apido:2007032

Available online at:
www.apidologie.org

Original article

Aggregations of male *Bombus muscorum* (Hymenoptera: Apidae) at mature nests. Incestuous brothers or amorous suitors?*

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Received 6 April 2006 – Accepted 20 June 2007

Abstract – Aggregations of male bumblebees are occasionally observed at the nest entrances of conspecifics. It has always been assumed that they originate from nearby nests and are hoping to mate with emerging gynes. However, it is possible that they are males from within the nest that have not yet dispersed, or are hoping to mate with their sisters. Inbreeding in Hymenoptera with single locus complimentary sex determination (sl-CSD) is costly and should be avoided. Nevertheless, other taxa with sl-CSD have been found to inbreed in this way. We use microsatellites to compare aggregating males with workers from within a *Bombus muscorum* nest. All males have genotypes inconsistent with origination from within the nest. Estimates of F_{IS} are near zero, indicating low rates of non-random mating. Clearly male bumblebees can detect mature nests, and congregate in the hope of mating with virgin queens. These data suggest that mechanisms may exist to avoid brother-sister matings at the nest, and perhaps beyond.

***Bombus* / mating / inbreeding avoidance / Hymenoptera / microsatellites**

1. INTRODUCTION

Much is still to be learned about the mating behaviour of many bumblebee species, with successful copulations only seldom observed (Goulson, 2003). A great diversity of male mating strategies exist, and are broadly grouped by Williams (1991) into 4 categories. *Patrolling* males leave scent marks on a number of prominent objects and visit them sequentially, hoping that a queen will be attracted by the pheromone. In *racing* behaviour, males choose a perch and pursue potential mates from this look-out, but do not compete with other males for preferred perches, unlike *territorial* males, who do compete. Finally, *cruising* males hover in mid-air, rather than perching, and pursue potential mates as they pass.

In addition to these strategies, a number of authors have documented the chance observation of aggregations of male bumblebees in close proximity to nest entrances (Smith, 1858; Tuck, 1897; Frison, 1930; Krüger, 1951; Alford, 1975; Lloyd, 1981; Free, 1987; Villalobos and Shelly, 1987; Foster, 1992). It has always been assumed that these males are individuals from other nests, hoping to mate with newly emerging gynes. It is, however, possible that these males have recently emerged from within the nest and are lingering prior to dispersal, or are waiting at the exit in the hope of mating with one of their sisters. One might predict that selection would act against this behaviour, due to the single-locus complementary sex determination system (sl-CSD) found in bumblebees. Brother-sister mating in monoandrous species (see Estoup et al., 1995; Schmid-Hempel and Schmid-Hempel, 2000) leads to nests producing sterile diploid-males in place of half of the work force, which is best viewed as 50%

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worker mortality (Cook and Crozier, 1995). These nests suffer significant costs and are likely to produce few reproductives, resulting in significant individual and population-wide fitness effects (Zayed and Packer, 2005). However, many more males are produced in any one season than are queens, so many males may fail to mate (reviewed in Bourke, 1997). Given that males can mate multiple times, mating with a sister prior to dispersal may well be a good insurance strategy (Cook and Crozier, 1995). Indeed, Cowan (1979) found that in the Vespid wasp, *Euodynerus foraminatus*, males routinely wait at their natal nest and mate with their sisters as they emerge. It was not clear whether the females were complicit in this behaviour, although those females that emerged in the male's absence dispersed immediately. Subsequent work has concluded that this species does have sl-CSD (Stahlhut and Cowan, 2004a), and that despite diploid male production, inbreeding is frequent (Stahlhut and Cowan, 2004b).

However, recent work suggests that, in addition to diploid male production, brother-sister mating has significant fitness costs for bumblebees. Hibernation survival and colony foundation success were both significantly reduced in inbred lines, and nest sizes were on average smaller (Gerloff and Schmid-Hempel, 2005). One might therefore predict that inbreeding would be avoided, and indeed some bumblebee species have been found to exhibit avoidance behaviour. Foster (1992) found that queens of *Bombus frigidus* and possibly *Bombus bifarius* avoided mating with nestmates based on individually-borne cues. However, the same study found that *Bombus californicus* and *Bombus rufocinctus* mated indiscriminately with nestmates. These latter species are thought to congregate at the entrances of conspecific nests and compete for access to emerging virgin queens. Foster (1992) argues that species with this mating system eject young males from the colony, and in so doing avoid inbreeding. However, this system does not preclude the possibility that males may linger in the region of the nest. Dispersal carries its own inherent costs and risks (Bengtsson, 1978), not least that no mates may ever be found.

Here we use molecular techniques to assess whether males of *Bombus muscorum* aggregated around a nest are brothers of the new gynes emerging from within, or are unrelated males from different nests.

