HIGH QUALITY DNA EXTRACTION FROM *HELICOVERPA ARMIGERA* FOR NEXT GENERATION SEQUENCING

Lauren K. Woolley, Yizhou Chen*, Kate L. Langfield and Grant A. Herron.

NSW Department of Primary Industries, Elizabeth Macarthur Agricultural Institute, Woodbridge Road, Menangle, NSW 2568, Australia.
*yizhou.chen@dpi.nsw.gov.au;

Summary

Modern sequencing technologies such as massively parallel sequencing with high throughput require input of high quality DNA. Numerous methods exist for the isolation of high quality DNA from insects, including commercially available kits such as the DNeasy® Blood & Tissue kit (Qiagen) and the Isolate II Genomic DNA kit (Bioline). Here we isolated DNA from *Helicoverpa armigera* (cotton bollworm or old world bollworm) using the DNeasy® method and the Isolate II Genomic DNA kit, and assessed the use of pure, high molecular weight extracts for genotyping by sequencing. High quality DNA suitable for genotyping by sequencing was only achieved using the DNeasy® Blood & Tissue kit. Furthermore, selective tissue sampling from the head only proved vital in obtaining high quality DNA, with extracts from the thorax and abdomen often degraded or containing low molecular weight contaminants.

Keywords: DNeasy®; selective tissue sampling; genotyping by sequencing

INTRODUCTION

Isolation of high molecular weight, contaminant-free DNA is a prerequisite for many molecular techniques including polymerase chain reaction (PCR), restriction digestion, southern blotting, DNA library preparation and NGS technologies. DNA extraction from insects can be problematic due to the presence of a hard chitinous exoskeleton that is difficult to homogenize (Hill and Gutierrez 2003, Hubbard et al. 1995). Furthermore, for unknown reasons DNA from some arthropods such as ixodid ticks is highly susceptible to degradation (Hill and Gutierrez 2003, Hubbard et al. 1995). The isolation of high quality DNA can also be complicated by the high concentration of plant phenolics and tannins in the digestive tracts of xylophagous insects (Calderón-Cortés et al. 2010). Nevertheless, there are a number of published methods for insect DNA isolation, the majority of which are sodium dodecyl sulfate (SDS)/proteinase K based protocols (Chen et al. 2010, Henry et al. 1990, Hill and Gutierrez 2003), various cetyltrimethylammonium bromide (CTAB) methods (Calderón-Cortés et al. 2010, Chen et al. 2010), and commercially available kits (Ammazzalorso et al. 2015, Chen et al. 2010, Gilligan et al. 2015). The SDS and CTAB methods are somewhat time-consuming and need a fume hood to operate because of hazardous reagents (i.e. phenol and chloroform). Commercial kits such as the DNeasy® Blood and Tissue kit and Isolate II Genomic DNA kit are more time-efficient, and use spin columns with DNA-binding silica membranes and a buffer system for cell lysis, DNA binding and elution. The various extraction methods differ in terms of quality and quantity of extracted DNA (Chen et al. 2010), and thus selection of an appropriate isolation technique is largely influenced by the requirements for intended downstream molecular use. For instance, NGS technologies require several micrograms of high quality DNA i.e. high molecular weight DNA with little contamination from RNA or protein (Healey et al. 2014).

H. armigera is a major agricultural pest in Australia, Asia and Africa with demonstrated resistance to a range of synthetic insecticides (Forrester et al. 1993). Successful isolation of DNA from *H. armigera* is the first step to understanding the molecular basis of resistance to synthetic insecticides and *Bt*-toxins. Here we investigate the use of the DNeasy® Blood & Tissue kit and the Isolate II Genomic DNA kit to extract high quality DNA suitable for GBS, from single *H. armigera* larvae and adults. Furthermore, we investigate whether the quality of isolated DNA is dependent upon selective tissue sampling of the head, thorax and abdominal segments.

