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DNA barcoding and real-time PCR detection of *Bactrocera xanthodes* (Tephritidae: Diptera) complex

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Abstract

Immature fruit fly stages of the family Tephritidae are commonly intercepted on breadfruit from Pacific countries at the New Zealand border but are unable to be identified to the species level using morphological characters. Subsequent molecular identification showed that they belong to *Bactrocera xanthodes*, which is part of a species complex that includes *Bactrocera paraxanthodes*, *Bactrocera neoxanthodes* and an undescribed species. To establish a more reliable molecular identification system for *B. xanthodes*, a reference database of DNA barcode sequences for the 5′-fragment of COI gene region was constructed for *B. xanthodes* from Fiji, Samoa and Tonga. To better understand the species complex, *B. neoxanthodes* from Vanuatu and *B. paraxanthodes* from New Caledonia were also barcoded. Using the results of this analysis, real-time TaqMan polymerase chain reaction (PCR) assays for the detection of *B. xanthodes* complex and for the three individual species of the complex were developed and validated. The assay showed high specificity for the target species, with no cross-reaction observed for closely related organisms. Each of the real-time PCR assays is sensitive, detecting the target sequences at concentrations as low as ten copies μl^{-1} and can be used as either singleplex or multiplex formats. This real-time PCR assay for *B. xanthodes* has been successfully applied at the borders in New Zealand, leading to the rapid identification of intercepted Tephritidae eggs and larvae. The developed assays will be useful biosecurity tools for rapid detection of species in the *B. xanthodes* complex worldwide.

Keywords: *B. xanthodes*, *B. neoxanthodes*, *B. paraxanthodes*, biosecurity, diagnostics, interceptions

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Introduction

New Zealand remains the only major fruit producing country in the world that is free from economically significant pest fruit flies (Stephenson *et al.*, 2003). Monitoring and surveillance programs have been put in place to ensure their early detection and to minimize pathway risk (Stephenson *et al.*, 2003; Armstrong & Ball, 2005). The ability to identify intercepted organisms to species level is essential for accurate interception data and assessment of pathway risk. Unfortunately, the interceptions at the borders in fruits from commercial consignments or accompanying overseas travellers are usually eggs or larvae, which cannot be identified morphologically beyond the family or genus level.

Bactrocera xanthodes (Broun) is regularly intercepted at the New Zealand border. It is part of a complex of four closely related

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species that together currently comprise the subgenus *Notodacus*, and also includes *Bactrocera paraxanthodes* Drew & Hancock from New Caledonia (Drew *et al*., 1997), *Bactrocera neoxanthodes* Drew & Romig from Vanuatu (Allwood *et al*., 1997) and an undescribed new species from Samoa (Heimoana *et al*., 1997). *B. xanthodes* is present in Fiji, Tonga, Niue, Samoa, American Samoa, Southern part of Cook Islands, and Wallis and Futuna (Plant Health Australia, 2016). It was detected on Nauru in 1992 and Raivavae (French Polynesia) in 1998 but eradicated by male annihilation (Leblanc *et al*., 2012; Plant Health Australia, 2016). *B. paraxanthodes* was initially described from New Caledonia, Vanuatu and Western Samoa (Drew & Hancock, 1995). Later data indicated that it is only present in New Caledonia (Drew *et al*., 1997). *B. neoxanthodes* (in Drew *et al*., 1997 as *B.* sp. n. No. 2) is only present in Vanuatu (Drew *et al*., 1997; Heimoana *et al*., 1997; Drew & Romig, 2001). To date, no detailed studies on the undescribed species from Samoa have been conducted.

Bactrocera xanthodes is a major fruit fly pest throughout the South Pacific region, where they infest a wide range of commercial fruit plants (Hoeben *et al*., 1996). The hosts of *B. xanthodes* have been recorded from 20 plant families, including Anacardiaceae, Annonaceae, Apocynaceae, Caricaceae, Combretaceae, Euphorbiaceae, Lauraceae, Lecythidaceae, Moraceae, Passifloraceae, Rutaceae and Sapotaceae (Leblanc *et al*., 2012). Host fruit survey showed that the abundant breadfruit, *Artocarpus altilis* (Moraceae) in the Pacific countries is a major host for *B. xanthodes* (Tora Vueti *et al*., 1997*a*). In addition, the wild fruits, *Ochrosia oppositifolia* (Apocynaceae) and *Barringtonia edulis* (Lecythidaceae) are also the common hosts for *B. xanthodes* (Tora Vueti *et al*., 1997*b*). In comparison, for the other three species in the complex, non-commercial fruits, i.e. only wild hosts, are recorded as hosts: *Schefflera* sp. for *B. paraxanthodes; Barringtonia edulis* (Lecythidaceae) and *Passiflora suberosa* (Passifloraceae) for *B. neoxanthodes* (Drew & Romig, 2001) and *Ficus* sp. (Araliaceae) for the undescribed species, *B.* sp. n. No. 1 (Drew *et al*., 1997; Leblanc *et al*., 2012).

