Comparison of manual and semi-automatic DNA extraction protocols for the barcoding characterization of hematophagous louse flies (Diptera: Hippoboscidae)

Rafael Gutiérrez-López✉, Josué Martínez-de la Puente, Laura Gangoso, Ramón C. Soriguer, and Jordi Figuerola

Estación Biológica de Doñana (EBD-CSIC), C/Américo Vespucio, s/n, E-41092, Seville, Spain, rgutierrez@ebd.csic.es

Received 13 May 2014; Accepted 16 September 2014

ABSTRACT: The barcoding of life initiative provides a universal molecular tool to distinguish animal species based on the amplification and sequencing of a fragment of the subunit 1 of the cytochrome oxidase (COI) gene. Obtaining good quality DNA for barcoding purposes is a limiting factor, especially in studies conducted on small-sized samples or those requiring the maintenance of the organism as a voucher. In this study, we compared the number of positive amplifications and the quality of the sequences obtained using DNA extraction methods that also differ in their economic costs and time requirements and we applied them for the genetic characterization of louse flies. Four DNA extraction methods were studied: chloroform/isoamyl alcohol, HotShot procedure, Qiagen DNeasy® Tissue and Blood Kit and DNA Kit Maxwell® 16LEV. All the louse flies were morphologically identified as *Ornithophila gestroi* and a single COI-based haplotype was identified. The number of positive amplifications did not differ significantly among DNA extraction procedures. However, the quality of the sequences was significantly lower for the case of the chloroform/isoamyl alcohol procedure with respect to the rest of methods tested here. These results may be useful for the genetic characterization of louse flies, leaving most of the remaining insect as a voucher. ***Journal of Vector Ecology* 40 (1): 11-15. 2015.**

Keyword Index: DNA extraction methods, Barcoding of life, COI, Hippoboscidae, *Ornithophila gestroi*, parasites.

INTRODUCTION

Taxonomy currently uses multidisciplinary approaches that combine both morphological and molecular techniques (Bisby et al. 2002, Besansky et al. 2003, Hajibabaei et al. 2007). DNA barcoding provides a useful tool for rapid and accurate identification of species applicable to a wide range of organisms from all fungi, plant, and animal kingdoms (Hebert et al. 2003a,b, Hajibabaei et al. 2007). In animals, this tool is based on the characterization of a 658bp fragment of a standardized region of the mitochondrial cytochrome c oxidase subunit I (COI) that shows low intraspecific but large interspecific variability (Hebert et al. 2003b, Ratnasingham and Hebert 2007, but see Meier et al. 2006, Shearer and Coffroth 2008).

DNA extraction has been recognized as a critical step for DNA barcode characterization (Ball and Armstrong 2008) but also may be important in studies using other approaches, including restriction fragment length polymorphism (RFLP) (Möller et al. 1992), amplified fragment length polymorphism (AFLP) (Reineke et al. 1998), or new generation sequencing (NGS) (Pompanon et al. 2012). Current DNA extraction methods can be differentiated into two main groups: commercial kits and standard/traditional methods. Most of these methods are constrained by factors such as the use of hazardous chemicals for human and environmental health (i.e., phenol, chloroform), the need of specialized laboratory equipment (automated DNA extraction), high costs (commercial kits (Petrigh and Fugassa 2013)), and/or time-consumption (Rohland et al. 2010). The latter may become an important factor for studies comprising large sample sizes, where automated DNA extraction protocols may significantly reduce manpower requirements (Lee et al. 2010). Therefore, it is necessary to evaluate the pros and cons of different DNA extraction procedures to characterize DNA barcodes.