MATERIALS and METHODS

Insects

H. armigera were obtained from a colony maintained at the Australian Cotton Research Institute, New South Wales Department of Primary Industries, Narrabri, Australia. Larvae were reared on an artificial diet of which soybean flour and wheat germ were the main constituents. Adults were supplemented with a 4% honey/sugar solution. Larvae and adults were collected alive and stored as a bulk sample or individually in RNA*later*® (Sigma-Aldrich, USA Lithuania) at -20 ºC until required.

DNA extractions

Initially DNA was extracted from random, unspecified segments of *H. armigera* larvae (third instar, ~ 8-13 mm) and adults using the DNeasy® Blood & Tissue kit (Qiagen, Germany), producing variable DNA yield and quality. To determine

whether such variation in DNA quality was associated with the use of specific tissues (i.e the head, thorax or abdomen) larvae were divided into eleven segments: head (H), prothorax (T1), mesothorax (T2), metathorax (T3), and seven abdominal segments (A1-A7); and the adult into seven segments: head (H), thorax (T1-T2), and four abdominal segments (A1-A4). DNA was extracted using the DNeasy® Blood & Tissue kit or the Isolate II Genomic DNA kit according to the manufacturer's instructions.

Briefly, the DNeasy® Blood & Tissue kit, required samples were homogenized using a disposable microtube pestle in 180 µL sterile 1 × phosphate buffered saline (PBS) (Sigma-Aldrich, USA), and lysed at 56 °C for 10 min in the presence of 20 µL proteinase K and 200 µL Buffer AL. DNA was precipitated with 200 µL of pure ethyl alcohol (Sigma-Aldrich, USA) and the mixture pipetted directly onto the silica-based membrane of a DNeasy® Mini spin column placed in a 2 mL collection tube. DNA was bound to the membrane by centrifugation at 16,300 × *g* for 1min. The column was washed with 500 µL of Buffer AW1 followed by 500 µL of Buffer AW2. To ensure any residual ethyl alcohol was removed from the column, a final centrifugation step was performed at 16,300 × *g* for 3 min. DNA was eluted in 200 µL Buffer AE at room temperature.

The Isolate II Genomic DNA Kit required larvae segments to be homogenized using a disposable microtube pestle in 50 µL of 1 × PBS, and incubated for 1 hr at 56 ºC in the presence of 180 µL Lysis Buffer GL and 25 µL proteinase K solution. Samples were lysed in 200 µL Lysis Buffer G3, vortexed and incubated at 70 ºC for 10 min. Pure ethyl alcohol (200 µL) was added and samples were vortexed before being pipetted into a Spin Column placed into a Collection Tube. DNA was bound to the column by centrifugation at 11,000 × *g* for 1 min. The column was washed with 500 µL Wash Buffer GW1 and 600 µL Wash Buffer GW2, and then dried by centrifugation at 11,000 × *g* for 1 min. DNA was eluted in 100 µL of Elution Buffer G (pre-heated to 70 ºC).

To increase DNA yield and quality, we included the following modifications to standard procedures: addition of 10 µL of RNase A (100 mg/mL) prior to lysis; doubling the amount of proteinase K, Buffer AL and pure ethyl alcohol; performing the lysis step overnight; and/or eluting DNA at room temperature versus 70ºC.

Evaluation of DNA quality

The yield and quality of extracted DNA was estimated using the Nanodrop™ 2000 Spectrophotometer. Samples were further analysed by gel electrophoresis on a 1% agarose gel in 1× Tris/Acetic Acid/Ethylenediaminetetraacetic Acid (TAE) Buffer (Bio-Rad, USA) and visualized using GelRed™ stain (Jomar Diagnostics, USA). The lambda DNA/HindIII marker (Life Technologies, Australia) with known band size and concentration was included on the gel. Samples were prepared for gel electrophoresis by adding 4 µL of 6 × DNA Gel Loading dye (Life Technologies, Lithuania) to 7 µL of undiluted DNA.

