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Specific detection of *Chrysomya bezziana* (SWF) in bulk trap catches using real-time PCR

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Abstract

The Old World screw-worm fly (SWF), *Chrysomya bezziana*, does not currently occur in Australia but it is a species of major concern to livestock in northern Australia because the larval stages cause primary cutaneous myiasis on warm blooded animals. Surveillance traps are currently screened by microscopic examination of species-specific morphological characters; however, morphologically similar flies are difficult to differentiate, especially when the condition of specimens is poor. A molecular based method to confirm or refute the presence of SWF in large Lucitrap® catches would greatly simplify Australia's monitoring program. The aim of this research was to develop a real-time PCR assay for the identification of SWF in bulk fly samples. A region of the ribosomal DNA ITS1 was chosen as the target for the assay after sequencing and comparing an alignment of ITS sequences from relevant *Chrysomya* and *Cochliomyia* species. The Taqman® assay is species-specific and sensitive to one SWF in 1,000 non-target species. A DNA extraction protocol has also been optimised to process trap catches of up to 1,000 flies. The optimal trapping period for DNA recovery was determined to be 10 days. The assay developed is sensitive and fast and will assist industry by providing early detection of a SWF incursion into Australia leading to a shorter response time and faster containment of this exotic pest.

Executive Summary

A sensitive *Chrysomya bezziana*-specific probe-based molecular assay has been developed and evaluated to improve detection of Old World screw-worm fly in bulk Lucitrap® catches. Buckets of flies are currently collected as part of Australia's screw-worm fly (SWF) monitoring program and small subsamples of flies are individually screened morphologically. A DNA extraction protocol, to be used in conjunction with the *C. bezziana*-specific real-time polymerase chain reaction (PCR) assay, has also been developed to process bulk Lucitrap® catches containing mixed fly species. The effect of trapping time on DNA recovery has been determined so that trapping protocols can be optimised to maximise assay sensitivity.

Why the work was done?
Old World screw-worm fly (SWF) myiasis, caused by the obligate myiasis blowfly *Chrysomya bezziana*, is considered to be one of the most serious animal disease threats facing Australia's livestock industries. An uncontrolled incursion of SWF into Australia would threaten the survival of the northern cattle industry (direct production losses would be in the order of $500 million per year). The policy of the AUSVETPLAN SWF Strategy (2007) is to eradicate SWF as soon as possible to minimise its economic and ecological impacts. This would initially involve chemical and other conventional controls such as quarantine and zoning. Eradication using the release of sterile flies would not be implemented for several years after the initial incursion. Once SWF has established in Australia eradication will be technically difficult and very expensive. The threat of an incursion from neighbouring countries to the north has been recognised and in 2002 Animal Health Australia (AHA) became responsible for managing Australia's SWF preparedness. Australia's SWF freedom assurance is heavily reliant on SWF surveillance activities. An early warning system has been established in high-risk areas for entry of Old World SWF into Australia as part of the Northern Australia Quarantine Strategy. Surveillance trapping is also conducted at shipping ports around northern Australia. The early warning system employs targeted quarantine surveillance, education, and a regular fly trapping program.

Fly trapping technology has advanced in this time with sticky traps being superseded by modified Lucitraps® and improved attractants. The new traps catch fewer non-target flies but are unable to exclude a number of species, including several close relatives of *C. bezziana*; thousands of morphologically similar flies can be present in the Lucitraps®. Screening technology had not advanced prior to this project requiring manual sorting of large trap catches followed by individual morphological examination. Suspicious flies were sent to expert entomologists for definitive identification. This labour intensive process could no longer keep up with the large trap catches resulting from the new trapping technology. The objective of this project was to improve the current Lucitrap® screening process to ensure adequate screening for early SWF detection. A molecular assay was chosen as the best strategy because DNA probes can be designed to be highly species-specific and PCR amplification permits detection of very low concentrations of DNA.

What was achieved?
A Taqman® real-time PCR assay has been designed that is specific to *C. bezziana*. The assay targets a small region of the ribosomal DNA internal transcribed space 1 (ITS1) that is 100% conserved in a DNA sequence alignment of 12 populations of *C. bezziana* originating from Indonesia, Malaysia, PNG, India, Oman, South Africa and the United Arab Emirates. The assay is sensitive enough that it can detect one *C. bezziana* in a sample of 1,000 flies. An extraction protocol capable of extracting DNA from 1,000 flies was developed. Up to twelve samples can be extracted simultaneously reducing the labour cost per sample. The process requires two days to complete and roughly one day of labour (for 12 samples). The extrapolated cost per 1,000 fly assay (screening two

lysate subsamples and including labour if 12 samples are simultaneously screened) is just under $55 each. A field trial was conducted that determined the optimal trapping period to maximise the sensitivity of the assay was 10 days.

When and how industry can benefit from the work?
The northern livestock industries greatest benefit from this work will be minimising the impact from an incursion of SWF into Australia, through early detection and ongoing monitoring. Implementing the assay will bring surveillance screening into better alignment with surveillance trapping, increasing the probability of early detection of SWF in Australia. If an incursion is detected, the modified Lucitraps® and molecular assay will be essential tools (rapid, sensitive and targeted screening) for monitoring fly numbers and movements.

Who can benefit from the results?
Early detection of SWF leading to rapid eradication of the pest will benefit Australia in general. SWF attacks all warm blooded animals, including humans. In areas with high SWF prevalence it is impossible to run livestock as they are particularly susceptible to infestation. The SWF preparedness strategy developed by Animal Health Australia (AHA) flagged that Australia needs to maintain adequate expertise in a range of SWF-related activities. Enhanced surveillance was a specific element of the new strategy. The Northern Australia Quarantine Strategy conducts regular surveillance trapping for early warning detection. This assay will permit more comprehensive screening of a greater number of trap catches with greater sensitivity for less labour. Early detection of an incursion permits a rapid response plan to limit the spread of SWF and earlier implementation of eradication strategies.

Recommendations for future actions
- AQIS and AHA evaluate suitability of new *C. bezziana*-specific real-time PCR assay for screening Lucitrap® catches in SWF surveillance trapping program
- Transfer technology to molecular facilities (Government or private) to conduct screening
- Conduct additional research to determine the cause of reduction in DNA recovery from flies in Lucitraps® with the aim of extending the optimal trapping period to more than 10 days
- Explore the application of this technology to other surveillance programs (e.g. fruit flies, fire ants).

Contents

1 Background

Chrysomya bezziana, (Old World screw-worm fly, SWF) is a parasitic blowfly that is found in subtropical and tropical climates including the coastal swamps of Papua New Guinea that border the Torres Strait. Characterised by their screw-like shaped larvae, *C. bezziana* differs from Australian blowfly species in that they only feed on living flesh. Infestations occur in open wounds and death occurs as a result of loss of tissue fluid and infection (AUSVETPLAN, 2007). Despite ideal tropical climatic conditions for establishment, Australia is the only continent with suitable habitat lacking SWF. In Australia the northern cattle industry would bear most of the impact from a SWF incursion. The cost of an uncontrolled incursion, in terms of direct producer losses, would be of the order of $500 million per year; this cost would seriously threaten the northern cattle industry (AQIS, 2007). Although never established in Australia, in the past eighteen years there have been two confirmed detections. The first occurred in an empty livestock vessel in Darwin harbor and the second in head wound of a person returning from an overseas trip (Atzeni *et al.*, 1997). Due to the close proximity of Papua New Guinea to Australia there is a greater risk of entry through either sea trade or a fly strike wound on animals or people (Strong *et al.*, 1991).

Accurate diagnosis involves the identification of adults and larvae by trained entomologists. The larvae of SWF can be confused with the larvae of other blowfly species. Confirmation of SWF relies on the recognition of a characteristic combination of spinulation, the number of lobes on the anterior spiracles, and pigmentation of secondary tracheal trunks (Kitching, 1974). The adult stage is up to 10 mm long and has a metallic blue, bluish-purple or blue-green colour. Adult SWF can be distinguished from other *Chrysomya* found in cases of myiasis by the combination of black-brown to dark-orange-coloured anterior thoracic spiracles (rather than pale yellow, creamy, or white), with waxy-white, lower squamae (rather than blackish-brown to dirty-grey) (Spradbery J.P., 1991).

More recent techniques for identification of SWF include cuticular hydrocarbon analysis (Brown *et al.*, 1998) and analysis of mitochondrial DNA (Hall *et al.*, 2001; Taylor *et al.*, 1996; Wells and Sperling, 2001; Harvey et al., 2003; Wells and Williams, 2007). All of these techniques, both morphological and molecular are based on the analysis of individual fly or larvae. Two methods are currently used by the Australian Quarantine and Inspection Service (AQIS) for surveillance for SWF. The first method is sentinel cattle that are periodically screened for maggots in naturally acquired wounds. Veterinarians throughout Northern Australia are also encouraged to submit maggots from fly struck animals for checking. This method results in manageable numbers of samples for individual visual or traditional molecular diagnostics. The second surveillance method involves modified Lucitraps® that contain chemical attractants that are used to lure adult SWF (AUSVETPLAN, 2007; Urech *et al.*, 2004). Although effective, they also lure other Calliphorids, making species identification difficult when population densities are high. Coupled with this is the fact that the identification of early stages of many blowfly species is almost impossible (Wallman *et al.*, 2001). Sticky traps become saturated with about 1,000 flies but the new modified Lucitrap® technology can trap tens of thousands of flies in a single collection. These sample sizes are unmanageable using existing diagnostic tools and are processed by sub-sampling a relatively small number of individuals for labour-intensive screening.

Molecular assays can be designed to be target specific enabling screening of mixed species DNA samples. The polymerase chain reaction (PCR) is accepted as the gold standard for detecting nucleic acids from a number of origins (Mackay, 2002) and the technology has become a useful tool for the identification of partial remains of specimens lacking morphological characters (Saigusa *et al.*, 2005). Most sequence data from the molecular analysis of SWF has involved mitochondrial DNA (mtDNA) with complete or partial sequences currently available for cytochrome oxidase one and two (COI, COII), cytochrome b and control region. These sequences have been used for species

identification and to investigate phylogenetic relationships particularly among the blowfly species used in forensic analyses such as *Chrysomya megacephala* (they are first flies to colonize carcasses so the life stages are used to age bodies) (Otranto and Stevens, 2002; Wallman, 2005; Wells and Williams, 2007). The mitochondrial markers have proven to be useful for taxonomic differentiation and evolutionary studies but an alignment of mitochondrial DNA of *C. bezziana* and its' two closest relatives, *C. megacephala* and *C. saffranea* indicated widely spaced mutations making this marker poorly suited to design species-specific primers and probes for a *C. bezziana* specific assay.

Several nuclear DNA genes have also been sequenced for *C. bezziana*, and made publically available through Genbank, including ribosomal DNA (rDNA) 5.8S and 28S, rDNA internal transcribed spacer 2 (ITS2), serine proteases, chitin synthase 1 and peritrophin-48 pre-cursor. Being multi-copy, non-protein coding, and with a high mutation rate (Hillis and Dixon, 1991), the rDNA internal transcribed spacers were considered the best candidate marker for a *C. bezziana* specific real-time PCR assay. The objective of this project is to simplify Australia's screw-worm fly monitoring program by developing a molecular assay capable of detecting the presence of Old World screw-worm fly (*Chrysomya bezziana*) in bulk fly trap catches.