Here, we compared the efficacy of four DNA extraction protocols for the genetic characterization of the barcoding region of hematophagous louse flies (Diptera: Hippoboscidae). In spite of the importance of louse flies as blood feeders and potential vectors of different blood parasites (Valkiunas 2005, Lehane 2008), precise information regarding the barcode characterization of this insect group is absent for the majority of the species. First, we identified the louse fly species on the basis of distinctive morphological features. Secondly, we used a small leg fragment of these louse flies that were preserved in ethanol during a relatively long period (over six years) to compare the efficacy of four DNA extraction protocols: two standard protocols, 1) based on the use of chloroform/isoamyl alcohol, and 2) the HotShot (Truett et al. 2000), and two commercial kits, 3) a Qiagen kit, and 4) a semi-automatic Maxwell Kit.

MATERIALS AND METHODS

We collected 32 louse flies during August and September, 2007 on the islet of Alegranza (10.5 km^2, 289 m a.s.l.) in the Canary Islands (27º 37' N, 13º 20' W), Spain. Louse flies were collected from 25-day-old Eleonora's falcon (*Falco eleonorae*) nestlings. Immediately after collection, each individual louse fly was transferred to a 2 ml Eppendorf tube with ethanol and stored at room temperature until molecular analyses in November, 2013.

Morphological identification of louse flies

Louse flies were identified to species level using available taxonomic keys (Hutson 1984, Muñoz et al. 1993). Nineteen morphological characters were measured in 16 louse flies using a stereo microscope connected to a camera and compared with

those previously reported (Muñoz et al. 1993).

DNA extraction

We separated the tibia and tarsomere from the middle and hind legs of each louse fly in individual Petri dishes using sterile blades, obtaining a tissue fragment weighing under 0.1 mg. Subsequently, each leg (including tibia and tarsomere) of each louse fly was assigned to one of each four DNA extraction treatments. As a result, 32 segments (eight from the right middle legs, eight from the left middle legs, eight from the right hind legs, and eight from the left hind legs) were assigned for each of the four DNA extraction treatments.

According to the chloroform/isoamyl alcohol procedure (Gemmell and Akiyama 1996), with minor modifications, each sample was introduced into individual tubes containing 300 µl of lysis buffer (100 mM NaCl, 50 mM Tris-HCl pH 8, 50 mM EDTA pH 8, 1% SDS), 5 µl of proteinase K (20 mg/ml), and 10 µl of DDT (1 M) and then kept on a shaker incubating at 55° C overnight. The following day, an equal volume (320 µl) of 5 M LiCl was added to each tube and then each sample was mixed by inversion for 1 min after adding 630 µl chloroform/isoamyl alcohol (24:1). After shaking the tubes, the samples were centrifuged for 15 min at 13,000 rpm and the supernatant (500 µl) was carefully removed and transferred into a new tube, where 1 ml of absolute ethanol was added to precipitate the DNA overnight at -18° C. The next day, the DNA was recovered by centrifugation at 13,000 rpm for 15 min. The pellet was dried and washed with 70% ethanol, and resuspended in 20 µl of milliQ water.

According to the HotShot procedure (Truett et al. 2000), each sample was introduced into individual tubes containing 50 µl of lysis solution (25 mM NaOH, 0.2 mM EDTA, pH 8) and then incubated at 95º C for 30 min. After incubation, the solution was put on ice for 5 min and 50 µl of neutralization solution (40 mM Tris-HCl) was added to each sample.

Manufacturer specifications were used for both commercial kits. These methods allow DNA extraction without organic extractions or ethanol precipitations. Qiagen kit method (DNeasy® Kit Tissue and Blood (Qiagen, Hilden, Germany)), involves enzymatic lysis using proteinase K followed by column purification of DNA using silica-gel-matrix. The semi-automatic Maxwell kit method (Maxwell®16 LEV system Research (Promega, Madison, WI)) involves an enzymatic lysis using proteinase K followed by a purification of DNA using magnetic beads that bind to DNA. The complete process was done in a robot for the simultaneous extraction of 16 samples. For Qiagen and Maxwell kits, DNA samples were diluted in 20µl milliQ water.

The average laboratory time requirement for each DNA extraction method was calculated based on our own measurements. The approximate cost per sample of each procedure was provided by the distributor in Spain (Table 1). Prices could vary depending on the country.

