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Accurate decontamination of insects from bulk samples does not affect DNA sequencing success

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Gut content analysis of lepidopteran caterpillars selected from bulk samples can lead to potentially biased results from contamination caused by plant DNA floating in the bulk sample. One method to minimize this error source is to first decontaminate the larvae by bleaching. In this study, we tested the efficiency of different bleaching protocols to decontaminate 190 lepidopteran larvae selected from 51 canopy fogging bulk samples, which were collected at the Panguana field station in the tropical lowland rainforest of Peru (Department Huanuco).

We show that bleaching with a 3 % solution of sodium hypochlorite for one minute does not reduce the sequencing success for the identification of the larvae (COI barcoding) or the identification of the gut contents (rbcL and ITS2 metabarcoding). For the rbcL genetic marker, sequencing reads significantly increased after one minute of bleaching compared to unbleached samples but significantly decreased after two minutes of bleaching. The number of reads for successful ITS2 sequencing did not differ significantly between bleaching treatments but tended to increase with exposure time. Based on the results of this study, we recommend the use of a 3% sodium hypochlorite solution for one minute to decontaminate lepidopteran larvae from bulk samples before gut content analysis.

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Introduction

After the first presentations of gut content analyses for lepidopteran larvae and other insects (Miller et al. 2007, Matheson et al. 2008), the rapid methodological improvements based on high-throughput sequencing (HTS) lead to increasing importance of gut content analyses of invertebrates for synecological research. Bulk samples, e. g., from canopy fogging or malaise traps, are a crucial source of data for this type of research (Hausmann et al. 2020 a,b). Molecular gut content analysis was proposed for unveiling insect-host plant associations, e. g., for beetles (Jurado-Rivera et al. 2009, Pinzón-Navarro et al. 2010, García-Robledo et al. 2013, Kitson et al. 2013) and for soil insects (Wallinger et al. 2013).

Hausmann et al. (2020 a,b) presented this method for caterpillars from canopy fogging bulk samples and discussed its huge potential for unveiling host-plant relationships in tropical biomes, where still little is known about trophic interactions between herbivores and plants.

In this novel field of research, there is still a certain lack of methodological background knowledge and little information on potential pitfalls. For instance, sequencing insects out of bulk samples potentially bears the risk of contamination due to DNA floating in the ethanol of the sample. Linville

& Wells (2002) showed that bleach treatment of maggots reduced the amount of external DNA contamination without interfering with subsequent mtDNA analysis of crop food consumed by the insect. Greenstone et al. (2012) investigated the effect of bleaching for removing external DNA contamination from arthropod predators (beetles, bugs) from bulk samples destined for molecular gut-content analysis. They used a bleaching protocol with exposure of the insects to a 2.5 % solution of commercial bleach over 40 minutes (containing 5.3 % sodium hypochlorite). Under this treatment, most external contaminating DNA was removed, but there was no effect on the sequencing success in the gut content. Meyer & Hoy (2008) successfully removed fungal contamination from hemipteran insects with exposure to 6 % sod ium hypochlorite solution for one minute. Passi et al. (2012) observed considerable DNA degradation caused by the bleaching agent sodium hypochlorite.

This study aimed at testing the sequencing success for (1) DNA barcoding of animal tissues and for (2) plant detection in larval guts after subjecting the larvae to a 3 % sodium hypochlorite solution for one and two-minute increments.

Material and methods

Collecting/canopy fogging

Canopy fogging was performed from the ground with a Swingfog SN 50 fogger, using natural Pyrethrum (in medical white oil solution) as the knock-down agent. For details of the fogging procedure see Floren (2010), for details of the collecting procedure at the ACP Panguana station, Peru (westernmost Amazonian Basin), see Hausmann et al. (2020a).

Tissue sampling and decontamination by bleaching

More than 1000 lepidopteran larvae were pre-sorted out of 94 fogging bulk samples taken in 2017 and 2018. 190 larger caterpillars (>20 mm) were selected from 51 fogging samples to fill two lysis plates. Of these, 88 were 'replicates' (pairs, triplets etc.) of apparently the same species and moulting stage, each from the same fogging sample. As these replicates were deemed comparable, one individual of a pair was subjected to bleaching and the other individual was used as a control.

