

Non-destructive sources of DNA used to genotype honey bee (*Apis mellifera*) queens

Pamela G. Gregory* & Thomas E. Rinderer

United States Department of Agriculture, Agricultural Research Service, Honey bee Breeding, Genetics & Physiology Laboratory, 1157 Ben Hur Road, Baton Rouge, Louisiana 70820-5502, USA

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Abstract

We describe a method for genotyping honey bee queens *Apis mellifera* L. (Hymenoptera: Apidae), using biological materials that are normally cast off during development (larval and pupal exuviae), or can be removed without apparent damage to queen longevity or acceptability to workers (wing clippings). Highly polymorphic microsatellite loci were successfully amplified from DNA from all of these sources, although with differing degrees of success. DNA was extracted using a simple Chelex 100® boiling procedure. Four microsatellite primers were used to amplify the DNA, and the PCR products were visualized on an ALFexpress Automated Sequencer. Genotypes created from these sources were consistent with those originating from tarsal tissue. Successful retrieval and amplification of DNA from the exuviae from immature queens allows potential breeding individuals to be genotyped and selected before they become adults. This procedure may therefore have value as DNA marker-assisted breeding programs are developed for honey bees.

Introduction

Honey bees *Apis mellifera* L. (Hymenoptera: Apidae), like other organisms beneficial to man, have been the target of genetic improvement through classical breeding. Breeding bees is hindered, however, by the labor-intensive processes of rearing and mating queen bees, and establishing colonies founded by those queens even before the new colonies can be tested for traits of interest. Early detection of desirable genes in queens would be beneficial, because the steps of mating queens and establishing test colonies could be minimized or even bypassed. Recent advances in knowledge of molecular genetics of bees underscore the potential for using genetic markers to select favored traits before much effort is expended on genetically inferior queens. Quantitative trait locus studies, for example, have been carried out on foraging (Hunt et al., 1995; Page et al., 2000), stinging (Hunt et al., 1998; Guzman-Novoa et al., 2002), learning (Chandra et al., 2001), and hygienic behavior (Lapidge et al., 2002). Mapping of honey bee genes has begun with the sex determination locus (Hunt et al., 1994; Beye & Moritz, 1996; Hasselmann et al., 2001). Beye et al. (2003)

have identified the gene responsible for sexual development in the honey bee; this is critical because of the deleterious effects of inbreeding in bees during selection programs (Mackensen, 1951). Furthermore, the Human Genome Sequencing Center at Baylor College of Medicine released the genome assembly of the honey bee in January 2004, and this will be important in future breeding applications.

Larval and pupal exuviae from queens could provide DNA for the detection of molecular markers. Queens, like other bees, are reared in individual cells in the beeswax combs of the honey bee nest. As the larval queen molts, its shed skin is pushed back toward the base of the cell where it mixes with yellow feces and can be found later as a light yellow flake (Bertholf, 1925). The pupal queen similarly sheds its skin, and creates the exuviae of a mature queen bee. Although the skins are mostly non-cellular, Bertholf (1925) described cellular components being shed during molting. The exuviae of both the larva and pupa are easily retrieved without damaging the developing queen.

We studied the reliability of the larval and pupal exuviae and wing clippings from adult queens as sources of DNA. Obtaining tissues as DNA sources from queens is problematic, because worker bees in a colony readily kill injured or inferior queens (Laidlaw & Eckert, 1962). However, the wings

*Correspondence: E-mail: pgregory@msa-stoneville.ars.usda.gov

of adult queens are commonly clipped by beekeepers to permanently mark the queen in a colony, and worker bees do not reject such queens. Wing clippings may thus provide a benign source of DNA for marker-assisted selection, although their genetic analysis would be delayed for several weeks until the adult queens completed their mating flights.

We chose to use Chelex 100® as a DNA extraction medium, and used the protocols of Singer-Sam et al. (1989) and Walsh et al. (1991) that were designed for forensic type samples. This extraction procedure was appealing because we only expected to harvest small amounts of DNA from the exuviae and wing pieces. The Chelex process is performed in a single tube and so prevents a loss of DNA that might occur during transfers. Four microsatellite regions of DNA from these three sources of DNA from each queen were amplified, and the genotypes for each source were compared to those created from the tarsal tissue of each queen. DNA from the tarsus served as a positive standard; other studies have routinely used the hind leg for genotyping honey bees (Franck et al., 2001; Clarke et al., 2002). Here we used only the tarsus, because we did not expect much DNA from wing and exuviae samples; thus, the amount of DNA extracted from the tarsus more realistically reflected the quantity of DNA that would be obtained from the innocuous tissues. For breeding purposes it is best not to use queens with damaged tarsi because they are more likely to be eliminated or superseded (Woyke, 1988; Lensky & Slabezki, 1981).