Genotyping by Sequencing

To confirm the suitability of DNA isolated from the head of *H. armigera* larvae and adults via the DNeasy® method for downstream NGS technologies, we carried out genotype by sequencing (GBS) for 95 DNA samples (73 larvae and 22 adults). The GBS library comprising 95 DNA samples and a negative (no DNA) control were processed by the Genomic Diversity Facility at Cornell based on Elshire et al. (2011). Each DNA sample was digested individually with PstI and ligated with unique adapters (barcodes) and a common adapter. Ligated DNA samples were purified, pooled and amplified by PCR. The pooled DNA library was sequenced on one lane of the Illumina HiSeq 2000 at the Genomic Diversity Facility at Cornell University (New York, USA). The raw sequence passed quality controls set by Illumina for downstream analysis. The criterion of GBS quality was valid tag counts defined as a unique sequence read which starts with a barcode containing the PstI restriction sequence and did not contain any uncalled bases. The number of valid tags was obtained by Tassel filter (Tassel 5) (Glaubitz *et al.* 2014).

RESULTS

DNA extractions

For the DNeasy® method, high quality DNA from larvae could only be recovered from the head and first thoracic segment (Fig. 1). Smearing indicative of degraded DNA and low molecular weight contaminants was evident in the larvae meso and metathorax as well as abdominal samples, especially those taken from the first to the fifth abdominal segment (Fig. 1). Smearing was less pronounced in adult segments compared with larvae, with only the thoracic adult segments proving unreliable (Fig. 1). In contrast to the DNeasy® method, the Isolate II Genomic DNA kit was inappropriate for extraction of DNA from *H. armigera* with smearing and the

presence of low molecular weight contaminants being evident in all larvae segments including the head (Fig. 1).

Attempts to improve quality of DNA isolated from the head of larvae using modified procedures to the standard DNeasy® method were unsuccessful (Fig. 2). Certainly, elution of DNA at 70ºC (Fig. 2, Lanes 9-12) resulted in the co-purification of low molecular weight contaminants (Fig. 2). Furthermore, there was no increase in the intensity of the high molecular weight band when double the amount of proteinase K, Buffer AL and ethyl alcohol were used (Fig. 2, Lanes 1-2), or when the lysis incubation step was increased from 10min (Fig. 2, Lanes 1-6) to overnight (Fig. 2, Lanes 7-12).

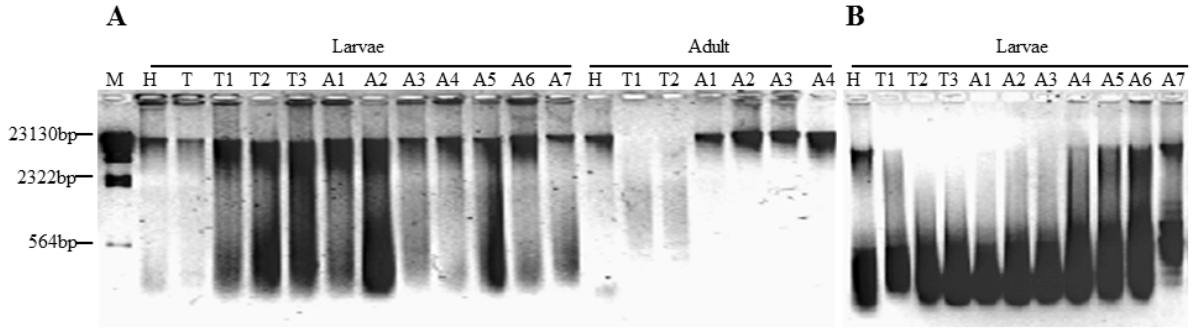

Figure 1. A) Genomic DNA preparations of representative *Helicoverpa armigera* larvae and adult segments (head: H; thorax: T; prothorax: T1; mesothorax: T2; metathorax: T3; abdominal segments: A1-A7) purified using the DNeasy® Blood & Tissue kit according to the manufacturer's supplementary protocol for purification of total DNA from insects and resolved by electrophoresis. M: Lambda DNA/HindIII Marker. **B)** Genomic DNA preparations of representative *Helicoverpa armigera* larvae segments (head: H; thorax: T; prothorax: T1; mesothorax: T2; metathorax: T3; abdominal segments: A1-A7) purified using the Isolate II Genomic DNA Kit according to the manufacturer's protocol for purifying genomic DNA from cultured cells and human or animal tissue.

