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Standardization of DNA extraction from sand flies: Application to genotyping by next generation sequencing

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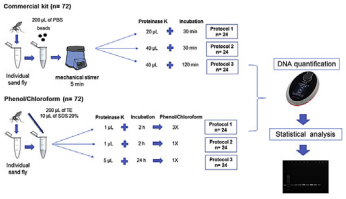
HIGHLIGHTS

- Three protocols developed for the commercial kit were found to be more practical.
- Phenol/chloroform protocols resulted in significantly higher quantities of sand fly gDNA.
- Higher quantities of proteinase K resulted in higher concentrations of extracted sand fly DNA.

GRAPHICAL ABSTRACT



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ABSTRACT

Standardization of the methods for extraction of DNA from sand flies is essential for obtaining high efficiency during subsequent molecular analyses, such as the new sequencing methods. Information obtained using these methods may contribute substantially to taxonomic, evolutionary, and eco-epidemiological studies. The aim of the present study was to standardize and compare two methods for the extraction of genomic DNA from sand flies for obtaining DNA in sufficient quantities for next-generation sequencing. Sand flies were collected from the municipalities of Campo Grande, Camapuã, Corumbá and Miranda, state of Mato Grosso do Sul, Brazil. Three protocols using a silica column-based commercial kit (*ReliaPrep™ Blood gDNA Miniprep System kit*, Promega®), and three protocols based on the classical phenol-chloroform extraction method (Uliana et al., 1991), were compared with respect to the yield and quality of the extracted DNA. DNA was quantified using a Qubit 2.0 fluorometer. The presence of sand fly DNA was confirmed by PCR amplification of the IVS6 region (constitutive gene), followed by electrophoresis on a 1.5% agarose gel. A total of 144 male specimens were analyzed, 72 per method. Significant differences were observed between the two methods tested. Protocols 2 and 3 of phenol-chloroform extraction presented significantly better performance than all commercial kit extraction protocols tested. For phenol-chloroform extraction, protocol 3 presented significantly better

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performance than protocols 1 and 2. The IVS6 region was detected in 70 of 72 (97.22%) samples extracted with phenol, including all samples for protocols 2 and 3. This is the first study on the standardization of methods for the extraction of DNA from sand flies for application to next-generation sequencing, which is a promising tool for entomological and molecular studies of sand flies.

1. Introduction

Sand flies (Diptera:Psychodidae) are vectors of several human and animal pathogens, such as *Bartonella bacilliformis*, viruses of the families Bunyaviridae and Rhadboviridae, and protozoa of the genera *Endotrypanum* and *Leishmania* (Adler and Theodor, 1957; Lainson and Shaw, 2005; Lutz and Neiva, 1912; Young and Duncan, 1994).

Cryptic sand fly species belonging to the species complexes *longipalpis*, *cortelezzii*, and *intermedia* present few distinguishable taxonomic characters, making their identification difficult, especially when living in sympatry (Bauzer et al., 2007; Galati et al., 1989; Marcondes, 1996). This may affect the identification of the species and of their parasite-vector-host relationship for a given area. Molecular tools can be used to help taxonomic identification of sand flies.

The number of studies on the use of molecular markers, based on mitochondrial DNA (Folmer et al., 1994; Zhang and Hewitt, 1997), microsatellites (Queller et al., 1993; Santos et al., 2013) and, more recently, Single Nucleotide Polymorphism (SNPs) (Brumfield et al., 2003; Soria-Carrasco et al., 2014; Vignal et al., 2002), for the identification and evaluation of intra- and inter-population diversity is increasing. Genotyping by traditional Sanger sequencing has also been used for the analysis of target regions but this approach proves costly for population genetics studies. Novel genotyping techniques termed Next-Generation Sequencing (Illumina, Roche 454, and AB SOLiD platforms), which can generate and analyze thousands of DNA fragments in short intervals of time, can be used to sequence dipteran genomes in studies with limited financial resources (Davey and Blaxter, 2011; Etter et al., 2011; Rubin et al., 2012).