Supplementary file 1 shows a list of all 190 larvae along with their bulk sample (fogged tree) number. For all 190 larvae, dissection and tissue sampling were performed on individual clean cellulose tissue using sterilized scissors and pincers. In order to avoid contamination among samples, these tools were carefully cleaned in standard 3 % chlorine bleach, then washed in water, cleaned again in 96 % ethanol and wiped off with a clean cellulose tissue after each sampling. Expecting the largest amount of muscle tissue and undigested plant material in the gut to be in the third thoracic segment of the larvae (cf. Woolley et al. 2017), this segment was cut as a vertical slice from each specimen. Smaller larvae required sampling of a larger section ranging from the first thoracic segment to parts of the abdomen. The resulting slice was transferred to a 96-well plate with 96 % ethanol, with one well used as a negative control.

For testing the effects of chlorine bleach decontamination, 77 caterpillars, were chosen for treatment with chlorine bleach prior to dissection (containing 44 larvae that had a replicate to be used as an unbleached control). They were removed from the ethanol, kept dry for about 15–20 minutes on cellulose paper (during the photographing process), and then bleached with a 3 % sodium hypochlorite ('Klorix') solution. By gently shaking the larva in a 15 ml tube with 5 ml chlorine bleach, 39 larvae were decontaminated for one minute, and 38 larvae for two minutes (see Table 1). After that, the bleach was discarded and the larva washed in another tube with distilled water three times for one minute each. A vertical tissue piece was cut for the DNA procedure as described above and re-immersed into 96 % ethanol on a 96-well plate.

Identification of larvae (DNA barcoding, COI)

Tissue samples were submitted to the Canadian Centre for DNA Barcoding (CCDB,Guelph, ON, Canada) for sequencing the mitochondrial 5' cytochrome oxidase gene, subunit 1 (COI), the standard marker for the identification of most animals. Following ethanol evaporation, samples were suspended in 50 µL lysis buffer (700 mM guanidine thiocyanate, 30 mM EDTA pH 8.0, 30 mM Tris-HCl pH 8.0, 0.5 % Triton X-100, 5 % Tween-20) and 2 mg/ml proteinase K (Promega), and then incubated overnight at 56 °C. DNA extraction was performed using a validated automated glass fiber protocol employed by Ivanova et al. (2006). DNA was eluted in 40 µL Elution Buffer (10 mM Tris-HCl, pH 8). PCR amplification was performed using primers LepF1 and LepR1 for the standard procedure and MLepF1 and MLepR1 for failure tracking (Supplementary file 2), and results were visualized using pre-cast 2 % Agarose E-gels (ThermoFisher). Amplified products were then cycle sequenced with a standardized commercially available BigDye Terminator v3.1 kit. Sequencing reactions were analyzed by high-voltage capillary electrophoresis on an automated ABI 3730xL DNA Analyzer. The sequences were compared against the full sequence database of the Barcode of Life Data systems (BOLD; Ratnasingham & Hebert 2007) in order to investigate the closest matches using the BOLD Identification Engine (http://www.boldsystems.org/index.php/IDS_OpenIdEngine). Morphology of larvae and genetic distance to the nearest neighbour

were also considered to verify the reliability of the results. Nomenclature of scientific species names follows the catalogue used on the BOLD database, which in many families is in accordance with the currently available catalogues (e. g. Scoble 1999 for Geometridae). Statistics were tested with ANOVA. Vouchers of larvae are stored at the Bavarian State Collection of Zoology, Germany. Sequences, images and related metadata are available open access on BOLD under the dataset DS-PANLARVA (https://dx.doi.org/10.5883/DS-PANLARVA).

