Specific detection of the Old World screwworm fly, *Chrysomya bezziana*, in bulk fly trap catches using real-time PCR

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Abstract. The Old World screwworm fly (OWS), *Chrysomya bezziana* Villeneuve (Diptera: Calliphoridae), is a myiasis-causing blowfly of major concern for both animals and humans. Surveillance traps are used in several countries for early detection of incursions and to monitor control strategies. Examination of surveillance trap catches is time-consuming and is complicated by the presence of morphologically similar flies that are difficult to differentiate from *Ch. bezziana*, especially when the condition of specimens is poor. A molecular-based method to confirm or refute the presence of *Ch. bezziana* in trap catches would greatly simplify monitoring programmes. A species-specific real-time polymerase chain reaction (PCR) assay was designed to target the ribosomal DNA internal transcribed spacer 1 (rDNA ITS1) of *Ch. bezziana*. The assay uses both species-specific primers and an OWS-specific Taqman® MGB probe. Specificity was confirmed against morphologically similar and related *Chrysomya* and *Cochliomyia* species. An optimal extraction protocol was developed to process trap catches of up to 1000 flies and the assay is sensitive enough to detect one *Ch. bezziana* in a sample of 1000 non-target species. Blind testing of 29 trap catches from Australia and Malaysia detected *Ch. bezziana* with 100% accuracy. The probability of detecting OWS in a trap catch of 50 000 flies when the OWS population prevalence is low (one in 1000 flies) is 63.6% for one extraction. For three extractions (3000 flies), the probability of detection increases to 95.5%. The real-time PCR assay, used in conjunction with morphology, will greatly increase screening capabilities in surveillance areas where OWS prevalence is low.

Key words. Blowfly, bulk DNA extraction, diagnostic assay, internal transcribed spacer 1, Old World screwworm fly, ribosomal DNA, surveillance.

Introduction

The Old World screwworm fly (OWS), *Chrysomya bezziana* Villeneuve, is a parasitic blowfly occurring in subtropical and tropical climates. Characterized by its screw-shaped larvae, *Ch. bezziana* differs from most other blowflies in that it only feeds on the living flesh of mammals and birds. The OWS is

widely distributed throughout Asia and is also found in tropical Africa, the Indian subcontinent, parts of the Middle East and Papua New Guinea (World Organization for Animal Health, 2008). The close proximity of Papua New Guinea to Australia poses a threat of *Ch. bezziana* incursion into Australia either via sea trade or fly strike wounds of animals or people (Strong & Mahon, 1991). Surveillance trapping is conducted at

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livestock shipping ports and other high-risk locations as part of the Northern Australia Quarantine Strategy. Suspect adults and larvae are identified using standard entomological techniques based on morphological keys. More recent techniques for the identification of OWS include cuticular hydrocarbon analysis (Brown *et al.*, 1998) and the analysis of mitochondrial DNA (mtDNA) (Taylor *et al.*, 1996; Hall *et al.*, 2001; Wells & Sperling, 2001; Harvey *et al.*, 2003; CSIRO, 2004; Wells & Williams, 2007). All of these techniques are based on the analysis of an individual fly or larva.

The surveillance of OWS requires the collection of either larvae or adult flies using different techniques. The first approach is to screen animals for larvae in naturally acquired wounds. This results in the collection of manageable numbers of larvae for individual visual or traditional molecular diagnostics. The second surveillance method, involving traps with chemical attractants, targets adult flies (Urech *et al.*, 2004; AUSVETPLAN Technical Review Group, 2007). The traps also catch other calliphorids, making species identification difficult when non-target fly population densities are high. Sticky traps (the older trapping technology) became saturated with a catch of 1000–2000 flies, but the new LuciTrap® technology can accommodate tens of thousands of flies in a single collection. A LuciTrap® consists of a plastic bucket with a removable lid that contains chemical attractants and entrance cones which allow blowflies to enter but not leave the trap (Urech *et al.*, 2009). Blowflies in the traps die of dehydration or starvation and fall to the bottom of the bucket where they dry out (drainage holes prevent water from accumulating in the bucket). Because the sample size may be large, identifications can be difficult using microscopic examination of individual flies. A species-specific molecular assay, capable of detecting the presence of OWS within a DNA sample extracted from a pool of mixed species, would greatly assist in surveillance programmes in which OWS densities are low compared with those of other calliphorids.

