ORIGINAL CONTRIBUTION

Effective molecular detection of *Diaphorina citri* Kuwayama (Hemiptera: Liviidae) in bulk insect samples from sticky traps

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Abstract

Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Liviidae), is the principal vector of citrus greening (huanglongbing) disease. Invasion of new areas by the vector increases the risk of further spread of the disease and has economic impacts on the global citrus industry. Effective implementation of vector surveys is essential to contain disease outbreaks. This is especially true in countries such as Japan, where most of the major citrus-producing areas are free from citrus greening. Recently, vector surveys have been routinely conducted to maintain 'disease-free' and 'disease- and vector-free' areas in Japan, and improvement of methods that can detect *D. citri* in native insect populations is imperative. Here, we developed a method of using conventional and real-time PCR to detect *D. citri* among bulk insects captured in sticky traps without the need for preliminary differentiation steps based on morphology. DNA fragments of *D. citri* were specifically detected by both conventional and real-time PCR in a mixture of a 10^{-3} dilution (ca. 0.008–0.009 ng/μl) of *D. citri* DNA and 100 ng/μl of bulk insect DNA, indicating that small body parts such as pieces of leg or parts of wings of *D. citri* were detectable in the bulk insect samples. No misleading amplification of fragments from the other psyllid species and citrus pests we used occurred under our PCR conditions. Our results suggest that the technique is applicable to extensive surveys of *D. citri* in early warning programmes.

Introduction

The Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Liviidae), is a phloem-feeding pest of citrus in many parts of tropical and subtropical zones and is the principal vector of one of the causal bacterial pathogens of citrus greening (huanglongbing) disease, namely 'Candidatus Liberibacter asiaticus'. This disease is a serious threat to commercial citrus production worldwide (Grafton-Cardwell et al. 2013). Feeding by *D. citri* causes little direct damage to host citrus trees. However, by sucking the sap of infected citrus trees, *D. citri* acquires the pathogen, which is then disseminated to healthy citrus trees when the psyllid sucks their sap. This vector-transmission process is the primary step in the spread of citrus greening disease and the most serious pathway of disease invasion (Bové 2006) – more serious than artificial transmission processes such as grafting. Infected citrus show leaf mottling and blotchiness, followed by defoliation, fruit drop, unseasonal flushing and blossoming, resulting in eventual tree death (Bové 2006; Gottwald et al. 2007). Control of the insect vector is a prerequisite for control of citrus greening. Reasonable practices for the control of citrus greening include using disease-free nursery stock, removing symptomatic trees and reducing *D. citri* populations by foliar or soil application of insecticides (Grafton-Cardwell et al. 2013). Furthermore, *D. citri* populations in areas where citrus greening disease has been introduced are surveyed

using yellow sticky traps (Hall et al. 2010) or other sampling methods (Monzo et al. 2015); this enables the monitoring of vector populations and assessment of the risks of spread of citrus greening disease (Da Graça et al. 2004; Bové 2006).

Monitoring of pest populations plays key roles in vector management and vector-borne disease control. Molecular-based techniques are now available for the rapid and reliable detection of vector insects using species-specific primers targeting several marker genes (Boykin et al. 2012; Udayanga et al. 2014). Boykin et al. (2012) have analysed worldwide diversity of *D. citri* using a *D. citri*-specific molecular marker and represented eight different haplotypes (D cit-1 through D cit-8), with D cit-2 being widespread in Asian countries. However, few studies have demonstrated the direct detection of vector insects from other psyllid species (Boykin et al. 2012) or from among native insect populations without the preliminary steps of differentiation based on morphology (Qureshi and Stansly 2007; Hall et al. 2010; Monzo et al. 2015). Methodologies for *D. citri*-specific detection need to distinguish the target species from other, non-target, insects such as other psyllid species and citrus pests and native insect populations. Here, we investigated the efficiency of *D. citri* detection in bulk insect samples. We used a *D. citri*-specific molecular marker (Boykin et al. 2012) to differentiate *D. citri* from other psyllid species, important citrus pests, and native insect populations present in areas of Japan that were classified as either 'disease- and vector-present areas', or 'disease-free but vector-present areas', or 'disease- and vector-free areas' (Figure S1). Our results indicate that the methods described here are rapid, convenient and reliable for detecting *D. citri* among native insect populations without the need for morphological examination.

