Detection of *Apis cerana* DNA from sugar syrup.

Background

In 2007, Biosecurity Qld developed a PCR based test for the identification of Asian honey bee DNA. The test was validated to provide identification of whole or part bee wings obtained from bee eater pellets. The test was known to be very sensitive and species specific.

Ongoing research into better surveillance techniques by Biosecurity Queensland scientists has produced sugar syrup based feeding stations in which bees are visually identified after feeding. It was hypothesised that effectiveness of identification may be enhanced using forensic molecular techniques to identify DNA left at the feeding station during feeding.

Technical advances in DNA technology have provided environmental management agencies around the world with the necessary tools for sight unseen detection of pests particularly in aquatic environments. Environmental DNA (eDNA) is a term associated with the background level of DNA in an ecosystem that is derived from all of the organisms that exist within the system. The DNA is released into the environment in shed tissues such as skin or gut lining, through the attack of pathogens that breakdown cell membranes or the degradation of dead organisms. The amount, durability and detectability of eDNA in the environment is necessarily subjective however studies have used detection of environmental DNA in detection of bacteria[1], fungi[2] bullfrogs[3] and asian carp[4]. A comprehensive study of environmental DNA detection as a tool for remote detection of asian carp is underway in the United States by the US Army corps of engineers, US fish and wildlife service and US geological survey[5]. It is the first national program to incorporate eDNA into a pest surveillance program with comprehensive calibration, and validation will lead the way for similar programs worldwide.

In the 1st half of 2012 a study looked into the feasibility of implementing the earlier test to look for DNA deposited in trap liquor during feeding. The report[6] concluded :

> **A test for the specific detection of *Apis cerana* DNA has been developed. The test is sensitive to approximately 0.5-5 ng of *Apis cerana* DNA and has shown no cross-reaction to *Apis mellifera* or any native insects that were trapped when lure syrup was placed in AHB free areas of far north Queensland. DNA extraction methods for whole bee, sugar syrup and wings removed from bee-eater pellets have been investigated and optimised for each sample type. This work provides proof of concept for a molecular surveillance program for AHB in far north Queensland.**

In the 2nd half on 2012 further work to determine the sensitivity of using trap liquor was conducted.

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[1] Leff et al. 1993
[2] Martin et al. 2005
[3] Ficetola et al. 2008
[4] Jerde et al. 2010
[5] http://www.asiancarp.us/index.htm
[6] Appendix 1.

Syrup samples were collected by Dr Anna Koetz in Cairns on 1 November 2012. Dr Koetz observed liquor traps allowing a limited number of bees to feed on the traps before sampling. Samples were received with the following information

Sample	Number of bees	Time of exposure
1	20	Unknown.
2	35	19
3	50	21
4	100	41
5	250	44

A new DNA extraction method for eDNA from trap liquor, based on alcohol precipitation, was used to allow for a greater sample volume at reduced cost ($5 down from $20 for consumables). 75ml of sugar syrup was extracted giving a final volume of 200uL. Four replicates were tested by PCR to allow for the redundancy as described by Ficetola et al. 2008.

Sample	Replicate	Result
20 bees	1	-
	2	+ *
	3	-
	4	-
35 bees	1	+/-
	2	+
	3	-
	4	-
50 bees	1	-
	2	-
	3	-
	4	-
100 bees	1	+
	2	+/-
	3	-
	4	-
250 bees	1	+ *
	2	+
	3	+
	4	-

* Representative positives were sequenced and found to be of *Apis cerana* origin.

Conclusions

These results indicate that by using the redundant testing regime a useful level of sight unseen detection can be achieved. As demonstrated by the 50 bee sample the test is capable of producing false negatives although this level can be reduced by testing more than one trap per location and raising the number of replicates to 5. Cost of processing per syrup sample is roughly $10 in consumables.

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Appendix 1 – June 2012 genetics report

Detection of *Apis cerana* DNA from bee eater pellets and trap liquor

Tasks

Biosecurity Queensland were tasked to develop a test for the detection of *Apis cerana* (asian honey bee or AHB) DNA. The test is required to assist in the surveillance program by providing either a more efficient or more sensitive detection tool than current methods. Advice from the surveillance team was to concentrate on two sample types:

1. Bee eater pellets, which could contain skeletal remains of consumed insects. Current work relies upon the presence of intact wings that can be morphologically identified. Wings can be quickly identified by an entomologist, the limiting factor being the necessity to find an intact wing. It would be advantageous if a DNA test could provide diagnosis in the absence of whole wings.