Molecular assays can be designed to be target-specific, thus enabling the screening of mixed-species DNA samples. Polymerase chain reaction (PCR) is accepted as the reference standard for detecting nucleic acids from a number of origins (Mackay *et al.*, 2002) and the technology has become a useful tool for the identification of partial remains of specimens lacking morphological characters (Saigusa *et al.*, 2005). Real-time PCR offers more sensitive and specific detection and is faster than standard PCR (Heid *et al.*, 1996). The use of real-time PCR Taqman® probes for the low-level detection of pathogens in the presence of non-target DNA is common in the food industry; commercial Taqman® Pathogen Detection Kits are available for *Salmonella enterica*, *Escherichia coli*, *Listeria monocytogenes* and *Campylobacter jejuni* (Applied Biosystems, Inc., Mulgrave, VIC, Australia). Other applications of the technology that require sensitive and specific DNA detection in mixed DNA samples include virus detection in cattle (Lew *et al.*, 2004) and tracking of the global spread of an emerging infectious fungal disease in frogs (Boyle *et al.*, 2004). To optimize OWS surveillance programmes that use the LuciTrap®, a bulk fly DNA extraction protocol and a highly sensitive *Ch. bezziana*-specific probe-based real-time PCR assay should be developed.

Most sequence comparisons from the molecular analysis of OWS have targeted mtDNA and complete or partial sequences are publicly available on GenBank for cytochrome oxidase I and II (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* (Fabricius) (these are the first flies to colonize carcasses so their life stages are used to estimate time since death) (Otranto & Stevens, 2002; Wallman *et al.*, 2005; Wells & Williams, 2007). Although mtDNA markers have proved useful for taxonomic differentiation and evolutionary studies, the mutations that differentiate *Ch. bezziana* from its two closest relatives, *Ch. megacephala* and *Chrysomya saffranea* (Bigot), are too widely distributed to enable the design of a robust OWS-specific real-time PCR assay. Several nuclear DNA genes have also been sequenced for *Ch. bezziana*, and made publicly 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. As the rDNA ITS regions are non-protein coding and present as multi-copies (Hillis & Dixon, 1991), they were considered the best candidate marker for the development of a sensitive and specific *Ch. bezziana* real-time PCR assay.

Commercial DNA extraction kits are easy to use and do not require the handling of hazardous substances (e.g. phenol and chloroform). Unfortunately the kits are not designed for bulk tissue extraction; the maximum recommended starting weight is 25 mg, less than the weight of a single fly. A LuciTrap® with newly developed Bezzilure-2 attractant (R. Urech, unpublished data, 2008) reduces the number of non-target fly species captured compared with the sticky trap. However, trap catch sizes can be large (tens of thousands of flies in a single collection) because the traps can be set for extended periods of time without servicing. To process such large trap catches with a commercial kit is both time- and cost-prohibitive. Protocols have been designed for extracting DNA from large amounts of plant tissue [which requires additives to digest cell walls (Ausubel *et al.*, 1987)] and for isolating DNA from soil samples [which requires the removal of PCR inhibitors (Vázquez-Marrufo *et al.*, 2002)], but little has been published on processing large amounts of animal tissue. A cost-effective DNA extraction protocol for high-throughput processing of large numbers of trap-caught flies is essential for practical and effective OWS surveillance. This report describes a sensitive *Ch. bezziana*-specific real-time PCR assay and DNA extraction protocol for up to 1000 flies, designed for the detection of OWS in bulk fly trap catches when OWS prevalence is low.

Materials and methods

Fly collections

Specimens of *Ch. bezziana* (12 laboratory and field populations from different geographic locations), other *Chrysomya* spp., *Cochliomyia hominivorax* and other fly genera typically

Table 1. Species and geographic populations used to construct the fly DNA bank.

