Bulletin of Entomological Research (2017) **107**, 332–339
doi:10.1017/S000748531600095X

Development and validation of a real-time PCR assay for the glassy-winged sharpshooter *Homalodisca vitripennis* (Hemiptera: Cicadellidae)

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Abstract

The glassy-winged sharpshooter (*Homalodisca vitripennis*) is an invasive pest organism, which is found throughout Central America and has recently invaded a few countries in the Pacific Islands. As a carrier of the highly virulent plant pathogenic bacterium *Xylella fastidiosa*, it is of great economic significance to horticulture and is estimated to cost Californian vineyards over US$100 million per year in control and losses. New Zealand is currently free from this pest, but its recent spread through the Pacific has raised concerns of it establishing in New Zealand, potentially as a result of introduction through human travel. We report here a real-time polymerase chain reaction assay for the rapid identification of *H. vitripennis*. The assay was extensively validated *in silico* then optimized and tested against a range of Cicadellidae species, both internationally collected and local to New Zealand. This assay was able to correctly identify *H. vitripennis* samples, and distinguish between *H. vitripennis* and close relatives, such as the smoke-tree sharpshooter (*Homalodisca liturata*) and will be of great benefit to New Zealand biosecurity.

Keywords: *Homalodisca vitripennis*, identification, qPCR, COI, biosecurity

(Accepted 12 September 2016; First published online 7 November 2016)

Introduction

The glassy-winged sharpshooter (GWSS), *Homalodisca vitripennis*, (previously known as *Homalodisca coagulata*) (Takiya *et al.*, 2006) is a member of the Cicadellidae family. *H. vitripennis* is native to the south-eastern United States, and north-eastern Mexico (Triapitsyn & Phillips, 2000), but is known for its spread into California in the 1980s (Sorensen & Gill, 1996). *H. vitripennis* is highly mobile and feeds on the xylem of over 100 species of plants (Turner & Pollard, 1959; Anderson *et al.*, 1989; Northfield *et al.*, 2009) of which it can process up to 300 times its body mass per day (Brodbeck *et al.*, 1993). *H. vitripennis* is a vector of the pathogenic bacterium, *Xylella fastidiosa* (Turner & Pollard, 1959; Almeida & Purcell, 2003), which is the causative agent of diseases in many plants, most notably Oleander Leaf Scorch disease, phony peach disease and Pierce's Disease (PD) in grapevine (Purcell *et al.*, 1999; Purcell & Feil, 2001; Hopkins & Purcell, 2002). *H. vitripennis* has been shown to penetrate deeper into vineyards than other sharpshooters which carry the disease (Blua & Morgan, 2003), allowing it to spread the disease further than other insect vectors. This attribute was linked to massive outbreaks of PD in California in the late 1990s (Almeida & Purcell, 2003; Blua & Morgan, 2003).

The virulence factors of *X. fastidiosa* are understood (Roper *et al.*, 2007; Pérez-Donoso *et al.*, 2010; Sun *et al.*, 2011) and diagnostic techniques for the detection of *X. fastidiosa* are well established (Minsavage *et al.*, 1994; Guan *et al.*, 2013), yet treatment of the disease is difficult (Dandekar *et al.*, 2012). Preventing the spread of PD and limiting the rate of infection by controlling *H. vitripennis* directly is the most efficient

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method of combating PD. Chemical and biological control of *H. vitripennis* has been tested using a variety of agents, including pesticides, predation, and parasitic wasps (Triapitsyn *et al.*, 1998; Bethke *et al.*, 2001; Grandgirard *et al.*, 2008; Guiterrez *et al.*, 2011), and efforts have even included attempts to inoculate the insect with benign strains of *X. fastidiosa* (Hopkins, 2005). The economic burden of these efforts is significant, in California alone the annual cost of monitoring, control and research is approximately US$50 million per year, and despite this effort *H. vitripennis* causes direct loses of an estimated US$60 million per year (Alston *et al.*, 2013; Tumber *et al.*, 2014). In the early 2000s *H. vitripennis* spread into the Pacific Islands, with reports of incursions into French Polynesia in 1999, Hawai'i in 2004, the Easter Islands in 2005 and the Cook Islands (Rarotonga) in 2007 (Grandgirard *et al.*, 2006; Gunawardana *et al.*, 2008; Petit *et al.*, 2008). In warmer climates, *H. vitripennis* flourishes, displaying an increased rate of feeding (Johnson *et al.*, 2006) and mating more frequently (Blua *et al.*, 1999; Grandgirard *et al.*, 2006), making it capable of achieving a much greater population density than that observed in California (Petit *et al.*, 2008; Wistrom *et al.*, 2010). Environmental modelling has suggested that *H. vitripennis* is capable of surviving in any climate that supports grape production, including that of Australasia (Hoddle, 2004; Rathé *et al.*, 2012). It has been speculated that the accidental transport of plants carrying *H. vitripennis* eggs was the original source of its introduction into California, French Polynesia, and Rarotonga (Sorensen & Gill, 1996; Grandgirard *et al.*, 2006; Gunawardana *et al.*, 2008; Petit *et al.*, 2008), and this mode of spread is considered the likeliest source of an incursion into Australasia (Grandgirard *et al.*, 2006; Rathé *et al.*, 2012).

New Zealand is currently free from *H. vitripennis* and *X. fastidiosa*, which are considered high-risk organisms for New Zealand's biosecurity sector. While *H. vitripennis* adults can be identified morphologically this approach does not scale well when many individuals require analysis. Further, when immature stages of *H. vitripennis* are intercepted, morphology is not capable of distinguishing between the eggs and nymphal stages of *H. vitripennis* and its close relative, such as the smoke-tree sharpshooter (*Homalodisca. liturata*) (S. Winterton, personal communication, 2007). Molecular and genetic identification tools have been developed for *H. vitripennis*, which overcome the morphology limitation, targeting protein and genetic markers (de León *et al.*, 2006; Fournier *et al.*, 2006), including a polymerase chain reaction (PCR) assay that targets the mitochondrial cytochrome c oxidase subunit 1 (COI) gene. The COI gene is a commonly used gene in molecular entomology as it can possess sufficient genetic resolution to distinguish between species and subspecies of organisms, as well as explore population genetics (Smith, 2005; Boykin *et al.*, 2007; Malausa *et al.*, 2011; Rakauskas *et al.*, 2011). The COI gene has previously been used to evaluate the phylogeny of several Hemiptera suborders (Park *et al.*, 2011; Li *et al.*, 2012; Foottit *et al.*, 2014), and the common occurrence of this gene in publically accessible data repositories makes it an excellent starting point for assay design.