Commercial DNA extraction kits are easy to use and do not require the handling of hazardous substances (eg. phenol and chloroform). Unfortunately the kits are not designed for bulk tissue extraction; the maximum recommended starting amount is 25 mg (less than one fly). The weight of 100 flies varies with moisture and species but when dry is roughly 1 g. Current surveillance methods can collect anything up to 50,000 flies in a single collection. A modified Lucitrap® with newly developed Bezzilure attractant (Rudolf Urech, pers. comm.) reduces the number of non-target fly species but sample sizes can still be very large. To completely process 100's of flies with a commercial kit is both time and cost prohibitive. We aimed to develop a DNA extraction protocol to cost effectively extract DNA from a large volume of starting material. Once the *C. bezziana*-specific real-time PCR assay had been developed and an extraction protocol optimised for extracting from a Lucitrap® of mixed species flies the assay was tested on trap captures of varying age to determine the best approach for surveillance sampling in Northern Australia.

2 Project Objectives

The project had three main objectives, each with target goals:

1. Develop a DNA-based real-time PCR assay specific to *C. bezziana* and validate the assay's specificity against other fly species, both closely related and those likely to be caught in the Australian surveillance traps.
    - Sequence ribosomal DNA internal transcribed spacers from several populations of *C. bezziana* as well as close relatives and other fly species likely to be caught in the Australian surveillance traps.
    - Design real-time PCR assay and optimise.
    - Test assay against DNA from target and non-target species.
    - Evaluate assay on non-target local fly DNA spiked with *C. bezziana* DNA.

2. Develop a DNA extraction protocol, to be used in conjunction with the *C. bezziana*-specific real-time PCR assay, to process bulk fly trap catches containing mixed fly species.
    - Optimise fly homogenisation protocol.
    - Determine best method for cell lysis and DNA release.
    - Assess efficiency and cost effectiveness of different DNA extraction protocols.

3. Test the *C. bezziana*-specific real-time PCR assay on field collected trap catches both with and without *C. bezziana* and assess if trapping time influences the sensitivity of the assay.
    - Screen trap catches from Australian surveillance program.
    - Screen trap catches from Malaysia where *C. bezziana* is endemic.
    - Conduct a field trial to determine optimal trapping time for maximum assay sensitivity.

3 Methodology

3.1 Fly collections

Different geographic populations of *C. bezziana* were collected as well as specimens of closely related *Chrysomya* species. In addition, flies representing species typically caught in Australian surveillance traps were collected to construct a bank of DNA sequences to further ensure the developed assay would be *C. bezziana*-specific. Flies were obtained by project entomologist (Peter Green) with additional specimens provided by collaborators Dr. Philip Spradbery at XCS Consulting in Canberra, Dr. James Wallman at the University of Wollongong and Dr. Sri (Nini) Muharsini from Bbalivet in Bogor, Indonesia.

3.2 Fly DNA bank

3.2.1 DNA extraction from single flies

DNA was extracted from fly legs only so that the head and body could be examined morphologically at a later date if required. Legs were removed under a dissecting microscope using sterile forceps. Samples preserved in ethanol were soaked in 1 ml of Tris-EDTA buffer overnight prior to extraction. The fly legs were homogenised with a micropestle in 180 μl of QIAamp Tissue kit (Qiagen, Valencia, CA, USA) extraction buffer ATL then DNA was extracted using a QIAamp Tissue kit as described by the manufacturer.

3.2.2 DNA amplification and sequence analysis

Internal transcribed spacer 1 was polymerase chain reaction (PCR) amplified between primers SWF-18SF 5' GTCGTAACAAGGTTTCCGTAGG and SWF-5.8SR 5' TCGATGTTCATGTGTCCTGCAGT. Internal transcribed spacer 2 was PCR amplified between primers L1 5' RRCGGYGGATCACTCGGCTC and SWF-28SR 5' CTCGCCGCTACTAAGAAAATCC. Unpublished primer L1 sequence was kindly provided by James T Wallman (University of Wollongong). The remaining primers were designed in conserved regions of ribosomal DNA based on a sequence alignment of related fly species that were publicly available from GenBank.

Each PCR reaction contained 0.5 μM of each primer pair, combined with 10-100 ng of extracted DNA, 10x HotMaster Taq buffer (Eppendorf, North Ryde, NSW, Australia, containing 25 mM magnesium), 0.8 mM dNTP, and 0.05 units/μl of HotMaster Taq DNA polymerase (Eppendorf, North Ryde, NSW, Australia). This mix was thermocycled in a Corbett Research Palmcycler (Version 2.0, Mortlake, NSW, Australia) for 30 cycles. Cycle 1 was 95ºC for 60 sec, 53ºC for 45 sec, and 72ºC for 90 sec. This was followed by 29 shorter cycles of 95º C for 30 sec, 53ºC for 30 sec, and 72ºC for 90 sec. The mix was held at 72ºC for 7 min to complete extension. PCR products were viewed on an ethidium bromide-stained 1.5% agarose and TBE gel. For species that produced multiple PCR products, dominant bands were cut from a 1% low melting point agarose gel and the DNA was extracted using a Mini-elute Gel Extraction Kit (Qiagen, Valencia, CA, USA) following manufacturers' instructions.

PCR products were cleaned prior to sequencing with Exosap-it® (USB Corporation distributed by GE Healthcare Bio-Sciences, Rydalmere NSW, Australia). The PCR products were sequenced using Big Dye Vers 3.1 technology (Applied Biosystems, Foster City, California) and were run on an Applied Biosystems 3130xl Genetic Analyser at Griffith University DNA Sequencing Facility (GUDSF). Sequences were edited and aligned in Sequencher (Vers 4.5 Gene Codes Corporation). Where possible (ie sequences were not too divergent) species were aligned in ClustalX (Vers 1.81, Thompson et al., 1997). Phylogenetic trees were constructed in PAUP* (Vers 4.0b8, Swofford, 2001) based on distance matrix analyses and neighbour-joining. Other settings used were Mulpars in effect, Maxtrees set to 100 (limited by computational time), one heuristic search repetition and tree-bisection-reconnection (TBR) branch swapping. Outgroups were determined from published mitochondrial DNA trees (Wallman, Leys and Hogendoorn, 2005).

3.3 Real-time PCR assay

3.3.1 Real-time PCR assay design

To ensure the specificity of the *C. bezziana* real-time PCR assay, both forward and reverse primers, as well as the fluorogenic probe, were designed in *C. bezziana* unique sequence. *Chrysomya bezziana* specific sequence was determined as regions of DNA where *C. bezziana* differed from sister species *C. megacephala* and *C. saffranea*. Due to limitations in the unique positioning of primers and probe they were designed by visual comparison and then adjusted in length to match desired melting temperatures calculated in Primer Express (Vers 2.0 Applied Biosystems). Primers were designed to be 15-30 bp long with a melting temperature (T_m) of 58-60°C and GC content between 30-80%. No more than two G and/or C's were permitted in the last 5 nucleotides at the 3' end of the primer to reduce non-specific priming. The length of the amplification product was kept under 200bp. The MGB (minor groove binding) probe was designed with a T_m of 10°C higher than the primers and GC content between 30-80%. The probe was labelled with FAM for detection in the assay.

Real-time PCR assays were conducted on a Rotor-Gene 3000 (Corbett Research). Total reaction volume was 20 µl containing 8 µl RealMasterMix Probe (Eppendorf), PCR primers at a concentration of 300 nM, the MGB probe at 200 nM and 5 µl of genomic DNA (tested over a 1,000 fold dilution series). A negative control replacing DNA with milliQ water, and a positive control were run alongside all samples. Amplification conditions were 2 min at 95°C followed by 45 cycles of 15 sec at 95°C, 20 sec at 60°C and 20 sec at 68°C, acquiring FAM at the end of this step. At the completion of the run the dynamic tube was turned on and the data was slope corrected. After initial testing the threshold line was fixed at 0.01.

3.3.2 Confirmation or real-time PCR assay specificity

The specificity of the *C. bezziana* real-time PCR assay was confirmed by individually testing the assay against DNA from the species listed in Table 1. In addition, samples of mixed species DNA were created and spiked with *C. bezziana* DNA to determine if low levels of target DNA could still be detected in the presence of large amounts of non-target fly DNA (two ratios, 1/10 and also 1/1,000 target DNA to non-target DNA, were tested). Species combined to make the non-target DNA were *C. megacephala, C. hominivorax, C. varipes, C. flavifrons, C. nigripes, C. latifrons, C. saffranea, C. incisuralis and C. putoria.*

3.4 DNA extraction from bulk trap catches

For method consistency (fly size and DNA content) and comparability (between different extraction protocols) the non-target DNA chosen to optimise the DNA extraction protocol was *Chrysomya*

megacephala. A colony of *C. megacephala* is maintained by DPI&F at the Animal Research Institute (ARI) and large numbers flies could be obtained of similar age and size. DNA extraction is a three step process; 1. fly homogenisation, 2. lysis of cells to release DNA and 3. removal of proteins and lipids to obtain clean DNA.

3.4.1 Fly homogenisation

Fly tissue has to be mechanically disrupted to assist with the release of DNA. Two commonly used protocols for large-scale homogenisation of tissue are 1. liquid nitrogen assisted grinding using a mortar and pestle, and 2. an electric blender.

Method 1 – Liquid Nitrogen

1. Transfer 99 *C. megacephala* plus 1 C. *bezziana* (approximately 4 g dry) into a sterilised mortar.
2. Add small volumes (5 ml) of liquid nitrogen then use a sterile pestle to grind the flies into a fine powder.

Method 2 – Waring Blender

1. Transfer 99 *C. megacephala* plus 1 C. *bezziana* (approximately 4 g dry) into a pre-sterilised stainless steel Waring blender jar.
2. Add 20 ml of distilled water to the jar, seal with the lid and homogenise for 30 seconds on the high speed setting of the blender.
3. Transfer homogenised mixture to a 50 ml falcon centrifuge tube.
4. Centrifuge at 4,000 rpm, at room temperature for 20 min.
5. Decant and discard supernatant.

3.4.2 Lysis of cells to release DNA

Extracting DNA (QIAamp Tissue kit, Qiagen, Valencia, CA, USA) from replicate sub-samples (200 µl sub-samples of recovered supernatant) of the homogenised flies proved too variable due to uneven dispersal of the *C. bezziana* tissue. For this reason a protocol to lyse the cells in the bulk fly preparation was determined prior to sub-sampling the soup for DNA extraction through a Qiagen kit. Scaling up lysis reagents in the Qiagen kit was uneconomical so three alternative lysis protocols were tested; 1. Boiling, 2. Boiling + Proteinase K and 3. Boiling + Proteinase K + SDS.

A fourth method using a commercial kit for bulk tissue extractions (Bioserve kit, Beltsville, MD, USA), capable of processing 500-1,000 mg tissue or up to 25 flies, was also tested. Unlike the Qiagen kit that cannot be scaled-up due to column saturation, the Bioserve kit is column free so the kit was tested to determine if it could be scaled-up to extract from 100 flies (method 4a) and also 250 flies (method 4b), each extraction spiked with just one C. *bezziana*.