Species	Origin	Source
Chrysomya bezziana	Lab	Bogor, Java, Indonesia
Chrysomya bezziana	Lab	East Sumba, Indonesia
Chrysomya bezziana	Lab	Maros, Indonesia
Chrysomya bezziana	Field	Gemas, Malaysia
Chrysomya bezziana	Lab	Papua New Guinea
Chrysomya bezziana	Field	Wau, Papua New Guinea
Chrysomya bezziana	Field	Goa-Usgao, India
Chrysomya bezziana	Field	Salalah, Oman
Chrysomya bezziana	Field	Muscat, Oman
Chrysomya bezziana	Lab hybrid	PNG × South Africa F1
Chrysomya bezziana	Lab hybrid	PNG × Sabah, Malaysia F1
Chrysomya bezziana	Lab hybrid	PNG × Fujairah, UAE F1
Chrysomya megacephala	Lab	Queensland, Australia
Chrysomya megacephala	Field	Malaysia
Chrysomya nigripes	Field	Queensland, Australia
Chrysomya putoria	Field	Grahamstown, South Africa
Chrysomya rufifacies	Lab	Queensland, Australia
Chrysomya saffranea	Field	Queensland, Australia
Chrysomya varipes	Field	Queensland, Australia
Cochliomyia hominivorax	Lab	Mexico
Hemipyrellia sp.	Field	Queensland, Australia
Lucilia cuprina	Lab	Canberra, Australia
Musca domestica	Lab	New South Wales, Australia
Sarcophagidae	Field	Queensland, Australia

caught in Australian surveillance traps were assembled and used to construct a DNA bank (Table 1).

DNA extraction from single flies

DNA was extracted from fly legs only so that flies could be retained for morphological examination. Legs were removed under a dissecting microscope using sterile forceps. Samples preserved in ethanol were soaked in 1 mL of TE buffer (10 mM Tris-Cl, 1 mM EDTA) overnight prior to extraction. The fly legs were diced and crushed with a micropestle prior to DNA extraction using the DNeasy Tissue Kit (Qiagen, Inc., Valencia, CA, U.S.A.).

DNA extraction from bulk trap catches

DNA extraction is a three-step process involving tissue disruption, cell lysis and finally protein and lipid removal. The cost of scaling up the first two steps is minor compared with the final DNA extraction. For this reason a protocol was optimized to accommodate the extraction of DNA from up to 1000 flies through the tissue disruption and cell lysis steps followed by subsampling (200 µL aliquots) and extraction to give consistent results using a commercial DNA extraction kit (QIAamp Tissue Kit; Qiagen, Inc.). Different extraction protocols were compared by extracting from uniform starting material consisting of 99 *Ch. megacephala* plus one *Ch. bezziana*.

Two techniques for mechanical tissue disruption and three protocols for cell lysis were compared, respectively. Mechanical disruption included the use of liquid nitrogen to grind the flies with a mortar and pestle and disruption in 15 mL of lysis buffer (10 mM EDTA, 20 mM Tris) using a sterile blender (Waring Laboratory Science, Torrington, CT, U.S.A.). The three cell lysis protocols tested were: (a) Protocol 1: homogenize flies and boil for 15 min; (b) Protocol 2: as Protocol 1, add 2 mg proteinase K and incubate the mixture overnight at 56°C, and (c) Protocol 3: as Protocol 1, add 2 mg proteinase K and 1% SDS (sodium dodecyl sulphate) and incubate overnight at 56°C. After lysis, the mix was centrifuged at 3220 **g** for 15 min and the supernatant was recovered and subsampled (200 µL) for extraction using the QIAamp Tissue Kit (Qiagen, Inc.), as per the manufacturer's instructions.

The optimal protocol was then applied to assess detection limits of larger fly samples containing a single *Ch. bezziana* (volumes were scaled). The range included one *Ch. bezziana* plus 249 non-target flies, one *Ch. bezziana* plus 499 non-target flies and one *Ch. bezziana* plus 999 non-target flies. Once detection was confirmed for one *Ch. bezziana* in a sample of 1000 flies, reagent concentrations were further optimized to reduce costs.

The following optimal protocol was developed and used for subsequent assays scaled to extract from 1000 flies: transfer 1000 flies (estimate by weight or volume) into a pre-sterilized stainless steel Waring blender jar; add 100 mL of lysis buffer (10 mM EDTA, 20 mM Tris) and homogenize at high speed for 3 min; transfer the homogenate to a 225-mL centrifuge tube and boil for 15 min; cool to room temperature, add 10 mg of proteinase K and mix by inversion; incubate overnight at 56°C; centrifuge at 3220 **g** for 15 min, subsample 200 µL of the lysate and extract DNA with a QIAamp Tissue Kit (Qiagen, Inc.) or equivalent.