In order to augment the existing entomological tools for the identification of *H. vitripennis*, we sought to develop a TaqMan based quantitative PCR (qPCR) protocol to rapidly and accurately identify *H. vitripennis* from both adult and egg life stages. The assay was based on the COI gene as it is a commonly used genetic marker in molecular entomology and thus provided a good range of reference organisms to test for non-specific binding. The specificity of the assay was examined extensively *in silico* and further validated through blind panel testing. Assay sensitivity was evaluated using controlled quantities of template DNA. Compared with conventional PCR techniques this assay is rapid, taking approximately 45 min to complete (compared with 2–3 h for PCR, followed by gel electrophoresis). Furthermore, we predict this assay will prove more robust than conventional PCR assays, due to the required annealing of a third oligonucleotide sequence.

Table 1. Cicadellidae samples obtained for blind panel testing.

Organism	Extraction source
Zeoliarus oppositus	Whole insect
Idiocerus decimaquartus	Whole insect
Eupteryx melissae	Whole insect
Idiocerus distinguendus	Whole insect
Unidentified *Cicadellidae*	Whole insect
Idiocerus sp.	Whole insect
Zygnia zealandica	Whole insect
Edwardsiana cratagi	Whole insect
Erythroneura elegantula	Whole insect
Graphocephala atropunctata	Leg
Homalodisca liturata	Leg
Homalodisca vitripennis	Leg, cast skin, egg

DNA was extracted from small or nymph-stage samples by means of non-destructively processing the entire insect. For the larger insects a single leg was removed from the insect and DNA extracted.

Materials and methods

Sample collection and DNA extraction

Samples of the glassy-winged sharpshooter (*H. vitripennis*) collected from the USA and Cook Islands, and smoketree sharpshooter (*H. liturata*), and blue-green sharpshooter (*Graphocephala atropunctata*) samples from the USA were used to develop the assay. *H. vitripennis* eggs were obtained from the USA. Additional cicadellid samples were obtained from the Plant Health and Environment Laboratory entomology reference collection (PANZ) for use in specificity testing. DNA was extracted from the leg or whole body of the insect (table 1). DNA extraction was performed using the DNeasy Blood and Tissue kit (Qiagen NV, Venlo, Netherlands) according to manufacturer instructions and stored at −80°C until required.

Analysis of phylogenetic relationships

Sequence data obtained from the COI gene for 164 Leafhoppers (162 Cicadellidae, 2 Fulgorodoidea) were downloaded from the GenBank nr (non-redundant) database (online table S1). Multiple sequence alignment was performed using MUSCLE version 3.8.31 (Edgar, 2004) with default parameters. Following alignment, a 370 bp subsection of the alignment common to all sequences was identified and extracted from the global alignment. Phylogenetic analysis was performed by constructing a maximum likelihood tree using RAxML version 8 (Stamatakis, 2014). The COI sequences of four species of the order Orthoptera were used as outgroup (online Supplemental table S1). Analysis of the phylogenetic tree was used to identify the closest genetic relatives to *H. vitripennis*, which were then extracted from the initial data set for use as references during primer design.

https://doi.org/10.1017/S000748531600095X Published online by Cambridge University Press

Table 2. Primer sequences used in the final assay.

Primer/probe	Sequence (5′→3′)	Length (bp)	Min. difference (%)	Median difference (%)
HcCOI-F	GGG CCG TAA ATT TTA TTA CC	20	5.0	20.0
GWSS_P1	AAT TGG AAT AAA TTT TGA CCG AAC ACC	27	7.4	29.6
GWSS_R1	GGT CAG TTA ATA ATA TAG TAA TTG C	25	4.0	16.0

The primer HcCOI-F was obtained from the previously reported *H. vitripennis* assay (de León *et al.*, 2006). Probe GWSS_P1 was synthesized with the reported fluorophore FAM and proprietary quencher BHQ. The minimum and average sequence differences between the appropriate regions of the leafhopper data set are reported.

qPCR design

The original COI sequences of *H. vitripennis* and *H. liturata* were re-aligned, and the primers and probe were designed using Beacon Designer version 8.01 (Premier Biosoft, Palo Alto, CA, USA). A reverse primer and probe were designed to work in conjunction with the forward primer of a previously published *H. vitripennis* PCR assay (table 2) (de León *et al.*, 2006) to take advantage of the previously established specificity of this target region. An additional 15,505 Cicadellidae COI sequences, six of which belonged to *H. vitripennis*, were downloaded from the Barcode of Life Data Systems (BOLD) (Ratnasingham & Hebert, 2007) for use *in silico* primer testing. The TaqMan probe was synthesized with a 5′ reporter fluorophore (FAM) and 3′ quencher molecule (BHQ) (Biosearch Technologies Inc, Petaluma, CA, USA).

qPCR optimization

Initial qPCR annealing conditions were tested in 10 µl reactions using the SsoFast Probes Supermix (BioRad, Hercules, CA, USA) with 0.8 µg µl^{-1} bovine serum albumin (Sigma-Aldrich Co., St Louis, MO, USA), 400 nM of each primer and 250 nM of probe. An annealing gradient of 55–65°C was used with the cycling conditions as follows; initial denaturing at 95°C for 2 min, followed by 40 cycles of 95°C for 20 s and annealing/extension for 30 s followed by a plate read step. Following the establishment of the optimal annealing temperature, the primer concentration was tested at 50, 100, 200 and 400 nM for each primer. For primer concentrations below 400 nM the probe concentration was reduced to 125 and 200 nM. Repeatability (intra-run variation) and reproducibility (inter-run variation) were tested with three qPCR master mix solutions; SsoFast Probes Supermix, SsoAdvanced Universal Probes mix (BioRad) and PerfectA qPCR Toughmix (Quanta Biosciences, Gaithersburg, MD, USA). All reactions were performed using a C1000 Touch™ thermocycler with CFX96 Optical Reaction Module™ (BioRad) and results were analyzed using CFX Manager version 3.1 (BioRad).