Method 1- Boiling

1. Resuspend homogenised flies (99 *C. megacephala* plus 1 C. *bezziana*) in 15 ml of lysis buffer (10mM EDTA, 20mM Tris).
2. Boil for 15 minutes. Cool to room temperature.
3. Centrifuge 15 min, 4,000 rpm
4. Transfer supernatant to a new tube. Discard pellet.

Method 2 – Boiling + Proteinase K

1. Resuspend homogenised flies (99 *C. megacephala* plus 1 C. *bezziana*) in 15 ml of lysis buffer (10mM EDTA, 20mM Tris).
2. Boil for 15 minutes. Cool to room temperature.

3. Add 100 μl of proteinase K (20 mg/ml). Mix well by gentle inversion.
4. Incubate overnight in a 56°C water bath.
5. Centrifuge 15 min, 4,000 rpm
6. Transfer supernatant to a new tube. Discard pellet.

Method 3 – Boiling + Proteinase K + SDS

1. Resuspend homogenised flies (99 *C. megacephala* plus 1 C. *bezziana*) in 15 mls of lysis buffer (10 mM EDTA, 20 mM Tris).
2. Boil for 15 minutes. Cool to room temperature.
3. Add 100 μl of proteinase K (20 mg/ml).
4. Add SDS to a final concentration of 1%. Mix well by gentle inversion.
5. Incubate overnight in a 56°C water bath.
6. Centrifuge 15 min, 4,000 rpm.
7. Transfer supernatant to a new tube. Discard pellet.

Method 4a –Bioserve large-scale purification kit - 99 *C. megacephala* plus 1 C. *bezziana*

1. Add 30 ml lysis buffer (kit) to 3g homogenised fly tissue (new cohort of smaller flies, 3 g = 100 flies) in a 50 ml centrifuge tube.
2. Add 60 μL of proteinase K (20 mg/ml)
3. Incubate overnight, 55°C water bath
4. Cool sample to 4°C on ice for 30 minutes
5. Add 9.9 ml cold Protein Out solution (kit)
6. Centrifuge 10 min, 4,000 rpm
7. Transfer supernatant to a new tube. Discard pellet.

Method 4b – Bioserve large-scale purification kit - 249 *C. megacephala* plus 1 C. *bezziana*

1. Add 75 ml lysis buffer (kit) to 7.5 g homogenised fly tissue (new cohort of smaller flies, 3 g = 100 flies) in a 250 ml centrifuge tube.
2. Add 150 μL of proteinase K (20 mg/ml)
3. Incubate overnight, 55°C water bath
4. Cool sample to 4°C on ice for 30 minutes
5. Add 24.7 ml cold Protein Out solution (kit)
6. Centrifuge 10 min, 4,000 rpm
7. Transfer supernatant to a new tube. Discard pellet.

3.4.3 Removal of proteins and lipids to obtain clean DNA

Two commercially available kits were compared. No PCR inhibitors were detected in the extracted DNA so virtually any extraction protocol could be substituted at this step. The Qiagen DNeasy Tissue kit was chosen because it is easy to use and doesn't require hazardous chemicals. The Bioserve kit was tested because it is marketed as a large-scale DNA purification kit.

1. QIAamp DNeasy Tissue kit (Qiagen, Valencia, CA, USA).
   - Transfer 200 μl sub-samples of recovered supernatant from lysis Methods 1-3 to new tubes.
   - Follow manufacturer's instructions to extract DNA.

2. Bioserve large-scale purification kit (Beltsville, MD, USA).
   - Continue processing entire recovered supernatant from lysis Method 4.
   - Follow manufacturers instructions scaled up 3 times for 100 flies and 7.5 times for 250 flies to extract DNA.

3.5 Assessing the sensitivity of the real-time PCR assay

Having determined the optimal homogenisation, lysis and extraction protocol for 100 flies the sample size was scaled up (homogenisation and lysis volumes adjusted proportionally) to assess the extraction efficiency and detection sensitivity of 1 C. *bezziana* fly plus 249 and 999 *C. megacephala* flies. In addition, the assay sensitivity of the 1 C. *bezziana* plus 999 *C. megacephala* flies was determined by diluting the extracted DNA by 0, 1/10 and 1/100. To detect DNA at the lowest dilution the assay is picking up a tiny fraction a C. *bezziana* fly (the reaction volume contains less than 1/100,000th of a fly).

3.6 Evaluation of trap catches by real-time PCR assay

An evaluation of the *C. bezziana* real-time PCR assay was carried out by screening DNA extracted from 28 trap samples. Each sample was counted and morphologically examined prior to extraction and the presence and number of *C. bezziana* was recorded. Fifteen of the trap samples were collected as part of the Australian SWF surveillance program and the remaining thirteen samples were collected from trapping programs in Malaysia, where *C. bezziana* is endemic. Genetic screening of all samples was done blind with Australian and Malaysian samples extracted concurrently. Trap samples were either air-dried or ethanol preserved. Large trap catches were sub-sampled (1,000 flies estimated by dry weight). Two 200µl sub-samples (replicates) of the cell lysis solution from each trap sample were extracted using a Qiagen DNeasy Tissue kit. Negative extraction and PCR controls were run alongside all samples.

3.7 Field trial to assess trap age on assay sensitivity

A preliminary field trial was conducted to determine if the Bezzilure chemicals might interfere with the recovery of DNA over time in the real-time PCR assay. Paired Lucitraps® containing 999 dead *C. megacephala* and 1 dead *C. bezziana* and either with or without Bezzilure chemicals, were exposed to the elements (no roof over the trap) in the full sun at the DPI&F Animal Research Institute (Brisbane, QLD) during the 2007/08 summer. Entry holes were covered with mesh to prevent additional flies from entering the Lucitrap®. Traps (two replicates per treatment) were collected at time points 0, 1, 4, 7, 10 and 15 days and screened using the molecular assay (with two sub-samples of the cell lysis solution extracted and screened per trap). As extraction volumes remained constant throughout the trial, Ct scores were used as a quantitative measure of DNA content; thus an increase in Ct score represented either DNA degradation or an increase in the level of PCR inhibitors.

The effect of trap catch age on the sensitivity of the real-time PCR assay was then assessed experimentally. Luciraps® containing 999 dead *C. megacephala* and 1 dead *C. bezziana* were exposed to the elements (no roof, and mesh to prevent additional flies entering) in the full sun at the DPI&F Animal Research Institute (Brisbane, QLD) during the 2007/08 summer (Figure 1). Three replicate Lucitraps® were collected at time points 0, 1, 4, 7 & 10 days plus 2, 4 & 6 weeks, and screened using the molecular assay (with two sub-samples of the cell lysis solution extracted and screened per trap). Three additional Lucitraps® containing 19,980 *C. megacephala* and 20 *C. bezziana* (1/1,000 ratio) were exposed for 4, 6 and 8 weeks respectively to assess a “worst case” sampling scenario. At each time point three samples of 1,000 flies were removed from a 20,000 fly bucket and screened (two cell lysis sub-samples were extracted for each 1,000 fly sample) to determine the effect of a full Lucitrap® left in the sun for an extended period of time.

Finally the effect of high temperature and humidity versus outdoor environmental variability on the ability to recover DNA from dead *C. bezziana* over time was assessed by storing paired samples of single flies in 70 ml containers with drainage holes and sealed with mesh either in a 28°C incubator at 89% humidity or outside exposed to the elements (no roof) in the full sun at the DPI&F Animal Research Institute (Brisbane, QLD) during the 2007/08 summer. Flies (three replicates per treatment) were screened after storage for 0, 1, 2, 4, 6 and 8 weeks. Fly legs only were extracted and mean Ct scores for each treatment were compared over time.

Figure 1. Arrangement of modified Lucitraps® containing either 1,000 or 20,000 flies for the field trial at the DPI&F Animal Research Institute in Brisbane, Queensland

4 Results and Discussion

4.1 Fly collections

The species and populations collected are listed in Table 1. DNA was extracted from all of the samples for screening with the real-time PCR assay. Stars indicate which individuals were sequenced (ribosomal DNA ITS1 and initially ITS2 also) to develop the assay.

Table 1. List of species and populations with DNA extracted for DNA bank

Species	Origin	Source	ITS1	ITS2
Chrysomya bezziana	Lab	Bogor, Java, Indonesia	*	*
Chrysomya bezziana	Lab	East Sumba, Indonesia	*	*
Chrysomya bezziana	Lab	Maros, Indonesia	*	*
Chrysomya bezziana	Field	Malaysia	*	*
Chrysomya bezziana	Lab	PNG	*	*
Chrysomya bezziana	Field	Wau, PNG	*	
Chrysomya bezziana	Field	Goa-Usgao, India	*	
Chrysomya bezziana	Field	Salalah, Oman	*	
Chrysomya bezziana	Field	Muscat, Oman	*	
Chrysomya bezziana	Hybrid	PNG x South Africa F1	*	
Chrysomya bezziana	Hybrid	PNG x Sabah, Malaysia F1	*	
Chrysomya bezziana	Hybrid	PNG x Fujairah UAE F1	*	
Chrysomya sp.	Field	Malaysia	*	*
Chrysomya flavifrons	Field	Kuranda, Qld		
Chrysomya incisuralis	Field	Kuranda, Qld		
Chrysomya latifrons	Field	Mt Keira, NSW		
Chrysomya megacephala	Lab	Yeerongpilly, Qld	*	*
Chrysomya megacephala	Field	Malaysia	*	*
Chrysomya nigripes	Field	Kuranda, Qld		
Chrysomya putoria	Field	Grahamstown, South Africa	*	*
Chrysomya rufifacies	Lab	Longreach, Qld	*	*
Chrysomya saffranea	Field	Yeerongpilly, Qld	*	*
Chrysomya semimetallica	Field	Kuranda, Qld		
Chrysomya varipes	Field	Kuranda, Qld		
Chrysomya varipes	Field	Yeerongpilly, Qld	*	*
Cochliomyia hominivorax	Lab	Mexico	poor	poor
Cochliomyia hominivorax	Field	Jamaica		
Hemipyrellia sp.	Field	Yeerongpilly, Qld	*	*
Lucilia cuprina	Lab	Canberra, ACT	*	*
Musca domestica	Lab	Novartis	*	*
Sarcophaginae	Field	Yeerongpilly, Qld	poor	*

4.2 Sequence analysis

For all of the species sequenced ITS1 was larger than ITS2 (*C. bezziana* ITS1 product = 907bp, ITS2 product = 584bp). Direct sequencing PCR products proved difficult for several species due to within individual variability (marked as poor in Table 1). A single nucleus contains many copies of the rDNA subunit (Figure 2). These copies are typically identical within a cell and also within a species due to a process called concerted evolution (Hillis and Dixon, 1991). Fortunately this appears to be the case for *C. bezziana*, all twelve geographic populations had 100% identical sequences indicating that a probe designed to target *C. bezziana* DNA should be effective at detecting populations originating from many different locations (including Indonesia, PNG, Malaysia, Oman, India and South Africa). Individual and or population differences were detected among sequences of *C. megacephala*, *C. saffranea* and *C. putoria*, their rDNA appears to be under different evolutionary pressure. The closest relatives to *C. bezziana* are *C. megacephala* and *C. saffranea*. An alignment of these species show far greater divergence through ITS1, 156 positions diverge (17.2%) than through ITS2, 21 positions diverge (3.6%). Some of the variation in ITS1 is explained by a 45bp repeat element present in *C. bezziana* that is lacking from *C. megacephala* and *C. saffranea*. It was not possible to align ITS1 sequences with any confidence for any but the closest relatives of *C. bezziana*. For this reason two phylogenetic trees were constructed, the first including all sequenced taxa but constructed with conserved and alignable ITS2 sequences only (Figure 3) and the second using partial ITS1 and targeting only *C. bezziana* and its closest relatives (Figure 4).