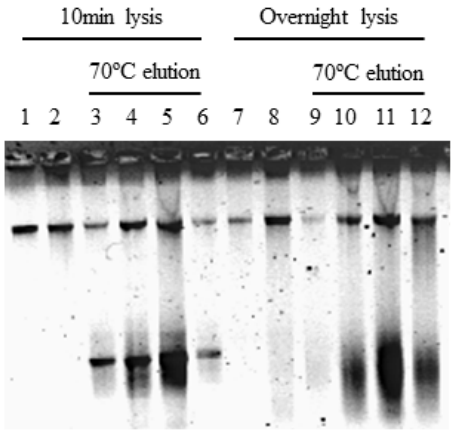

Figure 2. Genomic DNA preparations of *Helicoverpa armigera* larvae (head only) purified using the DNeasy® Blood & Tissue kit according to the manufacturer's supplementary protocol for purification of total DNA from insects and resolved by electrophoresis.

Evaluation of DNA quality

The 260/280 absorbance ratios for DNA isolated using the DNeasy® Blood & Tissue kit were indicative of relatively pure samples, whereas DNA extracted using the Isolate II Genomic DNA kit were less pure (Table 1). Whilst absorbance readings provided an estimate of DNA purity it cannot detect degraded DNA as was evident in the agarose gels for DNA isolated from the thorax and abdomen of larvae using the DNeasy® method, and for all samples extracted using the Isolate II Genomic DNA kit (Fig. 1).

Table 1. UV absorbance profiles and estimated DNA concentration for *H. armigera* genomic DNA extracts as measured using the Nanodrop™ 2000 Spectrophotometer. ND: concentration below 0ng/µL.

Sample	Extraction method	Concentration (ng/µL)	A260/A280	A260/A230
H (larvae, Fig.1)	DNeasy®	37.2	2.12	3.59
T (larvae, Fig. 1)	DNeasy®	39.3	1.94	2.44
T1 (larvae, Fig. 1)	DNeasy®	108.0	2.10	2.20
T2 (larvae, Fig. 1)	DNeasy®	144.7	2.16	2.33
T3 (larvae, Fig. 1)	DNeasy®	153.0	2.13	2.32
A1 (larvae, Fig. 1)	DNeasy®	109.6	2.14	1.95
A2 (larvae, Fig. 1)	DNeasy®	189.3	2.16	2.38
A3 (larvae, Fig. 1)	DNeasy®	264.0	2.13	2.23
A4 (larvae, Fig. 1)	DNeasy®	81.1	1.96	1.94
A5 (larvae, Fig. 1)	DNeasy®	400.1	2.13	2.19
A6 (larvae, Fig. 1)	DNeasy®	270.8	1.98	2.02
A7 (larvae, Fig. 1)	DNeasy®	170.5	2.12	2.28
H (adult, Fig. 1)	DNeasy®	105.5	1.78	0.73
T1 (adult, Fig. 1)	DNeasy®	8.3	1.93	20.73
T2 (adult, Fig. 1)	DNeasy®	11.3	2.03	0.99
A1 (adult, Fig. 1)	DNeasy®	18.2	2.04	1.36
A2 (adult, Fig. 1)	DNeasy®	22.7	1.87	1.61
A3 (adult, Fig. 1)	DNeasy®	32.7	1.83	1.29
A4 (adult, Fig. 1)	DNeasy®	41.4	1.71	1.02
1 (larvae, Fig. 2)	DNeasy®	19.8	2.02	1.40
2 (larvae, Fig. 2)	DNeasy®	26.1	2.11	2.31
3 (larvae, Fig. 2)	DNeasy®	39.2	2.13	4.32
4 (larvae, Fig. 2)	DNeasy®	67.5	2.00	1.69
5 (larvae, Fig. 2)	DNeasy®	146.4	2.17	2.10
6 (larvae, Fig. 2)	DNeasy®	27.0	2.17	1.83
7 (larvae, Fig. 2)	DNeasy®	14.3	2.08	1.25
8 (larvae, Fig. 2)	DNeasy®	35.3	2.12	2.14
9 (larvae, Fig. 2)	DNeasy®	22.0	2.23	1.66
10 (larvae, Fig. 2)	DNeasy®	53.6	2.20	1.89
11 (larvae, Fig. 2)	DNeasy®	161.7	2.16	2.29
12 (larvae, Fig. 2)	DNeasy®	78.3	2.12	2.12
H (larvae, Fig.1)	Isolate II Kit	40.0	-15.74	-0.21
T1 (larvae, Fig. 1)	Isolate II Kit	101.5	4.05	-0.59
T2 (larvae, Fig. 1)	Isolate II Kit	190.6	2.88	-1.44
T3 (larvae, Fig. 1)	Isolate II Kit	276.1	2.58	-2.99
A1 (larvae, Fig. 1)	Isolate II Kit	203.0	2.79	-1.56
A2 (larvae, Fig. 1)	Isolate II Kit	391.3	2.44	-9.54
A3 (larvae, Fig. 1)	Isolate II Kit	363.0	2.43	-7.41
A4 (larvae, Fig. 1)	Isolate II Kit	373.6	2.42	-7.45
A5 (larvae, Fig. 1)	Isolate II Kit	414.4	2.36	-18.79
A6 (larvae, Fig. 1)	Isolate II Kit	354.8	2.36	-8.07
A7 (larvae, Fig. 1)	Isolate II Kit	192.6	2.86	-1.54
A8 (larvae, Fig. 1)	Isolate II Kit	119.8	3.54	-0.74

