Non-destructive DNA sampling from bumblebee faeces

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Abstract

Genetic studies provide valuable data to inform conservation strategies for species with small or declining populations. In these circumstances obtaining DNA samples without harming the study organisms is highly desirable. Excrements are increasingly being used as a source of DNA in such studies, but such approaches have rarely been applied to arthropods. Bumblebees are ecologically and economically important as pollinators; however, some species have recently suffered severe declines and range contractions across much of Western Europe and North America. We investigated whether bumblebee faeces could be used for the extraction of DNA suitable for genotyping using microsatellite markers. We found that DNA could be extracted using a Chelex method from faecal samples collected either in microcapillary tubes or on filter paper, directly from captured individuals. Our results show that genotypes scored from faecal samples are identical to those from tissue samples. This study describes a reliable, consistent and efficient non-invasive method of obtaining DNA from bumblebees for use in population genetic studies. This approach should prove particularly useful in breeding and conservation programs for bumblebees and may be broadly applicable across insect taxa.

Introduction

Molecular genetic techniques are now commonly used to address questions in conservation, population and behavioural studies. For insects, these techniques have mostly been based on destructive methods that require the insect to be sacrificed. In population studies, genetic analysis can require sampling large numbers of individuals, which may reduce subsequent

population size or alter the population structure (Starks & Peters 2002). This is particularly undesirable when studying small or declining populations, yet often these are the ones of most interest (Hamm *et al.* 2010). In social insect species with large colonies, workers may be sampled with little impact on colonies, but for species such as bumblebees with small colony sizes the removal of workers is likely to reduce colony performance (Schmid-Hempel *et al.* 1993). In addition destructive methods are highly unsuitable for genotyping queens that are destined to found colonies (Chaline *et al.* 2004).

Bumblebees (*Bombus*: Hymenoptera, Apidae) are ecologically and economically important as pollinators (Goulson 2010; Velthuis & Van Doorn 2006). Some species have recently suffered severe declines and range contractions across much of Western Europe and North America (Cameron *et al.* 2011; Goulson *et al.* 2008). In the UK, seven out of the 27 species are listed on the Biodiversity Action Plan (BAP), a higher proportion than any other invertebrate group (Goulson 2010). Being social insects, bumblebees can have very small effective population sizes and suffer from population fragmentation and isolation (e.g. Estoup *et al.* 1996; Ellis *et al.* 2006; Goulson *et al.* 2011), which makes the conservation genetics of this group of particular interest and concern. Molecular tools have also proved to be useful in studying intractable aspects of bumblebee ecology, such as quantifying nest density, nest survival, and dispersal distances (Knight *et al.* 2005; Goulson *et al.* 2010). Non-destructive sampling would therefore be valuable in studies of bumblebees, especially of rare species and of queens involved in captive breeding or re-introduction programs. Any such sampling method should not interfere with the queen's ability to mate (Chaline *et al.* 2004), forage or found a colony.

A number of techniques have been used to non-lethally sample insect DNA such as extracting haemolymph from the defensive secretion of the forked fungus beetle, *Bolitotherus cornutus* (Donald *et al.* 2012), tibia removal in damselflies (Fincke & Hadrys 2001) and eusocial wasps (Starks & Peters 2002), wing clipping in butterflies (Hamm *et al.* 2010) and honeybees (Chaline *et al.* 2004) and tarsal clipping in bumblebees (Holehouse *et al.* 2003). Holehouse *et al.* (2003) do not recommend wing clipping as a method of non-lethally sampling DNA in bumblebees as reducing wing area most probably has an effect on flight ability and overall performance. On the other hand tarsal clipping was recommended as no significant effects on workers were detected but they concede that their analyses had relatively low power and a more extensive study could reveal significant effects of tarsal sampling. It seems likely that tarsal clipping may have more impact on queens. Bumblebee queens raise the first brood of workers alone, making this early stage in the life cycle, when she must incubate the brood but also forage regularly to provide a sufficient supply of pollen and replenish her nectar reserves, one of the most precarious (Goulson 2010). Moreover, there are situations when sampling of queen DNA is needed, such as when attempting to quantify queen dispersal (Lepais *et al.* 2010), or during reintroduction programmes.

Faeces have been shown to have the potential to provide a suitable source of DNA for genotyping individuals in mammals (Frantz et al. 2003; Goossens et al. 2000;Taberlet *et al.* 1997), birds (Idaghdour *et al.* 2003; Regnaut *et al*. 2006) and reptiles (Jones *et al.* 2008) but such non-invasive approaches have rarely been applied to studies of invertebrates. Monroe *et al.* (2010) found faecal pellets and shed exuviae from dragonfly larvae did not provide high enough quality DNA for microsatellite analyses but the frass of a phytophagous weevil, *Ceutorhynchus assimilis* (Fumanal *et al.* 2005), scarab beetles (Lefort *et al.* 2012) and butterfly caterpillars (Feinstein 2004) have been successfully used to differentiate between

morphocryptic entities and identify larvae to species. However, these studies used mitochondrial DNA and did not study genetic differences between individuals.