Gut content analysis (High-throughput sequencing, rbcL, ITS2)

Gut content analysis was performed on all 190 larvae for molecular identification of their 'true' diet (cf. Hausmann et al. 2020b). The DNA extracts used for COI barcoding were also used to analyze the gut contents. A two-stage fusion primer approach was used to prepare the amplicons for sequencing on an Ion Torrent S5 next-generation sequencer (Thermo Fisher). The first round of PCR amplification was performed using plant-specific primers tailed with M13 forward and reverse sequences. Primer pairs ITS-S2F_t1/ITS4_t1 and rbcLaF_t1/MrbcL-163R_t1 target an approximate 350 base pair (bp) fragment of the internal transcribed spacer, ITS2 barcode region, and a 163 bp fragment of the plastid genome sequence, rbcL barcode region, respectively (Supplementary file 2). First-round amplification products were visualized using pre-cast 2 % Agarose E-gels (ThermoFisher). The PCR products for each genetic marker were pooled for each plate of samples, diluted with water (1 : 1 v : v) and used as a template for the second round of amplification. The second round contained fusion primers composed of M13 forward and reverse sequences tailed with Ion Torrent sequencing adapters (Xpress A, Xpress trP1 respectively), and 96 Ion Xpress multiplex identifier (MID) tags (forward primers only; Thermo Fisher). To differentiate between the two 96-well plates, an additional identifier was used on the reverse sequences (Ion1-trP1 and Ion2-trP1). Samples were then pooled and purified using carboxylate modified magnetic beads as per the protocol described by Moran et al. (2019). The purified DNA was quantified using a Qubit 2.0 fluorometer dsDNA High Sensitivity kit and prepared to 1 ng/µl.

The NGS library was prepared to a concentration of 26 pM (5 µl of 1 ng/µl purified DNA and 1010 µl of dH2O), and loaded onto the Ion Chef System for clonal library amplification and loading onto an Ion 530 Chip. The S5 Torrent Browser automatically assigned the resulting reads to the samples using the unique MID tags. The demultiplexed data sets were then processed through a bioinformatics pipeline (Prosser & Hebert 2017). After further demultiplexing the two plates by the reverse sequence identifiers, the resulting sequence reads were associated to their source sample by the UMIs (with perfect matching), filtered to remove low quality reads (minimum quality of QV20), trimmed to remove primer and adapter sequences (reads lacking a forward primer were excluded from analysis while reads lacking a reverse primer were allowed to proceed to the next step), and then filtered for a minimum size of 100 bp. The processed reads were then compared to a comprehensive BOLD reference library (www.boldsystems.org) and assigned an identity using the BLAST algorithm. The BLAST results were collapsed into unique taxonomic identifications per sample and identifications were only accepted as genuine if they were supported by at least 100 reads that matched a reference sequence with at least 95 % identity across at least 100 bp. Statistics were tested with ANOVA. ANOVA and posthoc Tukey test were used to test if changes in exposure time to bleaching produced significantly different results.

Table 1. Sequencing success for COI barcoding (Sanger) and gut content analysis (rbcL and ITS2 markers; HTS approach; number of reads with minimum fragment lengths of 100 bp and > 95 % BLAST match.)

exposure time to bleaching	COI			rbcL			ITS2		
	success rate	mean sequence length	standard deviation	success rate	reads (>100 bp)	standard deviation	success rate	reads (>100 bp)	standard deviation
All larvae (190)									
unbleached (113)	79 %	634 bp	55	75 %	31850	18680	35 %	3119	3613
1 minute (39)	77 %	632 bp	49	64 %	41475	13400	18 %	3317	3698
2 minutes (38)	79 %	618 bp	79	61 %	19662	17886	26 %	6470	8540
Directly comparable larvae (88)									
unbleached (44)	75 %	642 bp	21	75 %	34710	20171	27 %	2803	2996
1 minute (18)	67 %	637 bp	29	72 %	40849	16673	17 %	5689	4524
2 minutes (26)	77 %	613 bp	93	58 %	17126	16233	23 %	6072	6941

Results

COI sequencing success for all 190 larvae

First pass sequencing with standard primers was successful in 149/90 cases (78 %), failure tracking with a primer set targeting overlapping 407 bp/306 bp amplicons added 30 sequences, resulting in an overall COI sequencing success of 94 %.

The COI sequencing success (first pass with standard primers) under different bleaching treatments (0/1/2 minutes) for the total of 190 lepidopteran larvae of this study (Table 1, Figure 1) was as follows:

For the 113 unbleached larvae, there were 24 failures (success: 79 %), 634 bp mean sequence length (of 89 successful sequences), 499 bp mean sequence length (of all 113 larvae).