DNA amplification and sequence analysis

Internal transcribed spacer 1 was PCR-amplified using primers OWS-18SF 5′ GTCGTAACAAGGTTTCCGTAGG and OWS-5.8SR 5′ TCGATGTTCATGTGTCCTGCAGT. ITS2 was PCR-amplified using primers L1 5′ RRCGGYGGAT-CACTCGGCTC and OWS-28SR 5′ CTCGCCGCTACTAA-GAAAATCC. Unpublished primer L1 sequence was kindly provided by Dr J. T. Wallman (University of Wollongong, Wollongong, NSW, Australia). The remaining primers were designed from conserved regions of rDNA based on a sequence alignment of fly species publicly available from the National Centre for Biotechnology Information (NCBI, Bethesda, MD, U.S.A.) (partial sequences available for representatives of *Lucilia*, *Drosophila*, *Calliphora*, *Musca*, *Melinda*, *Belvosia* and *Chrysomya*).

Each PCR reaction contained 0.5 µM of each primer pair, combined with 10–100 ng of extracted DNA, 10× HotMaster Taq buffer (5 Prime, containing 25 mM magnesium; Quantum Scientific Pty Ltd, Murrarie, QLD, Australia), 0.8 mM dNTP and 0.05 units/µL of HotMaster Taq DNA polymerase (5 Prime; Quantum Scientific Pty Ltd). The PCR reaction was

carried out using a Palmcycler (Version 2.0; Corbett Research Pty Ltd, Sydney, NSW, Australia) for one cycle of 95°C for 60 s, 53°C for 45 s and 72°C for 90 s. This was followed by 29 shorter cycles of 95°C for 30 s, 53°C for 30 s and 72°C for 90 s. The mix was held at 72°C for 7 min to complete extension. PCR products were cleaned prior to sequencing with Exosap-it® (USB Corp., Cleveland, OH, U.S.A.).

The PCR products were sequenced using Big Dye Version 3.1 technology (Applied Biosystems, Inc.) and were run on an Applied Biosystems 3130×1 Genetic Analyser (Griffith University DNA Sequencing Facility, School of Biomolecular and Biomedical Science, Griffith University, QLD, Australia). Sequences were edited and aligned in Sequencher Version 4.8 (Gene Codes Corp., Ann Arbor, MI, U.S.A.).

Chrysomya bezziana-*specific real-time PCR*

To ensure assay specificity, both forward and reverse primers and the carboxyfluorescein (FAM)-labelled TaqMan® MGB (minor groove binding) probe were designed to target *Ch. bezziana* unique sequences (Table 2). A species-specific sequence was identified as regions of DNA where *Ch. bezziana* differed from its phylogenetic sister species *Ch. megacephala* and *Ch. saffranea*. Because of 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 Version 2.0 (Applied Biosystems, Inc.).

Real-time PCR assays were conducted on a Rotor-Gene 3000 (Corbett Research Pty Ltd). Total reaction volume was 20 μL containing 8 μL RealMasterMix Probe (5 Prime; Quantum Scientific Pty Ltd), 300 nM of PCR primers, 200 nM of freshly diluted TaqMan® MGB probe and 5 μL of genomic DNA (tested over a 1000-fold dilution series). A negative (water) and a positive (*Ch. bezziana* DNA) control were included in all assays. Amplification conditions were 2 min at 95°C followed by 45 cycles of 15 s at 95°C, 20 s at 60°C and 20 s at 68°C, acquiring FAM at the end of this step. At the completion of the run, the dynamic tube was turned on, the data were slope-corrected and the threshold line was set to 0.01. A cycle threshold (C_T) score was determined for each assay as the cycle number at which the DNA amplification curve crossed the threshold line. The assay was considered positive for *Ch. bezziana* if the C_T score was ≤35, suspicious if the C_T was >35 and negative if C_T = no amplification (no amp).

Confirmation or real-time PCR assay specificity and sensitivity

The specificity of the *Ch. bezziana* real-time PCR assay was confirmed by individually testing the assay against the species in Table 2. In addition, samples of mixed-species DNA were prepared and spiked with *Ch. 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 of target DNA to non-target DNA, 1 : 9 and 1 : 999, were tested). Species combined to prepare the non-target DNA were *Ch. megacephala*, *Cochliomyia hominivorax* (Coquerel), *Chrysomya varipes* (Macquart), *Chrysomya flavifrons* (Aldrich), *Chrysomya nigripes* (Aubertin), *Chrysomya latifrons* (Malloch), *Ch. saffranea*, *Chrysomya incisuralis* (Macquart) and *Chrysomya putoria* (Wiedemann) (all Diptera: Calliphoridae).