The purpose of this study was therefore to determine whether bumblebee faeces could be used for the extraction of DNA suitable for genotyping individuals with microsatellite markers for use in population genetic studies.

Materials and methods

Sampling

The common Palearctic bumblebee species *Bombus terrestris* queens and workers collected in and around Stirling were captured and maintained in ventilated, clear plastic containers with access to sugar water. These containers had been cleaned with bleach, to ensure they could not be contaminated with DNA from other individuals, and were checked for faeces several times a day. A single faecal sample, usually all that is required, can be obtained rapidly, usually within 30 minutes of capturing an individual. Retaining individuals in this study allowed us to collect multiple samples per individual and thus assess the repeatability of our results.

Several sample storage, DNA extraction and amplification methods were used to determine which were the most suitable. Two methods of faecal collection were tested (i) using microcapillary tubes and (ii) using filter paper. The drops of liquid that form bumblebee faeces were drawn up into sterilised capillary tubes by capillary action, or gentle sucking if necessary, and then sealed with electrical tape at either end. These were used in an extraction protocol either fresh or stored immediately at -18°C. Otherwise, drops were absorbed onto

small strips of Whatman Grade 3 filter paper, approximately 2-2.5cm x 0.5-1cm. Each strip was placed into an Eppendorf tube ensuring no contamination. They were then either used in an extraction protocol fresh or allocated to one of three storage methods: (1) immediate storage at -18°C, (2) in 0.5 or 1ml of absolute ethanol at room temperature or (3) dry (dried overnight) at room temperature. In order to determine whether a single filter paper sample could be used for several extractions, some were cut in half or quarters before extraction was carried out.

DNA extraction and amplification

Two methods of DNA extraction were tested (i) using a HotShot protocol (Truett *et al.* 2000) and (ii) a Chelex® 100 protocol (Walsh *et al*. 1991). For the extractions from capillary tube samples, the faeces were gently blown from the microcapillary tubes into an eppendorf tube. Extractions from filter paper samples were carried out directly on the strips of filter paper. When testing the HotShot extraction protocol, different amounts of the buffers were tested according to the nature of the sample: 100 µl or 200 µl of both the alkaline lysis reagent and the Tris HCl buffer for the filter paper samples and 35 µl or 75 µl of each buffer for the microcapillary tube samples. All samples were incubated in the alkaline lysis reagent at 95°C for 30 min before the addition of Tris HCl buffer. In the Chelex extractions of capillary tube samples 200 µl of 5% Chelex solution, 7 µl Dithiothreitol and 2µl proteinase K were used per sample. These volumes were doubled for the filter paper samples. All samples were incubated at 56 °C for 70 min and then centrifuged at 14,000 rpm for 3 min. One hundred µl of supernatant was placed into new tubes and incubated for a further 10 min at 95 °C. DNA from tarsal tips of the queens and workers that produced the faecal samples was used to verify that

the genotypes obtained from the faecal samples were correct. This was extracted using the Chelex method under the same conditions as for the microcapillary tube samples.

To investigate the effectiveness of the different collection, storage and extraction methods we initially amplified a single microsatellite locus (B118; Estoup *et al.* 1995; Estoup *et al.* 1996) for all sampled individuals under the same conditions. PCR was performed in a reaction volume of 10µl containing 1 or 2 µl of template DNA, 0.2 µM of the primer, 1x QIAGEN Multiplex Master Mix and 0.5x Q-solution. All reactions were initially heated to 95°C for 15 minutes to activate the HotStarTaq DNA polymerase, before 35 cycles of 94°C for 30 s, 49°C for 90s and 72°C for 90 s followed by a final extension period of 10 min at 72°C. Amplification success was determined by electrophoresis on 2.5% agarose gels.

Tarsal tip and faecal DNA from 23 individuals that successfully amplified with B118 was then genotyped at 4 microsatellite loci: B118, B124, B11 and B10 (Estoup *et al.* 1995; Estoup *et al.* 1996). Multiplex PCRs were performed using QIAGEN Multiplex PCR Kits. Each 10µl reaction volume contained 1x QIAGEN Multiplex Master Mix, 0.5x Q-solution, 0.2µM of primers for the loci B118, B124, B11 and 0.4µM of primers for B10 (all with the forward primer fluorescently labelled), and 2µl of template DNA. The thermocycler conditions were the same as for amplification of the single locus B118. All PCR reactions were performed using both negative (water) and positive controls (DNA extracted from worker wing muscle using HotShot technique). PCR products were analysed on a 3730 automated capillary DNA sequencer (Applied Biosystems) and scored with reference to an internal size-standard (GeneScan500 ROX; Applied Biosystems Inc.) using GeneMarker software version 1.97 (SoftGenetics). Amplification and analysis was carried out twice for each faecal sample to check for consistency.