The trees clearly separate *C. megacephala* and *C. saffranea* as sister taxa to *C. bezziana*. Both trees show *C. megacephala* and *C. saffranea* grouping together extremely closely; this genetic marker cannot reliably distinguish the two species from each other. The grouping of unidentified *Chrysomya* sp. with *C. megacephala* and *C. saffranea* suggests this sample belongs to one of these two species, it is clearly not *C. bezziana* although its' morphologically was considered suspicious.

The ITS2 tree (Figure 3) suggests that the genus *Chrysomya* may not be a monophyletic assemblage as both *Lucilia cuprina* and *Cochliomyia hominivorax* fall within the *Chrysomya* clade. This result is not supported in mitochondrial DNA trees and may be an artefact of long-branch attraction (taxa with long branches group together as an artefact of substitution saturation not because of true evolutionary relationships) (Stiller and Hall, 1999). Sequencing additional representatives of each genus would help to resolve this disparity but was not within the scope of this project.

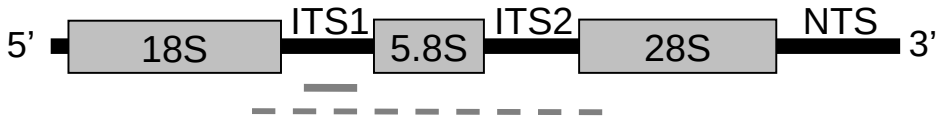


Figure 2. Nuclear ribosomal DNA subunit indicating region sequenced (dashed grey line) and site in ITS1 targeted for the *C. bezziana* real-time PCR assay (solid grey line)

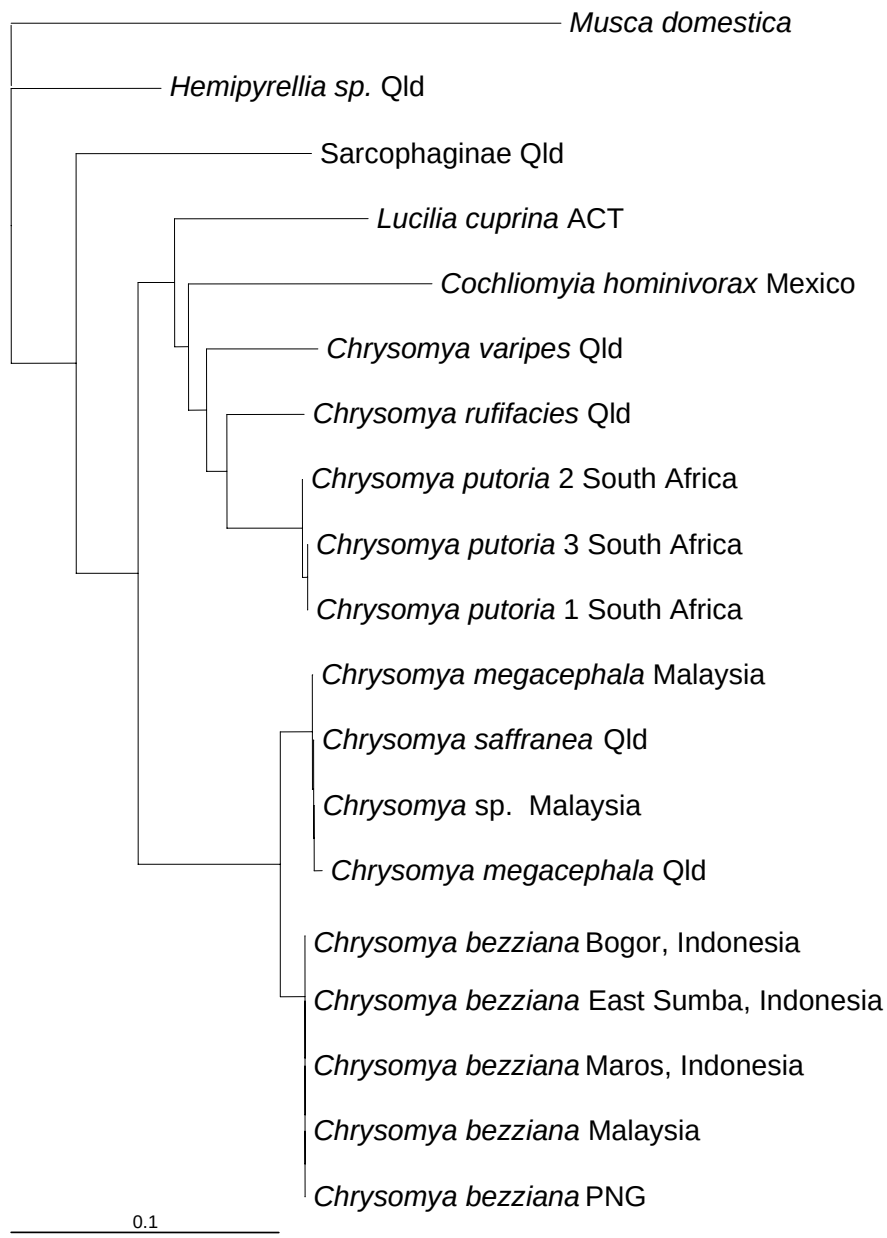


Figure 3. Neighbour joining distance tree of rDNA ITS2 sequences for *C. bezziana* and related fly species

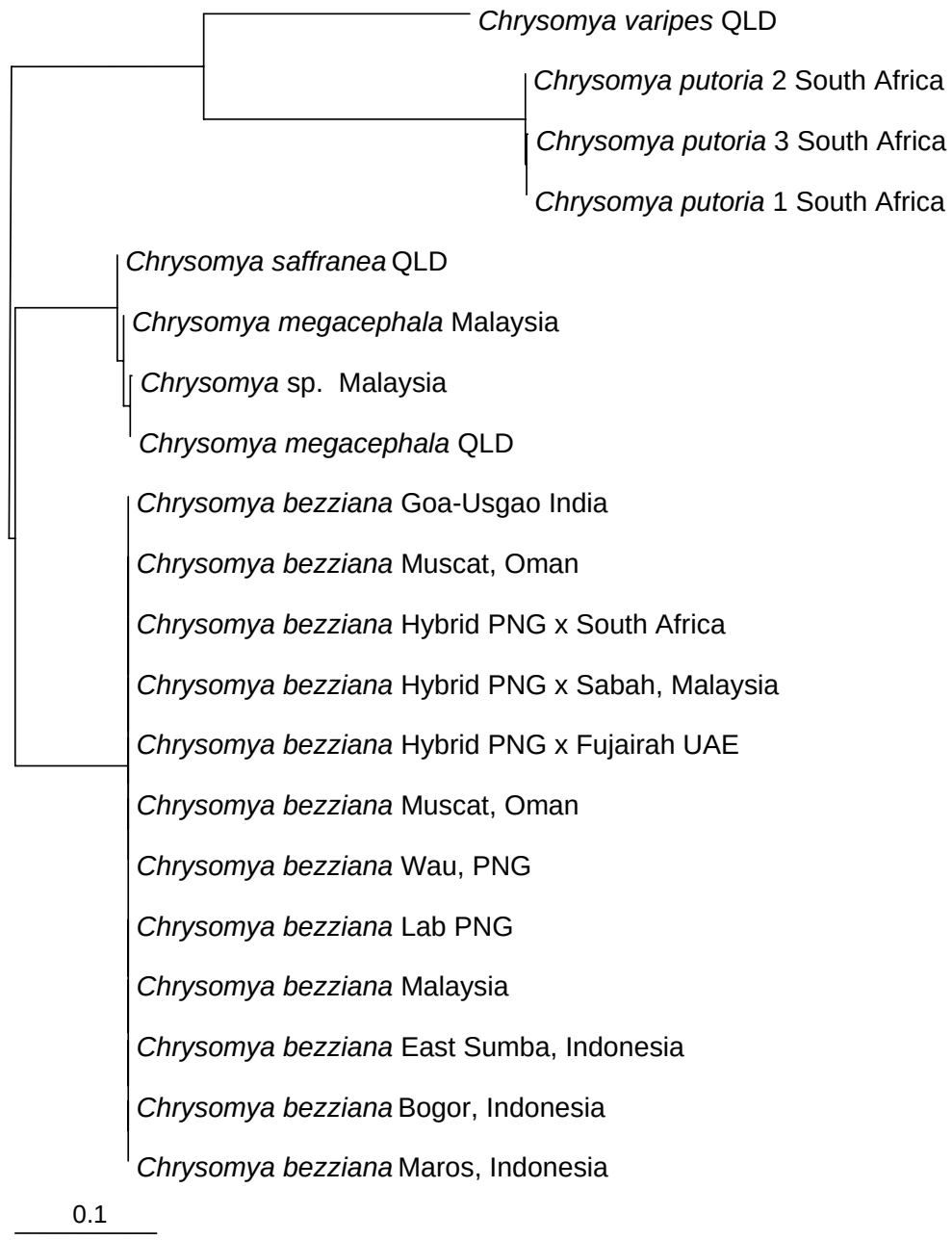


Figure 4. Neighbour joining distance tree of partial rDNA ITS1 sequences for *C. bezziana* and closely related *Chrysomya* species

4.3 Real-time PCR assay design

The ITS1 was the only region of rDNA sequence found to be variable enough among the sister species *C. bezziana*, *C. megacephala* and *C. saffranea* to achieve both primer and probe specificity, see Figure 2. As primers are relatively inexpensive compared to probes, one forward primer and three reverse primers were designed flanking one probe position to optimise the assay (Table 2). Care had to be taken to avoid a repeating sequence element, 12-45bp long and present 5 times within the *C. bezziana* sequence. A minor groove binding (MGB) probe was selected for the real-time PCR assay as this moiety gives greater stability to the hybridized probe. The fluorophore FAM was selected as the reporter dye as it typically gives a reliable and strong signal.

Table 2. Real-time PCR assay primer and probe sequences, the optimised assay uses reverse primer 2

	Sequence	Tm (°C)	Position in *C. bez* sequence
Forward primer	5' GACACAAACAAAAACATAGAATAGATCTTG	58	206
Reverse primer 1	5' TCATTAGTAGGGTAAACCAACAATCATC	58.8	292
Reverse Primer 2	5' TCTTTTTGCCAATAGTAGGGTAAGACTA	58.2	257
Reverse Primer 3	5' CAATGTATGAATATTTTCATATTCAGTACATCA	59	452
Probe MGB, FAM	5' AGCAAATTTCATTCTTGACA	69.9	236

Reverse primer 2 amplified the strongest product during testing of the new *C. bezziana* specific real-time PCR assay. This result is consistent with real-time PCR technology that favours smaller amplicon products. The assay easily detected DNA extracted from the legs of single *C. bezziana* and was also successful amplifying the same DNA diluted 10x in water (Figure 5).

