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# Rapid method for DNA extraction from the honey bee *Apis mellifera* and the parasitic bee mite *Varroa destructor* using lysis buffer and proteinase K

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**ABSTRACT.** We developed a rapid method for extraction of DNA from honey bees, *Apis mellifera*, and from the parasitic bee mite, *Varroa destructor*. The advantages include fast processing and low toxicity of the substances that are utilized. We used lysis buffer with nonionic detergents to lyse cell walls and proteinase K to digest proteins. We tested whole thorax, thoracic muscle mass, legs, and antennae from individual bees; the mites were processed whole (1 mite/sample). Each thorax was incubated whole, without cutting, because exocuticle color pigment darkened the extraction solution, interfering with PCR results. The procedure was performed with autoclaved equipment and laboratory gloves. For each sample, we used 100 µL lysis buffer (2 mL stock solution of 0.5 M Tris/HCl, pH 8.5, 10 mL stock solution of 2 M KCl, 500 µL solution of 1 M $MgCl_2$, 2 mL NP40, and 27.6 g sucrose,

 

completed to 200 mL with bidistilled water and autoclaved) and 2 µL proteinase K (10 mg/mL in bidistilled water previously autoclaved, as proteinase K cannot be autoclaved). Tissues were incubated in the solutions for 1-2 h in a water bath (62°-68°C) or overnight at 37°C. After incubation, the tissues were removed from the extraction solution (lysis buffer + proteinase K) and the solution heated to 92°C for 10 min, for proteinase K inactivation. Then, the solution with the extracted DNA was stored in a refrigerator (4°-8°C) or a freezer (-20°C). This method does not require centrifugation or phenol/chloroform extraction. The reduced number of steps allowed us to sample many individuals/day. Whole mites and bee antennae were the most rapidly processed. All bee tissues gave the same quality DNA. This method, even using a single bee antenna or a single mite, was adequate for extraction and analysis of bee genomic and mitochondrial DNA and mite genomic DNA.

**Key words:** DNA extraction; *Varroa destructor*; *Apis mellifera*; Proteinase K; Lysis buffer; Polyacrylamide gel

## INTRODUCTION

Laboratory procedures for extracting DNA should be optimized to provide the most satisfactory results. For each species and type of tissue, methods should be adapted to maximize output, with minimum cost and time requirements, while maintaining quality.

We have frequently needed to identify the genetic origin of a large number specimens of the bee *Apis mellifera* and the parasitic bee mite *Varroa destructor*, through DNA fragment analysis. We searched for techniques that use low toxicity reagents for DNA extraction. Initial procedures used phenol, chloroform, and isoamyl alcohol (Oldroyd et al., 1992); they gave good results, but we found them time-consuming. Later, we adapted more rapid techniques using a lysis buffer with nonionic detergents and proteinase K (PK) (Higuchi, 1989; Kawasaki, 1990; Wright and Manos, 1990). We have used these techniques for DNA extraction from honey bee fat bodies (Maiostre et al., 1996); however, DNA extraction from this tissue is also time-consuming. Over time, we made changes to the procedures (Issa and Simões, 1998; Issa et al., 2004).

Additionally, we needed to extract both genomic and mitochondrial DNA. This led to additional adaptations in the extraction method, in order to ensure that only a single extraction of a sample provides sufficient quantities of both DNA types.

## MATERIAL AND METHODS

### Biological material

We extracted DNA from cut thorax, whole thorax, thoracic muscle mass, antennae or legs of adult worker bees (*A. mellifera*), and whole adult female mites (*V. destructor*). The bees and mites were collected from apiaries in the transition zone between Africanized and European honey bees, parallel 30°-35° South (Kerr et al., 1982) in Rio Grande do Sul, Brazil,

 

Entre Rios, Argentina, and Uruguay. They were transported live by post office to our laboratory in plastic vials, normally used to store medicines (Issa and De Jong, 1999). The bees and mites were then stored at -20°C.

Some worker bees and mites were also collected in 95 or 70% ethanol. Before the procedures, the ethanol was completely removed, by blotting the specimens on a piece of filter paper for at least 15 min at room temperature, allowing evaporation of the ethanol. They could then be used to extract DNA or be stored at -20°C.