Materials and Methods

Insects and sample collection

Psyllid and citrus pest samples were collected from Honshu, Shikoku, Kyushu, Amami islands, and the Ryukyus, Japan, between 1998 and 2015 and preserved in 99.5% ethanol at –20°C until DNA extraction. Native insect samples were collected in cultivated citrus fields in three distinct areas of southeast Japan (Figure S1), namely: (i) the disease- and vector-present area of Okinawa Island in the Ryukyus, where *D. citri* often harbours '*Ca.* L. asiaticus' (at approximately 9.8% (Inoue, unpublished data));

(ii) the disease-free but vector-present area of Amami-Oshima island in Amami islands, where *D. citri* does not carry the pathogen; and (iii) the disease- and vector-free area of Kuchinotsu in Kyushu, where neither *D. citri* nor citrus greening disease is present. We set up 10-cm wide × 25-cm long yellow sticky traps and collected them every 2 months from April 2013 to November 2015. All sampling traps were stored at –20°C until further processing.

DNA extraction and amplification

Traps containing insect samples were soaked in hexane for 1 h to recover insect remains. Bulk insect samples were carefully decanted and then filtered through a plastic mesh (pore size of 0.05 mm × 0.05 mm) and rinsed with sterilized water three times. The resulting insect pellets were subjected to DNA extraction. Total DNA was extracted from psyllid species, citrus pests and the insect pellets using a DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA, USA). In the case of psyllids and citrus pests (excluding Thripidae), DNA was extracted individually. All samples were first ground with a mortar and pestle; the instructions of the kit manufacturer were then followed. DNA of Thripidae was extracted in accordance with the method of Toda and Komazaki (2002). DNA concentration and quality were analysed with a Qubit 2.0 Fluorometer (Life Technologies, Thermo Fisher Scientific Inc., Waltham, MA, USA). DNA extracted from the insect pellets was precipitated with ethanol and ethachinmate (Ethachinmate; Nippon Gene, Tokyo, Japan) and suspended in 20 μl of nuclease-free water.

Conventional PCR was performed in a 20-μl reaction mixture with 1 × PCR buffer containing 2 μl of extracted DNA, 0.2 m$_M$ deoxyribonucleotide triphosphates, 0.5 U *ExTaq* polymerase (Takara Bio Inc., Shiga, Japan) and 0.25 μM DCITRI COI primers (Boykin et al. 2012), which generated an 821-base pair fragment of the mtCOI coding region of *D. citri*. PCR reaction mixtures were heated at 94°C for 3 min; this was followed by 35 cycles of denaturation of 94°C for 30 s, annealing at 60°C for 30 s, extension at 72°C for 1 min and a final extension at 72°C for 10 min. Ten microlitres of PCR products was analysed by 1.5% agarose gel electrophoresis containing 2 μl of RE-DYE (Toyobo, Osaka, Japan) and 0.5 mg/ml ethidium bromide. Real-time PCR analysis was performed in a StepOnePlus real-time PCR system (Life Technologies, Thermo Fisher Scientific Inc.), using a SYBR *Premix Ex Taq* II (Tli RNaseH Plus) kit (Takara Bio Inc.) to label and amplify templates in accordance with the

manufacturer's protocol; 42 cycles of amplification were used.

Limits of detection of *D. citri* in bulk insect samples

To estimate the limits of detection of *D. citri* in bulk insect samples, we prepared mixtures of dilution series of the respective DNAs from *D. citri* and from insect pellets from the disease- and vector-free area. *Diaphorina citri* DNA diluted in serial 10-fold ranges was artificially mixed with bulk insect DNA (original concentration 100 ng/μl) diluted to 50 ng/μl, 10 ng/μl or 1 ng/μl; this was obtained from 5 to 10 sampling traps, depending on the number of insects caught on each trap. Detection limits were assessed from the results of conventional and real-time PCR.