The sensitivity of the real-time PCR assay and extraction protocol was determined by extracting and screening one *Ch. bezziana* plus 99 *Ch. megacephala*, one *Ch. bezziana* plus 249 *Ch. megacephala* and one *Ch. bezziana* plus 999 *Ch. megacephala* flies. In addition, the assay sensitivity was determined by diluting the extracted DNA by 10- and 100-fold. The lowest dilution contains only a fraction of a *Ch. bezziana* fly as the reaction volume contains less than 1/100 000th of a fly. Two subsamples from each of the homogenized fly samples were extracted and assayed.

Assay evaluation screening trap catches

An evaluation of the *Ch. bezziana* real-time PCR assay was carried out by screening DNA extracted from 29 trap catches, mainly from LuciTraps® but including two sticky trap catches (flies from half of the sticky sheet were extracted and assayed). Each catch was morphologically examined prior to extraction

Table 2. *Chrysomya bezziana*-specific real-time PCR primer and probe sequences and alignment against *Chrysomya megacephala* and *Chrysomya saffranea* (identical).

Region and species	Sequence and alignment
RTBezF1 forward primer	
Ch. bezziana (5′ position 163)	GACACAAACAAAAACAT–––AGAATAGATCT–TG
Ch. saffranea/Ch. megacephala	…T………..T..GGT….ACC..G.C.A
RTBezR1 reverse primer	
Ch. bezziana (5′ position 305)	TCATT–AGTAGGGTAAACCAACAATCA–TC
Ch. saffranea/Ch. megacephala	A….T….A.A……….T.T.A.T
Probe (TaqMan® MGB, FAM)	
Ch. bezziana (5′ position 241)	AGCAAATTTCATTCTTGACA
Ch. saffranea/Ch. megacephala	.–..T.G.–––….ACCAT

A dot (.) corresponds to a base match to the *Ch. bezziana* sequence in the alignment. A dash (–) corresponds to a gap in the alignment resulting from an historical nucleotide deletion/insertion event.

and the presence and number of *Ch. bezziana* was recorded. Sixteen of the trap samples were collected as part of the Australian OWS surveillance programme and the remaining 13 samples were collected from trapping programmes in Malaysia, where *Ch. bezziana* is endemic. Genetic screening of all samples was undertaken in a blind manner and Australian and Malaysian samples were extracted concurrently. Traps were left in the field for 7–14 days and catches were either air-dried or ethanol-preserved. Large trap catches were subsampled (1000 flies estimated by dry weight). Two 200-μL subsamples (replicates) of the cell lysis solution from each trap sample were extracted using a Qiagen DNeasy Tissue Kit. Negative extraction and PCR controls were included.

Probability of detecting low-prevalence OWS

The probability of detecting OWS in trap catches of varying size (5000–50 000), containing large numbers of non-target fly species, a new incursion scenario, was predicted. These probabilities were based on a binomial distribution (presence or absence of OWS in a sequence of independent extractions) and the assumption that the real-time PCR assay is 100% sensitive at detecting one OWS in 1000 flies. Three levels of OWS prevalence were compared: one in 100, one in 1000 and one in 10 000 flies. The effect of increasing the number of flies screened per trap (increasing the number of DNA extractions from one to three) was also assessed. Probability estimates were calculated using EpiTools with Herd-level sensitivity for testing in a finite population (Sergeant, 2009).

Results

DNA amplification and sequence analysis

All of the *Ch. bezziana* populations had 100% identical ITS1 region sequences. By contrast, individual and/or population differences were detected among ITS sequences of *Ch. megacephala*, *Ch. saffranea* and *Ch. putoria*. An alignment of these species' DNA sequences showed a far greater divergence from *Ch. bezziana* in ITS1 (156 nucleotide differences, 17.2%) compared with ITS2 (21 nucleotide differences, 3.6%). Some of this variation reflects the presence of a 45-bp repeat element in the ITS1 of *Ch. bezziana* (two perfect copies plus a further four smaller 11–21-bp fragments of the repeat) that is absent in the other species. The ITS1 sequences for *Ch. bezziana*, *Ch. megacephala* (Australian and Malaysian strains), *Ch. saffranea* and *Ch. putoria* have been submitted to GenBank (accession nos FJ824825, FJ830686–FJ830689).

