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TECHNICAL NOTE

An economical and effective high-throughput DNA extraction protocol for molecular marker analysis in honeybees

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Introduction

The honeybee is becoming an increasingly important organism in the realm of laboratory-based genetics. As honey bee health around the world is declining, measures are being taken to understand the genetic basis of a multitude of traits including behavior, disease resistance, survivability, honey production, and pollination efficiency. Quantitative Trait Loci (QTL) studies and mapping projects are elucidating the complex interactions of genetic loci that dictate the important traits being bred for by beekeepers, queen producers, and researchers alike. Quantitative Trait Loci for foraging behavior and aggression were identified nearly 15 years ago (Hunt et al., 1995, 1998), long before the advancements of the current and ongoing honey bee genome project (Honey Bee Genome Sequencing Consortium, Baylor College of Medicine, Houston, TX, USA). More recent studies have identified a single QTL for chalkbrood disease resistance (Holloway et al., 2012) and have utilized the genome data to fine map the interval to contain just two genes of potential interest (Holloway et al., 2013). As QTL studies are becoming more commonplace in the understanding of honey bee genetics, DNA extraction methods are needing to be faster, more effective, and more economical to keep pace with the analyses of the populations being studied.

Honey bee QTL studies are generating information on potential gene or allele functionality relevant to mapping populations. Yet, narrowing the intervals and pinpointing the genes of interest for eventual marker-assisted selection requires hundreds or even thousands of phenotyped bees to be processed for DNA extraction and genotyping. Typically, the extraction processes yield good qualities and quantities of DNA per individual sample, yet are costly in time and materials. Commercial DNA extraction kits allow for consistent and reproducible yields, yet can cost several dollars per sample. Chelating agents purify DNA with high quality but may require long incubations and preparations with additional proteinase K (Giraffa et al., 2000; Casquet et al., 2012). Traditional phenol-chloroform extractions require handling, storage, and disposal of hazardous organic solvents. Honey bee DNA has recently been effectively extracted and purified by homogenization and utilizing proteinase K and 'salting-out' methods to remove proteins (Bourgeois et al., 2008, 2010; Bourgeois & Rinderer, 2009). However, we developed a simple, cost-effective, and fast method using only sodium chloride and sodium dodecylsulfate (SDS) to extract clean DNA directly useable for PCR without additional dilution as is typically required. This method is particularly useful for high-throughput extraction of DNA from large numbers of individual bees while minimizing labor, plastic consumables, and reagent requirements. The resulting DNA is of a high enough quality and quantity to directly amplify bands for molecular marker analysis.

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Materials and methods

Bee samples

Freshly emerged adult bees or purple-eyed pupae individually pulled from honeycombs were frozen at −20 °C. Bee samples for aged DNA were maintained at −20 °C for 1 year or fresh samples were processed immediately after a lethal freezing. Body segments from fresh or aged samples were separated and processed with any associated appendages (head with antennae, thorax with legs and wings, abdomen).

Processing methodology

1. Individual bees, pupae, or body segments (or 100 μl of 250 mg ml^{-1} control BSA) were placed in 96-well racked 1.2-ml microtiter tubes (Genesee Scientific, San Diego, CA, USA) containing a single 3.2-mm stainless steel bead (Next Advanced, Averill Park, NY, USA). A

second 3.2-mm steel bead was loaded into the tubes on top of the tissue and tubes were capped with a silicone cap mat (USA Scientific, Ocala, FL, USA).