DNA amplification and sequencing

The primer pair LCO1490 (5'- GGT CAA CAA ATC ATA AAG ATA TTG G -3') and HCO2198 (5'- TAA CTT CAG GGT GAC CAA AAA ATC A -3') (Folmer et al. 1994) was used to amplify a 658 bp fragment of the COI gene. PCRs were performed with a final volume of 50 µl containing 0.3 mM each deoxynucleoside triphosphate (dNTP), 0.6 µM of each primer, 2.5 mM $MgCl_2$, 1x PCR buffer (Applied Biosystem, Foster City, CA), 0.6 units of Taq DNA polymerase, and 3 µl of DNA. Following Whiteman et al. (2006), PCRs conditions were: an initial denaturation for 4 min (94° C), followed by 35 cycles of 94° C for 1 min, 40° C for 1 min ,and 70° C for 1 min with a final extension at 72° C for 7 min. The presence of amplicons was verified on 1.8% agarose gels.

Table 1. Estimation of economic costs (€) of components used in each DNA extraction method and time necessary for the extraction of DNA from 16 samples. Laboratory equipment is not included.

Extraction method	Ease of operation	Cost (per sample)	Time
DNeasy® Kit Tissue and Blood (Qiagen)	Manual	€ 5.71	5 h
Maxwell®16 LEV system Research (Promega)	Semi-automatic	€ 3.79	1.25 h
HotShot	Manual	< € 1.00	1.5 h
Chloroform/ isoamyl alcohol	Manual	< € 2.50	6 h in 3 days

Sequencing reactions were performed according to the BigDye technology (Applied Biosystems). Positive PCR fragments were resolved in both directions through a 3130xl ABI automated sequencer (Applied Biosystems) using the same primers employed in PCR reactions. Sequences were edited using the SequencherTM v4.9 software (Gene Codes Corp., ©1991-2009, Ann Arbor, MI 48108). Subsequently, Sequencher software was used to quantify the quality value of each sequence obtained by each DNA extraction method after removal of the primer. The quality was measured as the percentage of bases in each sequence with quality scores >20 (see Fazekas et al. 2010).

Statistical analyses

Statistical analyses were conducted using General Linear Mixed Models (GLMMs) in SAS (GLIMMIX procedure, SAS Institute Inc., Cary, NC), including a random factor to account for non-independence of samples coming from the same louse fly. First, we fitted a GLMM with binomial error and logistic link function for success (1) or failure (0) of positive amplification of the COI gene as the response variable and extraction method as explanatory factor. Secondly, we fitted a GLMM with normal error and identity link function for the quality of the sequence obtained as the response variable. The DNA extraction method, the sequence direction (forward or reverse), and their interaction were included as fixed factors. In both analyses, louse fly identity was included as a random factor.

RESULTS

All louse flies were identified as *Ornithophila gestroi* on the basis of morphological characters, in particular the patterns of wing venation. In addition, most morphometric measures of louse flies were within the range previously reported for this species (Table 2). A single genetic haplotype of the COI gene was isolated from the 32 louse flies [GenBank accession number: KJ174684]. Three *O. gestroi* were deposited in the collection of the Museo Nacional de Ciencias Naturales (Madrid, Spain) (accession numbers: MNCN/ADN: 65231 - 65233).

The DNA extraction method used did not affect significantly the number of positive amplifications ($F_{3, 93}$ = 0.43; P = 0.73). Amplification was successful for all the samples (n=32) extracted with the Qiagen kit, whereas 29 were successfully amplified using the HotShot procedure and Maxwell kit extraction method and only 26 when using the chloroform/isoamyl alcohol procedure. However, the quality of the sequence obtained was strongly affected by the DNA extraction method ($F_{3, 194}$ = 8.69; P < 0.0001), while both the sequence direction ($F_{1, 194}$ = 0.85; P = 0.36) and the interaction between the method and the sequence direction ($F_{3, 194}$ = 0.44; P = 0.72) had no effect on the sequence quality. The sequence quality obtained when using DNA extracted with the Qiagen kit, the Maxwell kit, and the HotShot procedure was similar (post-hoc tests, p>0.61). The quality of the sequences obtained using the chloroform/isoamyl alcohol procedure was significantly lower than that obtained using the other three methods. (post-hoc tests, p<0.0001; Figure 1).