2. Material from the surveillance feeding traps. The traps of sugar syrup and sand rely upon a bee being caught in the syrup and morphologically identified. Ineffective capture rates left many traps empty despite bees feeding in them. It would be advantageous if a DNA test could detect when AHB have fed though not been caught.

History

In 2007 BQ were tasked with development of a simple PCR that would react with DNA from *A cerana* but not DNA from any other species. The test material was intended to be wings and wing fragments within honey-eater pellets. The resulting test was shown to detect approx. 5-50 pg *A. cerana* DNA and did not react with *Apis mellifera*. The test necessitates the separation of wings and other insect parts from the pellets prior to analysis and it was suspected the pellet material contained some PCR inhibitors. Wings are separated from the pellets with a simple floating technique. This test is ready for operational use, pending confirmation that reaction components that have superseded those from 2007 perform sufficiently.

Samples

1. Bee eater pellets were obtained from bee eater roosting sites in areas of known AHB infestation in far north Queensland. Some pellets were identified as positive by BQ entomologists while others were tested without prior processing.

   Pellet 1 – Machan's beach. Location (-16.85221,145.74518)
   Pellet 2 – McLeod St. Location (-16.92386, 145.77218)
   Pellet 3 – Kuranda. Sample processed at TAAHL and positive for AHB wings.

2. Trap samples were obtained from BQ staff in Cairns, traps were exposed to feeding bees for an hour then the top 5 mm of sand and all of the liquor were removed and stored at 4 degrees until processed. Negative samples were exposed to uninfested sites overnight prior to sampling.

3. *Apis cerana* samples were obtained from infested sources in far north Queensland. Two samples were taken from the following locations:

   IP8 – Nest located at Greenhill, Qld 4865
   IP9 – swarm located Greenhill, Qld 4865
   IP10 – Nest located at Aloomba, Qld 4871
   IP15 – Nest located at Portsmith, Qld 4870

4. *Apis mellifera* samples were obtained from the Varroa mite detection team at the Biosecurity Sciences Laboratory.

Confirmation and validation of test designed in previous study by BQ

The test designed in a previous study by Biosecurity Queensland was used as a starting point. The study had designed primers to the cytochrome B gene located on the mitochondrial DNA of *Apis cerana*. Comparison of this work and later published[7] sequence of the entire mitochondrial DNA from *Apis cerana* (Chinese origin) indicated a possible redesign of one of the two primers and a change of reagents may yield a more sensitive test.

The two sets of primers and two reagent chemistries were tested with respect to annealing temperature and serial dilution of template to determine the optimal combination for detection. Detection of AHB DNA was found to be most sensitive and robust using MyTaq DNA polymerase (Bioline, Alexandria, NSW) and the redesigned primers at 63 °C annealing. No cross reaction with *Apis mellifera* was observed and sensitivity was increased by 1 order of magnitude to detect approximately 0.5-5pg of DNA.

Bee eater pellets

A bee wing identified as *A. cerana* from a pellet was used as template to determine the viability of DNA having passed through the gut of the bee eater. Two single wings were extracted using the standard insect DNA extraction procedure developed for the Electric ant eradication program (Biosecurity Queensland) requiring a modification of the manufacturers recommended procedure applied to DNEasy Blood & Tissue Kit (Qiagen, Doncaster, VIC). Viable DNA was extracted and tested positive to *Apis cerana* by PCR and identification was confirmed through DNA sequencing of the amplicon. Wings found in bee eater pellets collected in AHB-free areas produced a negative result.

The insect extraction method was compared with the DNeasy Stool DNA extraction kit, commonly used where PCR inhibitors are likely to be present. One pellet sample was spiked with an *Apis cerana* wing. Minor changes were made to the manufacturers recommended procedure to allow for a large volume of sample, and 1 gram of pellet was used. The increased starting volume was required to accommodate homogenisation of the sample to ensure that subsampling did not exclude the small amount of *Apis cerana* skeletal tissue expected to be in the sample. It was not possible using these methods to sufficiently reduce inhibitors to allow downstream PCR processing, therefore the test can currently be used only on separated wing samples. Information has been provided by Qiagen as to a possible alternative procedure however due to a short timeframe and limited number of samples available this investigative direction has not been fully exhausted.