2. Tissues were homogenized by a TissueLyser (Qiagen, La Jolla, CA, USA) in 285 μl of 6 M NaCl (or in water, 0.5, 1, 2, 3, 4, or 5 M NaCl) (Fisher Scientific, Pittsburgh, PA, USA). Tissue maceration was attained by homogenizing at 30 strokes per s for 2 min; microtiter plates were rotated, and the homogenization repeated.
3. Plates were briefly centrifuged (Beckman Coulter Allegra X-15 Centrifuge; Beckman Coulter, Brea, CA, USA) to collect the homogenate away from the cap mat. A volume of 15 μl of 20% SDS (Amresco, Solon, OH, USA) was added, tubes re-capped, and homogenized at 20 strokes per s for 30 s, microtiter plates were rotated, and the homogenization repeated.
4. Homogenates were incubated at room temperature for 15 min, then centrifuged at 4 800 ***g*** for 20 min at 4 °C.
5. Aliquots of 150 μl of cleared supernatant were transferred to a 96-well PCR plate containing 150 μl of −20 °C isopropanol (Fisher Scientific), and gently pipetted up and down several times.
6. Plates were centrifuged as in step 4 to pellet the DNA.
7. Supernatant was removed by inverting the plates to decant the liquid and floating gelatinous conglomerate from the wells.
8. DNA pellets were washed twice with 200 μl of −20 °C 70% ethanol (Fisher Scientific), followed by centrifugations as above in step 4 but modified to 10 min per spin, and supernatant decanted.
9. DNA pellets were dried for 10 min in a 37 °C incubator, then resuspended in 25 μl purified water.

DNA quantification and qualification

Resuspended DNA was analyzed by NanoDrop (NanoDrop, Willimgton, DE, USA) for absorbance at λ = 260/280 nm ratios, dsDNA yield at μg μl^{-1}, and protein contamination by absorbance 280 = 1 mg ml^{-1}. Statistical analyses were performed using JMP 8.0 (Cary, NC, USA).

PCR amplification

Extracted DNA using 6 M NaCl from frozen samples, either undiluted or diluted in water (1:20) regardless of yield, was amplified by standard PCR methods. A 155-base segment of honey bee *beta-actin* was amplified using primers F-TGCCAACACTGTCCTTTCTG and R-AGAATTGACCCACCAATCCA, then electrophoresed on a 2% agarose gel, and imaged.

Statistical analysis

All statistical analyses were performed using JMP software (Version 8). A two-way ANOVA, with adjusted least squares (LS) means, was used to distinguish differences among treatments. Tukey's HSD test was used for separating means by treatment.

Results and discussion

Increased sodium chloride concentrations more effectively 'salt-out' proteins

It is known that the anion products of dissolved salts individually affect the aggregation or precipitation of proteins from solutions such that the efficacies can be described by the Hofmeister series (Zhang & Cremer, 2006; Kunz, 2010). Although many DNA extraction protocols (Bourgeois et al., 2008, 2010; Bourgeois & Rinderer, 2009) and kits use acetate salts (potassium or ammonium), we chose to focus on sodium chloride due to its availability and universal use in a variety of laboratories. Chloride anion is considered a marginally functional salting-out factor, somewhat less effective than acetate on the Hofmeister series. Typical NaCl concentrations used for fast DNA extraction range from less than 1 M (Chen et al., 2010; Margam et al., 2010) to upward of 4 M (Aljanabi & Martinez, 1997). We homogenized whole adult bees, whole pupae, and BSA control protein in a concentration series of 0–6 M NaCl (where 0.5 M served as a comparison for the protocol presented in Margam et al., 2010). During this process, we noted an interesting phenomenon following the mixing of bee supernatant with isopropanol: the aggregated protein formed a gelatinous disk at the surface of the supernatant rather than as a pellet at the bottom of the well, once initial NaCl concentrations reached 5 M. This resulted in an overall decrease in protein contamination of the extracted DNA and an easily removable conglomerate during decanting. The BSA control samples from the NaCl concentration series were analyzed for protein contamination by measuring absorbance at 280 nm, where 1 absorbance is equal to 1 mg ml^{-1} of protein. The protein contamination sharply decreases as NaCl concentration increases to 2 M (Figure 1). Bee DNA quantity and quality measurements following resuspension of the dried DNA show that the overall DNA yield also decreases somewhat (Figure 2). However, it remains unclear if the 260/280 nm absorbance measurements used to determine extraction efficiency are artificially skewed toward higher DNA yield when protein contamination is high. Regardless, the quantity and quality are more than sufficient for downstream PCR techniques.