Assay repeatability and reproducibility

Eight samples of *H. vitripennis* DNA were tested in triplicate in two independent machine runs. Repeatability and reproducibility were quantified using the percentage coefficient of variation (%CV) of the detection threshold value (*Cq*). All statistical analysis and plotting were performed in the R software environment (R Core Team, 2013).

Assay sensitivity

Sensitivity of the assay was determined through the use of qPCR against a controlled number of target copies. A single-gene target sequence was constructed by amplifying a 485 bp fragment of the *H. vitripennis* COI gene using the primer pair C1-J-1718 (forward, 5′- GGA GGA TTT GGA AAT TGA TTA GTT CC -3′) and C1-J-2191 (reverse, 5′-CCC GGT AAA ATT AAA ATA TAA ACT TC-3′) (Simon *et al.*, 1994). Reactions were performed in 20 µl reactions using the GoTaq master mix (Promega, Madison, WI, USA) with 0.4 µg µl^{-1} bovine serum albumin (Sigma-Aldrich Co.) and 500 nM of each primer. Cycling conditions were as follows: initial denaturing at 94°C for 3 min followed by 40 cycles of denaturing at 94°C, annealing at 50°C and extension at 72°C for 45 s each, then a final extension at 72°C for 5 min. PCR product was evaluated by running on a 1% (w/v) agarose gel stained with Invitrogen SYBR Safe (Life Technologies, Auckland, New Zealand) and results were visualized using a GelDoc XR+ system (BioRad). PCR product was purified using the illustra Microspin column (GE Healthcare, Pittsburgh, PA, USA) kit according to manufacturer instructions and the purified product cloned using the Invitrogen TOPO TA vector cloning kit (Life Technologies). Successfully transformed clones were selected and the insert examined by amplifying with the M13 primer pair, designed to amplify the insert sequence (provided as part of the TOPO TA vector cloning kit). Inserts were sequenced by EcoGene® (Auckland, New Zealand) to confirm that the correct sequence had been cloned. A single clone was then grown in overnight culture and plasmid DNA extracted using the Wizard Plus SV Miniprep kit (Promega) and linearized by incubating overnight with *Pst* I (BioLab Inc, Lawrenceville, GA, USA). Linearized plasmid DNA was then quantified using NanoDrop (Thermo Fisher Scientific, Auckland, New Zealand) and normalized to a concentration of 10^9 copies µl^{-1}. Triplicate qPCR reactions were performed against a range of target gene concentrations (10^7–10 copies per reaction) using the previously reported conditions. The detection threshold of each reaction was plotted against the $\log_{10}$ of the template count and linear regression performed, measuring the fit as r^2. The amplification efficiency was calculated from the linear trend line using the equation $E = 10^{|1/\text{slope}|}$ and was converted to a percentage value using $E_{\%} = (E-1) \times 100$.

Assay specificity

Specificity of the assay was tested using a blind panel procedure, whereby 24 samples were tested using the developed assay by a diagnostician. The sample collection contained a mixture of Cicadellidae species including those native to New Zealand and commonly intercepted at the borders, as

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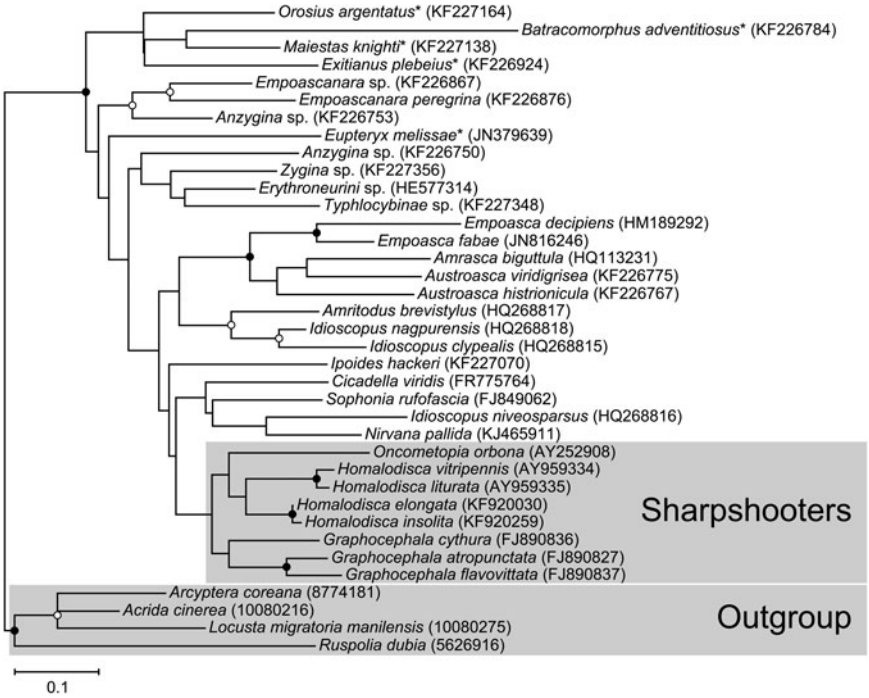

Fig. 1. Phylogenetic analysis of Cicadellidae-derived COI gene sequences. COI sequences were obtained from GenBank and a 370 bp region of sequence, common to 33 close relatives of *H. vitripennis*, aligned. Asterisk (*) denotes species found in New Zealand. Branch lengths were calculated using the maximum-likelihood method RAxML using a general time-reversible model with gamma rate distribution. Node support was calculated based on 1000 bootstrap iterations. Solid junctions denote nodes with ≥85% support and hollow junctions ≥65% support.

well as *Graphocephala actropunctata* and *H. liturata*, with *H. vitripennis* DNA randomly interspersed. Assay results were interpreted without knowledge of the sample identity and results were recorded as 'GWSS' (positive identification) or 'Not GWSS'.