Results

Specificity of detection of *D. citri* by conventional and real-time PCR

A total of 43 psyllid species and 13 citrus pests were used in the study (Tables S1 and S2). When we used PCR templates diluted in serial 10-fold ranges for real-time PCR, *D. citri* was detectable in dilutions up to 10^{-5}, corresponding to a threshold cycle (Ct) value of 32.92 (± 0.93) (table 1). The concentration of total DNA extracted from individual *D. citri* was measured at 1.46 ng/μl (= quantity of 1 in table 1); the calculated values of DNA in 10-fold dilutions from 10^{-1} to 10^{-5} ranged from 0.146 ng/μl to 0.0000146 ng/μl. In addition, we measured the concentrations of total DNA extracted from various parts of *D. citri*. The concentrations of DNA extracts derived from the legs and wings roughly corresponded to a dilution of 10^{-3} (table 1). There was either no PCR amplification or a Ct higher than 36 in the case of the other psyllid species and citrus pests (Table S1 and Table S2), confirming the specificity of the primer set for *D. citri* detection in real-time PCR.

Diaphorina citri was detected effectively, with a prominent band, using conventional PCR conditions with 35 cycles of amplification. Four other psyllid species – *Cacopsylla albigena*, *Cacopsylla elaeagnicola*, *Cacopsylla japonica* and *Trioza cinnamomi* – were also detected, although these psyllids gave unclear bands that were much fainter than those of *D. citri* (Figure S2 and Table S1). PCR amplification did not occur in the case of the citrus pests (Table S2). PCR products from those non-target psyllid species were analysed by a sequence and resulted in the distinguishment from *D. citri* (data not shown). By taking into account the

DNA concentrations and Ct values in relation to *D. citri* morphological status, we used these results to set 30 cycles as the upper limit of amplification, demonstrating that *D. citri* detection was confirmable at dilutions up to 10^{-4} (fig. 1) and that no PCR amplification of the DNA of any psyllid or citrus pest candidate other than *D. citri* occurred under conventional PCR conditions (Table S1 and Table S2).

Diaphorina citri detection in bulk insect samples by conventional and real-time PCR

Even when *D. citri* DNA at low concentrations was mixed with bulk insect DNA at high concentrations, *D. citri* was effectively detected using real-time PCR (table 2). However, the detection sensitivity decreased as a decreasing amount of *D. citri* DNA was mixed with an increasing amount of bulk insect DNA. The largest Ct value was 38.05 (± 3.01), in a mixture of a 10^{-5} dilution of *D. citri* DNA and 100 ng/μl of bulk insect DNA. Real-time PCR of bulk insect samples excluding *D. citri* was negative. Real-time PCR was positive for *C. elaeagnicola* and *C. japonica*, at 37.69 Ct and 36.56 Ct, respectively. By taking into account the volume of the bulk insect samples, which varied from approximately 19.7–55 mg of the bulk insect remains obtained from each sticky trap, providing DNA at approximately 10.38–28.92 ng/μl, *D. citri* detection in bulk insect samples was more reliable in real-time PCR if less than 50 ng/μl bulk insect DNA originating from fewer than six sampling traps was used. These results suggested that the detection limit for *D. citri* should be taken as less than 35 Ct in real-time PCR to exclude false positive results from other psyllids.

Diaphorina citri in bulk insect samples was effectively detected and distinguished from other pests under conventional PCR conditions with 30 cycles of amplification. The detection limit was reached with a mixture of a 10^{-3} dilution of *D. citri* DNA with 100 ng/μl of bulk insect DNA (fig. 2). No amplification was detected in the bulk insect samples or with the four psyllid species listed above (data not shown). Altogether, *D. citri* was detectable upon both real-time and conventional PCR when small tissue fragments of *D. citri* (such as a leg piece or wing part) were present in bulk insect samples.