Comparable quantities and qualities of DNA of fresh or historical tissue samples

Comparisons were made between DNA extractions using 6 M NaCl on bees frozen for 1 year and freshly collected

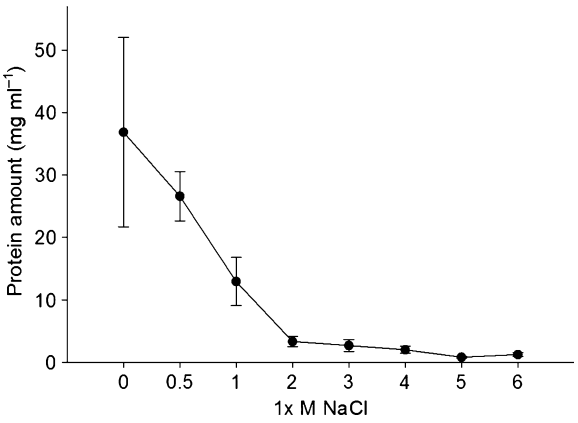

Figure 1 Average (± SD; n = 12) protein content (mg ml^{-1}) remaining after extracting 25 mg of BSA using a concentration series of NaCl [0.5, 1, 2, 3, 4, 5, 6 M, and 0 (water)].

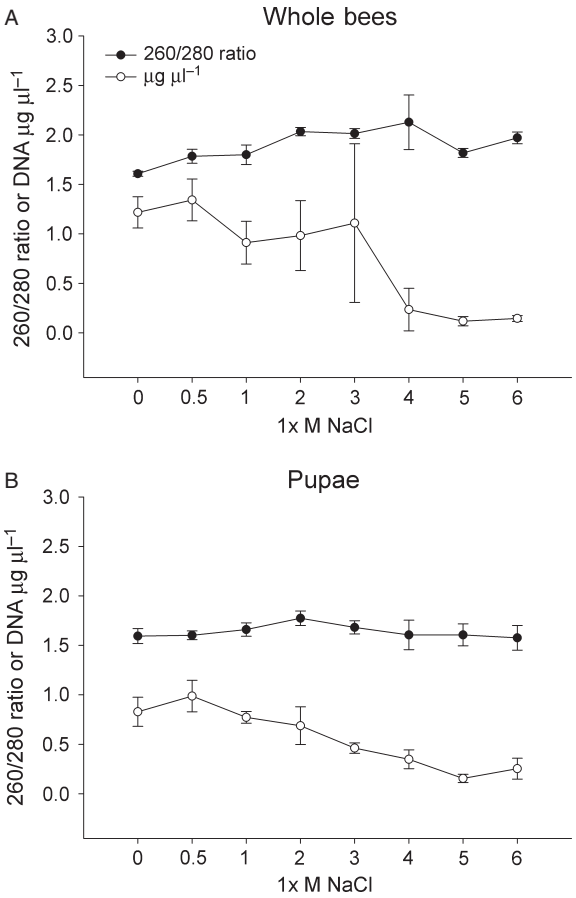

Figure 2 Average (± SD; n = 6) quality (solid symbols) and quantity (open symbols) of DNA extracted from (A) whole bees and (B) pupae using a NaCl concentration series. DNA quality remains relatively constant regardless of the NaCl concentration used during the extraction, whereas overall DNA yield decreases as the NaCl concentration increases.

bees. In addition to whole bees, body segments were processed to determine whether the yield and quality are dependent on the type of sample. In a tissue-specific manner, the extraction protocol yielded comparable DNA regardless of the age of the sample (Figure 3); however, only in whole bee or abdomen tissues did the disk form, float, and easily decant (data not shown). Quantity and quality of DNA extracted in the different samples were significantly different across tissue types (ANOVA; DNA amount retrieved: whole model, $F_{4,95} = 9.53$, $P<0.0001$; condition, $F_{1,95} = 0.0021$, $P = 0.96$; body region, $F_{3,95} = 12.7$, $P<0.0001$; DNA quality retrieved: whole model, $F_{4,95} = 50.69$, $P<0.001$; condition, $F_{1,95} = 0.988$, $P = 0.32$; body region, $F_{3,95} = 67.27$, $P<0.0001$; protein contamination: whole model, $F_{4,85} = 2.78$, $P = 0.032$; condition, $F_{1,85} = 0.11$, $P = 0.74$; body region, $F_{3,85} = 3.65$, $P = 0.015$. In all cases, n = 12). Despite the remaining extraneous protein in the head and thorax samples, the quality and quantity of the extracted DNA is sufficient for subsequent PCR applications only when diluted. The head and thorax DNA samples failed (or partially failed) to directly amplify and required dilution for successful amplification (Figure 4A and B), thereby requiring additional steps and plastic consumables to make such dilutions. The extraction from abdomens or whole bees resulted in relatively low yield, yet highly purified DNA such that no dilution step was needed to perform PCR (Figure 4C and D).

