BARCODING: A TOOL TO ASSIST THE INDUSTRY TO MANAGE INSECT INCURSIONS

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Abstract

THE AUSTRALIAN SUGARCANE industry has been fortunate not to have several significant insect pests that exist overseas. Moth borers are a particular concern to the Australian sugar industry due to the heavy losses they cause overseas and their potential to colonise new regions. Other high risk exotic pests include whiteflies, planthoppers and aphids. The biosecurity group in SRA is involved in many activities to prepare for possible incursions, including the development of diagnostic methods. Since the adult stage (which is required for correct taxonomic identification) may not be present in the early stage of an incursion, it is important to develop diagnostic techniques which apply to all insect stages or any available field material. The presence of frass may be the only indication that stalk damage is due to a moth borer. In such cases, identification can be greatly assisted by DNA-based techniques. For almost all animals, the universal barcode is the cytochrome oxidase I gene (COI), and this universality has led to the Barcode of Life, a project to promote DNA barcoding as a global standard for sequence-based identification. As several important exotic pests do not have publically available barcodes, SRA is assembling its own reference collection. Using the minor Australian sugarcane pest *Bathytricha truncata* as an experimental system, we attempted to develop methods to extract DNA and perform barcoding on frass. The method was then applied to detect three exotic borers in stalk material from PNG. Accurate detection of insect DNA from the complex stalk/frass environment is challenging, but can be achieved.

Introduction

Several damaging insect pests of sugarcane exist to the immediate north of Australia, particularly moth borers. Australia is fortunate not to have any of these devastating species. Species of *Chilo, Scirpophaga* and *Sesamia* are widely distributed across South East Asia and Papua New Guinea (PNG) and are major constraints to sugar production. For example, *Sesamia grisescens* is responsible for commercial cane losses of 31 t/ha and sugar losses of 18% in PNG (Kuniata *et al.,* 2001).

SRA conducts many activities as part of its biosecurity planning. The first step is to be aware of what is out there, and this is achieved through surveys and collaborations with organisations such as Ramu Agri-Industries Limited (RAIL) and the Indonesian Sugar Research Institute (ISRI).

The next step is to establish quick and reliable pest identification methods to promptly deploy in case of an incursion. If a high risk pest is suspected to be present in Australia, there needs to be a rapid diagnostic capacity to identify the pest and confirm whether it is an indigenous/endemic problem or an exotic one. The final stage is the development of incursion management plans. These plans outline our response, being either eradication or containment (i.e. long term management strategies if eradication is unsuccessful).

Correct identification of insects often requires the adult stage, which is usually difficult to obtain, while the larval or pupal stages of moth borers are the more likely stages to be found in damaged stalks. Larvae can be difficult to separate morphologically and there can also be situations where only eggs or frass (insect excrement) are present. In these cases, identification can be greatly assisted by DNA-based techniques.

The term 'barcode' refers to a short standardised fragment of genomic DNA, so called because it is like a barcode tag for each taxon (Jinbo *et al.,* 2011). The procedure involves a generic Polymerase Chain Reaction (PCR) test, followed by DNA sequencing, and then identification through association with DNA sequences stored in databases.

DNA barcoding

The current worldwide standard for insect identification (and in fact all animals) is the upstream half of the mitochondrial cytochrome c oxidase subunit I (COI or *cox*1). COI barcoding is used in the project *Barcode of Life* (BOL). The International Barcode of Life (iBOL) is a global collaboration that aims to build DNA barcode libraries for each animal group including insects.

Plants, fungi and bacteria are also included in iBOL but are identified using other standardised genes. The Barcode of Life Data Systems (BOLD) is an informatics workbench aiding the acquisition, storage, analysis and publication of DNA records.

As described by Mitchell (2008), the strength of DNA barcoding comes from its combination of four main factors:

1. standardisation on a particular gene region, with the COI gene being used for insects;

2. the large scale of operation, where the global consortiums depositing data use high-throughput techniques to process thousands of samples per day;

3. compulsory deposition of voucher specimens in museums or herbaria to facilitate integrated morphological and molecular taxonomy and

4. enforcement of data standards including specimen collection records, photographs, numbers of specimens that must be processed and minimum quality scores for sequence trace files, combined with the review and curation of the records. This last factor should lead to a degree of rigour that is currently not found in existing public domain primary databases, such as GenBank. Despite this, many BOLD records are simply mined from GenBank.