Results

Analysis of phylogenetic relationships and qPCR design

Phylogenetic analysis of publically available COI sequence fragments confirmed that *H. vitripennis* forms a deep-branching lineage separate from other Cicadellidae. The closest neighbour to *H. vitripennis* was *H. liturata* and these two organisms formed a strongly supported clade apart from other species of *Homalodisca* (fig. 1, online Supplemental table S1). We therefore sought to maximize the differences between these organisms while keeping within the constraints of the TaqMan chemistry. Primers from an existing PCR assay of the *H. vitripennis* COI gene were used as starting points during the design process (primer pair HcCOI-F/HcCOI-R) (de León *et al.*, 2006). A stable probe and reverse primer were predicted to function with the HcCOI-F primer while still providing diagnostic specificity for *H. vitripennis* (table 2), which amplified a 163 bp fragment. Primers and probe sequence were mapped against the BOLD reference data using default methods, yielding no hits for either primer or probe to sequences that did not belong to *H. vitripennis*. Optimal reaction conditions were identified as annealing/extension temperature of 62°C and primer/probe concentrations of 200 nM per reaction.

Assay repeatability, reproducibility and sensitivity

Repeatability and reproducibility of the assay were high; with the lowest median %CV reported for the SsoAdvanced universal probes mix (fig. 2), which was used exclusively for the sensitivity and specificity. The limit of detection for the assay was identified as 10^2 copies/reaction with a linear dynamic range of 10^7–10^2 copies. The efficiency for the assay was 89.9%, with an r^2 of 0.99 (fig. 3). A template concentration of 10 copies/reaction was sporadically detected in the assay with an average *Cq* of ∼36 cycles. Due to inconsistent detection at this concentration it was excluded from the linear dynamic range, but was used to set an upper limit on the maximum allowable *Cq* in the assay (36 cycles), with any signal detected after this point considered a late-amplification false positive and treated as a negative result.

Assay specificity

Twenty-four samples were tested with the assay, tested by a diagnostician who was unaware of the source of each DNA sample (table 3). A single *H. vitripennis* sample, extracted from a poorly preserved nymph cast skin, was not correctly identified (table 3, Sample 3). Further analysis revealed that this sample yielded an extremely low DNA quality. One additional sample was identified late in the reaction (table 3, Sample 21); this sample had been extracted from an old and poorly preserved specimen and the DNA obtained from this sample possessed an extremely low quality, with a 260/280 ratio of 0.98. Running the assay against DNA extracted from another individual, obtained from the same location but preserved in

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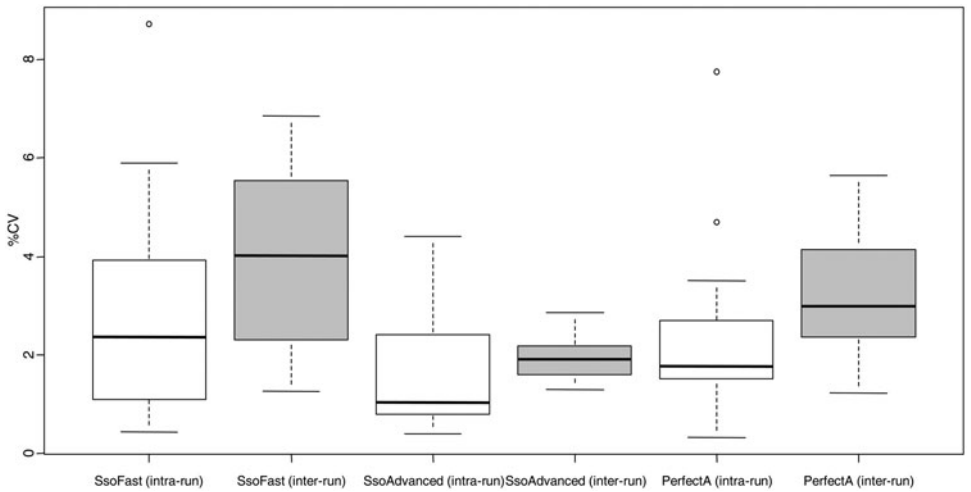

Fig. 2. Intra- and inter-run variation using three qPCR master-mix solutions. The median %CV for all solutions was low, with the inter-run % CV being slightly higher than intra-run for each solution. The SsoAdvanced solution was selected due to its low overall %CV, and narrower range of variance between runs.

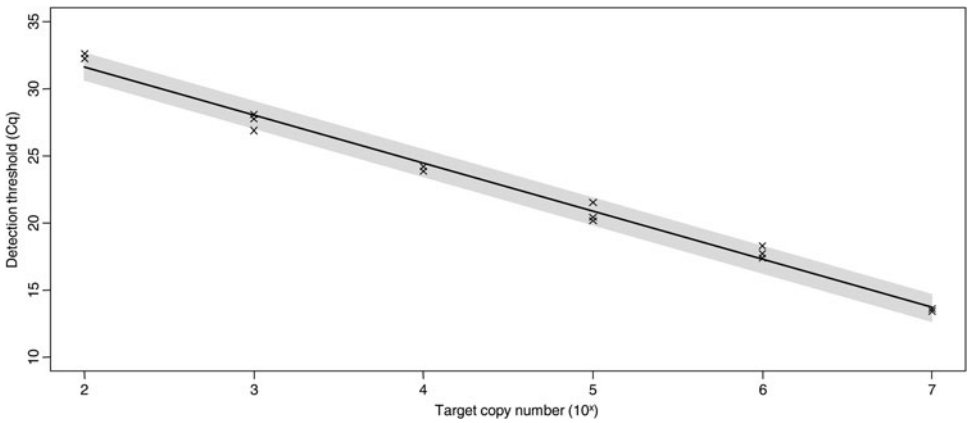

Fig. 3. Linear dynamic range of the qPCR assay. Efficiency of the assay was derived from the linear regression using standard methods. Slope of the regression curve yielded an efficiency of 89.9%, with a fit of $r^2 = 0.99$. Shading denotes the 95% confidence interval of regression.

ethanol prior to extraction, provided a more conclusive result (median $Cq = 22.3$, data not shown). DNA obtained from *H. vitripennis* eggs were all identified correctly using the assay. DNA extracted from *H. vitripennis* egg masses was identified in all tests performed (median $Cq = 26.4$).