Materials and methods

Forty-two queen cells were produced from brood grafted from 8 widely varied stocks (Table 1). Newly grafted cells were placed in a cell builder for 24 hours then placed in a swarm box. By the 7th day, the cells were capped by the nurse bees, removed from the swarm box and placed in an incubator (35 °C, 50% r.h.) with each cell inserted into a vial. Eight days after grafting, the cells were hinged open and the light yellow exuviae from the last larval instar was removed and frozen at -20 °C for DNA extraction. After removal of exuviae of a single larva the cell was put back together for the pupa to finish development and emerge from the cell as an adult. Twelve days after grafting adult queens emerged from their cells. Approximately half of both of the wings from one side were clipped, one hind tarsus was removed and the pupal exuvia (which can also be found at the base of the cell as a light yellow flake) was collected. Each tissue was placed in a separate microcentrifuge tube and frozen at -20 °C.

Using a modification of the Chelex 100® method described by Walsh et al. (1991), DNA was extracted from the exuviae

Table 1 Stocks (variety) and number (n) of queen bees genotyped

| Variety | n |
|---------------------|----|
| Buckfast | 4 |
| Italian | 4 |
| Koehnen | 4 |
| New World Carniolan | 4 |
| SMR | 2 |
| Russian | 16 |
| Wooten | 4 |
| Yugoslavian | 4 |
| Total | 42 |

of the mature larva (LE), exuviae of the pupa (PE), wing clippings (WC), and tarsus (TAR). Each sample was placed in a separate 0.5 ml microcentrifuge tube. A tube with a sample and a 0.5 ml pestle were dipped into liquid nitrogen, the frozen sample was ground, 60 µl of 20% Chelex 100® (Bio-Rad, Hercules, CA) was added, and the mixture was ground again. The Chelex was transferred with a wide bore, 200-µl micropipette tip while a stir bar kept the resin beads suspended. The sample was vortexed, incubated in a dry block bath incubator (Fisher Scientific, Pittsburgh, PA) at 56 °C for 30 min, vortexed again, incubated in a dry block bath at 99 °C for 8 min, centrifuged at 14 000 g for 3 min, and then frozen.

The amount of DNA in one µl was determined from 10 different queens for each tissue (LE, PE, WC, and TAR) using a Genesys 5 spectrophotometer (ThermoSpectronic, Rochester, NY). The concentration was calculated by the following equation: $[DNA] = (\text{Absorbance}_{260}) \times (\text{dilution factor}) \times (\text{extinction coefficient } 0.050 \mu\text{g/ml for ssDNA}) \times \text{optical path length (1 cm)}$.

Approximately 2.7–10 ng of template DNA from the larval and pupal exuviae, wing clippings, and tarsus of each queen were amplified using the following four microsatellite loci primers: A107, A14 (Estoup et al., 1994), IC1 (Rowe et al., 1997), and A81 (Rinderer et al., 1998). The 5' end of the forward primer of each microsatellite was labeled with Cy5 for fragment detection in an ALFexpress Automated Sequencer (Amersham Biosciences, Piscataway, NJ). Polymerase chain reactions (PCRs) were carried out in 10-µl volumes containing 0.6 µl of template DNA, 164 µM dNTP mix (Invitrogen, Carlsbad, CA), 0.8 µM of each forward and reverse primer, 1.5 mM MgCl₂, 100 ng bovine serum albumen, 10 × reaction buffer (Invitrogen, Carlsbad, CA), and 0.2 units of Taq polymerase (Invitrogen, Carlsbad, CA). After a denaturing step of 3 min at 94 °C, the samples were cycled for 30 or 35 times (see Table 2) through denaturing (94 °C for 45 s), annealing (for 45 s; see Table 2)