There are also disadvantages. Barcoding relies on matching a test sequence against databases of sequences, but if that insect is yet to be sequenced, or the sequence has been kept private, or the reference insect was misidentified, then correct matching will not occur.

Many large consortiums keep their data private until project completion. Also, due to the BOLD requirement for vouchered taxonomic specimens, larval sequences can be difficult to deposit into the database in the absence of adult moths.

For this reason, many SRA derived sequences have not been submitted to BOLD, only GenBank, or exist as a small in-house data set in the event of an incursion. We also have access to data collected by our collaborators, such as Dr A. Mitchell from the Australian Museum (Sallam and Mitchell, 2015).

High priority pests of the Australian sugar industry

The Plant Health Australia Biosecurity Plan for the Sugarcane Industry lists more than 240 exotic plant pests and the potential biosecurity threat that they represent to the Australian sugarcane industry.

Each pest is given an overall risk rating based on four criteria: entry, establishment and spread potential and economic impact. Table 1 shows the high risk and extreme risk insect threats likely to come from Indonesia and PNG.

The current barcode status of some important exotic pests, including several examples of borers, is shown in Table 2. Many borers of interest to the Australian sugar industry have been successfully barcoded with two genes; the mitochondrial cytochrome oxidase II (COII) and the

mitochondrial large ribosomal subunit (16S) by Lange *et al.* (2004) and the sequences are publically available.

Table 1—High priority pests likely to enter Australia from Indonesia and PNG. Information is extracted from the Plant Health Australia Biosecurity Plan for the Sugarcane Industry. The nearest known location to Australia and entry potential of the pest is shown. Overall threat risk is a measure of entry, establishment and spread potential and economic impact.

Common name	Scientific name	Closest known location	Entry potential	Overall risk
Hemiptera				
Sugarcane whitefly	*Aleurolobus barodensis*	Java	high	high
Sugarcane woolly aphid	*Ceratovacuna lanigera*	PNG	high	high
Island sugarcane planthopper (vector of Ramu stunt)	*Eumetopina flavipes*	PNG	high	extreme
Sugarcane planthopper (vector of Fiji leaf gall)	*Perkinsiella vastatrix* and *P. vitiensis*	PNG	high	extreme
Sugarcane pyrilla	*Pyrilla perpusilla*	Indonesia	medium	high
Lepidoptera				
Sugarcane stalk borer	*Chilo auricilius*	PNG	medium	extreme
Yellow top borer	*Chilo infuscatellus*	PNG	medium	extreme
Sugarcane internode borer	*Chilo sacchariphagus*	Indonesia	medium	extreme
Stem borer	*Chilo terrenellus*	Torres Strait	high	extreme
Top shoot borer	*Scirpophaga excerptalis*	PNG	high	extreme
Ramu shoot borer	*Sesamia grisescens*	PNG	high	extreme

Table 2—Barcoding status of some high priority exotic pests. For 16S and COII, GenBank accessions provided by Lange *et al.* (2004) are included if they exist, otherwise the number of other publically available records are shown. For COI, the GenBank accessions obtained by SRA and the number of other publically available records are shown.