General remarks

We developed a fast, affordable, and eco-friendly DNA extraction protocol for high throughput analysis of molecular markers in honeybees. The goal of developing this DNA extraction protocol was to reduce the costs and wastes for reagents and consumables, while reducing the hazards associated with handling organic solvents. This protocol essentially uses common, inexpensive, disposable, and non-hazardous chemicals (NaCl, SDS, isopropanol, and ethanol). The price of consumables and reagents per 96-well plate of processed DNA is about USD 8.00, or 8.5 ¢ per individual bee [with the majority of the price (ca. 80%) determined by the plastic consumables], as compared to several dollars per extract with commercial kits. In addition, sample preparation time is decreased because whole bees can be processed as opposed to individual body segments. The protocol presented here employs a modification to other protocols in that the saturated concentration of NaCl functions to reduce protein contamination to negligible amounts. Importantly, this method allows high-throughput 96-well-platform extractions as are necessary

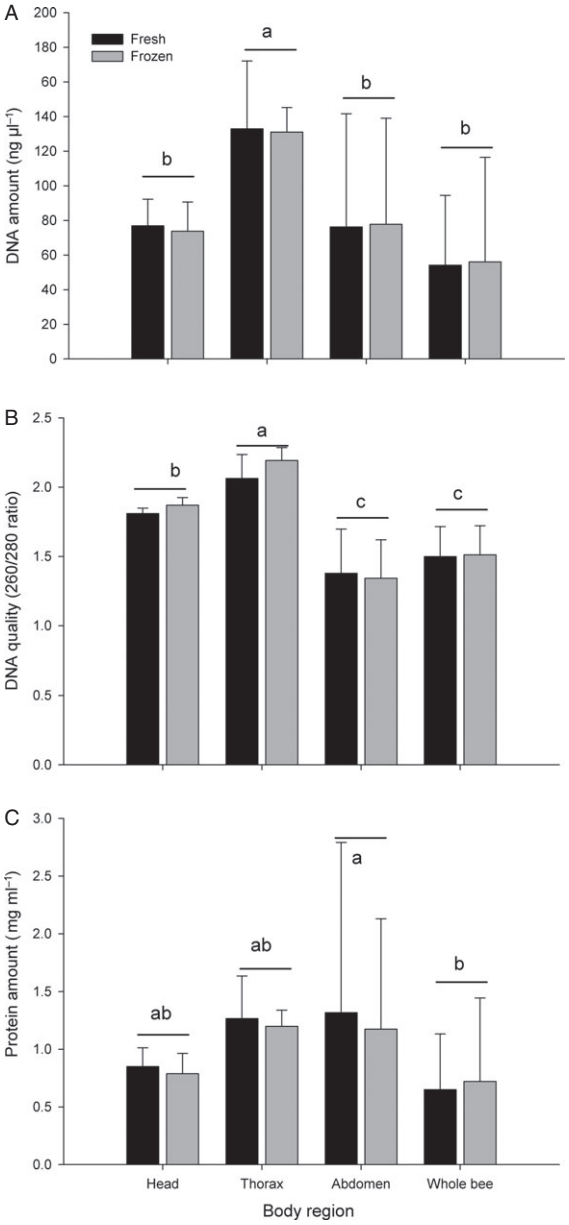

Figure 3 The time from sample collection to processing does not affect the (A) overall yield, (B) quality, or (C) purity (protein contamination) of the extracted DNA after purification with 6 M NaCl. No statistical differences in DNA yield, quality, or purity were found between frozen (stored for 1 year at −20 °C) and freshly collected samples for any of the tissue types (head, thorax, abdomen, or whole body). Quantity and quality of DNA extracted were significantly different across tissue types; different letters on bars within a panel indicate significant differences (Tukey's HSD test: $P<0.05$).