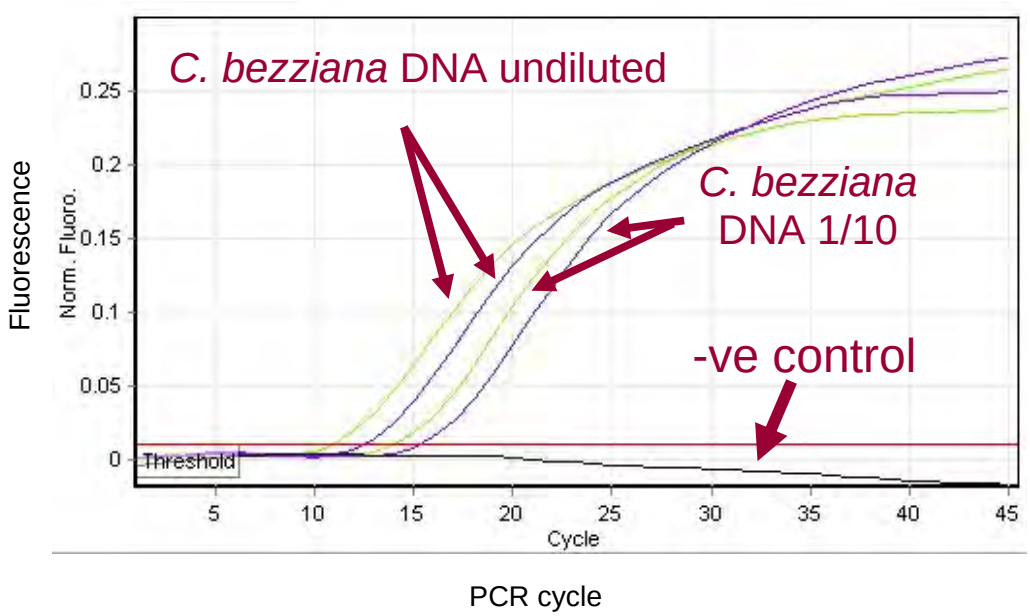


Figure 5. Real-time PCR assay showing successful amplification of two *C. bezziana* DNA samples each at undiluted and 1/10 diluted concentrations

4.4 Confirmation of real-time PCR assay specificity

Results of the specificity tests of the *C. bezziana* real-time PCR assay against non-target fly DNA are shown in Table 3. The assay shows no cross-reactivity with closely related flies or with fly species likely to be caught in screw-worm fly surveillance traps. This result means that *C. bezziana* free trap samples should not give false-positive results due to probe cross-reactivity. Using spiked DNA samples with large amounts of non-target DNA (non-target to *C. bezziana* DNA 10:1 and 1,000:1) the *C. bezziana* real-time PCR assay displayed no inhibition and was still able to detect low levels of target *C. bezziana* DNA. All twelve populations of *C. bezziana* from Table 1 tested positive with the assay (results not shown).

Table 3. *Chrysomya* species, related flies, and species likely to be included in Australian trap catches, which were tested for cross reactivity with the C. *bezziana* specific real-time PCR assay

Species	Ct score*
Chrysomya bezziana (1/100 dilution of DNA with water)	19.6
C. flavifrons	No amp
C. incisuralis	No amp
C. latifrons	No amp
C. megacephala	No amp
C. nigripes	No amp
C. putoria	No amp
C. rufifacies	No amp
C. saffranea	No amp
C. semimetallica	No amp
C. varipes	No amp
Cochliomyia hominivorax	No amp
Hemipyrellia sp.	No amp
Lucilia cuprina	No amp
Musca domestica	No amp
Sarcophaginae	No amp
C. bezziana plus *C. megacephala* (1:1 ratio, 1/100 dilution)	19.41
C. bezziana plus mixed non-target DNA† (1:10 ratio, 1/100 dilution)	18.46
Mixed non-target DNA†	No amp
C. bezziana (1/1,000 dilution)	21.8
C. bezziana plus mixed non-target DNA† (1:1,000 ratio, 1/1,000 dilution)	22.15
Mixed non-target DNA†	No amp

* Ct score = number of cycles in real-time PCR assay required before amplification reaches required threshold (the smaller the number, the more starting DNA).
† mixed non-target DNA from *C. megacephala, C. hominivorax, C. varipes, C. flavifrons, C. nigripes, C. latifrons, C. saffranea, C. incisuralis and C. putoria.*

4.5 Optimising DNA extraction from bulk trap catches

4.5.1 Fly homogenisation

Liquid Nitrogen versus Waring Blender
The liquid nitrogen method was better at mincing the flies into smaller pieces than the Waring blender method. Although more effective at homogenising the flies the liquid nitrogen method was a manual process that increased the potential risk of contamination due to the increased length of time that the samples were exposed to air as they were crushed. In contrast the Waring blender provided

a completely enclosed environment with a fixed rotor speed making the process consistent and repeatable.

Sub-sampling in triplicate from the homogenised flies then lysing and extracting the DNA with a Qiagen DNeasy tissue kit produced highly variable results for both methods, see Figure 6. The liquid nitrogen method replicates gave both the best and worst Ct scores while the Waring blended replicates displayed less variance but a higher mean Ct score (higher Ct indicates later amplification thus less DNA). Neither homogenisation method was able to disperse the *C. bezziana* tissue evenly throughout the sample. Based on these results the extraction protocol was modified to include a large-scale DNA lysis step prior to sub-sampling for DNA extraction.

Despite producing a courser end product, the Warning blender method was chosen over the liquid nitrogen method for the advantage of less sample exposure, less handling time, less expense and greater reproducibility.

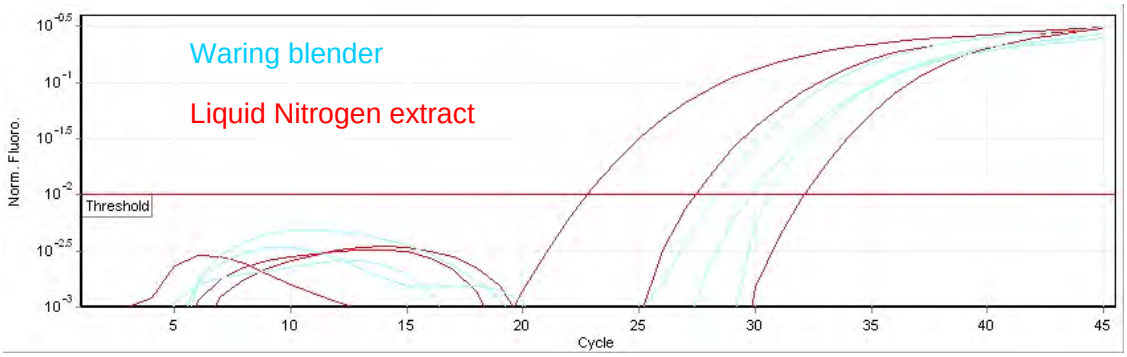


Figure 6. Real-time PCR amplification results of the homogenisation experiment comparing tissue disruption by manually grinding 100 flies spiked with one *C. bezziana* in liquid nitrogen versus a Waring blender. The effect of sub-sampling prior to lysing the cells is marked by replicate heterogeneity within the treatments.

Method 1 – Boiling
Method 2 – Boiling + Proteinase K
Method 3 – Boiling + Proteinase K + SDS
Method 4 – Bioserve commercial large-scale purification kit

Lysing the homogenised flies prior to sub-sampling produced much more consistent results between replicates (Figure 7, Table 4). Interestingly the most complex and costly boiling protocol (Method 3) produced the weakest results. The optimal boiling lysis method was Method 2 involving boiling and the addition of Proteinase K to help break down proteins. Results of method 4, the Bioserve kit, are compared under the next subheading.

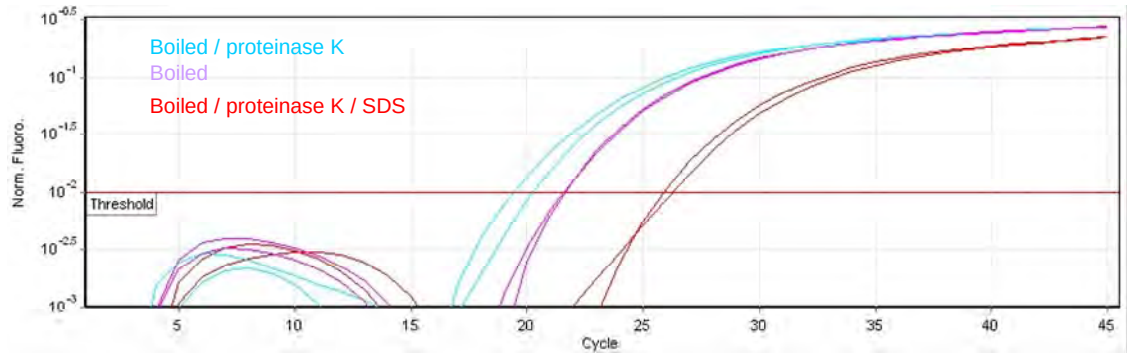


Figure 7. Real-time PCR amplification curve showing results of lysis experiment using boiled preparations with different additions. (fewer cycles indicates more DNA)

Table 4. Tabulated results of Ct scores from real-time PCR amplification showing results of lysis experiment using boiled preparations with different additions

Extraction Method	Ct score*	
	Sub-sample 1	Sub-sample 2
Boiled	21.60	21.56
Boiled / proteinase K	19.41	20.27
Boiled / proteinase K / SDS	25.87	26.27

* Ct score = number of cycles in real-time PCR assay required before amplification reaches required threshold (the smaller the number, the more starting DNA).

4.5.3 Removal of proteins and lipids to obtain clean DNA

Both the Qiagen DNeasy Tissue kit and the Bioserve large-scale purification kit were successful at extracting DNA from fly tissue. Neither kit was capable of processing 1,000 flies without sub-sampling. At maximum recommended concentrations the Bioserve large-scale purification kit could process up to 25 flies (1,000 mg) while the Qiagen DNeasy Tissue kit was limited to less than 1 fly (25 mg).

Scaling up the Bioserve kit protocol amplified more target DNA than the comparable extraction through the Qiagen kit (1+99 flies Bioserve Ct Score = 20.53, Qiagen kit Ct score = 25.74; 1+249 flies Bioserve Ct Score = 24.06, Qiagen kit Ct score = 29.79). By holistic sampling the Bioserve kit produced a better result than sub-sampling into a Qiagen kit, however, scaling up the Bioserve kit proved to be extremely expensive ($110 per 250 flies) and for this reason this kit was not considered a practical option for screw-worm fly surveillance.

Replicate sub-samples extracted using the Qiagen DNeasy Tissue kit were consistent and extraction results were repeatable, see Table 5. The Qiagen DNeasy Tissue kit proved cost and time effective and was deemed the most practical option for screw-worm fly surveillance. The optimised protocol (Appendix 1) was chosen to reduce cost ($35 in reagents per 1,000 flies), to minimise sample exposure to potential contaminants, to avoid the handling of hazardous chemicals and to maximise DNA release.