The results have proven the ability of the test to definitively distinguish *Apis cerana* wings from *Apis mellifera*. The test is more expensive and time consuming than morphological identification therefore has little application in its present form, though will provide a secondary analysis confirmation when morphological characteristics such as a brown streak, vein length and spurs are obscured or conflicting. Further investigation into inhibitor removal would enable the test to be undertaken without the necessity of wing removal. Should this prove successful the test could be used to screen large numbers of samples efficiently. As the DNA extraction preserves the morphology of the wings this would enable both forms of identification to be used, and a record of the find to be retained.

[7] Tan et al. PLoS ONE 6 (8), E23008 (2011) The Complete Mitochondrial Genome of the Asiatic Cavity-Nesting Honeybee Apis cerana

Trap samples

The traps essentially consist of a "takeaway food container" that is half filled with sand to provide a landing area for the bees and an amount of sugar syrup with floral attractant for the bees to feed on at the other end.

Initially, it was reported that bees were unidentifiable due to degradation as they lay trapped in sugar syrup in the field for up to a week before collection. Attempts to recreate this scenario in the lab proved unsuccessful. A fresh bee was placed in the sugar syrup and incubated at 37 °C for a month. While organism growth was observed through the increase in biomass and the production of gases, the bee itself did not require DNA analysis as it remained morphologically identifiable after this time and may have been preserved by the sugar concentration in the syrup. Field trials were setup by Cairns field staff although were impeded by wet weather so no results are available.

It was decided to apply the DNA test to the scenario in which AHB have landed on the trap, fed on the syrup and flown away. Samples of sugar syrup and sand that had been placed in a AHB-free area were used to prove the concept. Syrup sample #1 was spiked with 2 µL (1µg) of AHB DNA; syrup #2, with 10µL (5µg) of AHB DNA; syrup #3, with a single leg of an AHB; and syrup #4 remained AHB free, though would have contained native insect DNA collected in the field. Sand sample #1 was spiked with 2µL (1µg) AHB DNA; Sand #2 was unexposed sand; and sand #3 was sand that had absorbed syrup but not exposed to AHB. Samples were extracted with DNeasy blood and tissue kit. It was found that all spiked syrup and sand samples provided positive reactions.

To challenge the DNA test with field samples BQ staff prepared a trap and allowed AHB from a research colony to feed freely for a period of an hour, it was estimated that over fifty bees fed on the trap. Three replicates of 3 mL of AHB exposed syrup and a 3mL sample of unexposed AHB syrup were extracted with the DNeasy blood and tissue kit. The top 5mm of sand that had AHB land on it was washed in buffer EB (DNEasy kit component) and this was extracted using the blood and tissue kit. All replicates of the syrup tested positive for AHB DNA, however both sand samples tested negative, controls indicated reliable results.
These results demonstrate the DNA test can be used to determine if AHB have fed on a syrup sample. Further field trials to test the limitations of the test in terms of number of bees required to feed and duration of feeding have been hindered by recent weather conditions and results are not available at present. When this has been completed, the results to date will be used to develop a standardised testing regime based on DNA detection from syrup samples that can be applied in the surveillance program.

Conclusions

A test for the specific detection of *Apis cerana* DNA has been developed. The test is sensitive to approximately 0.5-5 ng of *Apis cerana* DNA and has shown no cross-reaction to *Apis mellifera* or any native insects that were trapped when lure syrup was placed in AHB free areas of far north Queensland. DNA extraction methods for whole bee, sugar syrup and wings removed from bee-eater pellets have been investigated and optimised for each sample type. This work provides proof of concept for a molecular surveillance program for AHB in far north Queensland. The test has been optimised on the following sample types:

1. Whole bee (Diagnostic speciation) - $9 per sample (includes bidirectional sequence)

2. Bee wing extracted from bee-eater pellet (Diagnostic speciation) - $9 per sample

3. Sugar syrup (Detection of presence/ absence) $22 per sample + $6 sequence for positives