Table 2 Primer sequences and PCR parameters for annealing temperature and number of cycles for four microsatellite loci

| Locus | Sequence of primers | Annealing temperature (°C) | Number of cycles |
|-------|--|----------------------------|------------------|
| A107 | 5'-GCCGTGGGAGGTTTATTGTTCG-3' 5'-CCTTCGTAACGGATGACACC-3' | 50 | 35 |
| A81 | 5'-GCCGAGTTCTTCGACTCCC-3' 5'-GGACTTTGCCAAATGGGTC-3' | 55 | 35 |
| A14 | 5'-GTGTCGCAATCGACGTAACC-3' 5'-GTCGATTACCGATCGTGACG-3' | 58 | 30 |
| IC1 | 5'-GGTTTGATGCTCGTAAGGG-3' 5'-GGCACCTCTTGCCATCTG-3' | 58 | 30 |

and extension (72 °C for 45 s) steps. A final extension of 72 °C for 10 min followed the last cycle.

PCR products of the four tissues (LE, PE, WC, and TAR) were run on 5% Page + polyacrylamide gels (Amresco, Solon, OH) in the automated sequencer. A sequencing reaction of M13 DNA was used to calibrate the fragment sizes as well as an internal sizer (100, 150, 200, or 250 bp) in each lane with the PCR product. Amplified DNA fragment sizes were determined using DNA Fragment Manager V1.2[®] (1995) a computer software program from Pharmacia Biotech.

Results

The mean amount of DNA was 16.9 ng μL^{-1} in LE, 13.3 ng μL^{-1} in PE, 7.5 ng μL^{-1} in WC, and 4.5 ng μL^{-1} in TAR. Microsatellite loci were successfully amplified for all four tissues at the four loci examined (Figure 1). However, there was variation among the tissues and primers in the number of samples successfully amplified (Table 3). Virtually all of the WC and TAR samples were successfully amplified for each of the four primers tested. There was variability in the success of amplification of LE and PE for different primers. LE and PE amplified as well as WC for primer A81 and almost as well for primer IC1. For primer A14, DNA did not amplify for 17% of the LE and 39% of the PE samples. LE and PE responded similarly for primer A107, for which approximately 38% of the samples failed to amplify. For the 28 queens from which we obtained both LE and PE, we found that either one or both of these tissues successfully amplified for 82% of the queens for primer A107, 96% of the queens for primer A14, and for 100% of the queens for primers A81 and IC1.

PCR reactions were re-done for the six LE samples that did not amplify initially for primer A14, along with other samples that had been amplified previously, and four of these six were successfully amplified. Sometimes there were false negatives; however, no false positives were detected when previously amplified products were used in the new PCR and electrophoresed. When amplification

occurred, the products were of the same size as the TAR control. No re-runs were incorporated in Table 3, and therefore the 'percentage amplified' is an underestimate.

Discussion

DNA was obtained easily and without significant injury to honey bee queens by extracting exuviae from immature stages and wing pieces from adults. The Chelex extraction method proved to be easy, inexpensive, fast (requiring

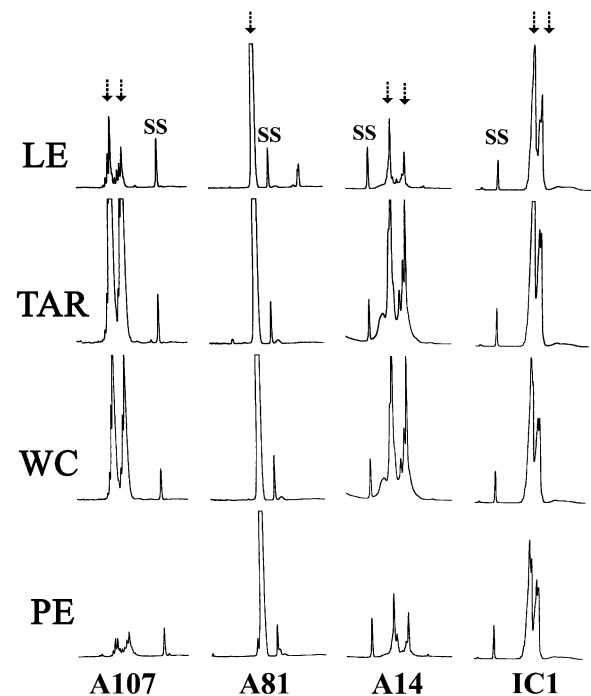


Figure 1 Electropherogram showing amplified alleles at four microsatellite loci (A107, A81, A14, and IC1) for each DNA source. Each peak representing an allele is indicated with an arrow, and the SS peaks correspond to the size standard. The other annotations represent larval exuviae (LE), pupal exuviae (PE), wing clippings (WC), and tarsus (TAR).