Discussion

We successfully used a DCITRI COI primer set that generates an 821-base pair fragment of the mtCOI coding region of *D. citri* (Boykin et al. 2012) for *D. citri*

Species	Quantity	Ct (SD)	Rank	DNA (ng/μl)
D. citri	1	15.46 ($\pm$0.12)	Individual	1.46 ($\pm$0.49)
	10^{-1}	18.93 ($\pm$0.57)	Prothorax	0.27 ($\pm$0.038)
			Abdomen	0.49 ($\pm$0.082)
	10^{-2}	22.40 ($\pm$0.52)	Head	0.05 ($\pm$0.0025)
	10^{-3}	26.18 ($\pm$0.44)	Leg	0.008 ($\pm$0.00083)
			Hindwing	0.009 ($\pm$0.0015)
			Forewing	0.008 ($\pm$0.00076)
	10^{-4}	30.38 ($\pm$0.50)	n/a	n/a
	10^{-5}	32.92 ($\pm$0.93)	n/a	n/a
	10^{-6}	Undetermined	n/a	n/a

Table 1 Sensitivity of detection of *Diaphorina citri* by real-time PCR

Ct: threshold cycle. SD: standard deviation. n/a: not applicable. DNA concentrations were measured with a Qubit 2.0 Fluorometer.
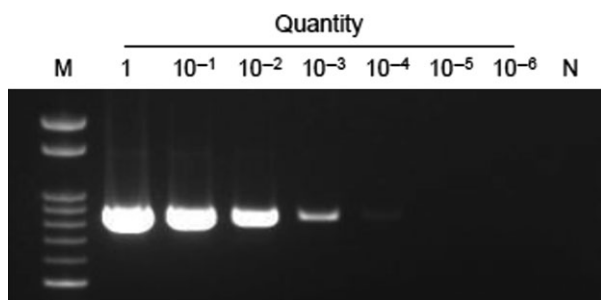

Fig. 1 Sensitivity of detection of *Diaphorina citri* in conventional PCR. The dilution series of *D. citri* DNA ranged from 1 to 10^{-6}. The original DNA concentration of *D. citri* (shown as 1) represents 1.46 ng/μl. N: negative control. M: Marker.
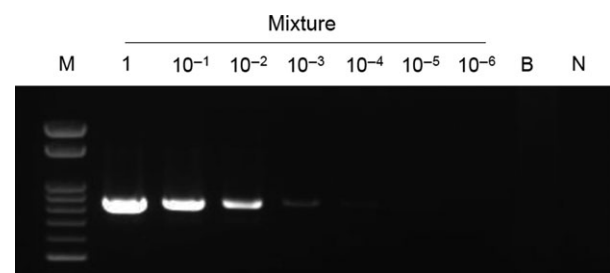

Fig. 2 Limits of detection of *Diaphorina citri* in bulk insect samples, as determined using conventional PCR. Mixtures consisted of a dilution series of *D. citri* DNA from 1 to 10^{-6} and bulk insect DNA at 100 ng/μl. The original DNA concentration of *D. citri* (shown as 1) represents 1.46 ng/μl. B: bulk insect DNA only. N: negative control. M: Marker.

Table 2 Limits of detection of *Diaphorina citri* in bulk insect samples by real-time PCR

		D. citri (quantity)							
		0	1	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Bulk sample DNA (ng/μl)	100	*u.d.*	36.16 ($\pm$1.42)	33.37 ($\pm$0.61)	34.18 ($\pm$0.42)	33.18 ($\pm$1.30)	34.88 ($\pm$1.55)	38.05 ($\pm$3.01)	*u.d.*
	50	*u.d.*	18.86 ($\pm$0.97)	21.23 ($\pm$0.33)	25.77 ($\pm$0.47)	31.18 ($\pm$2.21)	33.84 ($\pm$0.78)	36.22 ($\pm$4.55)	*u.d.*
	10	*u.d.*	16.16 ($\pm$0.50)	19.77 ($\pm$0.86)	23.08 ($\pm$0.07)	27.21 ($\pm$0.42)	30.70 ($\pm$0.79)	35.90 ($\pm$1.98)	*u.d.*
	1	*u.d.*	15.86 ($\pm$0.48)	19.11 ($\pm$0.42)	23.59 ($\pm$1.50)	26.60 ($\pm$0.28)	30.33 ($\pm$1.19)	32.23 ($\pm$0.08)	*u.d.*

u.d.: undetermined.