Discussion

The spread of *H. vitripennis* in the Pacific in recent years has been attributed primarily to poor quarantine procedures that resulted in the transport of plants carrying *H. vitripennis* eggs between islands (Grandgirard *et al.*, 2006; Petit *et al.*, 2008). There is lack of pattern to the spread of *H. vitripennis* in the Pacific Islands, which is consistent with random spread due to human activity rather than a progressive migration of the species, and human activity is considered to be the likeliest source of an incursion into New Zealand (Grandgirard *et al.*, 2006; Rathé *et al.*, 2012). It is therefore important to develop a rapid diagnostic tool for the identification of *H. vitripennis* to prevent the spread of the pests in New Zealand once it is found.

DNA-based identification via qPCR is a technique commonly performed in virology, mycology, and bacteriology and is increasingly becoming popular within entomology (Jones *et al.*, 2008; Huang *et al.*, 2010; Dhami & Kumarasinghe, 2014; ven de Vossenberg & van der Straten, 2014). Comparisons of an existing *H. vitripennis* assay based on enzyme-linked immunosorbent assay (ELISA) of egg proteins and the previously mentioned COI gene PCR assay have shown that the PCR assay is able to detect *H. vitripennis* tissue in a higher number of samples than the ELISA method, primarily due to the universal presence of DNA compared with the gender-specific expression of the protein target (Fournier *et al.*, 2008; Hagler *et al.*, 2013). Within DNA-based identification techniques, qPCR assays are significantly faster than their conventional counter parts. The qPCR assay designed in this study required approximately 45 min to complete, compared with 3 h for the conventional assay. Rapid identification is the

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Table 3. Results of the blind panel testing procedure.

Sample	Identity	*Cq*	Diagnostic result
1	*Zeoliarus oppositus*	–	Not GWSS
2	*Idiocerus decimaquartus*	–	Not GWSS
3	*Homalodisca vitripennis*	39.4	Not GWSS
4	*Eupteryx melissae*	–	Not GWSS
5	*Edwardsiana cratagi*	–	Not GWSS
6	*Eupteryx melissae*	–	Not GWSS
7	*Homalodisca vitripennis*	18.4	GWSS
8	*Idiocerus distinguendus*	38.6	Not GWSS
9	*Cicadellidae* indet.	–	Not GWSS
10	*Homalodisca vitripennis*	15.9	GWSS
11	*Graphocephala atropunctata*	–	Not GWSS
12	*Idiocerus* sp.	38.8	Not GWSS
13	*Homolidosca liturata*	–	Not GWSS
14	*Zygnia zealandica*	–	Not GWSS
15	*Homolidosca liturata*	–	Not GWSS
16	*Edwardsiana cratagi*	37.6	Not GWSS
17	*Cicadellidae* indet.	–	Not GWSS
18	*Erythroneura elegantula*	39.5	Not GWSS
19	*Homalodisca vitripennis*	26.0	GWSS
20	*Idiocerus* sp.	–	Not GWSS
21	*Homalodisca vitripennis*	34.3	GWSS
22	*Idiocerus* sp.	–	Not GWSS
23	*Cicadellidae* indet.	–	Not GWSS
24	*Homalodisca liturata*	–	Not GWSS

Mean detection *Cq* values are reported, dash (–) indicates no signal was generated during the assay. *H. vitripennis* samples are shaded. A cut-off of 36 cycles was used when interpreting *Cq*, based on the sensitivity limit of the assay. Results were recorded simply as an *H. vitripennis* hit (GWSS) or no signal (Not GWSS). Sample 3 produced a false negative likely due to originating from poorly preserved tissue sample, as discussed in the main article.

cornerstone of biosecurity when dealing with invasive organisms as delays in identification, however minor, have drastic impacts on the success of quarantine and containment protocols.

The qPCR developed can be used to detect *H. vitripennis* of all life stages, however, as all the other tests there are limitations for this real-time PCR assay. A universal limitation to molecular diagnostic techniques is that there is always the risk that a genetic sequence not foreseen during the design process may react with the assay causing a false positive reaction. New Zealand possesses a diverse insect population, of which it is estimated half is currently undescribed (Cranston, 2010). Of the described insect fauna, New Zealand hosts over 544 species of Hemiptera, at least 444 of which are endemic, including 80 Cicadellidae (51 endemic) (Larivière, 2005). As these fauna are poorly represented in GenBank (online table S1) a variety of these insects were included in testing in order to ensure that false positive reactions would not be likely to occur with insects that are already present in New Zealand. Phylogenetic analysis of the short COI gene region was sufficient to demonstrate previously reported findings obtained from the complete mitochondrial genome; that *Homalodisca* forms a distinct clade apart from the other Cicadellidae (Song *et al.*, 2012). This does not guarantee that no Cicadellidae COI gene is capable of generating a false positive result, it gives confidence that Cicadellidae COI genes sequenced in the future will fall further away from *H. vitripennis*.