Results

The Chelex 100 extraction method allowed amplification of the B118 locus from fresh samples collected on filter paper and using capillary tubes (12/13 fresh samples), whereas the amplification of DNA extracted using the HotShot method yielded very poor results regardless of the volume of buffers used (2/12). Using 2 µl of template DNA appeared to yield more PCR product than just 1 µl. Given that both sample collection methods gave positive results when amplifying a single microsatellite locus, it was decided to use the simpler method, filter paper, as the collection method for the subsequent samples.

After storage on filter paper at -18 °C, preliminary testing showed amplification of the microsatellite locus B118 to be successful (10/10) as was microsatellite amplification when a half or a quarter of a filter paper sample was used for the extraction. Dry storage of the samples at room temperature was not successful; none of the eight samples that were tested amplified.

Following microsatellite analysis at four loci, samples collected on filter paper or in capillary tubes and extracted immediately gave 100% and 80% successful amplification at all loci respectively (Table 1) after a single amplification. Storing filter paper samples at -18 °C was revealed to be the most effective storage method (Table 1). Only 45% of samples stored in 1 ml of 100% ethanol for two weeks could be genotyped at all four loci after two repeats, compared to 100% of samples frozen for two weeks. None of the samples stored in 0.5 ml of ethanol could be correctly genotyped. Four of five samples stored frozen for two months amplified successfully at all four loci with two repeats. Using fragments of each filter paper sample did not reduce the genotyping success with 100% accuracy at all loci after a single amplification.

As several faecal samples from each individual, as well as tarsal tips, were genotyped to test the different methods, we were able to verify the reliability of genotypes obtained from the faeces samples and show that the quantities of DNA obtained from the fresh and frozen samples did not cause allelic dropout during the amplifications as can sometimes occur when using very small amounts of DNA (Taberlet & Luikart 1999). All of the positive controls amplified successfully and the negative controls were always 'blank'. Sufficient DNA was extracted using the Chelex protocol from both filter paper and capillary tube samples to perform at least 50 PCR amplifications.

Discussion

These results show that it is possible to extract DNA from bumblebee faeces using standard and simple techniques and that the quality of the DNA is high enough to allow PCR amplification of microsatellites permitting reliable genotyping of individuals.

We found that DNA could be extracted from faecal samples collected in either microcapillary tubes or on filter paper, but the latter was much easier. The microcapillary tubes were more difficult to fill and to seal and very easy to break unintentionally, which consequently means that they would require careful storage and be more problematic to transport than samples on filter paper. The best results were achieved with DNA obtained from samples freshly collected on filter paper strips and extracted using the Chelex extraction method. Samples collected on filter paper strips can be stored frozen and still yield accurate results but the success rate may decrease with the length of storage time, testing with a larger sample size would verify this. The filter paper strips can also be divided into fragments (halved or quartered) before extraction without any negative impact on amplification success.

We obtained these positive results using very simple and inexpensive extraction methods. Further testing using more advanced extraction approaches, such as column-based techniques, could improve the method, potentially permitting consistent DNA extraction from ethanol-stored samples or the amplification of other molecular markers with alternative applications.

In this study, individual bumblebees were captured and faecal collection was carried out in the laboratory. This is, however, not a requirement; individuals may be captured and held in small containers in the field until they defecate, whereupon the faecal samples can be collected using the preferred method. If microcapillary tubes are kept sealed or filter paper samples prevented from drying out in sealed tubes, they can be kept for several hours in this way before freezing. However, this method would probably not be suitable for sampling in remote situations where access to a freezer was not available.

This study describes a reliable, consistent and efficient non-invasive method of obtaining DNA from bumblebees. Although excrements are increasingly being used as a source of DNA in molecular and ecological studies (Beja-Pereira et al. 2009), such approaches have rarely been applied to arthropods. These results demonstrate that this procedure is effective both in terms of amplification success and scoring reliability. This method is ideal when no impact on survival or behaviour is required making it a particularly useful approach in breeding and conservation programs. Despite Monroe *et al*. (2010) failing to obtain DNA of sufficiently high quality for genotyping from non-invasive samples from the dragonfly, *Somatochlora hineana*, we have shown that it is possible for bumblebees and therefore it seems likely that the approach may also be applicable to other insect species.

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328 Table 1. Success rate of amplification of all four microsatellite loci for each preservation
329 technique tested after each repeat. The cumulative total is the sum of the success rate for both
330 repeat amplifications combined.

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Sample Treatment	Number of samples	Genotyping success (%)		
		Repeat 1	Repeat 2	Cumulative Total
Fresh filter paper samples	7	100	100	100
Filter paper stored frozen for 2 weeks	17	76	76	100
Filter paper stored frozen for 2 months	5	60	80	80
Filter paper stored in 1 ml ethanol for 2 weeks	11	45	45	45
Filter paper stored in 0.5 ml ethanol for 2 weeks	3	0	0	0
Half or quarter filter paper fragments stored frozen for 2 weeks	8	100	100	100
Fresh capillary tube samples	5	80	80	80
Tarsal samples	9	100	100	100

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