Table 5. Results of Qiagen DNeasy Tissue kit extractions showing real-time PCR amplification consistency both within and between extractions

Ct scores	Qiagen extraction 1		Qiagen extraction 2	
	Sub-sample 1	Sub-sample 2	Sub-sample 1	Sub-sample 2
1 *C. bezziana* + 99 *C. megacephala*	20.27	19.41	19.43	19.54

4.6 Assessing the sensitivity of the real-time PCR assay

The sensitivity of the real-time PCR assay and extraction protocol was measured with extracted samples of 99, 249 and 999 non-target *C. megacephala* flies spiked with a single C. *bezziana* fly (Table 6). Target DNA was amplified in all of the samples indicating that the assay is extremely sensitive to very low concentrations of target DNA even in the presence of large amounts of non-target fly DNA. The increasing Ct scores with increasing fly numbers reflect the subsequent dilution of target DNA as more non-target flies were added to the extraction and extraction volumes were

accordingly scaled. Target DNA could still be detected even after diluting the extracted DNA 100 fold with water prior to analysing.

Table 6. Results of real-time PCR screen of scaled up extraction volumes ranging from 100 to 1,000 flies

Sample	Mean Ct Score*		
	No dilution	1 in 10 dilution	1 in 100 dilution
1 *C. bezziana* + 99 *C. megacephala*	20.27	23.50	25.74
1 *C. bezziana* + 249 *C. megacephala*	23.09	26.15	29.79
1 *C. bezziana* + 999 *C. megacephala*	30.40†	31.21	35.15

* Ct score = number of cycles in real-time PCR assay required before amplification reaches threshold (the smaller the number, the more starting DNA). Mean taken of duplicate subsamples.
† Improvements to the assay during the course of the study increased sensitivity such that by the end of the project 1 *C. bezziana* plus 999 *C. megacephala* gave an average Ct = 25.

4.7 Evaluation of trap catches by real-time PCR assay

Results of the specificity of the *C. bezziana* real-time PCR assay against non-target fly DNA isolated from both local and overseas trap catch populations are shown in Table 7. Accuracy of the real-time PCR assay in the blind screen of 28 trap catches was 100%. All trap catches morphologically identified as containing *C. bezziana* tested positive with the molecular assay with levels as low as one *C. bezziana* in 1,000 non-target flies successfully identified. Ct scores ranged from 18.63 (5 *C. bezziana* in a trap of 110 flies) to 34.75 (1 *C. bezziana* in a trap of 629 flies). Although real-time PCR provides a quantitative measure of DNA content, scores for the *C. bezziana* real-time PCR assay will not necessarily scale due to the changes in extraction volume associated with initial trap size and a DNA saturation effect on the Qiagen DNA extraction columns. Scores lower than Ct = 35 should be considered positive for *C. bezziana*, those with Ct > 35 should be treated as suspicious positives and be re-tested. Samples that amplify repeatedly and consistently with Ct >35 are probably positive for *C. bezziana*, however, care should be taken to confirm these samples by screening any remaining trap catch morphologically. The Australian surveillance traps collected from Qld (Brisbane and Thursday Is) and WA (Geraldton, Fremantle and Port Hedland) were all negative for SWF.

Table 7. Results of real-time PCR assay on National and International fly trap catches

Trap Country of origin	**Location**	**Fly numbers in trap**	**No. of *C. bezziana* identified using morphology**	**Real-time PCR molecular assay Ct score* subsample 1**	**Real-time PCR molecular assay Ct score* subsample 2**
Malaysia	Jelai Gemas	37	1	21.48	21.20
Malaysia	Jelai Gemas	270	0	No amp	No amp
Malaysia	Jelai Gemas	276	1	28.41	27.51
Malaysia	Jelai Gemas	1020	1	24.25	24.14
Malaysia	Jelai Gemas	282	2	21.40	21.14
Malaysia	Ulu Lepar	21	1	18.90	20.20
Malaysia	Jelai Gemas	261	0	No amp	No amp
Malaysia	Jelai Gemas	691	1	31.33	31.30
Malaysia	Jelai Gemas	48	11	19.82	19.63

Trap Country of origin	Location	Fly numbers in trap	No. of *C. bezziana* identified using morphology	Real-time PCR molecular assay Ct score* subsample 1	Real-time PCR molecular assay Ct score* subsample 2
Malaysia	Jelai Gemas	110	5	18.89	18.63
Malaysia	Jelai Gemas	65	0	No amp	No amp
Malaysia	Jelai Gemas	629	1	34.75	33.08
Malaysia	Jelai Gemas	77	1	29.00	27.43
Australia	Port Hedland WA	1/2 sheet	0	No amp	No amp
Australia	Port Hedland WA	15,000	0	No amp	No amp
Australia	Port Hedland WA	50,000	0	No amp	No amp
Australia	Geraldton WA	1/2 sheet	0	No amp	No amp
Australia	Geraldton WA	1,500	0	No amp	No amp
Australia	Geraldton WA	120	0	No amp	No amp
Australia	Fremantle WA	2,000	0	No amp	No amp
Australia	Fremantle WA	400	0	No amp	No amp
Australia	Fremantle WA	80	0	No amp	No amp
Australia	Thursday Island	2,000	0	No amp	No amp
Australia	Thursday Island	2,000	0	No amp	No amp
Australia	Brisbane QLD	516	0	No amp	No amp
Australia	Brisbane QLD	913	0	No amp	No amp
Australia	Brisbane QLD	2	0	No amp	No amp
Australia	Brisbane QLD	30	0	No amp	No amp
Australia	Brisbane QLD	16	0	No amp	No amp

* Ct score = number of cycles in real-time PCR assay required before amplification reaches required threshold (the smaller the number, the more starting DNA).

4.8 Field trial to assess trap age on assay sensitivity

The Bezzilure chemicals were found to have no effect on DNA recovery over time, Figure 8. Both chemical and chemical-free traps showed a decrease in DNA recovery by 15 days.

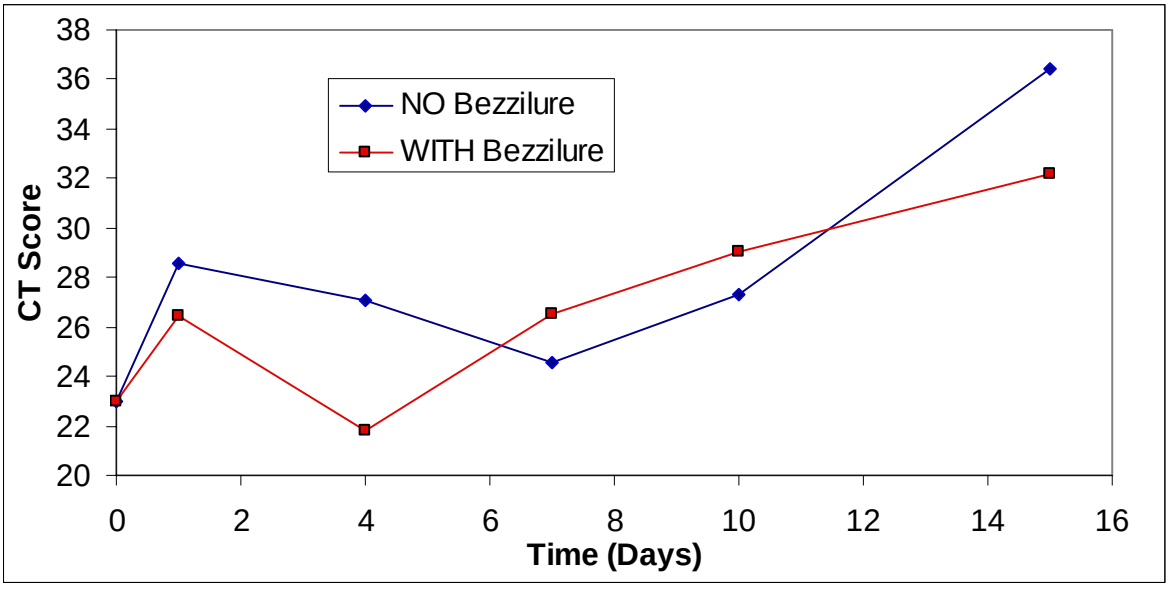


Figure 8. Effect of Bezzilure chemicals on DNA recovery (1 C. *bezziana* plus 999 *C. megacephala*) from Lucitraps® left in full sun for up to 15 days

Length of trapping time had an effect on DNA recovery, after 14 days one of the three replicate Lucitraps® failed to amplify, and a second gave an uncertain score with mean Ct > 35, Figure 9. All Lucitraps® were positive up to 10 days and *C. bezziana* was detected in at least some traps (2 out of 3) for the entire 6-week trial. Within Lucitrap® sub-samples amplified Ct scores within +/-1 (sub-samples given as mean in Figure 9) indicating the bulk sample cell lysis step was effective. In contrast, replicate Lucitraps® were highly variable, even at time point 0 suggesting that the homogenisation step (blending) should be based on homogenate consistency not a fixed length of time. Some of the observed variability may also be due to fly to fly differences in DNA content. Over time the position of *C. bezziana* in the Lucitrap® may also affect its' rate of decomposition. The three extreme Lucitraps® representing the "worst case" sampling scenario (19,980 *C. megacephala* and 20 *C. bezziana*) gave uncertain positives at 4, 6 and 8 weeks. Two samples, one at week 4 and the other week 8, failed to amplify at all indicating that either the *C. bezziana* DNA had degraded beyond detection or that the 1,000 fly sub-sample failed to include the target species. Similarly 2 sub-samples in week 6 gave positive scores well within range suggesting that more than 1 *C. bezziana* were sub-sampled in the random 1,000 flies. This result highlighted the risk of missing low abundance *C. bezziana* in very large Lucitrap® catches.