On the basis that *H. liturata* was the closest relative over the sequence fragment we aimed to maximize the differences between these two organisms in the assay. *H. liturata* possessed fewer mismatches across its three binding sites than any other sequence, including those with sequence data that only accounted for two binding sites (online Supplemental table S1). Our results showed that the samples of *H. liturata* were tested negative in the qPCR assay developed in this study (tables 1 and 3). Samples of two additional related species in the genus, *Homalodisca elongata* and *H. insolata* were not obtained for testing the qPCR assay despite multiple attempts to obtain specimens. However the *in silico* analysis showed that the degree of mismatches in the primers and probe sites (online Supplemental table S1) make it extremely unlikely that the qPCR assay for *H. vitripennis* will cross react with the *H. elongata* and *H. insolata*.

Quantitative PCR assays-based are subject to specific parameters for the assay to perform optimally. Factors such as the melting temperature (Tm) profile and GC content of the primers and probe and, in the case of TaqMan, the relative positioning of the oligonucleotides on the target sequence dictate the design of qPCR assays. As a consequence, the functionality of a conventional PCR assay cannot be directly extrapolated to a qPCR counterpart. For example, the HcCOI-F/HcCOI-R assay developed by de León *et al.* (2006) possessed an limit of detection (LOD) of 6 pg of genomic DNA, and the assay developed in this study possessed an LOD of 100 copies per reaction (using cloned plasmid DNA containing a COI insert). Since there are multiple copies of COI in genomic DNA and a single copy in each plasmid these numbers cannot be directly compared. The degree of sensitivity of de Leon's primers was tested as a real-time PCR using SYBR chemistry with the plasmid construct, and a lower detection sensitivity was observed than for conventional PCR (data not shown). In additional, testing the blind panel samples using the original HcCOI-F/HcCOI-R assay yielded a sporadic, weak false-positive reaction with the New Zealand endemic fulgoroid *Zeoliarus oppositus* (data not shown), which was not observed using the qPCR assay. This result is likely due to the additional *H. vitripennis*-specific mutations introduced by the TaqMan probe as well as the cut-off threshold for accepting a positive result, determined by the linear dynamic range of the assay. The testing regime used during the development of this assay ensures that our protocol is conformant with minimum information for publication of quantitative real-time PCR experiments (MIQE) guidelines for qualitative assays development (Bustin *et al.*, 2009).

Within blind panel testing, late-stage amplification was observed in several samples from organisms endemic to New Zealand (table 3, *Edwardsiana cratagi, Erythroneura elegantula, Idiocerus distinguendus*) yet these signals could be ruled out using the cut-off threshold. During blind panel testing, the qPCR assay was unable to correctly identify a single *H. vitripennis* sample (table 3, Sample 3). This sample was an aged cast skin sample and further testing showed that the negative results for the sample was due to the low quality of DNA extracted. We concluded that this false-negative result was an artefact of DNA extraction due to the sample storage and did not reflect a lack of sensitivity in the assay. Although this finding does not detract from the sensitivity of the assay it does highlight an important limitation of molecular diagnostic approaches.

In summary we have designed, optimized, and validated a real-time PCR assay for the rapid and accurate identification of *H. vitripennis* to be employed as part of New Zealand biosecurity practices. This assay is much faster to perform than conventional PCR equivalents. The primers and probe have been

https://doi.org/10.1017/S000748531600095X Published online by Cambridge University Press

extensively validated *in silico* and tested against a range of closely and distantly related samples to simulate a real diagnostic scenario. This assay has proven to be accurate and sensitive, which is essential for the future diagnostic applications.

Supplementary material

The supplementary material for this article can be found at https://doi.org/10.1017/S000748531600095X.

Acknowledgements

We are grateful to Dr Mark Hoddle, Dr David Morgan, Dr Youngsoo Son, and Humesh Kumar (California Department of Food and Agriculture, USA), Dr Matt Daugherty (University of California, USA) and Dr Maja Poeschko (Ministry of Agriculture, Cook Islands) for providing sharpshooter samples for assay validation. This work was funded by the Ministry for Primary Industries Operational Research Programme, New Zealand.

References

Almeida, R. & Purcell, A. (2003) Transmission of *Xylella fastidiosa* to grapevines by *Homalodisca coagulata* (Hemiptera: Cicadellidae). *Journal of Economic Entomology* **96**, 264–271.

Alston, J., Fuller, K., Kaplan, J. & Tumber, K. (2013) Economic consequences of Pierce's Disease and related policy in the California winegrape industry. *Journal of Agricultural and Resource Economics* **38**, 269–297.

Anderson, P., Brodbeck, B. & Mizell, R. III (1989) Metabolism of amino acids, organics acids and sugars extracted from the xylem fluid of four host plants by adult *Homalodisca coagulata*. *Entomologia Experimentalis et Applicata* **50**, 149–159.

Bethke, J., Blua, M. & Redak, R. (2001) Effect of selected insecticides on *Homalodisca coagulata* (Homoptera: Cicadellidae) and transmission of Oleander Leaf Scorch in a greenhouse study. *Journal of Economic Entomology* **94**, 1031–1036.

Blua, M. & Morgan, D. (2003) Dispersion of *Homalodisca coagulata* (Hemiptera: Cicadellidae), a vector of *Xylella fastidiosa*, into vineyards in Southern California. *Journal of Economic Entomology* **96**, 1369–1374.

Blua, M., Phillips, P. & Redak, R. (1999) A new sharpshooter threatens both crops and ornamentals. *California Agriculture* **53**, 22–25.

Boykin, L., Shatters, R. Jr., Rosell, R., McKenzie, C., Bagnall, R., De Barro, P. & Frohlich, D. (2007) Global relationships of *Bemisia tabaci* (Hemiptera: Aleyrodidae) revealed using Bayesian analysis of mitochondrial COI DNA sequences. *Molecular Phylogenetics and Evolution* **44**, 1306–1319.

Brodbeck, B., Mizell, R. III & Andersen, P. (1993) Physiological and behavioral adaptations of three species of leafhoppers in response to the dilute nutrient content of xylem fluid. *Journal of Insect Physiology* **39**, 73–81.

Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G. L., Vandesompele, J. & Wittwer, C.T. (2009) The MIQE Guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clinical Chemistry* **55**, 611–622.