2. MATERIALS AND METHODS

2.1. Study species

Once widespread on the mainland, *B. muscorum* (L.) now survives only in a series of small fragmented populations. It is, however, still relatively abundant on some Scottish islands (Edwards and Broad, 2005) where it thrives on heath and machair (Goulson et al., 2005). Within the UK, a number of different subspecies are recognised, differentiated on the basis of coat colour, with the race found on the island of Pabbay being *B. muscorum agricolae* (Baker).

2.2. Sample collection

On the 17th August 2003, whilst visiting the island of Pabbay (Hebrides, NW Scotland – Fig. 1) an aggregation of up to 20 male *B. muscorum* was discovered concentrated within a few square metres. Upon closer inspection it became evident that they were localised around the entrance to a mature *B. muscorum* nest. Their behaviour was quite distinctive, with each bee attempting to perch close to the nest entrance. When another bee flew close to a perched male, the in-situ male would set off in pursuit and a chase lasting several seconds would ensue. It was not possible to tell whether the objective was to drive away a competitor, or whether it was an attempt to catch and mount a potential mate. Shortly after the chase had ended one of the males would land back at a spot close to the nest.

The chance discovery of this rare phenomenon offered a unique opportunity to investigate the relatedness of the male bees to the nest. As many of the swarming males as possible were caught using a butterfly net before the disturbance caused them to disperse. Nine were caught in total. Non-lethal tissue samples were taken for DNA analysis following the method of Holehouse et al. (2003). A sample of 12 workers was then collected as they left the nest to forage. In addition, a population genetic sample was taken by collecting DNA samples from 36 foraging workers from random locations across the island.

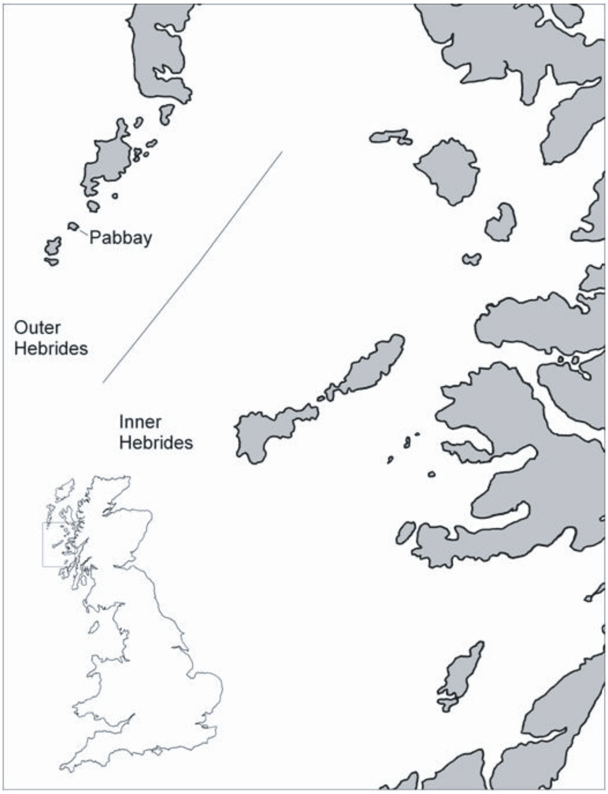

Figure 1. A map of the study area, showing the location of the island of Pabbay.

This sample was used to assess the suitability of the chosen molecular markers for use in this study, and to quantify certain population genetic parameters. Samples were preserved in pure ethanol and stored at ambient temperature.

2.3. Molecular methods

DNA was extracted using the HotShot protocol (Truett et al., 2000). Workers were genotyped at 7 microsatellite loci: B132, B118, B96, B10, B11, B124, B126 (Estoup et al., 1995, 1996). B11 was found to be monomorphic. 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 visualised on an ABI PRISM^{TM} 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 any samples that had failed to amplify clearly.

2.4. Statistical methods

The dataset was first checked for unexpected mutation steps, large gaps in the data or unusually sized alleles using MSA (Dieringer and Schlotterer, 2003). Tests for genotypic linkage disequilibrium and departure from Hardy-Weinberg equilibrium (HWE) were performed using GENEPOP version 3.4 (Raymond and Rousset, 1995). Sequential Bonferroni corrections (Rice, 1989) were applied to minimise Type I errors. Population genetic parameters were calculated using FSTAT version 2.9.3

(Goudet, 2001). The coefficient F_{IS} (Wright, 1951) was calculated using Weir and Cockerham's (1984) estimator (f).