The entire procedure was performed with sterilized instruments, using laboratory gloves, to avoid contamination (Kawasaki, 1990). The biological material (cool or frozen) was prepared on ice, using autoclaved tweezers, scalpels, scissors, spatulas, solutions, and pipette tips. Since we also wanted to extract both genomic and mitochondrial DNA, the bees were initially prepared as follows: a) halves and quarters of the thorax, cut with a scalpel and a pair of scissors, and b) thoracic muscle mass, a yellow-colored tissue, collected from inside the thorax. The results were satisfactory with thoracic muscle mass, but pieces of the thorax cuticle, which contain exocuticle pigment, caused darkening of the extract, hampering PCR results. Consequently, the thorax was used whole, without cutting the cuticle.

The procedure consisted of the following steps: the whole thorax was obtained by holding the bees with a forceps; scissors were used to remove the abdomen, legs, wings, and head, taking care not to damage the cuticle of the thorax. This represented 1 unit/sample. The thoracic muscle mass was collected, with fine tweezers and a spatula, from inside the thorax, which had been cut in half with a scalpel. A portion of this mass was used. Antennae were pulled from the bees with fine tweezers. These represented 1 unit/sample. The first pair of legs were also pulled from the bees with fine tweezers. These represented 1 unit/sample. The whole (adult female) mite was used, which represented 1 unit/sample. Between specimens, laboratory materials were cleaned to avoid contamination between tissues. Tweezers, scissors, scalpels, and spatulas were washed twice in commercial grade 92% ethanol (placed in 2 beakers), dried with tissue paper and cotton soaked with commercial grade 92% ethanol. The tips of the instruments were passed through the flame of an alcohol lamp. The tissues were prepared on ice and used immediately for DNA extraction.

## DNA preparation of *A. mellifera* bees and *V. destructor* mites

### *Extraction protocol*

We analyzed genomic (PCR of microsatellites A7, A113, A88, A24; Estoup et al., 1995) and mitochondrial DNA (amplification of the cytochrome B region, cut with the restriction enzyme *Bgl*II; Crozier et al., 1991) of *A. mellifera* bees and genomic DNA of *V. destructor* mites (RAPD locus OPE-07; de Guzman et al., 1997; Guerra et al., 2010).

### *Reagents and solutions*

Products purchased:

- 1 M $MgCl_2$ (Sigma M-1028)
- Nonidet P40 (NP40, Amersham-Pharmacia US19628)
- Sucrose (Merck 1.07687)

Stock solutions prepared:

- 0.5 M Tris-HCl, pH 8.5 (Amersham-Pharmacia 17-1321.01 or US75825)
- 2.0 M KCl (Merck 4936)
- Both solutions were autoclaved and stored in a freezer (-20°C) in 50-mL aliquots.

Lysis buffer:
Preparation:

- 2 mL 0.5 M Tris-HCl, pH 8.5 (final concentration: 5 mM Tris-HCl)
- 10 mL 2.0 M KCl (final concentration: 100 mM KCl)
- 500 µL 1.0 M $MgCl_2$ (final concentration: 2.5 mM $MgCl_2$)
- 2 mL NP40 (final concentration: 1% NP40)
- 27.6 g sucrose (final concentration: 0.40 M sucrose)
- Bidistilled $H_2O$ was added to complete volume to 200 mL. Sucrose was first dissolved in 100 mL distilled water (use of warm water bath to dissolve easier); the other components were then added.

Lysis buffer was autoclaved and stored frozen (-20°C) in 50-mL aliquots. The portion to be used for DNA extraction prepared within a period of 15 to 30 days did not need to be refrozen and could be stored in a refrigerator (4°-8°C).

Proteinase K:

- 10 mg PK (Amersham-Pharmacia E76230Y) in 1 mL autoclaved bidistilled water

Autoclaved Eppendorf tubes (1.5 mL) and autoclaved bidistilled water were used, because PK cannot be autoclaved. PK was stored in the same Eppendorf tubes in a freezer (-20°C) in 1-mL aliquots. The portion used in extractions was stored in a refrigerator (4°-8°C). PK was checked regularly for fungal growth.