Table 3 Amplification success for DNA extracted from four sources [larval exuviae (LE), pupal exuviae (PE), wing clippings (WC), and tarsus (TAR)] for four microsatellite loci (A107, A81, A14, and ICI) (see Table 2 for microsatellite primer information). LE + PE represents successful amplification of one or both of these tissues for 28 queens

| Tissue | A107 | | | A81 | | | A14 | | | ICI | | |
|---------|------|------------------|-------------|-----|------------------|-------------|-----|------------------|-------------|-----|------------------|-------------|
| | n | Number amplified | % amplified | n | Number amplified | % amplified | n | Number amplified | % amplified | n | Number amplified | % amplified |
| LE | 42 | 26 | 62 | 42 | 41 | 98 | 42 | 35 | 83 | 42 | 38 | 90 |
| PE | 28 | 17 | 61 | 28 | 28 | 100 | 28 | 17 | 61 | 28 | 24 | 86 |
| LE + PE | 28 | 23 | 82 | 28 | 27 | 100 | 28 | 28 | 100 | 28 | 28 | 100 |
| WC | 42 | 41 | 98 | 42 | 42 | 100 | 42 | 42 | 100 | 42 | 41 | 98 |
| TAR | 42 | 42 | 100 | 42 | 42 | 100 | 42 | 42 | 100 | 42 | 42 | 100 |

only 40 min to complete after grinding the samples) and reliable for obtaining DNA for PCR from insect exoskeletal sources. The WC were very reliable, and gave good amplification for the four tested primers in 98–100% of cases. The LE and PE were not as reliable, but provided amplification in 61–100% of cases (Table 3).

Insect exoskeletons are non-cellular glycoproteins produced by the hypodermis. Because of the non-cellular nature of the tissues that were candidates for non-injurious and early evaluation of honey bee queen genotypes, we used tarsal DNA as a positive control to assure that the DNA extracted from candidate tissues were derived from the queen, and not from contaminating sources (e.g., bacteria, molds, or pollen). The exuviae and wing samples gave genotypes consistent with the genotypes from DNA extracted from tarsi (Table 3; Figure 1). When molting occurs, the lining of the foregut, hindgut, and all the tracheae pull out and become deposited with the molted skin (Bertholf, 1925), adding some cells with DNA to the exuviae. The DNA from the wings probably came from dried haemolymph deposited in the wing veins.

The primers we used appear to be highly conservative and species specific. We recently genotyped mother queens and their unmated daughters using WC, LE, and PE, and each drone (using a hind leg) that was used to inseminate each mother queen. The PCR products of the daughters for each tissue were consistent with those of the parents, suggesting that the DNA being amplified was from bees and not from extraneous sources such as microbes or pollen. However, it appears that there is a considerable amount of contamination in exuviae. From spectrophotometric measurements we found the mean quantity of DNA in LE and PE to be considerably higher than that in WC and TAR. Nonetheless, microsatellite data from LE and PC in every case matched data from WC and TAR.

Some primers (e.g., A81) were very robust and amplified nearly all the extracts. Given the availability of many

more candidate microsatellites, it is clear that optimizing several loci is possible in the future. We found that when the LE of one individual did not amplify, the PE from that individual most likely would. Thus, larval and pupal exuviae taken together show excellent promise in providing a source of DNA for use in early genotyping in a marker-assisted selection program for the breeding of honey bees.

Early screening and selection of breeder queens based on DNA markers is made possible by using DNA obtained from exuviae. There is thus ample time to obtain and evaluate genotypes from LE, even before the queen emerges from the cell. Such early genotyping allows a selection of desirable individuals before any major beekeeping resources are expended to care for adult queens. Honey bee breeders invest much time in selecting and testing stocks of honey bees, and finding stock differences requires the screening of many colonies in order to select those that express the most desirable traits. Recent advances in the knowledge of molecular genetics of honey bees suggest that genetic markers will soon be important in breeding bees. Queen sources and drone sources can be genotyped to ensure that enough heterozygosity is maintained in breeding populations to prevent inbreeding depression arising at the sex locus. By efficiently culling queens that do not possess desirable traits, costs in human effort and equipment can be minimized. The work presented here lays the foundation for an early genotyping of queens, assuring that only those queens possessing specific traits identified molecularly will be placed in a colony.

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