Pest	16S Genbank	COII GenBank	COI public BOLD or GenBank
Fulmekiola serrata (Kobus) Oriental sugarcane thrips	Only public records are SRA sequences KC505476, KC505477, KC505478 (Sallam *et al.*, 2013)	No public records	1 public record + SRA sequences KC505480, KC505481, KC505482 (Sallam *et al.*, 2013)
Eumetopina flavipes (Muir) Island sugarcane plant hopper	No public records	No public records	Only public record is SRA sequence KU525010
Chilo sacchariphagus (Bojer) Sugarcane internode borer	AY320436, AY320437, AY320438, AY320439 (Lange *et al.*, 2004)	AY32083, AY320484, AY320485, AY320486(Lange *et al.*, 2004)	1 public record + SRA sequence KU525011
Chilo terrenellus (Pagenstecher) Stem borer	AY320440 (Lange *et al.*, 2004)	AY320487 (Lange *et al.*, 2004)	Only public record is SRA sequence KU525012
Scirpophaga excerptalis (Walker) Top shoot borer	AY320460, AY320461 (Lange *et al.*, 2004)	AY320507, AY320508 (Lange *et al.*, 2004)	2 public records + SRA sequence KU525013
Sesamia grisescens (Warren) Ramu shoot borer	AY320466 (Lange *et al.*, 2004)	AY320513 (Lange *et al.*, 2004)	Only public record is SRA sequence KU525014
Sesamia inferens (Walker) Pink stemborer	2 public records	9 public records	24 public records + SRA sequence KU525015

Although the study included many species of *Chilo* and *Diatraea*, it had limited species of others, such as *Sesamia*. Also, the primer combination used to amplify the COII region (A-298 and B-tLys from Liu and Beckenbach, 1992) works very poorly on many insects other than borers. A major benefit of COI barcoding is that the primer combination LCO1490 and HCO2198 from Folmer *et al*. (1994) is much more universal.

Where no publically available barcoding data are available for species that are important to the Australian sugar industry, we have begun compiling our own SRA data set and the sequences are lodged with GenBank when possible. An example of the value of this approach occurred in 2012 during a potential Oriental sugarcane thrips, *Fulmekiola serrata* Kobus (Thysanoptera: Thripidae), incursion.

Although the outbreak was later shown to be due to Oriental rice thrips, *Stenchaetothrips biformis* Bagnall (Thysanoptera: Thripidae), the barcoding process was slow because the publically available sequences were of poor quality and we had to generate our own (Sallam *et al*., 2013). This was time consuming since the reference insects had to be obtained from overseas, which is not an ideal situation in the event of an incursion.

Barcoding procedure

In the event of a potential incursion, pest identification must be prompt, so the equipment, reagents, protocols and expertise must be on hand. Samples of priority pests (Table 2) have been assembled at the SRA laboratory in Brisbane and used to test the procedures. Various methods of DNA extraction are available but the Qiagen QIAamp Micro DNA kit is ideal for very small amounts of tissue.

For borers, a single leg is optimal to minimise the chance of simultaneously detecting a parasite or a parasitoid. But for very small insects such as thrips, the entire organism is used. The COI gene is amplified with the primers LCO1490 and HCO2198 (Folmer *et al.*, 1994) and the PCR products are excised from agarose gels, purified and sequenced.

The edited sequences are then checked against publically available databases; GenBank, operated by NCBI (National Centre for Biotechnology Information) using the program BlastN (Basic Local Alignment Search Tool) and BOLD (http://www.boldsystems.org).

Can borers be identified by their frass?

DNA-based identification is ideal for situations where there are only larvae, pupae, eggs, exuviae or frass present. For borers, often the only evidence remaining in damaged cane is frass. DNA analysis of frass has been used successfully for other insects where non-invasive sampling is required, for example with high value insects such as queen bees (Scriven et al., 2013) or where the larva is required to proceed to the adult stage or for behavioural studies (Lefort et al., 2012). PCR testing of frass has also detected plant pathogens vectored by insects (Mitchell and Hanks, 2009).

In many published reports, the insect was kept in an isolated environment and fed artificially. However in the event of a potential sugarcane borer incursion, the frass within the stalk would be mixed with digested sugarcane, bacteria, fungi and other microorganisms, which makes extracting good quality insect DNA challenging. Our aim was see if a practical biosecurity procedure could be developed to determine if residual frass in sugarcane stalks was due to an endemic or exotic borer.

Our experimental system was the large moth borer *Bathytricha truncata* (Walker; Lepidoptera: Noctuidae), one of only two lepidopterous borers of sugarcane recorded in cane fields in Australia. It is a minor pest of cane in Australia, rarely causing significant damage. *B. truncata* is distributed in NSW and Qld in rice, sugarcane, *Echinochloa* spp., *Typha* spp., *Cyperus* spp., paspalum and water couch (Jones, 1966). Larval instars feed inside the growing point of young cane plants causing dead hearts (Figure 1A). Specimens have previously been assigned 16S and COII barcodes by Lange *et al.* (2004) (AY320424, AY320425, AY320426, AY320471, AY320472, AY320473) and nine COI sequences are available in the BOLD database.