for a growing array of honey bee genetic studies such as QTL and fine mapping, phylogenetic studies, population studies, and the like. The quality of the DNA retrieved is

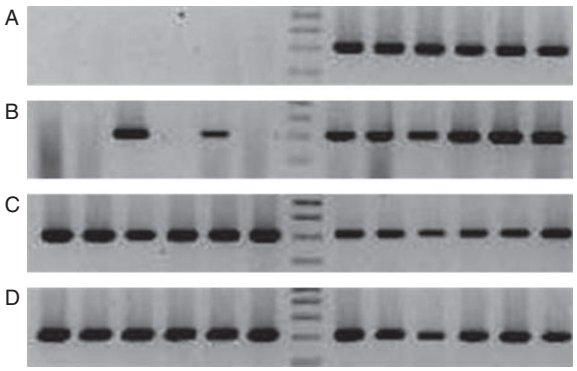

Figure 4 PCR amplification of a 155-bp *beta-actin* fragment using DNA extracted using 6 M NaCl from individual tissue samples either directly from the resuspended pellet (six lanes on left) or a 1:20 dilution of the same samples (six lanes on right) of frozen (A) heads, (B) thoraxes, (C) abdomens, or (D) whole bees. Center lanes are 50-bp ladder. The amplified bands align approximately with the 150-bp band of the ladder.

sufficient to perform downstream standard PCR reactions that enable marker analyses such as cleaved amplified polymorphisms (Holloway et al., 2013), amplicon sequencing, and cloning with honey bee DNA.

The quantity of the DNA retrieved is more than sufficient for standard practices. A return of >5 µg of DNA from one total individual bee can be expected that is more than enough to perform dozens or hundreds of PCR reactions. Calculations of the total available DNA present in a whole honeybee suggest that this protocol retrieves only a portion. Based on the average mass of a nucleotide base-pair (1.029×10^{-9} pg) (Doležel et al., 2003), the widely accepted typical dimensions of a eukaryotic cell (assume 10-µm-diameter sphere, or ca. 0.5 pl), the diploidy of female bees, and the volume of a newly emerged worker bee (ca. 100 µl), if the bee were a solid mass of cells (ca. 800 000 cells) then the total mass of DNA contained would be ca. 92 µg, an extremely gross overestimation based on the volume of bee that is non-cellularized (hemolymph) or proteinaceous (exoskeleton) in nature. Regardless, the minimum amount of DNA retrieved is likely to be at least 5–10% of the total, which is plenty for standard PCR use. However, because we did not perform mass spectrometry or any other means of purity measurements of the pelleted DNA, we cannot assume that the quantity of DNA calculated by the NanoDrop is entirely accurate as contaminants may also affect the absorbance at 260 nm used for the determination. Understanding the formation of the gelatinous floating disk is beyond the scope of our research, yet the components of it may be sequestering some of the DNA that is lost during the decanting.

This protocol helps to achieve an important balance between purifying ample DNA while preventing the need for additional consumables and time to prepare dilutions. Samples that were obtained using head and thorax samples required dilution to amplify DNA. Likely, the dilution is important to minimize the contaminating protein concentrations remaining due to the lack of the gelatinous disk formation. The excess protein and other contaminants would therefore inhibit the activity of the polymerase during normal PCR. In addition, the protein pellet remaining in the head and thorax samples may be sequestering extraneous NaCl, which is otherwise removed in the abdomen or whole bee samples. Following the addition of water for resuspension of the DNA pellet, NaCl may be released from the protein aggregate which could also inhibit PCR. The reason for the discrepancy in the formation of the disk dependent on tissue type is yet not clear; however, it may be due to a protein-interacting compound in the gut, reproductive tract, or other structure in the abdomen. Alternatively, the abdomen may contain a particular protein (or proteins) that aggregates with the other debris, yet is overall much less buoyant in the salt: SDS:isopropanol milieu and therefore causes all other contaminating proteins to float. On the basis of the results presented, we believe that the most effective and most inexpensive (in both labor and resources) method for honey bee DNA extraction uses abdomens or whole bees in combination with 5–6 M NaCl to remove protein contamination. This purifies the DNA while allowing for direct downstream molecular analysis.

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