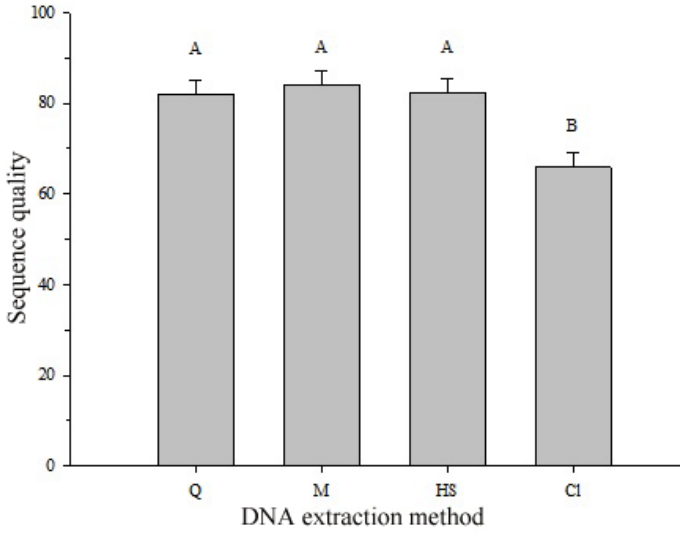

Figure 1. Percentage (± SE) of sequence quality from DNA samples obtained with four different extraction methods (Q= DNeasy® Kit Tissue and Blood (Qiagen); M= Maxwell®16 LEV system Research (Promega); HS= HotShot; Cl= Chloroform/isoamyl alcohol). Dissimilar letters over bars represent significant differences in sequence quality.

Table 2. Measurements (mm) of different morphological characters of 16 *Ornithophila gestroi* (W= width; L= length).

Structure	Mean (SD)	Range
Body length	7.94 (1.02)	6.69-9.80
Wing length	6.62 (0.43)	5.76-7.13
Antennae (W)	0.29 (0.03)	0.26-0.34
Lunula (L)	0.32 (0.08)	0.47-0.23
Lunula (W)	0.68 (0.07)	0.54-0.79
Internal orbital width (medium vertex level)	0.19 (0.02)	0.15-0.20
Eye (L)	0.88 (0.08)	0.73-0.97
Eye (W)	0.51 (0.07)	0.38-0.61
Head (L)	1.46 (0.29)	1.34-1.90
Head (W)	2.03 (0.09)	1.88-2.17
Postvertex (L)	0.31 (0.05)	0.23-0.40
Postvertex (W)	0.88 (0.09)	0.77-1.05
Mediovertex (L)	0.52 (0.1)	0.36-0.62
Mediovertex (W)	0.55 (0.05)	0.48-0.63
Prescutum (L)	0.95 (0.11)	0.79-1.12
Scutellum (L)	0.63 (0.07)	0.51-0.72
Scutellum (W)	1.38 (0.18)	1.07-1.67
Palpi lenght	0.32 (0.1)	0.16-0.43
Minimal distance between ocular margins	0.94 (0.06)	0.84-1.01

DISCUSSION

Genetic characterization of louse flies

Ornithophila gestroi, the species genetically characterized here for the first time, parasitizes different raptor species belonging to the genus *Falco*, that includes species like the Common Kestrel (*Falco tinnunculus*), the Lesser Kestrel (*Falco naumanni*), and the Eleonora's Falcon (Gil Collado 1932, Walter 1979, Beaucournu et al. 1985, Gangoso et al. 2010), thus representing an important piece for studies on host-pathogen interactions on this avian group. Our results showed the presence of a single genetic haplotype in the louse fly population studied in the Canary Islands. This pattern of low variability at this gene had been previously reported in the louse fly *Trichobius major* (Wilson et al. 2007). We cannot discriminate whether this lack of variation is due to a generally low divergence at the COI gene, the fact that samples were obtained from a single island, or to demographic

constraints associated with the geographic isolation of the studied population (e.g., Dasmahapatra and Mallet 2006). Further studies on the genetic diversity of this species, considering samples from different localities, would be necessary to clarify this issue.