Molecular diagnostic techniques, such as polymerase chain reacation-restriction fragment length polymorphism (PCR–RFLP) (Armstrong *et al*., 1997) and DNA barcoding have been used for fruit fly identification (Hebert *et al*., 2003; Armstrong & Ball, 2005). More rapid identification has been achieved by real-time PCR assays, several of which have been developed for fruit fly species, i.e. *Bactrocera tryoni* complex (including *Bactrocera tryoni, Bactrocera aquilionis* and *Bactrocera neohumeralis*), *Bactrocera invadens, Ceratitis capitata* and *Dirioxa pornia* (Dhami *et al*., 2016) and are used in the routine diagnostics at New Zealand's borders. However, there is no reported real-time PCR protocol for *B. xanthodes*, which is common interceptions at New Zealand borders, therefore, there is the need to develop a real-time PCR assay for rapid detection of the common border interceptions. Here we report the DNA barcoding data for *B. xanthodes*, *B. paraxanthodes* and *B. neoxanthodes*, and also the development and validation of the real-time PCR protocols targeting the *B. xanthodes* species complex, including their sensitivity, specificity and blind panel test. The assay for *B. xanthodes* has been tested on the interceptions at New Zealand borders, and provided accurate and rapid identification of the intercepted eggs and larvae from the Pacific countries.

Materials and methods

Sampling

Eggs and larval fruit fly specimens used in this study were stored at −20°C freezer or 96% ethanol prior to DNA extraction (table 1). These specimens were intercepted at New Zealand borders from various countries. In addition, dry mounted adult specimens of *B. neoxanthodes* from Vanuatu, and adults of *B. paraxanthodes* in 96% ethanol from New Caledonia were obtained from their country collections (tables 1 and 2). Identifications of the adult specimens were done by morphological characters and confirmed by Professor Dick Drew from Griffith University, Australia.

DNA extraction and barcoding

Total DNA from individual specimens was extracted using the DNeasy for Blood and Tissue kit (Qiagen, Valencia, CA, USA) as per the manufacturer's instructions. An adult-leg, piece of larva or egg was used for each extraction and physical disruption was performed by micro-pestles, with DNA eluted in 100 µl of warmed AE buffer. For some samples, DNA was also extracted using crude extraction methods: 6% Chelex 100 (Sigma- Aldrich Co. Aldrich Co.) and prepGem DNA kit (ZyGem Corporation Ltd., Hamilton, New Zealand).

All the DNA extracted was used for PCR amplification with primers, LCO1490 and HCO2198 (Folmer *et al*., 1994). For all the PCR reactions, each 20 µl reaction consisted of 1 × GoTaq master mix (Promega, Madison, WI), 250 nM of each primer, 0.5 µg μl^{-1} Bovine Serum Albumin (BSA, Sigma-Aldrich Co.), and 2 µl of DNA extract. Cycling conditions were: initial denaturation at 94°C for 2 min, 40 cycles of 94°C for 15 s, 52°C for 30 s and 72°C for 45 s, followed by a final extension step of 7 min at 72°C. However, no amplification was observed with the DNA extracted from the dried aged specimens of *B. neoxanthodes*. Testing on 18S qPCR assay, positive amplification was observed, indicating that DNA was extracted from the specimens, thus two sets of primers were designed to amplify 200–300 bp PCR products each targeting different regions of the COI sequences (DQ116354-DQ116356): BNX211F: TACCTCCTTCCCTGA CCCTG; BNX345R: GTGAGAAAATTGCAAGATCAACGG; BNX345F: GCATCCGTTGATCTTGCAA and BNX_607R: TCAAAATAGGTGCTGATAAAGAATAGG. The same PCR compositions and cycling conditions as above were applied using the two pairs of primers. All the PCR products and DNA sequences were analyzed as per Li *et al*. (2015). The DNA sequences were submitted to BOLD under the project of Barcode of *Bactrocera* Specimens (BBS) at BOLD and with accession numbers MF742414-MF742457 for *B. xanthodes*, MF742409-MF742411 for *B. neoxanthodes* and MF742412-MF742413 for *B. paraxanthodes* under GenBank.