For the 39 larvae bleached for one minute, there were 9 failures (success: 77 %), 632 bp mean sequence length (of 30 successful sequences), 486 bp mean sequence length (of all 39 larvae).

For the 38 larvae bleached for two minutes, there were 8 failures (success: 79 %), 618 bp mean sequence length (of 30 successful sequences), 488 bp mean sequence length (of all 38 larvae).

The sequence lengths for successful sequences do not show a statistically significant difference under the different bleaching treatments ($p = 0.44$).

COI sequencing success for directly comparable larvae

For 88 larvae (44 pairs) the COI sequencing success under different treatments can be directly compared, as the larvae belong to the same species, same size class and moulting stage, and came from the same fogging sample:

44 unbleached larvae: 11 failures (success: 75 %), 642 bp mean sequence length (of 33 successful sequences), 481 bp mean sequence length (of all 44 larvae).

18 larvae bleached for one minute: 6 failures (success: 67 %), 637 bp mean sequence length (of 12 successful sequences), 425 bp mean sequence length (of all 18 larvae).

26 larvae bleached for two minutes: 6 failures (success: 77 %), 613 bp mean sequence length (of 30 successful sequences), 471 bp mean sequence length (of all 26 larvae).

The sequence lengths for successful sequences do not show a statistically significant difference under the different bleaching treatments ($p = 0.17$).

Effects on gut content analysis for all 190 larvae

For the following analysis, only those NGS reads are counted which exceed 100 reads per sample record (plant OTU) and which exceed 95 % similarity with another sequence in the BOLD reference library.

For the 113 unbleached larvae, there were 80 records with failures for one or both markers (71 %). The average read counts were 31 850 + 3043 reads per larva (for 85 successful rbcL and 41 successful ITS2 runs), and 23 958 + 1104 reads per larva (rbcL and ITS2; of all 113 larvae).

For the 39 larvae bleached for one minute, there were 32 records with failures for one or both markers (82 %). The average read counts were 41 475 + 2902 reads per larva (for 25 successful rbcL and 8 successful ITS2 runs), and 26 587 + 595 reads per larva (rbcL + ITS2; of all 39 larvae).

For the 38 larvae bleached for two minutes, there were 28 records with failures for one or both markers (74 %). The average read counts were 19 662 + 6470 reads per larva (for 23 successful rbcL and 10 successful ITS2 runs), and 11 900 + 1703 reads per larva (rbcL + ITS2; of all 38 larvae).

Reads for successful rbcL-sequencing are significantly different with different exposure to bleaching ($p < 0.001$). Bleaching for one minute significantly increases reads per larva compared to unbleached ($p < 0.05$), whereas bleaching for two minutes significantly decreases the reads compared to unbleached ($p < 0.05$) and bleaching for one minute ($p = 0.001$). The number of reads for successful ITS2-sequencing does not differ significantly between bleaching treatments ($p = 0.15$) but tend to increase with exposure time.

Effects on gut content analysis for directly comparable larvae

For 88 larvae (44 pairs), the NGS sequencing success of gut content under different treatments can be compared directly (see above):

For the 44 unbleached larvae, there were 35 records with failures for one or both markers (80 %). The average read counts were 34 724 + 2803 reads per larva (for 33 successful rbcL and 12 successful ITS2 runs), and 26 043 + 765 reads per larva (rbcL + ITS2; of all 44 larvae).

For the 18 larvae bleached for one minute, there were 15 records with failures for one or both markers (83 %). The average read counts were 40 849 + 5689 reads per larva (for 13 successful rbcL and 3 successful ITS2 runs), and 29 502 + 948 reads per larva (rbcL + ITS2; of all 18 larvae).