Efficacy of DNA extraction methods

By comparing four different DNA extraction procedures, we found that there were no significant differences in the number of amplifications obtained. However, the quality of the sequences was strongly affected by the method used, with the chloroform/isoamyl alcohol procedure resulting in significantly lower sequence qualities than the other three methods. By using the Qiagen kit, we successfully amplified the 658 bp fragments of all louse flies with high sequence quality. These results are in accordance with previous studies comparing DNA extraction procedures from samples with poorly preserved or degraded DNA (Yang et al. 1996, Martínez-de la Puente et al. 2013). These results might be especially useful for studies on valuable specimens held in museums, as only a small fragment of tissue was necessary for barcoding while retaining the rest of the specimen as a voucher. However, this procedure is the most expensive of the four methods compared here, which probably may hinder its widespread use (Table 1). To reduce the overall costs of DNA extractions, cleaning methods could be employed to remove any remaining DNA from silica-gel-columns used (Siddappa et al. 2007), although this could result in traces of contamination (Fogel and McNally 2000).

Furthermore, we found that the semi-automatic Maxwell kit presented a similar efficacy than the Qiagen kit in terms of the sequence quality, although the amplification success was slightly, but not significantly, lower. These results support those previously obtained by Khokhar et al. (2012), who reported that the Maxwell kit is suitable for the extraction of small-size DNA fragments and has the advantage that it requires a limited sample handling (Silva et al. 2013). The Hotshot procedure presented similar results to those obtained with the Maxwell kit. Previous studies have already demonstrated the utility of the Hotshot procedure for DNA barcoding using complete individuals (Montero-Pau et al. 2008, Lassaad et al. 2013). Our results confirmed that the Hotshot procedure yields enough DNA of high quality for barcoding even when using very small quantities of tissue, consequently retaining most of the individual as a voucher.

Finally, we obtained the lowest efficacy, in terms of sequence quality but not in terms of amplification success, using the chloroform/isoamyl alcohol method. This result was unexpected because this method is considered one of the best to obtain DNA of high quality and yield and has been used in studies on barcoding characterization of insects (Gilbert et al. 2007). However, the lower performance could be due to the handling of the extremely small samples in our study, which may result in DNA loss and degradation through the DNA extraction process that involves several steps transferring the supernatant from one tube to another. In this respect, this method may be considered useful in those studies requiring organism identification to the species level, where it is not necessary to obtain a complete barcoding sequence (Vesterinen et al. 2013).

In conclusion, the commercial Qiagen kit was the most suitable method of DNA extraction of the four tested here. Additionally, the Maxwell method (due to its reduced manpower requirements) and the Hotshot procedure (due to their lower cost) provided similar performance but at significantly lower economic costs. The usefulness of the chloroform/isoamyl alcohol method for the characterization of louse fly barcodes is poorly supported by our results.

Acknowledgments

This study was partially supported by the Cabildo de Lanzarote and project CGL2012-30759 from the Spanish Ministry of Science and Innovation. RGL was first supported by a grant from the Doñana 21 Foundation and later by an FPI grant. JMP is supported by a Juan de la Cierva contract and LG by a postdoctoral contract under the Excellence Projects from Junta de Andalucía (RNM-6400). The Regional Government of the Canary Islands approved the research protocols. We thank J. J. Moreno for his help during fieldwork and also I. Martín and F. J. Oficialdegui for their help with molecular analysis and graph design, respectively. Members of the bank of tissues and DNA of the Museo Nacional de Ciencias Naturales de Madrid (MNCN-CSIC) also helped in the deposition of samples.

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