2.5. Assessing relatedness

Bumblebees exhibit haplodiploidy, with diploid queens producing diploid workers and new queens from fertilised eggs, and haploid males from unfertilised eggs. The vast majority of bumblebees so far studied are monoandrous, with the queen mating only once (Estoup et al., 1995; Schmid-Hempel and Schmid-Hempel, 2000; Sauter et al., 2001; Payne et al., 2003). Maternal and paternal genotypes can therefore be estimated based on the frequencies of alleles in a sample of worker offspring. Parental genotypes can be deduced in most cases, with the exception being when the queen is homozygous, but for a different allele to that found in the male genotype. In this case both alleles will be present at a frequency of 0.5, and it will not be possible to determine which allele came from which parent.

As males are produced from an unfertilised egg, if any alleles are present in a male genotype that are not present in the nest-queen genotype then it is certain that the male is not the offspring of that queen. Workers are also able to lay unfertilised eggs, which develop into males, so it was also necessary to determine whether congregating males could be their offspring. Males were excluded as being potential workers-sons if they exhibited any alleles not present in the inferred queen or paternal genotypes.

3. RESULTS

Neither a global test nor tests by locus detected any significant deviation from HWE. Similarly, no two locus-pairs demonstrated significant linkage disequilibrium.

Based on the alleles found at each locus in the sample of workers, the parental genotypes were estimated (Tab. I). At locus B132, it was not possible to determine whether the queen was homozygous for allele 155 or 157, so both possibilities were considered.

The genotypes of all of the sampled males contained alleles *not* present in the maternal genotype (Tab. II) confirming that the males were not the offspring of the nest-queen. Similarly, no male had a genotype consistent with being the offspring of a worker from the nest. Within the male genotypes, the most polyallelic locus (B96) contained 4 alleles, suggesting that the males originated from at least 2 different nests.

Of the 7 microsatellite loci amplified, 6 were found to be polymorphic with a range of 2–6 alleles per locus (Tab. III). Average expected heterozygosity (H_E) for the polymorphic loci was 0.465 ± 0.12 and the estimate of F_{IS} (f) was −0.004 ± 0.039.

4. DISCUSSION

To date it was not known whether male bumblebees congregating outside mature nests were the offspring of that nest, or males from other nests in the area. In this case, none of the 9 sampled males had genotypes consistent with being the offspring of either the queen or the workers from the nest. They must, therefore, have emerged from other nests on the island, and congregated at the entrance. Analysis of their genotypes suggests that the males originated from at least 2 different nests. Males must therefore be receptive to as yet unidentified cues emitted from mature colonies.

Based on this small study it is not possible to say whether male bumblebees avoid congregating at their own nests in all cases. However, the population as a whole was found to be in Hardy-Weinberg equilibrium, and estimates of the inbreeding co-efficient (f) were very low (–0.004), both of which are consistent with the avoidance of frequent brother-sister matings. Cameron et al. (2004) recently studied male congregations of eusocial Asian stingless bees (*Trigona collina*), which are well known for gathering outside nests. They found that none of the males in drone aggregations of up to 42 males were flying in front of their natal nest. Given the high costs of inbreeding depression (Gerloff and Schmid-Hempel, 2005) and the production of diploid males (Zayed and Packer, 2005) it is perhaps unsurprising that brother-sister matings are avoided. Whether this behaviour is mediated by the willing dispersal of males or is policed by workers remains unknown. If the costs of inbreeding are sufficiently high then male bumblebees may choose to focus their

Table I. The alleles present in the sample of workers from the nest, and the parental genotypes estimated on the basis of their frequencies.

Locus	**B132**		**B118**		**B96**		**B10**		**B11**		**B124**		**B126**	
Alleles	155	157	201	203	226	228	176		128		250	254	141	
Frequency	0.5	0.5	0.75	0.25	0.625	0.375	1		1		0.25	0.75	1	
QUEEN	155/155 ***or*** 157/157		201	203	226	228	176	176	128	128	250	254	141	141
MALE	155 ***or*** 157		201		226		176		128		254		141	

Table II. The genotypes of the sampled males found aggregated around the nest.

Locus	B132	B118	B96	B10	B11	B124	B126
Male 1	157	207*	226	176	128	258*	141
Male 2	157	207*	226	176	128	250	141
Male 3	165*	203	228	176	128	250	141
Male 4	157	207*	228	176	128	250	141
Male 5	157	207*	232*	176	128	258*	141
Male 6	155	201	232*	176	128	258*	141
Male 7	157	201	226	176	128	258*	139*
Male 8	157	201	224*	176	128	256*	141
Male 9	157	201	232*	176	128	250	141

* Alleles not present in the maternal or paternal genotype which indicate that these males did not originate from the sampled nest.