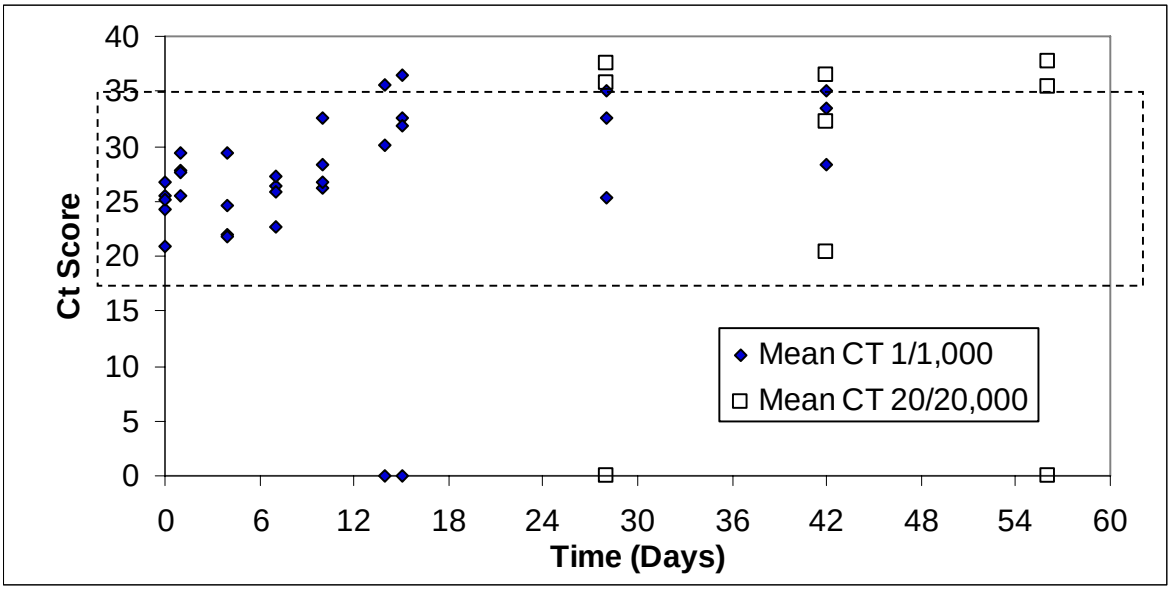


Figure 9. Time trial to determine best trap age for maximum assay sensitivity under poor (trap catch = 1,000 flies, diamonds) and worst (trap catch = 20,000 flies, open squares) sampling conditions. The dashed line box indicates *C. bezziana* positive Ct scores, those above the box are uncertain and those at 0 failed to amplify

The combined effect of high temperature (28°C) and humidity (89%) could not account for the observed reduction in *C. bezziana* DNA recovery, Figure 10. Flies left outdoors (representing trap samples) began degrading by 14 days compared to incubator flies that retained high Ct scores for twice this length of time. This result suggests that environmental fluctuations (i.e. wet/dry cycle; high/low temperature; UV/visible light) may have a greater impact on DNA recovery than high temperature and humidity alone. The outdoor flies were susceptible to rain, a problem that is overcome in the field, on standard surveillance traps, with the addition of a roof over the trap. The

roof may also provide shade and some protection from UV damage. The cause of low DNA recovery is not known; further work to determine the mechanisms involved could lead to longer trapping periods increasing the probability of detecting low prevalence SWF.

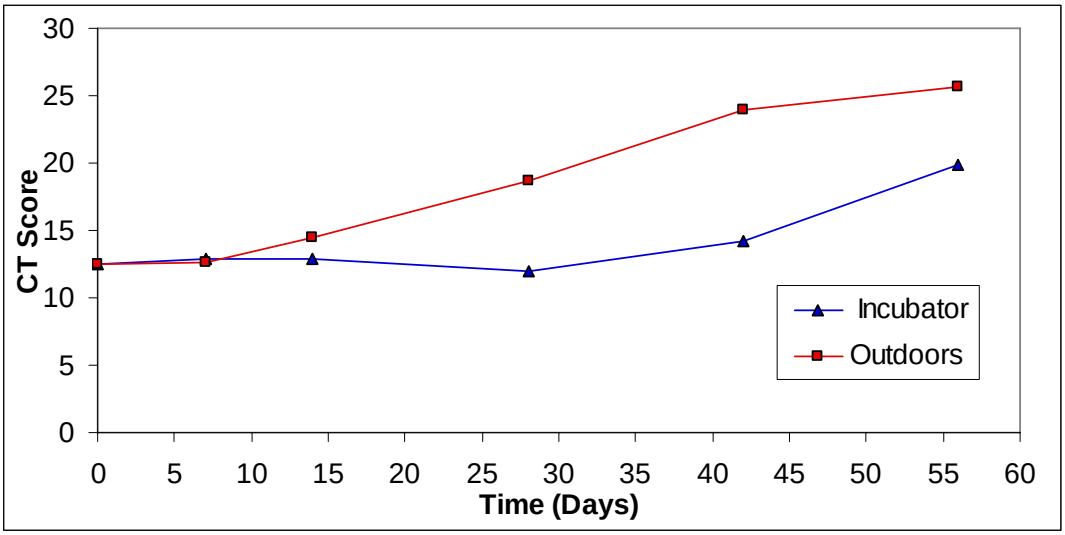


Figure 10. Effect of incubation at high temperature (28°C) and humidity (89%) on DNA degradation of single *C. bezziana* flies compared to outdoor exposure

4.9 Probability of detecting low prevalence SWF

Budgets and average catch sizes will determine the number of assays that can be performed for surveillance purposes. At a prevalence of one SWF in 1,000 flies, real-time PCR screening three subsamples of 1,000 flies, from a trap containing 50,000 flies, has over a 95% probability of detecting SWF if it is present in the trap, Table 8. These probabilities, based on a binomial distribution, assume the real-time PCR assay has 100% sensitivity at detecting one SWF in 1,000 flies, a valid assumption based on optimisation results and trap testing outcomes. The trade-off of screening more than one subsample from a catch is that fewer Lucitraps® overall can be screened. For general surveillance widespread geographic representation might be prioritised over complete coverage of a single trap. At high risk sites (i.e. large cattle yards at shipping ports) screening a greater proportion of the catch (three subsamples versus one) might be advantageous.

Table 8. Probability estimates of detecting SWF (if present) using real-time PCR assay, given different SWF prevalence levels and trap catch sizes

SWF prevalence in population	Number of flies in trap catch	1 subsample of 1,000 flies screened	2 subsamples of 1,000 flies screened	3 subsamples of 1,000 flies screened
1/100	1,000	100%	-	-
	10,000	100%	100%	100%
	20,000	100%	100%	100%
	50,000	100%	100%	100%
1/1,000	1,000	100%	-	-
	10,000	65.1%	89.3%	97.2%
	20,000	64.2%	87.8%	96.1%
	50,000	63.6%	87%	95.5%
1/10,000	1,000	100%	-	-
	10,000	10%	20%	30%

	20,000	9.8%	19%	27.8%
	50,000	9.6%	18.5%	26.6%

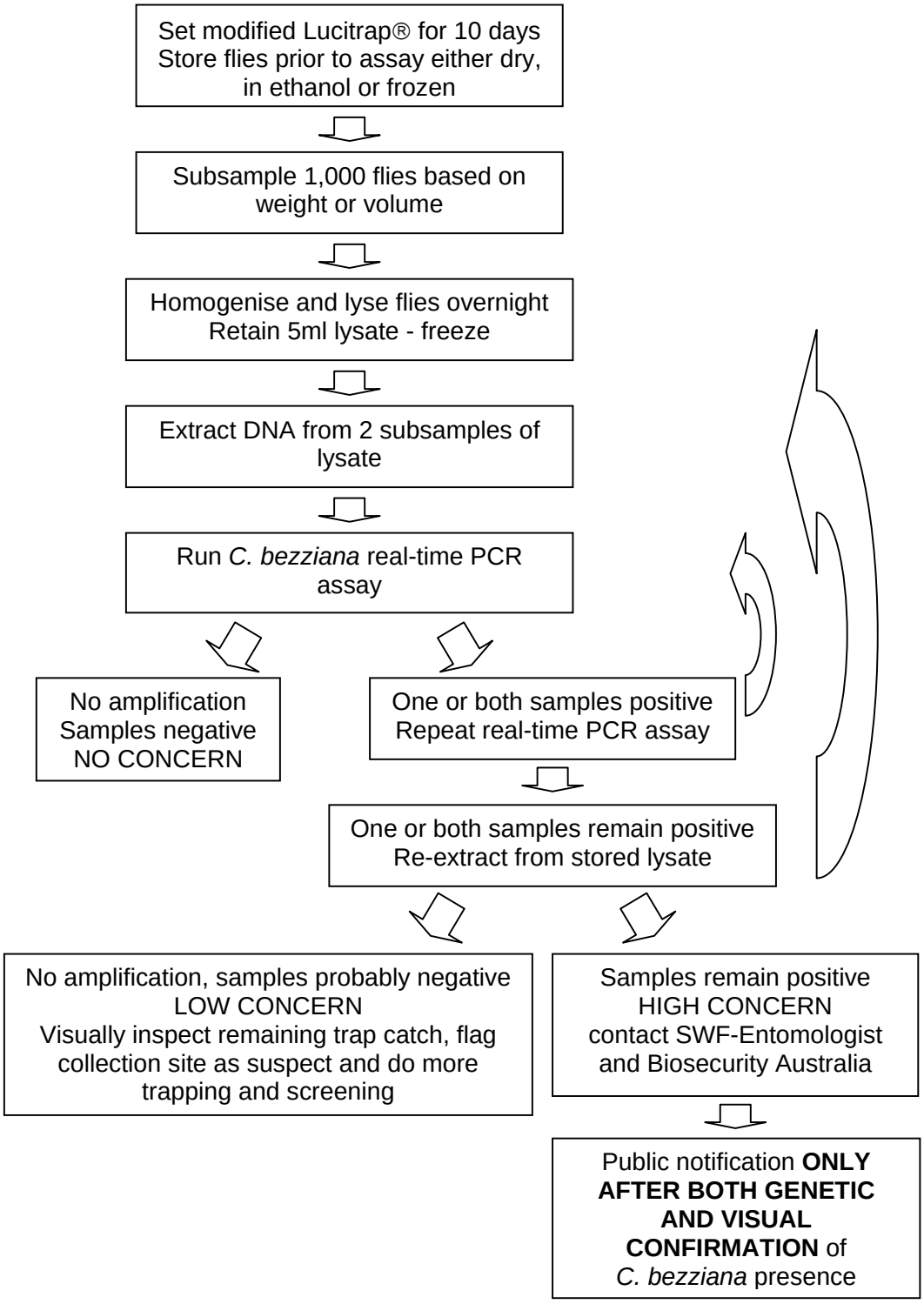
4.10 Screening small trap catches

Trap catches smaller than 1,000 flies can still be screened with the real-time PCR assay. Extraction volumes should be scaled accordingly although the volume of lysis buffer added for the homogenisation step needs to be sufficient to cover the flies in the Waring blender (different sized blender jars are available). A single *C. bezziana* fly extracted using the 1,000 fly bulk extraction protocol remains detectable in the real-time PCR assay so volumes do not need to be changed if it is inconvenient to do so for practical purposes.

A further consideration of screening an entire trap catch is that there are no remaining flies left in the sample to examine if a positive real-time PCR result occurs. Definitive confirmation of SWF presence in Australia should not be based on genetic evidence alone, but on both molecular AND visual confirmation. One approach is to extract and screen only half of a catch if fewer than 1,000 flies are caught. Alternatively the entire trap could be screened to maximise detection capabilities; if a positive trap is detected, re-trapping at the site will obtain more flies.

If trap catches are small, and the geographic sampling area is limited, a more cost effective approach to screening may be to pool catches. This will enable more flies and sites to be screened but at a lower resolution i.e. if the assay returns a SWF positive result more trapping will be required to determine the source.

4.11 Flow chart of real-time PCR assay application



4.12 Assay costs

A breakdown of the costs involved in screening a Lucitrap® sample of 1,000 flies is given in Table 9. The most cost efficient approach to minimise labour costs is to process 12 samples of 1,000 flies concurrently. This reduces the final cost per sample, including labour, to just under $55.