Cranston, P. (2010) Insect biodiversity and conservation in Australia. *Annual Review of Entomology* **55**, 55–75.

Dandekar, A., Ibáñez, A., Gouran, H., Phu, M., Rao, B. & Chakraborty, S. (2012) Building a next generation chimeric antimicrobial protein to provide rootstock-mediated resistance to Pierce's disease in grapevines. pp. 89–192 *in* Esser, T. and Randhawa, R. (*Eds*) *Pierce's Disease Research Progress Reports*. Sacramento, CA, California Department of Food and Agriculture.

de León, J., Fournier, V., Hagler, J. & Daane, K. (2006) Development of molecular diagnostic markers for sharpshooters *Homalodisca coagulata* and *Homalodisca liturata* for use in predator gut content examinations. *Entomologia Experimentalis et Applicata* **119**, 109–119.

Dhami, M. & Kumarasinghe, L. (2014) A HRM real-time PCR assay for rapid and specific identification of the emerging pest spotted-wing drosophila (*Drosophila suzukii*). *PLoS ONE* **9**, e98934.

Edgar, R.C. (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research* **32**, 1792–1797.

Foottit, R., Maw, E. & Hebert, P. (2014) DNA barcodes for Nearctic *Auchenorrhyncha* (Insecta: Hemiptera). *PLoS ONE* **9**, e101385.

Fournier, V., Hagler, J., Daane, K., de León, J., Groves, R., Costa, H. & Henneberry, T. (2006) Development and application of a glassy-winged and smoke-tree sharpshooter egg-specific predator gut content ELISA. *Biological Control* **37**, 108–118.

Fournier, V., Hagler, J., Daane, K., de León, J. & Groves, R. (2008) Identifying the predator complex of *Homalodisca vitripennis* (Hemiptera: Cicadellidae): a comparative study of the efficacy of an ELISA and PCR gut content assay. *Oecologia* **157**, 629–640.

Grandgirard, J., Hoddle, M., Roderick, G., Petit, J., Percy, D., Putoa, R., Garnier, C. & Davies, N. (2006) Invasion of French Polynesia by the glassy-winged sharpshooter, *Homalodisca coagulata* (Hemiptera: Cicadellidae): a new threat to the South Pacific. *Pacific Science* **60**, 429–438.

Grandgirard, J., Hoddle, M., Petit, J., Roderick, G. & Davies, N. (2008) Engineering an invasion: classical biological control of the glassy-winged sharpshooter, *Homalodisca vitripennis*, by the egg parasitoid *Gonatocerus ashmeadi* in Tahiti and Moorea, French Polynesia. *Biological Invasions* **10**, 135–148.

Guan, W., Shao, J., Singh, R., Davis, R., Zhao, T. & Huang, Q. (2013) A TaqMan-based real time PCR assay for specific detection and quantification of *Xylella fastidiosa* strains causing bacterial leaf scorch in oleander. *Journal of Microbiological Methods* **92**, 108–112.

Guiterrez, A., Ponti, L., Hoddle, M., Almeida, R. & Irvin, N. (2011) Geographic distribution and relative abundance of the invasive glassy-winged sharpshooter: effects of temperature and egg parasitoids. *Environmental Entomology* **40**, 755–769.

Gunawardana, D., Ashcroft, T., Braithwaite, M. & Poeschko, M. (2008) Bio-control for glassy-winged sharpshooter in Cook Islands. *Biosecurity Magazine* **85**, 12–13.

Hagler, J., Blackmer, F., Krugner, R., Groves, R., Morse, J. & Johnson, M. (2013) Gut content examination of the citrus predator assemblage for the presence of *Homalodisca vitripennis* remains. *BioControl* **58**, 341–349.

Hoddle, M. (2004) The potential adventive geographic range of glassy-winged sharpshooter, *Homalodisca coagulata* and the grape pathogen *Xylella fastidiosa*: implications for California and other grape growing regions of the world. *Crop Protection* **23**, 691–699.

Hopkins, D. (2005) Biological control of Pierce's Disease in the vineyard with strains of *Xylella fastidiosa* benign to grapevine. *Plant Disease* **89**, 1348–1352.

Hopkins, D. & Purcell, A. (2002) *Xylella fastidiosa*: cause of Pierce's disease in grapevine and other emergent diseases. *Plant Disease* **86**, 1056–1066.

Huang, K., Lee, S., Yeh, Y., Shen, G., Mei, E. & Chang, C. (2010) Taqman real-time quantitative PCR for identification of western flower thrip (*Frankliniella occidentalis*) for plant quarantine. *Biology Letters* **6**, 555–557.

Johnson, M., Daane, K., Groves, R. & Backus, E. (2006) Spatial population dynamics and overwintering biology of the glassy-winged sharpshooter in California's San Joaquin Valley. pp. 12–15 in *Pierce's Disease Research Symposium Proceedings*. Sacramento CA, California Department of Food and Agriculture.

Jones, C., Gorman, K., Denholm, I. & Williamson, M. (2008) High-throughput allelic discrimination of B and Q biotypes of the whitefly, *Bemisia tabaci*, using TaqMan allele-selective PCR. *Pest Management Science* **64**, 12–15.

Larivière, M.-C. (Ed.) (2005 and updates) *Checklist of New Zealand Hemiptera (excluding Sternorrhyncha)*. Available online at http://hemiptera.landcareresearch.co.nz/ (accessed 2 April 2016).

Li, M., Tian, Y., Zhao, Y. & Bu, W. (2012) Higher level phylogeny and the first divergence time estimation of *Heteroptera* (Insecta: Hemiptera) based on multiple genes. *PLoS ONE* **7**, e32152.

Malausa, T., Fenis, A., Warot, S., Garmain, J., Ris, N., Prado, E., Botton, M., Vanlerberghe-Masutti, F., Sforza, R., Cruaud, C., Couloux, A. & Kreiter, P. (2011) DNA markers to disentangle complexes of cryptic taxa in mealybugs (Hemiptera: Pseudococcidae). *Journal of Applied Entomology* **135**, 142–155.