Table III. Observed and expected heterozygosity for the 6 loci used, along with the number of alleles per locus and f, Weir & Cockerham's (1984) estimator of F_{IS}.

	b132	b118	b96	b10	b124	b126	Mean	Std. Error
H_E	0.413	0.597	0.721	0.237	0.769	0.053	0.465	± 0.115
H_O	0.405	0.541	0.757	0.216	0.892	0.054	0.478	± 0.130
No. of alleles	4	4	6	2	5	2	3.83	± 0.654
f	0.018	0.096	–0.051	0.089	–0.162	–0.014	–0.004	± 0.039

efforts elsewhere. Bumblebees are known to mark their nest entrances with colony-specific odours (Foster and Gamboa, 1989; Pouvreau, 1996) and one function of this may be to help males avoid congregating at their own nests.

As part of a wider study of the population genetics of *B. muscorum* (Darvill et al., 2006), samples from several small isolated islands were collected. Some islands were as small as 0.5 km^2, but despite this no diploid males were found on most islands. It is possible that diploid male producing nests are very short-lived and therefore diploid males are at very low frequency. However, it is also possible that the absence of diploid males may be indirect evidence for an inbreeding avoidance mechanism. In the field cricket (*Gryllus bimaculatus*), females reared in isolation have been found to show a clear preference for mating with unrelated individuals, with mating preference increasing as relatedness decreased (Simmons, 1991). Simmons (1989) suggests that females use their own cuticular compounds as a template. Pheromone composition is known to correlate with kinship in primitively eusocial sweat bees (*Lasioglossum* spp.) (Smith and Ayasse, 1987). It would be of great interest to determine whether bumblebees are similarly able to determine relatedness on the basis of cuticular compounds and avoid inbreeding on this basis.

ACKNOWLEDGEMENTS

We thank the Leverhulme Trust (Grant No. F/00180/M) for providing funding to support this work. The National Trust for Scotland for allowing access to the field site; Mike Edwards and Murdo MacDonald for advice; and Lara Derwent for carrying out DNA extractions.

Les agrégations de mâles de *Bombus muscorum* (Hymenoptera : Apidae) devant les entrées de nids. Frères incestueux ou soupirants amoureux ?

***Bombus* / accouplement / évitement de la consanguinité / microsatellite**

Zusammenfassung – **Aggregationen von *Bombus muscorum*-Männchen (Hymenoptera: Apidae) vor Nesteingängen. Inzestuöse Brüder oder liebestolle Freier?** Gelegentlich findet man Ansammlungen von männlichen Hummeln an den Ausgängen von voll entwickelten Hummelnestern. Obwohl dies keine bevorzugte Paarungsstrategie der Hummeln darstellt, wurde trotzdem angenommen, dass diese Männchen von anderen Nestern der Umgebung stammen in der Hoffnung, sich mit einer unbegatteten Königin zu paaren, wenn diese das Nest verlässt. Es gibt aber auch die Möglichkeit, dass diese Männchen aus eben diesem Nest stammen und vor dem Ausfliegen hier verweilen, möglicherweise in der Hoffnung sich mit einer ihrer Schwestern zu paaren. Es wird allerdings angenommen, dass sich Inzucht gerade bei Hymenopteren negativ auswirkt und daher vermieden werden sollte. Trotzdem ist bei verschiedenen Hymenopteren-Arten die Paarung zwischen Geschwistern weit verbreitet.

In dieser Arbeit verwendeten wir genetische Marker um festzustellen, ob Männchen, die sich um ein reifes Nest herum sammeln, mit den Individuen innerhalb des Nestes verwandt sind. Die Untersuchungen wurden an zufällig gefundenen Nestern auf einer isolierten schottischen Insel durchgeführt. Zusätzlich überprüften wir anhand des Inzuchtgrades innerhalb der gesamten Population, ob Geschwister-Paarungen üblich sind. Unsere Ergebnisse lassen darauf schließen, dass keines der vor den Nesteingängen versammelten Männchen aus dem jeweiligen Nest stammt. Die Männchen müssen daher aus der Umgebung durch bisher unbekannte Reize angelockt werden. Der Inzuchtgrad innerhalb der Population lag nahe Null, was mit den sehr seltenen Bruder-Schwester-Paarungen übereinstimmt. Unsere Ergebnisse lassen vermuten, dass die Mehrzahl der Männchen sich vom Nest entfernt, um Paarungen mit den eigenen Schwestern zu vermeiden. Wir diskutieren frühere Arbeiten bei verwandten Taxa, die eine bevorzugte Paarung mit nicht verwandten Partnern nachgewiesen haben. Wir vermuten, dass Hummeln in der Lage sind, den Verwandtschaftsgrad anhand von kutikulären Duftstoffmustern festzustellen und diese Fähigkeit zur Vermeidung von Inzucht verwenden.

***Bombus* / Paarung / Inzuchtvermeidung / Hymenoptera / Mikrosatelliten**

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