## *Procedures*

All laboratory materials used in DNA extractions were autoclaved. Gloves were used to avoid contamination. Aliquots of PK and lysis buffer were thawed on ice. The autoclaved Eppendorf tube caps were labeled with numbers of samples and the 0.5-mL Eppendorf tubes were placed in a metal rack with appropriate sized holes. This material was kept on ice. The lysis buffer + PK extraction solution was prepared for each sample in an autoclaved Eppendorf tube (1.5 mL).

Lysis buffer was prepared (2 mL stock solution of 0.5 M Tris-HCl, pH 8.5, 10 mL stock solution of 2 M KCl, 500 µL solution of 1 M $MgCl_2$, 2 mL NP40, and 27.6 g sucrose, with bidistilled water added to 200 mL), autoclaved and then 2 µL 10 mg/mL PK was added. This mixture (lysis buffer + PK extraction solution) was gently shaken. Aliquots of 100 µL were then distributed in the 0.5-mL autoclaved Eppendorf tubes and identified with the number of each sample. Tissue fragments prepared on ice (antenna, mite, whole thorax, leg,

or a small amount of thoracic muscle mass), with fine tweezers, were separately placed in the 0.5-mL Eppendorf tubes containing lysis buffer + PK extraction solution. Between each tissue sample, tweezers and spatulas were cleaned twice with cotton soaked in ethanol and theirs tips were quickly flamed to avoid contamination. The Eppendorf tubes were capped, placed in a metal rack, and incubated. This material was incubated in two ways (according to the two different times used for the procedure): in a hot water bath at 62°-68°C for 1-2 h or in a hot water bath (or laboratory incubator) at 37°C overnight. After incubation, the tissues were removed from the extraction solution.

Mites, whole thoraxes, antennae, and legs were removed from the Eppendorf tubes with the aid of tweezers and discarded, leaving the extraction solution. When we processed thoracic muscle mass, the supernatant was transferred (without centrifugation) to a fresh 0.5-mL Eppendorf tube with the aid of a micropipette (using autoclaved tips). Between specimens, the tweezers were cleaned with cotton soaked in ethanol, and the ends were quickly flamed to avoid contamination. The extract was then heated at 92°C for 10 min to inactivate PK, with the tubes in the same metal rack. The extracted DNA was stored in a refrigerator (4°-8°C) for immediate use or in a freezer (-20°C) in the same Eppendorf tubes.

## RESULTS

All tissues gave the same quality PCR results, as determined by 6% polyacrylamide gel electrophoresis (data not shown). The results also did not vary with changes in incubation time and temperature: 1-2 h at 62°-68°C or overnight at 37°C. Good PCR results were obtained when using bee whole thoraxes and thoracic muscle mass, but not when the thorax was cut in halves and quarters, with pieces of thorax cuticle present in the extract becoming darkener. There was no contamination of any sort with any of the tissues, not even the whole mites, bee antennae or legs (which are external appendages). There was also no need to rinse them in water or alcohol before being used for DNA extraction. The whole mites and bee antennae and legs required the shortest working time. The thoracic muscle mass had the longest working time because of its collection and preparation. This technique worked well for bee and mite samples that were fresh, frozen (-20°C), or collected in alcohol (95 or 70%), but the alcohol needed to be completely evaporated away.

This method required only 2 or 3 h of working time, unlike most other extraction methods that need up to 2-3 days to complete all procedures. It allowed the sampling of many bees and mites in a day. DNA extractions could also be started on one day and finished on another. We tested different kinds of biological material (thoracic muscle mass, whole thorax, antenna, and leg) to determine which of them were optimal in the laboratory. We found that any of them could be used to extract DNA. Most of the material used (whole mite and bee antennae) allowed for faster isolation of DNA fragments and the use of lower toxicity reagents. A volume of 200-300 µL extraction solution per Eppendorf tube could also be used, if it was necessary to work with different PCR analyses. A single extraction provided enough DNA to work with both mitochondrial and genomic DNA. This method was suitable for the analysis of microsatellites A7, A113, A88, and A24 of genomic DNA (Figure 1 - microsatellite A7) and the cytochrome B region of mitochondrial DNA, cut with the restriction enzyme *Bgl*II (Figure 2), of *A. mellifera* bees and genomic DNA of the mite *V. destructor* (RAPD locus OPE-07).