Samples of borers and accompanying frass were collected from Ashfield Road, Bundaberg in August 2015. Each individual borer and its frass were placed into microfuge tubes, 100% ethanol was added for preservation and sent to the Brisbane SRA laboratory.

Frass test results

The initial step was to optimise the DNA extraction procedure. As an incursion response must be prompt, the kits and reagents need to be those commonly available in any molecular lab. Three well known commercial DNA extraction kits were trialled: Qiagen QIAamp Micro DNA kit (routinely used for insect barcoding as described previously), Qiagen DNeasy Plant Mini kit (routinely used in the SRA Biosecurity group for plants and fungi) and MoBio PowerSoil kit (which contains reagents to eliminate humic substances and other PCR inhibitors).

For each of the three kits, three frass samples of various sizes were tested. The DNA was extracted according to the methods supplied with the kits. COI PCR amplification used the primers LCO1490 and HCO2198 and the presence of a 700bp PCR product on an agarose gel indicated successful PCR. The frass extractions were also checked for the presence of sugarcane DNA using PCR primers for sugarcane phosphofructokinase (PFK5F1-R1; Botha, personal communication), for the presence of fungi using universal ribosomal internal transcribed spacer (ITS) primers (ITS1F-4; White *et al.*, 1990) and for the presence of bacteria using universal ribosomal 16S primers (fD1-rP1; Weisburg *et al.*, 1991).

Results are presented in Table 3. Not surprisingly, PCR products for sugarcane, fungi and bacteria could be obtained from many frass samples. These PCR products were not further characterised. Regardless of the extraction method used, the PCR success rate for insect COI was very low. As it would simplify procedures during a potential incursion if insect and frass methods were the same, the Qiagen QIAamp Micro DNA kit was selected and further optimisation focussed on those samples.

Table 3—Comparison between different DNA extraction kits in their ability to generate PCR-quality DNA from frass. Each kit was used to extract DNA from three different *Bathytricha truncata* frass samples and the number of DNA extractions that could amplify a PCR product is shown.

Extraction kit	Frass sample	Insect COI	Sugarcane PFK	Fungi ITS	Bacteria 16S
Qiagen QIAamp Micro DNA	F1-F3	2/3	1/3	1/3	2/3
Qiagen DNeasy Plant Mini kit	F4-F6	1/3	1/3	2/3	2/3
MoBio PowerSoil kit	F7-F9	0/3	3/3	3/3	3/3

To improve the reactions we incorporated several common techniques to deal with poor amplification, namely diluting the template, lowering the PCR annealing temperature from 50 °C to 45 °C and performing nested PCR. Nested PCR was achieved by redesigning the primers LCO1490 and HCO2198 (Folmer *et al.*, 1994) with M13 extensions to use as first round primers, with the original as the nested primers. All three techniques appeared to improve the amplification success rate for some of the samples (data not shown), but as they could represent non-specific amplification products, the PCR products needed to be sequenced to confirm that *B. truncata* DNA was being amplified. Included in the sequencing were three *B. truncata* borers (B1-B3, matching frass F1-F3), extracted and amplified using the standard procedure for insects, and two more frass samples, extracted and amplified using some of the techniques trialled during the optimisation. Table 4 shows that mixed results were obtained.

Only three of the five frass samples could be shown to have *B. truncata* DNA. One DNA sample (F10) could not give any COI PCR product, while the PCR products generated from sample F3 were not insect DNA. While lowering the annealing temperature to 45 °C initially appeared to improve the PCR success, it was allowing DNA from the non-insect organisms living in the frass to be amplified instead. A wide range of microorganisms could be detected in the frass, including fungi, insects and bacteria.

Table 4—COI sequencing results for *Bathytricha truncata* borer and frass samples extracted with the Qiagen QIAamp Micro DNA kit.