Genotyping By Sequencing

DNA extracted from the head of *H. armigera* larvae and adults using the DNeasy® method generated 258,825,273 valid tag (reads starting with barcode plus cut site remnant sequence). The number of tag generated per sample ranged from 285,389 to 6,607,756 (Fig. 3) and is sufficient for genotype calling.

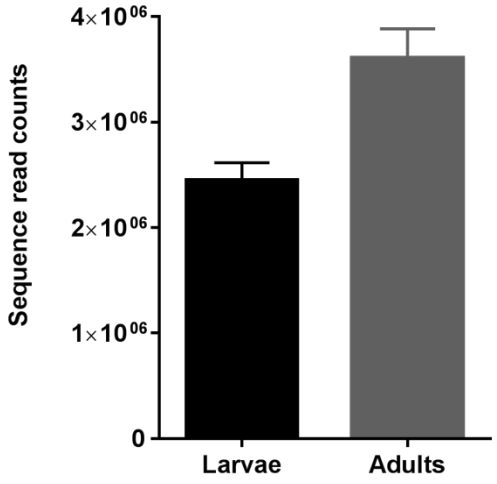

Figure 3. Distribution of sequence reads which contained the barcode and restriction enzyme cut sequence. Mean ± sem.

DISCUSSION

In general low molecular weights and/or DNA smearing were less in adult segments than in larvae. It is uncertain why tissue sampling was less problematic in adults compared with larvae. However, previous studies have used head, thorax, wings or legs to avoid phenolic compounds and other plant contaminants in the digestive tract which can co-precipitate with genomic DNA during extraction (Calderon-Cortes et al. 2010, Horne et al. 2004, Juen and Traugott, 2006, Wong et al. 2014). Additionally in the larvae apoptosis may have been a contributing factor to obtaining lower-quality DNA from abdominal segments compared with the head. Apoptosis (programmed cell death) plays a central role in insect development regulating molting and metamorphosis, cell proliferation, differentiation and death (Truman and Riddiford 2002). Apoptosis caused by the regulatory caspase gene (Hearm caspase-1) is known to occur in the embryos, haemocytes, fat body and midgut of *H. armigera* larvae (Yang et al. 2008). Similarly, expression of another caspase gene (*Cs*caspase-1) was shown to be higher in the midgut, hindgut and Malpighian tubules compared with the foregut and head of *Chilo suppressalis* (striped rice stemborer) (Lu et al. 2013).

In conclusion, we consider the extraction of high quality DNA from *H. armigera* larvae and adults preserved in RNA*later*® is largely dependent on use of an appropriate isolation method and selective tissue sampling. If precise sample selection is followed by avoiding thorax and abdominal section in larvae, the DNeasy® method can be used to isolate high quality genomic DNA suitable for use in downstream GBS applications.