It is important to obtain DNA free from contaminants and in sufficient quantities to allow high efficiency of subsequent molecular analyses (Oliveira et al., 2007). The aims of the present study were to compare two methods for the extraction of genomic DNA from sand flies (Diptera, Psychodidae, Phlebotominae), and to standardize the protocol for obtaining DNA with better quality and quantity.

2. Material and methods

2.1. Study area and period

Sand flies were captured between August 2014 and July 2015 from the municipalities of Camapuã (19° 31′ 51″ S; 54° 02′ 38″ W), Campo Grande (20° 27′ 5,5″ S; 54° 37′ 25,7″ W), Corumbá (18° 59′ 44″ S; 57° 19′ 36″ W) and Miranda (20° 14′ 27″ S; 56° 22’ 40”), state of Mato Grosso do Sul, Brazil (IBGE, 2016).

2.2. Identification

Male *Lutzomyia cruzi* and *Lutzomyia longipalpis* flies were selected, and identified in wet mount slides by observing the last abdominal tergite according to the classification proposed by Galati (2014). Head, thorax and the remaining part of the abdomen of the sand flies were then stored separately at −20 °C.

2.3. Extraction methods

Two extraction methods were tested: a commercial kit (*ReliaPrep™ Blood gDNA Miniprep System kit*, Promega®) and the classical phenol-chloroform extraction method as described by Uliana et al. (1991); two modifications were tested per method.

2.3.1. Genomic DNA extraction by commercial kit (Method 1)

Extraction of genomic DNA (gDNA) was performed using a commercial kit at the Genetic Research and Analysis Laboratory of the State University of São Paulo, Botucatu campus (PANGENE/UNESP). Twenty-four male *L. longipalpis* and *L. cruzi* sand flies were used for each protocol. The first protocol was based on the recommendations of the manufacturer of the commercial kit (*ReliaPrep™ Blood gDNA Miniprep System kit*, Promega®): Zirconium oxide microbeads and 200 μL of PBS were added to tubes containing one male sand fly, and homogenized in a mechanical stirrer (Bullet Blender® Homogenizer) for 5 min at ambient temperature. Twenty microliters of proteinase K and 200 μL of cellular lysis buffer were added; the mix was stirred for 10 s and incubated at 56 °C for 30 min. Following incubation, 250 μL of binding buffer were added and stirred for 10 s. The contents were transferred to a kit column and centrifuged for 1 min at 13000 rpm. The kit column was transferred into a new collection tube, 500 μL of washing solution were added, and centrifuged for 3 min at 13000 rpm. This stage was repeated three times. Fifty microliters of nuclease-free water were added to the column, and centrifuged for 1 min at 13000 rpm. The column was discarded and the pellet was stored at −20 °C.

The remaining protocols were developed by modifying the first protocol (i.e., the standard protocol). The modifications for each protocol have been presented in Table 1.

2.3.2. Genomic DNA extraction by Phenol-Chloroform (Method 2)

Extraction of gDNA by phenol-chloroform method was performed at the laboratory of Molecular Biology and Cell Culture of the Federal University of Mato Grosso do Sul. Twenty-four sand fly specimens were analyzed per protocol. The first protocol was based on Uliana et al. (1991). Ten microliters of 20% sodium dodecyl sulfate (SDS) and 200 μL of Tris-EDTA (TE) were added to tubes containing the specimens, and homogenized using plastic pestles. One microliter of proteinase K (20 mg/mL) was added and the contents homogenized. The tubes were placed in a dry-bath at 42 °C for 2 h, and 200 μL of phenol:chloroform:isoamyl alcohol (25:24:1) were added. The samples were centrifuged for 5 min at

Table 1
Protocols of sand flies' genomic DNA extraction using a commercial kit according to the manufacturer and modified protocols (n = 72).

	Protocol 1 (n = 24)	Protocol 2 (n = 24)	Protocol 3 (n = 24)
V proteinase K (μL)	20	40	40
Incubation period (min)	30	30	120