Table 9. Breakdown of DNA extraction and real-time PCR assay costs

Reagents	Cost/Unit	Cost to extract 1,000 flies and screen two subsamples
TE buffer	$150 / kg	$0.05
Proteinase K	$725 / g	$7.25
Probe	$520 / 500 reactions	$2.10
Primers, forward and reverse	$20 / 500 reactions	$0.20
Real-time PCR master mix	$539 / 100 reactions	$10.80
Qiagen DNA easy kit	$1200 / 250 columns	$9.60
Consumables (tips, tubes, gloves)		$5.00
Total Reagents		$35.00
Reagent cost to screen 12 samples		$420.00
Labour to screen 12 samples	$30/hour	$217.50
Total cost to screen 12 samples		$637.50
Extrapolated cost per sample		$53.13

5 Success in Achieving Objectives

All three objectives were achieved within this project. A DNA-based real-time PCR assay specific to *C. bezziana* has been developed targeting the ribosomal DNA ITS1. The assay is specific to *C. bezziana* with no cross reactivity detected against other closely related *Chrysomya* species, or against species likely to be caught in Australian SWF surveillance traps. A DNA extraction protocol has also been developed to be used in conjunction with the *C. bezziana*-specific real-time PCR assay, to process large Lucitrap® catches containing mixed fly species. The optimal protocol can process up to 1,000 flies and is affordable (under $55 per 1,000 flies), minimises sample exposure to contaminants, avoids the handling of hazardous chemicals and maximises DNA recovery with consistent results. Finally the assay has been blind tested on field collected Lucitrap® catches from both Australia and Malaysia with outstanding results, *C. bezziana* was detected with 100% accuracy. Based on a field trial designed under extreme sampling and weather conditions the optimal trapping period for maximising the chance of SWF detection has been determined to be 10 days.

6 Impact on Meat and Livestock Industry

6.1 Impact on Meat and Livestock Industry now

The immediate impact of this assay is to increase the sensitivity of Australia's SWF surveillance screening. To date the advances in trapping technologies have not been paired with processing technology and as a result currently only a fraction of flies caught in surveillance traps are screened using morphological examination because the process involves the handling of individual flies. The new molecular assay permits 1,000 flies to be screened in a single batch. Extraction and amplification steps can be done simultaneously so that 12 samples (12,000 flies) can be processed within two days. Even in areas with low prevalence (1/1,000) and high catches (50,000 flies) just

three real-time PCR assays have a 95% probability of detecting SWF in the trap. AQIS staff will now have the technology to keep up with the necessary processing required to handle large trap catches.

6.2 Impact on Meat and Livestock Industry in five years time

The impact of this technology on the Meat and Livestock Industry, particularly the northern cattle industry, in five years time could be dramatic. If SWF were to establish in Australia tomorrow either through sea trade or fly strike wound then early detection and monitoring would be essential. Uncontrolled this pest could cost the industry $500 million per year. Manual morphological processing of flies is already unsustainable; this assay gives the Meat and Livestock Industry the best technology for early detection and thus the best chance to control an outbreak before it devastates the northern cattle industry. Once eradication is complete ongoing monitoring and surveillance will ensure the molecular assay remains an essential procedure in the AQIS toolbox.

7 Conclusions and Recommendations

7.1 Conclusions

The bulk-fly extraction protocol and *C. bezziana*-specific real-time PCR assay have been designed to compliment the new modified Lucitrap® used in Australia's SWF surveillance program. The assay can also be used on single flies (extraction from fly legs alone provides sufficient DNA), eggs or larvae for genetic confirmation of species identity. The cost per assay to screen 1,000 flies (extracting two subsamples) including labour is under $55. The time required to process up to 12 samples is two days. Screening requires a facility with real-time PCR capabilities and basic training in molecular biology. By substantially increasing the number of flies checked the molecular assay will greatly improve Australia's SWF screening capabilities. The assay should only be used in conjunction with morphology for definitive identification of *C. bezziana*. Using morphology alone to screen the Lucitraps® is too labour-intensive, instead molecular screening will filter out negative traps and identify potential positives, and suspicious samples can then be confirmed or rejected using morphology. The following steps describe the recommended process for future SWF monitoring from Lucitraps® in Australia.

7.2 Recommendations

- Adopt the new *C. bezziana*-specific real-time PCR assay for screening Lucitrap® catches in SWF surveillance trapping program
- Explore facilities and capacity of various government and private laboratories to conduct screening
- Carry out additional research on the causes for the reduction in DNA recovery from flies in Lucitraps® with the aim of extending the optimal trapping period to more than 10 days.
- Explore the application of this technology to other surveillance programs

Adoption of assay
The new *C. bezziana*-specific real-time PCR assay is cost effective, very sensitive (one *C. bezziana* can be detected in sample of 1,000 flies) and will assist industry by providing early detection of a SWF incursion into Australia leading to a shorter response time and faster containment.

Transfer of technology
Screening Lucitrap® samples using the new technology requires a facility with real-time PCR capabilities and basic training in molecular biology. Both government and private laboratories containing molecular facilities with suitably trained staff exist around Australia. Transfer of the

technology for bulk DNA extraction and the *C. bezziana*-specific real-time PCR assay should be straight forward as both protocols will be published (manuscript in prep) and publically available.

Future research
Through further research to investigate the cause of DNA degradation in the traps it might be possible to extend the trapping period, and further optimise surveillance trapping. The effect of high heat and humidity has been tested. Other variables to investigate include UV light (Lucitraps® can be made in different colours), high and low temperature fluctuations and wet and dry cycles.

Application of technology
Surveillance screening for insect pests around Australia is common (fruit fly, bumble bee, European wasp, fire ant, whitefly, khapra beetle). Trap screening insects followed by visual examination and sorting still remains the most common method for surveillance screening. Where catch rates of non-target species are high and morphological characters to distinguish them are few, molecular assay based screening may provide a more cost effective solution. A good example is the invasive fire ant. Two incursions have been detected in Queensland and millions of dollars have been spent on eradicating the pest. Huge amounts of time and effort are currently spent trapping and sorting ants looking for small remaining populations. Over 250 native ant species can also be found in the surveillance areas and many end up in the traps and have to be manually sorted. The scope of molecular screening to assist in Australia's pest surveillance programs is far reaching.

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9 Appendices

9.1 Appendix 1. Protocols for SWF assay

9.1.1 DNA extraction Reagents & Materials

Sterilised Glass Waring blender jars (1 / trap)
Waring blender
Boiling water bath
56°C water bath
70°C water bath
Microcentrifuge (adjustable speed to 14000 rpm)
225 ml centrifuge tubes (BD Falcon cat no. 352075) (1 / trap)
1.5 ml microcentrifuge tubes (6 / trap)
5 ml sterile specimen vial (1 / trap)
Lysis buffer (10mM EDTA, 20mM Tris) (100 ml / trap)
Proteinase K (20 mg/ml) (500 µl / trap)
Qiagen DNeasy Tissue Kit (cat no. 69504) (2 columns / trap)

9.1.2 Extraction protocol

Homogenise flies in a Waring blender

1. Transfer 1,000 flies (estimate by weight or volume) into a pre-sterilised stainless steel Waring blender jar.
2. Add 100 ml of lysis buffer to the blender, seal with lid and homogenise at high speed until there are no large fly pieces remaining (1 - 3 minutes).
3. Transfer homogenate to a 225 ml centrifuge tube.

Lyse cells to release DNA

4. Boil homogenate for 15 minutes.
5. Cool to room temperature.
6. Add 500 µl of proteinase K (20 mg/ml). Mix well by gentle inversion.
7. Incubate overnight at 56°C (water bath).
8. Mix by gentle inversion then take two sub-samples of 200 µl lysate into sterile 1.5 ml microcentrifuge tubes for DNA extraction.
9. Retain 5 ml of lysate in a well labelled liquid specimen container, (store frozen at -20°C).

DNA extraction - Qiagen QIAamp DNeasy DNA mini kit

10. Add 200 µl buffer AL to sample and vortex for 15 seconds
11. Incubate at 70°C for 10 minutes
12. Briefly centrifuge
13. Add 200 µl 100% Ethanol
14. Vortex for 15 seconds, briefly centrifuge
15. Place mixture into spin column
16. Centrifuge at full speed for 1 minute
17. Replace collection tube
18. Add 500 µl Buffer AW1 to spin column
19. Centrifuge at 8000rpm for 1 minute
20. Replace collection tube
21. Add 500 µl Buffer AW2 to spin column

22. Centrifuge at full speed for 3 minutes
23. Trnasfer QIAamp spin column to a clean 1.5 ml microcentrifuge tube
24. Add 100 µl Buffer AE to spin column
25. Incubate at room temperature for 1 minute
26. Centrifuge at 8000 rpm for 1 minute
27. Store DNA at -20C

9.1.3 Real Time PCR assay

	Volume per sample
Extracted fly DNA	5 µl
RealMasterMix Probe (Eppendorf)	8 µl
Forward primer (5µM)	1.2 µl
5’ GACACAAACAAAAACATAGAATAGATCTTG	
Reverse primer (5µM)	1.2 µl
5’ TCTTTTTGCCAATAGTAGGGTAAGACTA	
MGB-FAM Taqman probe (5µM)*	0.8 µl
5' AGCAAATTTCATTCTTGACA	
Milli-Q water	3.8 µl
Total reaction volume	20.0 µl

*Probe must be diluted from 100 µM stock solution immediately prior to run. Storage and re-use of diluted probe can give false signal in the assay.

Always include a negative extraction control and positive and negative PCR controls with every run.

Thermocycle in a Rotor-Gene 3000 (Corbett Research) or equivalent.
Amplification conditions
95°C for 2 minutes
45 cycles of
95°C for 15 seconds
60°C for 20 seconds
68°C for 20 seconds
Acquire FAM at end of extension step

At the completion of the run
Dynamic tube turned on
Data slope corrected
Threshold line set to 0.01

9.2 Appendix 2. Abstract for National conference presentation

Morgan JAT, SJ Jarrett, BM Wlodek, GW Brown, R Urech, PE Green and AE Lew (2008) DNA based detection of Old World screw-worm fly, *Chrysomya bezziana*, in bulk fly trap catches using real-time PCR. ASP & ARC/NHMRC Research Network for Parasitology Annual Conference 6-9 July, Stamford Grand Hotel, Glenelg, South Australia.

Abstract: Old World screw-worm fly (OWS), *Chrysomya bezziana*, is a species of major concern to the Australian cattle industry because the larval stages cause cutaneous myiasis as they feed on their hosts' living tissue. Australia is the only continent with tropical regions lacking OWS fly. Monitoring in Australia currently involves morphological screening of trap catches. Morphologically similar flies are difficult to differentiate especially when the condition of specimens is poor. A molecular based method to identify or refute the presence of OWS fly in bulk trap catches would greatly simplify Australia's monitoring program. The aim of this research is to develop a real-time PCR assay for the identification of OWS fly in bulk fly traps. A region of the ribosomal DNA ITS1 was chosen for the assay after sequencing and comparing an alignment of ITS sequences from relevant *Chrysomya* and *Cochliomyia* species. The assay is species-specific and sensitive to 1 OWS fly in 1,000 non-target species. A DNA extraction protocol has also been optimised to process trap catches of up to 1,000 flies.

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