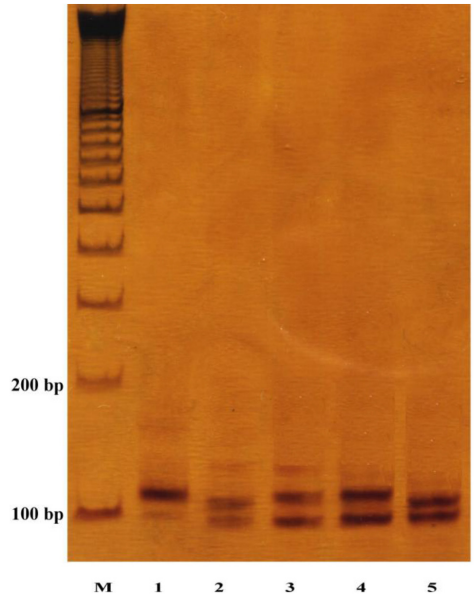


**Figure 1.** Electrophoretic pattern for locus A7 from honey bees (6% polyacrylamide gel silver stained). *Lane 1* = 6/6. *Lane 2* = 5/2. *Lanes 3* and *4* = 6/2. *Lane 5* = 5/2. *Lane M* = a 100-bp DNA marker.

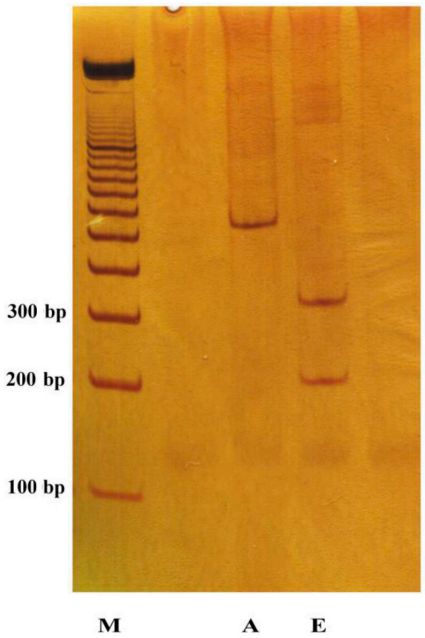


**Figure 2.** Mitochondrial DNA fragments from honey bees. Amplification of the cytochrome B region, digested with the restriction endonuclease *Bgl*II. A = Africanized bees; E = European bees. *Lane M* = a 100-bp DNA marker (6% polyacrylamide gel silver stained).

## DISCUSSION

Initially, we opted for methods that had traditionally been used to process honey bee tissues (Sheppard and McPheron, 1991; Oldroyd et al., 1992), involving the use of phenol, chloroform, and isoamyl alcohol. These techniques gave good results, but were time-consuming and required toxic substances. Consequently, we adapted a technique that uses a lysis buffer with nonionic detergents, which breaks the cell wall, and PK, which digests the proteins present in the extract. A number of extraction methods served as the basis of our procedure (Berger and Kimmel, 1987; Higuchi, 1989; Kawasaki, 1990; Wright and Manos, 1990; Sheppard and McPheron, 1991; Walsh et al., 1991; Oldroyd et al., 1992). Kawasaki (1990) formulated a DNA extraction protocol using lysis buffer with nonionic detergents (Laureth 12, NP-40, and Tween-20) and PK, without gelatin, to analyze blood, cells, and other fluids; this publication gave some suggestions as how to proceed to avoid contamination in DNA/RNA extraction. Wright and Manos (1990) used nonionic detergents (Laureth 12 and Tween 20) and PK to analyze paraffin-embedded tissues. Higuchi (1989) originally proposed this for the study of mononuclear cells, whole blood, clinical swabs, and plucked hairs from humans, aiming to find conditions to release DNA and/or RNA from a large number of cells, adequate for PCR, while preserving Taq DNA polymerase activity with certain nonionic detergents that do not significantly affect Taq DNA polymerase, and PK, which can be inactivated at 92°C. He used a PCR buffer with PK, gelatin, NP40 and Tween 20, and a lysis buffer with sucrose and Triton-X-100. Accordingly, we developed a protocol with a single lysis buffer composed of nonionic detergents, removing the detergent Tween 20 and slightly increasing the concentration of the detergent NP40 (from 0.45 to 1%), while changing the pH of Tris-HCl (from 8.3 to 8.5) and the concentrations of Tris-HCl (from 10 to 5 mM) and KCl (50 to 100 mM). Sucrose at a concentration of 0.40 M was added to the new lysis buffer and gelatin was eliminated because otherwise we had problems with fungal growth in the buffer.