-specific detection using both conventional and real-time PCR. Our results proved that the primer set, which was designed for *D. citri* identification *in silico*, was applicable to the detection of *D. citri* DNA without interference from potential PCR inhibitors derived from the total DNA of the bulk insect samples collected from 'the disease- and vector-free area'. Similarly, DNA from bulk insect samples from 'the disease-free but vector-present area' or 'the disease- and vector-free area' had no inhibitory effects on *D. citri* detection (data not shown). On the other hand, our PCR methods had a potential drawback in that the primer we used had the potential to yield false positive results. We detected four psyllid species (*C. albigena*, *C. elaeagnicola*, *C. japonica* and *T. cinnamomi*), although they were clearly distinguishable from *D. citri* by the use of different PCR conditions such as different annealing temperature and time. Similarly, *D. citri* detection in bulk insect samples using real-time PCR was susceptible to error due to contamination by *C. elaeagnicola* and *C. japonica* when bulk insect DNA at 100 ng/μl was used in the mix-

ture. This problem disappeared by adding smaller concentrations of bulk insect DNA. Furthermore, post-PCR approaches screening for non-target amplification needed to be considered. In this study, four non-target psyllid species were effectively distinguished from *D. citri* by a sequencing using those PCR products from conventional and real-time PCR methods (data not shown). Other methodologies such as restriction fragment length polymorphism (RFLP) or microsatellite analysis would also be helpful to avoid potentially misleading source of error (Jenkins et al. 2012). Hence, our approaches can be adapted to optimize *D. citri* monitoring efforts in Japan, and the information obtained here should be useful for enhancing citrus greening management worldwide. To enhance *D. citri* detection specificity, further investigations of native insect populations and a greater understanding of population diversity in each part of the world are required.

The introduction of a vector pest management programme for *D. citri* into Japan surprisingly revealed *D. citri* invasion of 'disease- and vector-free areas', namely Yakushima Island in 2002 and the city of Ibusuki in Kyushu in 2006, while the vector was pathogen free (Ushimaki 2002; Inoue 2007). As successful eradication programmes were implemented, these areas have reverted to being disease and vector free. Current measures against citrus greening are focused on three distinct areas of south-west Japan: (i) disease- and vector-present areas, namely the Okinawa Islands and three of the Amami Islands of Kagoshima Prefecture (Tokunoshima Island, Okinoerabu Island and Yoron Island), where *D. citri* often harbours '*Ca.* L. asiaticus'; (ii) disease-free but vector-present areas, namely the northern Amami Islands (Amami-Oshima and Kikai Island), where *D. citri* is free from '*Ca.* L. asiaticus'; and (iii) disease- and vector-free areas, namely the other islands of Kagoshima Prefecture and the whole of Kyushu, where neither *D. citri* nor citrus greening disease is present. The management strategy aims not only to control *D. citri* in 'disease- and vector-present areas' and 'disease-free but vector-present areas', but also to enhance the monitoring of *D. citri* in 'disease- and vector-free areas'. Historically, risk management of vector-borne diseases in 'disease- and vector-free areas' may have a considerable role in disease control and management (Ushimaki 2002; Da Graça et al. 2004; Inoue 2007; Grafton-Cardwell et al. 2013). Thus far, *D. citri* detection and identification have relied on expert morphological observation. However, these approaches may fail to detect *D. citri* when only fragmented body parts are available. The

methodology described here should help solve this problem and provide convenient and reliable detection of *D. citri* without the need for preliminary morphology-based differentiation. For practical use in *D. citri* management programs, it would be suitable for extensive monitoring of native insect populations in areas that are currently disease and vector free; any positive detections would mean that further investigation and effective measures for control and management of both *D. citri* and citrus greening disease would be warranted.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Distribution of citrus greening disease and *Diaphorina citri* in Japan.

Figure S2. Detection of *Cacopsylla albigena*, *Cacopsylla elaeagnicola*, *Cacopsylla japonica*, and *Trioza cinnamomi* under conventional PCR conditions with 35 cycles of amplification.

Table S1. Psyllid species used in the study.

Table S2. Citrus pests used in the study.