Sample	PCR conditions	Top BlastN match in GenBank and accession number	Top BOLD match and Probability of Placement (%)
Borer 1	Standard conditions	*Bathytricha truncata* HQ950779	*Bathytricha truncata* 98%
Borer 2	Standard conditions	*Bathytricha truncata* HQ950779	*Bathytricha truncate* 98%
Borer 3	Standard conditions	*Bathytricha truncata* HQ950779	*Bathytricha truncata* 98%
Frass 1	Standard conditions	*Bathytricha truncata* HQ950779	*Bathytricha truncata* 98%
	50°C, nested PCR	*Bathytricha truncata* HQ950779	*Bathytricha truncata* 98%
	45°C, nested PCR	*Bathytricha truncata* HQ950779	*Bathytricha truncata* 92%
Frass 2	50°C, nested PCR	*Bathytricha truncata* HQ950779	*Bathytricha truncata* 98%
	45°C, nested PCR	Bacteria	Arthropod 50%
Frass 3	Standard conditions	No significant match	Hymenoptera 54%
	50°C, nested PCR	Hymenoptera	Arthropod 75%
	45°C, nested PCR	Hymenoptera	Oomycete 75%
Frass 10	No PCR product	–	–
Frass 11	Standard conditions	*Bathytricha truncata* HQ950779	*Bathytricha truncata* 93%
	50°C, nested PCR	Bacteria	Bacteria 73%

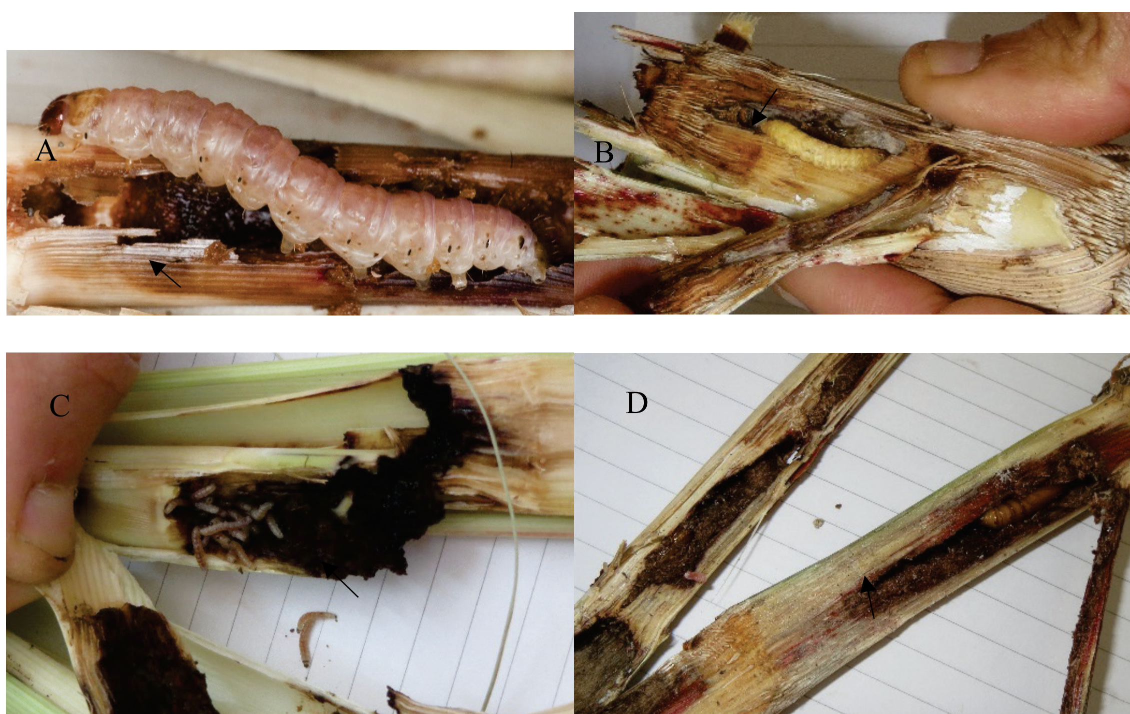

Fig. 1—The appearance of the frass and borer samples used in this study. A: *Bathytricha truncata;* B: *Scirpophaga excerptalis* C: *Sesamia grisescens;* and D: *Chilo terrenellus*. Examples of the type of material sampled for the DNA extractions are indicated by the arrows.