Minsavage, G., Thompson, C., Hopkins, D., Leite, R. & Stall, R. (1994) Development of a polymerase chain reaction protocol for detection of *Xylella fastidiosa* in plant tissue. *Phytopathy* **84**, 456–461.

Northfield, T., Mizel, R. III, Paini, D., Andersen, P., Brodbeck, B., Riddle, T. & Hunter, W. (2009) Dispersal, patch leaving, and distribution of *Homalodisca vitripennis* (Hemiptera: Cicadellidae). *Environmental Entomology* **38**, 183–191.

Park, D.-S., Foottit, R., Hebert, P. (2011) Barcoding bugs: DNA-based identification of the true bugs (Insecta: Hemiptera: Heteroptera). *PLoS ONE* **6**, e18749.

Pérez-Donoso, A., Sun, Q., Roper, M., Greve, L., Kirkpatrick, B. & Labavitch, J. (2010) Cell wall-degrading enzymes enlarge the pore size of intervessel pit membranes in healthy and *Xylella fastidiosa*-infected grapevines. *Plant Physiology* **152**, 1748–1759.

Petit, J., Hoddle, M., Grandgirard, J., Roderick, G. & Davies, N. (2008) Invasion dynamics of the glassy-winged sharpshooter *Homalodisca vitripennis* (Germar) (Hemiptera: Cicadellidae) in French Polynesia. *Biological Invasions* **10**, 955–967.

Purcell, A. & Feil, H. (2001) Glassy-winged sharpshooter. *Pesticide Outlook* **12**, 199–203.

Purcell, A., Saunders, S., Hendson, M., Grebus, M. & Henry, M. (1999) Causal role of *Xylella fastidiosa* in Oleander Leaf Scorch disease. *Phytopathology* **89**, 53–58.

Rakauskas, R., Turčinavičeine, J. & Bašilova, J. (2011) How many species are there in the subgenus *Bursaphis* (Hemiptera: Sternorrhyncha: Aphididae)? CO-I evidence. *European Journal of Entomology* **108**, 469–479.

Rathé, A., Pilkington, L., Gurr, G., Hoddle, M., Daugherty, M., Constable, F., Luck, J., Powell, K., Fletcher, M. & Edwards, O. (2012) Incursion preparedness: anticipating the arrival of an economically important plant pathogen *Xylella fastidiosa* Wells (Proteobacteria: Xanthomonadaceae) and the insect vector *Homalodisca vitripennis* (Germar) (Hemiptera: Cicadellidae) in Australia. *Australian Journal of Entomology* **51**, 209–220.

Ratnasingham, S. & Hebert, P. (2007) BOLD: the barcode of life data system (www.barcodinglife.org). *Molecular Ecology Notes* **7**, 355–364.

R Core Team (2013) R: a language and environment for statistical computing.

Roper, M., Creve, L., Warren, J., Labavitch, J. & Kirkpatrick, B. (2007) *Xylella fastidiosa* requires polygalacturonase for colonization and pathogenicity in *Vitis vinifera* grapevines. *Molecular Plant-Microbe Interactions* **20**, 411–419.

Simon, C., Frati, F., Beckenbach, A., Crespi, B., Liu, H. & Flook, P. (1994) Evolution, weighting and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. *Annals of the Entomological Society of America* **87**, 651–701.

Smith, P. (2005) Mitochondrial DNA variation among populations of the glassy-winged sharpshooter, *Homalodisca coagulata*. *Journal of Insect Science* **5**, 41–49.

Song, N., Liang, A.-P. & Bu, C.-P. (2012) A molecular phylogeny of *Hemiptera* inferred from mitochondrial genome sequences. *PLoS ONE* **7**, e48778.

Sorensen, J. & Gill, R. (1996) A range extension of *Homalodisca coagulata* (Say) (Hemiptera: Clypeorrhyncha: Cicadellidae) to southern California. *Pan-Pacific Entomologist* **72**, 160–161.

Stamatakis, A. (2014) RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics* **30**, 1312–1313.

Sun, Q., Greve, L. & Labavitch, J. (2011) Polysaccharide compositions of intervessel pit membranes contribute to Pierce's Disease resistance of grapevines. *Plant Physiology* **155**, 1676–1687.

Takiya, D., McKamey, S. & Cavichioli, R. (2006) Validity of *Homalodisca* and of *H. vitripennis* as the name for glassy-winged sharpshooter (Hemiptera: Cicadellidae: Cicadellinae). *Annals of the Entomological Society of America* **99**, 648–655.

Triapitsyn, S. & Phillips, P. (2000) First record of *Gonatocerus triguttatus* (Hymenoptera: Mymaridae) from eggs of *Homalodisca coagulata* (Homoptera: Cicadellidae) with notes on the distribution of the host. *Florida Entomologist* **83**, 200–203.

Triapitsyn, S., Mizell, R. III, Bossart, J. & Carlton, C. (1998) Egg parasitoids of *Homalodisca coagulata* (Homoptera: Cicadellidae). *Florida Entomologist* **81**, 241–243.

Tumber, K., Alston, J. & Fuller, K. (2014) Pierce's disease costs California $104 million per year. *California Agriculture* **68**, 20–29.

Turner, W. & Pollard, H. (1959) Life histories and behavior of five insect vectors of phony peach disease. *US Department of Agriculture Technical Bulletin* **1188**, 1–27.

ven de Vossenberg, B. & van der Straten, M. (2014) Development and validation of real-time PCR tests for the identification of four *Spodoptera* species: *Spodoptera eridania, Spodoptera frugiperda, Spodoptera littoralis*, and *Spodoptera litura* (Lepidoptera: Noctuidae). *Journal of Economic Entomology* **107**, 1643–1654.

Wistrom, C., Sisterson, M., Pryor, M., Hashim-Buckey, J. & Daane, K. (2010) Distribution of glassy-winged sharpshooter and three cornered alfalfa hopper on plant hosts in the San Joaquin Valley, California. *Journal of Economic Entomology* **103**, 1051–1059.