Chrysomya bezziana-*specific real-time PCR assay*

The ITS1 was the only region of the rDNA sequence found to be variable enough among the phylogenetic sister species *Ch. bezziana*, *Ch. megacephala* and *Ch. saffranea* to achieve both primer and probe specificity (Table 2). Care was taken to avoid mispriming by positioning the assay upstream of the repeating sequence element identified in the *Ch. bezziana* ITS1 sequence.

DNA extraction from bulk trap catches

The most effective method for completely homogenizing 100 flies, based on particle uniformity and C_T score, was manual grinding in liquid nitrogen using a mortar and pestle. Although blending in buffer solution gave a coarser homogenate than manual grinding, it was considered to be a more consistent and repeatable method (fixed rotor speed) and as the samples were enclosed within the blender the opportunity for cross-sample contamination was reduced. Of the three cell lysis methods tested, the protocol which gave the lowest C_T scores and thus recovered the most DNA was Protocol 2, which involved boiling the flies followed by proteinase K treatment (Protocol 1: boiled, mean C_T score = 21.6; Protocol 2: boiled + proteinase K, mean C_T score = 19.9; Protocol 3: boiled + proteinase K + SDS, mean C_T score = 26.1). The optimal DNA extraction protocol for processing up to 1000 flies was developed from these results.

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

Species	C_T score*
Ch. bezziana (1 : 100 dilution of DNA with water)	19.6
Ch. bezziana (1 : 1000 dilution of DNA with water)	21.8
Ch. bezziana + *Chrysomya megacephala* (1 : 1 ratio, 1 : 100 dilution)	19.4
Ch. bezziana + mixed non-target DNA† (1 : 10 ratio, 1 : 100 dilution)	18.5
Ch. bezziana + mixed non-target DNA† (1 : 1000 ratio, 1 : 1000 dilution)	22.2
Mixed non-target DNA†	No amp
Chrysomya flavifrons	No amp
Chrysomya incisuralis	No amp
Chrysomya latifrons	No amp
Chrysomya megacephala	No amp
Chrysomya nigripes	No amp
Chrysomya putoria	No amp
Chrysomya rufifacies	No amp
Chrysomya saffranea	No amp
Chrysomya semimetallica	No amp
Chrysomya varipes	No amp
Cochliomyia hominivorax	No amp
Hemipyrrellia sp.	No amp
Lucilia cuprina	No amp
Musca domestica	No amp
Sarcophagidae	No amp

*C_T score = number of cycles in real-time PCR assay required before amplification reaches required the threshold.
†Mixed non-target DNA from *Ch. megacephala*, *C. hominivorax*, *Ch. varipes*, *Ch. flavifrons*, *Ch. nigripes*, *Ch. latifrons*, *Ch. saffranea*, *Ch. incisuralis* and *Ch. putoria*.
No amp, no amplification.

Table 4. Results of *Chrysomya bezziana*-specific real-time PCR screen of DNA extractions from varying fly numbers (*Ch. bezziana* and *Chrysomya megacephala*) and from different DNA dilutions.

Sample	Mean C_T score*		
	No dilution	1 : 10 dilution	1 : 100 dilution
One *Ch. bezziana* + 99 *Ch. megacephala*	20.3	23.5	25.7
One *Ch. bezziana* + 249 *Ch. megacephala*	23.1	26.2	29.8
One *Ch. bezziana* + 999 *Ch. megacephala*	30.4	31.2	35.2

*C_T score = number of cycles in real-time PCR assay required before amplification reaches threshold. The value given is the mean of duplicate subsamples (subsample scores were within ± 1 of one another).

Confirmation of real-time PCR assay specificity and sensitivity

Results of the specificity tests of the *Ch. bezziana* real-time PCR assay are shown in Table 3. All of the samples containing *Ch. bezziana* gave positive results with C_T scores of ≤22.

The assay shows no cross-reactivity with closely related flies or with fly species likely to be caught in OWS surveillance traps. Using spiked DNA samples with large amounts of non-target DNA (ratios of *Ch. bezziana* DNA to non-target DNA 1 : 10 and 1 : 1000) the *Ch. bezziana* real-time PCR assay displayed no inhibition and was still able to detect low levels of target *Ch. bezziana* DNA. Extracted DNA from all 12 populations of *Ch. bezziana* (Table 2) tested positive with the assay (results not shown).