Application of the technique to exotic borers

To test if barcoding would work on exotic borer species, we collected samples of *Sesamia grisescens* (Lepidoptera: Noctuidae), *Chilo terrenellus* (Lepidoptera: Crambidae) and *Scirpophaga excerptalis* (Lepidoptera: Pyralidae), which are the main moth borer species at Ramu Agri-Industries, PNG (Kuniata *et al.,* 2001). Frass and larvae samples were taken from three separate stalk pieces (Figure 1B-D) during October 2015. The borers were identified by Dr Lastus Kuniata, RAIL entomologist and Head of Research. Samples were preserved under ethanol and declared on arrival into Australia. DNA was extracted with the QiaAmp kit and the COI gene was amplified and sequenced as described above.

The sequences obtained were matched against publically available sequences in GenBank and BOLD (Table 5). The *S. grisescens* and *C. terrenellus* borers could not be correctly identified

through BOLD because there are no publically available records in that database for them. However the borer sequences do match previously obtained SRA sequences now deposited in GenBank. Of the three frass samples, only one sample matched its insect species (*Sc. excerptalis*). The sequences amplified from *S. grisescens* and *C. terrenellus* frass matched bacteria and nematodes, respectively.

Table 5—COI sequencing results for three borer and frass samples collected from PNG.

Borer	Top BlastN match in GenBank and accession number	Top BOLD match and Probability of Placement (%)
Sesamia grisescens		
Borer	SRA *S. grisescens* sequence KU525014	*Sesamia inferens* 94%
Frass	Bacteria (e.g. CP003538)	Bacteria 75%
Chilo terrenellus		
Borer	SRA *C. terrenellus* sequence KU525012	*Chilo crypsimetalla* 92%
Frass	Nematode (e.g. FN397792)	Nematode 86%
Scirpophaga excerptalis		
Borer	SRA *Sc. Excerptalis* sequence KU525013	*Sc. excerptalis* 95%
Frass	SRA *Sc. Excerptalis* sequence KU525013	*Sc. excerptalis* 95%

Discussion

Barcoding is an important tool in the SRA biosecurity toolbox. It is a useful technique for insect identification providing the species has been previously correctly identified and the barcode sequence is available to end users. Over time, other high priority insects without publically available barcodes will be sequenced by SRA and made available on GenBank if it is not possible to submit them to BOLD.

To extend the usefulness of barcoding we have attempted to develop procedures to identify borers from frass taken from inside sugarcane stalks. While many published reports describe successful DNA extractions from frass, those insects are often kept in isolated environments and are fed artificially. However, isolating insect DNA from the complex matrix that is a borer-damaged sugarcane stalk is challenging.

Even after attempting to optimise the techniques with the Australian large moth borer (*B. truncata*), two out of three field obtained frass samples did not lead to the insect being identified. Close inspection of stalks shown in Figure 1 indicates that *B. truncata* and *Sc. excerptalis* frass is relatively light in colour and texture in comparison to the dense, dark wet mass produced by S. *grisescens* and the extensive rotting produced by *C. terrenellus.*

While some progress has been made, it is clear that further work is required to optimise the DNA extractions and PCR conditions. Extraction methods that focus on removing PCR inhibitors may be more successful.

Replicate sampling is required to deal with the low success rate. That was not possible on this occasion because Ramu Agri-Industries was experiencing a severe drought and it was difficult to find mature stalks containing the three borer types. The poor condition of the cane at Ramu most likely caused the borer damaged stalks to be more susceptible to secondary infection by microorganisms.

The age of the frass may be important and optimal sampling age needs to be determined. Age would influence the ratio of insect DNA relative to microorganism DNA. In the event of an incursion threat to our industry where the only evidence was frass in damaged cane, it would be advisable to take replicate samples and to try a range of PCR amplification techniques to maximise success.

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