For the 26 larvae bleached for two minutes, there were 20 records with failures for one or both markers

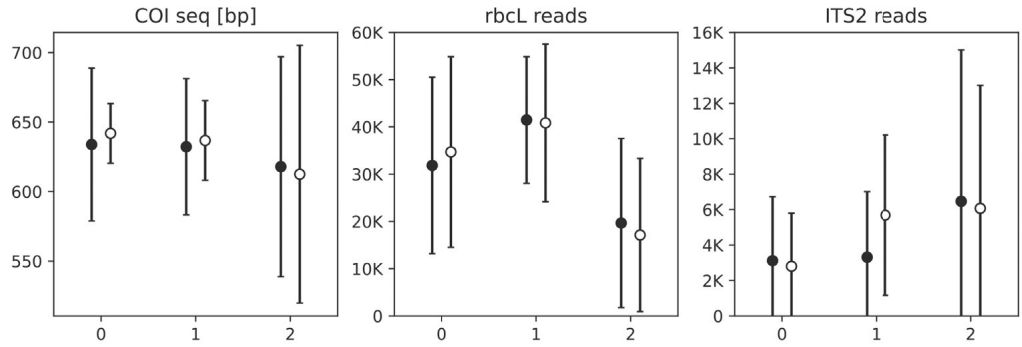

Fig. 1. Sequencing success for COI barcoding (Sanger) and gut content analysis (rbcL and ITS2 markers; HTS approach). ●,○, mean values; bars, standard deviation; "seq", sequence length; ●, sequencing success for all 190 larvae; ○, sequencing success for the 88 directly comparable larvae.

(77 %). The average read counts were 19120 + 6072 reads per larva (for 15 successful rbcL and 6 successful ITS2 runs), average of 11031 + 1401 reads per larva (rbcL + ITS2; of all 26 larvae).

Analogous to the analysis of all 190 larvae, read counts for successful rbcL-sequencing are significantly different with different exposure to bleaching ($p < 0.01$). Bleaching for one minute, however, does not significantly increase reads per larva compared to unbleached ($p = 0.57$), whereas bleaching for two minutes significantly decreases the reads compared to unbleached ($p < .01$) and bleaching for one minute ($p < 0.01$). The number of reads for successful ITS2-sequencing does not differ significantly between bleaching treatments ($p = 0.32$).

Discussion

Sodium bleaching procedures with exposure for one and two minutes did not show any effect on sequencing success in COI barcoding, measured by failure rates and sequence lengths. In the HTS-based gut content analysis, 2-minutes-bleaching reduced the sequencing success for the rbcL marker whilst it was increasing the success for the ITS2 marker. Since increasing sequencing success can hardly be explained by the bleaching procedure, this may be due to the accidental effects of the small sample size. Since the success rate for ITS2 was low and the standard deviation high, the statistical significance may be reduced due to the low sample size. Based on our results we suggest that decontamination by sodium bleaching (3 % solution of sodium hypochlorite) for one minute is the best solution for COI barcoding and gut content analyses of insect vouchers after selection from bulk samples if comparable to our samples (larvae of 20–40 mm length). If the target insects can be selected and singularized in the field, decontamination may not be needed at all.

In our study, gut content analyses of directly comparable larvae (same species, same size, fogged from the same tree) in the vast majority of cases are pointing to the same plant family, underpinning the correctness of the results from gut content analysis without any nuisance through contamination (Hausmann et al. in prep.). In the case of larva no. 82, the gut content analysis of the unbleached larva seemed to have been contaminated (*Pistacia* instead of *Guarea / Aglaia*, the latter being the fogged tree and the gut content confirmed in a 'replicate', second larva of the same sample, after decontamination). The same may have happened in larva no. 88 (*Piper* instead of *Palaqium*) and no. 132 (*Cucurbita* instead of *Zollernia*). Polyphagy may be another explanation for such inconsistencies, but a detailed analysis of the gut content analyses was beyond the scope of this study and will be provided in a subsequent paper (Hausmann et al. in prep.).

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Supplementary file 1

Sample IDs (BOLD datasystems) for the 190 larvae from canopy fogging in Panguana (Peru) with exposure times to sodium hypochlorite bleaching (in minutes). Sequencing success for the COI barcode region (fragment length in base pairs; first pass with standard primers, second pass/failure tracking targeting overlapping 306 bp/407 bp amplicons) and for rcbL and ITS2 gene fragments (number of reads) in the High Throughput Sequencing (HTS) approach. For the 88 directly comparable larvae (same tree, same species, same moulting stage) the sample ID is marked by an asterisk.

Supplementary file 2

Primers used for DNA amplification of larval tissues and gut contents.