The sensitivity of the real-time PCR assay was measured with extracted samples of 99, 249 and 999 non-target *Ch. megacephala* flies spiked with a single *Ch. bezziana* fly (Table 4). Target DNA was amplified in all of the samples, indicating that the assay is extremely sensitive and provides positive results to low concentrations of *Ch. bezziana* DNA, even in the presence of large amounts of non-target fly DNA. The increasing C_T scores reflect the dilution of target DNA as more non-target flies were added to the samples. *Ch. bezziana* could still be detected even after diluting the extracted DNA 100-fold with water prior to analysing.

Table 5. Results of *Chrysomya bezziana* real-time PCR assay screen of fly trap catches from Australia and Malaysia.

Trap country of origin	Location	Total number of flies per trap†	Number of *Ch. bezziana* (morphological examination)	Real-time PCR assay C_T score*
Malaysia	Jelai Gemas	37	1	21.3
Malaysia	Jelai Gemas	270	0	No amp
Malaysia	Jelai Gemas	276	1	28.0
Malaysia	Jelai Gemas	1020	1	24.2
Malaysia	Jelai Gemas	282	2	21.3
Malaysia	Ulu Lepar	21	1	19.6
Malaysia	Jelai Gemas	261	0	No amp
Malaysia	Jelai Gemas	691	1	31.3
Malaysia	Jelai Gemas	48	11	19.7
Malaysia	Jelai Gemas	110	5	18.8
Malaysia	Jelai Gemas	65	0	No amp
Malaysia	Jelai Gemas	629	1	33.9
Malaysia	Jelai Gemas	77	1	28.2
Australia	Port Hedland, WA	46†	0	No amp
Australia	Port Hedland, WA	15 000	0	No amp
Australia	Port Hedland, WA	50 000	0	No amp
Australia	Geraldton, WA	63†	0	No amp
Australia	Geraldton, WA	1500	0	No amp
Australia	Geraldton, WA	120	0	No amp
Australia	Fremantle, WA	2000	0	No amp
Australia	Fremantle, WA	400	0	No amp
Australia	Fremantle, WA	80	0	No amp
Australia	Thursday Island	2000	0	No amp
Australia	Thursday Island	2000	0	No amp
Australia	Brisbane, Qld	516	0	No amp
Australia	Brisbane, Qld	913	0	No amp
Australia	Brisbane, Qld	2	0	No amp
Australia	Brisbane, Qld	30	0	No amp
Australia	Brisbane, Qld	16	0	No amp

*C_T score = number of cycles in real-time PCR assay required before amplification reaches the required threshold.
†Traps were conventional sticky sheet traps rather than LuciTraps®.
No amp, no amplification.

Table 6. Probability estimates (%) of detecting *Chrysomya bezziana* (if present) using the real-time PCR assay, given different prevalence levels, trap catch sizes and numbers of flies screened.

Ch. bezziana prevalence in trap	Number of flies in trap catch	Probability of detection, %		
		1000 flies screened (one extraction)	2000 flies screened (two extractions)	3000 flies screened (three extractions)
1 : 100	5000	100.0	100.0	100.0
	10 000	100.0	100.0	100.0
	20 000	100.0	100.0	100.0
	50 000	100.0	100.0	100.0
1 : 1000	5000	67.2	92.2	99.0
	10 000	65.1	89.3	97.2
	20 000	64.2	87.8	96.1
	50 000	63.6	87.0	95.5
1 : 10 000	5000	20.0	40.0	60.0
	10 000	10.0	20.0	30.0
	20 000	9.8	19.0	27.8
	50 000	9.6	18.5	26.6

Assay evaluation screening trap catches

Of the 29 trap catches tested in the blind screen, 10 tested positive for *Ch. bezziana*. All of the Malaysian trap catches that were morphologically identified as containing *Ch. bezziana* tested positive using the real-time PCR assay with levels as low as one *Ch. bezziana* in 1020 non-target flies successfully identified (Table 5). The C_T scores ranged from 18.8 (five *Ch. bezziana* in a trap of 110 flies) to 33.9 (one *Ch. bezziana* in a trap of 629 flies). The Australian surveillance traps collected from Queensland (Brisbane and Thursday Island) and Western Australia (Geraldton, Fremantle and Port Hedland) were all negative for *Ch. bezziana*.