The DNA extraction procedure only requires 2 to 3 h when we used the smaller incubation time (1-2 h at 62°-68°C). Otherwise, the material can be incubated at 37°C overnight.

Since we wanted to extract mitochondrial DNA, we initially chose to work with the thorax (Issa and Simões, 1998), because of the large muscles it contains (Snodgrass, 1956). We referred to this thorax tissue as “thoracic muscle mass”, and considered it to be a tissue from which we could obtain larger quantities of DNA compared to the fat body (Maiostre et al., 1996). We then abandoned the procedure of extracting DNA from the bee fat body because it was very time-consuming.

When cut thoraxes were used for DNA extraction, the extraction solution darkened and produced poor PCR results. We considered various possibilities to “clean up” the extract by precipitating the DNA with 5 M NaCl (we also developed a protocol to “clean-up” the extract in this case), but the pigment co-precipitated. Probably, when thorax cuticle releases exocuticle pigment, this reacts with the extract and makes it dark. The body wall of arthropods has a layered structure, formed by cellular epithelium internally and the cuticle externally. The cuticle is also stratified, consisting of two major layers, the endocuticle and the exocuticle, and a thin outer layer, the epicuticle. The cuticle is essentially formed of chitin; but there are other substances in the exocuticle, which is distinguished from endocuticle by its black color (Snodgrass, 1935). Thus, we used the whole thorax and thoracic muscle mass.

None of the bee tissues (or mites) showed any kind of contamination, and there was no need to rinse them in water or alcohol before their use for DNA extraction. We expected some contamination when we used whole mites and bee antennae and legs (external appendages), but this did not occur. Thoracic muscle mass had the lowest risk of contamination from the environment, since it was collected from inside the thorax with sterilized instruments. We also found it to be important that the whole procedure was performed with autoclaved and sterilized instruments and reagents and that all laboratory materials were cleaned with alcohol and their ends quickly flamed between procedures to avoid contamination between tissues.

As we were also using whole mites and getting PCR results for genomic and mitochondrial DNA, we tried other bee parts for which processing would be faster compared to the thorax or thoracic muscle mass. Thus, we used antennae and legs of the 1st pair, which were simply removed from the bees with tweezers and placed directly into lysis buffer + PK extraction solution for an easier extraction process. PCR results for both genomic and mitochondrial DNA were the same for all tissues tested. Thus, any of them can be used to extract DNA for genetic analysis of the bees.

Since the PCR results were the same for all tissues and the use of the antennae involved less time-consuming, the majority of our extractions were done with antennae, reserving the bee thorax for isoenzyme analysis.

This method is rapid and simple, without a need for centrifugations; different kinds of biological material can be chosen to extract DNA. There is no need to change Eppendorf tubes or go through a process phenol/chloroform extraction. It involves few steps, and it allows the sampling of many bees and mites per day. DNA extraction can also be done in one day, or started on one day and finished on another, still requiring only 2 or 3 h of working time, unlike most other extraction methods, which require up to 2-3 days to complete procedures. Finally, with this method, even a single bee antenna (or a mite) was adequate for the analysis of both genomic and mitochondrial DNA, which were separated by 6% polyacrylamide gel electrophoresis (data not shown) and stained with silver (Sanguinetti et al., 1994). Good results were obtained for analysis of genomic DNA, with regard to the PCR microsatellites A7, A113, A88, and A24 (A7 - Figure 1) and mitochondrial DNA, with regard to the amplification of cytochrome B, cut with the restriction enzyme *Bgl*II (Figure 2), of *A. mellifera* bees and genomic DNA of the bee mite parasite *V. destructor* (RAPD locus OPE-07).

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