ACKNOWLEDGEMENTS

We thank L.J. Bird and L. Drynan for providing *H. armigera* larvae and adults. This work was funded by the Australian Cotton Research and Development Corporation (CRDC) as project DAN1507.

REFERENCES

Ammazzalorso, A.D., Zolnik, C.P., Daniels, T.J. and Kolokotronis, S-O. (2015) To beat or not to beat a tick: comparison of DNA extraction methods for ticks (*Ixodes scapularis*). *PeerJ* 13: doi: 10.7717/peerj.1147

Calderón-Cortés, N., Quesada, M., Cano-Camacho, H. and Zavala-Páramo, G. (2010) A Simple and Rapid Method for DNA Isolation from Xylophagous Insects. *International Journal of Molecular Science* **11**: 5056–5064.

Chen, H., Rangasamy, M., Tan, S.Y., Wang, H. and Siegfried, B.D. (2010). Evaluation of Five Methods for Total DNA Extraction from Western Corn Rootworm Beetles. *PLoS ONE* 5: doi: 10.1371/journal.pone.0011963.

Forrester, N.W., Cahill, M.,Bird, L.J. and Layland, K. (1993). Management of pyrethroid and endosulfan resistance in Helicoverpa armigera. *Bulletin of Entomological Research* **1**: 1-132.

Gilligan, T.M., Tembrock, L.R., Farris, R.E., Barr, N.B., van der Straten, M.J., et al (2015). A Multiplex Real-Time PCR Assay to Diagnose and Separate *Helicoverpa armigera* and *H. zea* (Lepidoptera: Noctuidae) in the New World. *PLoS ONE* **10:** e0142912.

Healey, A., Furtado, A., Cooper, T. and Henry, R.J. (2014). Protocol: a simple method for extracting next-generation sequencing quality genomic DNA from recalcitrant plant species. *Plant Methods* **10**. doi: 10.1186/1746-4811-10-21.

Henry, J.M., Raina, A.K. and Ridgway, R.L. (1990). Isolation of high-molecular-weight DNA from insects. *Analytical Biochemistry* **185**: 147-150.

Hill, C.A. and Gutierrez, J.A. (2003). A method for extraction and analysis of high quality genomic DNA from ixodid ticks. *Medical and Veterinary Entomology* **17**: 224-227.

Horne, E.C., Kumpatla, S.P., Patterson, K.A., Gupta, M. and Thompson, S.A. (2004). Improved high-throughput sunflower and cotton genomic DNA extraction and PCR fidelity. *Plant Molecular Biology Reporter* **22**: 83a–83i.

Hubbard, M.J., Cann, K.J. and Wright, D.J. (1995). Validation and rapid extraction of nucleic acids from alcohol-preserved ticks. *Experimental and Applied Acarology* **19**: 473-478.

Juen, A. and Traugott, M. (2006). Amplification facilitators and multiplex PCR: Tools to overcome PCR-inhibition in DNA-gut-content analysis of soil-living invertebrates. *Soil Biology and Biochemistry* **38:** 1872-1879.

Lu, M.-X., Du, Y.-Z., Cao, S.-S., Liu, P. and Li, J. (2013) Molecular Cloning and Characterization of the First Caspase in the Striped Stem Borer *Chilo suppressalis*. *International Journal of Molecular Sciences* **14**: 10229–10241.

Truman, J.W. and Riddiford, L.M. (2002). Endocrine Insights into the Evolution of Metamorphosis in Insects. *Annual Review of Entomology* **47**: 467-500.

Wong, W.H., Tay, Y.C., Puniamoorthy, J., Balke, M., Cranston, P.S. and Meier, R. (2014) 'Direct PCR' optimization yields a rapid, cost-effective, nondestructive and efficient method for obtaining DNA barcodes without DNA extraction. *Molecular Ecology Resources* **14:** 1271–1280.

Yang, D., Chai, L., Wang, J. et al. (2008). *Molecular Biology Reports* **35**: 405.