Probability of detecting low-prevalence OWS

At a trap prevalence of one in 100 flies, the probability of detecting *Ch. bezziana* in any size trap catch is 100% (Table 6). At a lower prevalence (one in 1000 flies) the probability of OWS detection is 63.6–65.1% and can be increased to >95% by testing three extractions (1000 flies each). At an extremely low trap prevalence (one in 10 000 flies) the probability of detecting *Ch. bezziana* (if present) in a single extraction of 1000 flies is approximately 10%.

Discussion

The Australian strategy for preparing for an OWS incursion includes early detection followed by rapid implementation of control and eradication strategies (AUSVETPLAN Technical Review Group, 2007). Surveillance trapping is a key element of early detection and ongoing monitoring (Vargas-Terán *et al.*, 2005). Until now, advances in trapping technologies have not been paired with equivalent advances in processing technology and, as a result, screening a surveillance trap can be an extremely labour-intensive process involving the handling and morphological examination of large numbers of individual flies. The molecular assay described here permits 1000 flies to be screened in a single sample and multiple samples can be extracted and screened concurrently.

The real-time PCR assay amplified samples of *Ch. bezziana* originating from Indonesia, Malaysia, Papua New Guinea, India, Oman, South Africa and the United Arab Emirates. The specificity of the assay was confirmed by testing against DNA from six other species of *Chrysomya*, including the phylogenetic sister species *Ch. megacephala* and *Ch. saffranea*. In addition, DNA from five other fly genera commonly caught in Australian surveillance traps was tested and no cross-reactivity was detected.

The specificity of the assay permits screening of mixed-species trap catches. A time- and cost-effective protocol was developed to extract DNA from up to 1000 flies. The protocol was tested and shown to be effective at extracting real-time PCR-detectable DNA from a sample of 1000 flies containing a single *Ch. bezziana*.

The extraction protocol and real-time PCR assay were blind-tested on 29 field-collected trap catches from both Australia and Malaysia with completely congruent results (i.e. *Ch. bezziana* detected with the real-time PCR assay matched those detected by microscopic examination with 100% accuracy). A real-time PCR reaction provides a C_T (cycle threshold) score that gives an indication of the amount of starting DNA present in a sample. Although quantification of the number of OWS present in a sample of 1000 flies would be useful, it is not within the scope of this assay to give more than a presence/absence score. The difficulty with quantification relates to extracting DNA from bulk fly catches. Flies can vary greatly in size (within and between species) and DNA content. In addition, the age and degree of decomposition of flies after capture can also influence DNA recovery. The condition of flies recovered from traps after a 2-week collection period was generally very good, but moisture in the environment did impact DNA recovery from trap samples older than 2 weeks.

The detection of low-prevalence OWS is important for early warning of an incursion. Although the LuciTrap® with Bezzilure-2 attractant is much more selective for *Ch. bezziana*

than the sticky trap (R. Urech, unpublished data, 2008), large numbers of flies can be caught in the plastic bucket, particularly if extended trapping periods are used. The ability to screen up to 1000 flies for the presence of *Ch. bezziana* with one real-time PCR assay and the fact that many assays can be run simultaneously will accelerate the detection process and improve *Ch. bezziana* surveillance.

The trade-off between extracting and screening >1000 flies from a catch (i.e. more than one extraction per trap) is that fewer traps overall can be screened. For general surveillance, widespread geographic representation might be prioritized 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 extractions vs. one) might be advantageous to increase the probability of detection. Budgets and average catch sizes will ultimately determine the number of traps and samples that can be assayed.

Development of the assay brings surveillance screening into better alignment with surveillance trapping capability, thereby increasing the probability of early detection. If an incursion is detected, the rapid, sensitive and targeted screening afforded by the LuciTraps® and molecular assay will be essential tools for monitoring fly populations while eradication strategies are implemented.

Although the DNA-based real-time PCR assay can be used to confirm the identity of single flies, eggs or larvae, it is more specifically designed to detect OWS in large trap catches of mixed species. Used in conjunction with morphology, for definitive identification of *Ch. bezziana*, the real-time PCR assay will greatly increase screening capabilities in surveillance areas with low OWS prevalence, especially when trap catches of non-target species are high.

Acknowledgements

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