Real-time PCR design

DNA sequences of COI from *B. xanthodes, B. neoxanthodes, B. paraxanthodes* and the closely related species were aligned in Geneious 7.1.5 (Biomatters, Auckland, New Zealand). Regions that showed differences specific to *B. xanthodes, B. paraxanthodes* and *B. neoxanthodes* were selected manually and used as the target region for the specific probes and primers. The primers and probe were manually designed and their suitability examined in the web-based RealTimeDesign qPCR assay design software from Biosearch. Their secondary structures and thermodynamic properties were checked using Geneious and OligoAnalyzer 3.1 (OligoAnalyzer, 2012). The specificity of the designed primers and probes were also checked in Geneious and BLAST searches in GenBank database (Altschul *et al*., 1990).

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Table 1. Information of the target species tested in the real-time PCR assays.

Samples ID	Numbers of individuals	Life stage	Country	Year	Host	DNA extraction[1]
B. neoxanthodes[2]	1	Larva	Vanuatu	2012	*Barringtonia edulis*	PG
B. neoxanthodes	5	Adult	Vanuatu	1995	Trap	QC
B. paraxanthodes	2	Adult	New Caledonia	2015	Lab colony (from *Schefflera gabrielae*)	QC
B. xanthodes	4	Egg	Fiji	2012–2013	*Artocarpus altilis* (breadfruit)	QC
B. xanthodes	4	Egg	Unknown Pacific country	2015–2017	*Artocarpus altilis* (breadfruit)	QC
B. xanthodes	9	Egg	Samoa	2012–2017	*Artocarpus altilis* (breadfruit)	PG/QC
B. xanthodes	2	Larva	Samoa	2015–2017	*Artocarpus altilis* (breadfruit)	QC
B. xanthodes	2	Adult	Samoa	1996	*Mangifera indica* (mango)	QC
B. xanthodes	15	Egg	Tonga	2013–2017	*Artocarpus altilis* (breadfruit)	PG/QC
B. xanthodes	8	Larva	Tonga	2011–2017	*Artocarpus altilis*(breadfruit)	CH/QC
B. xanthodes	2	Larva	Tonga	2016	*Citrullus lanatus* (watermelon)[3]	QC

[1]DNA extraction method of Qiagen Blood and Tissue kit (QC), PrepGEM (PG) and Chelex 100 (CH) were used for the samples.
[2]The DNA sequence has 99% identities with *B. neoxanthodes* from Vanuatu, ~89% to *B. xanthodes* of other locations obtained from this study.
[3]Only two larvae were intercepted from watermelon (*Citrullus lanatus*), no breeding population were observed, thus whether the watermelon is the original host needs to be further confirmed.

Real-time PCR optimization

All real-time PCR reactions were set up on a CFX96™ Touch Real-time platform (BioRad). Optimization were conducted on a temperature gradient of 55–66°C, using primer concentrations (200 and 300 nM), probe gradients (125–300 nM), and with or without the addition of Mg^{2+} (1.5 mM). To select an appropriate real-time mastermix for the assay, the real-time PCR tests were run with mastermixes: PerfeCTa® qPCR ToughMix® (Quanta Bioscience) and SsoAdvanced™ Universal Probes Supermix (BioRad). The real-time PCR was also tested in singleplex and multiplex formats and with 18S ribosomal RNA (rRNA) gene internal control real-time PCR (Applied Biosystems, CA, USA).

Specificity

The specificity of the real-time PCR assay was tested using the DNA extraction from different *Bactrocera* species both the target (table 1) and non-target (table 2) species. These samples included *B. xanthodes*, *B. neoxanthodes*, *B. paraxanthodes*, *B. passiflorae*, *B. cucumis*, *B. dorsalis*, *B. jarvisi*, *B. latifrons*, *B. tryoni* and other flies (table 2).

Sensitivity

To evaluate the analytical sensitivity of the developed assay, 710, 708 and 709 bp of the COI gene, respectively for *B. xanthodes*, *B. paraxanthodes* and B. *neoxanthodes*, were used to prepare plasmid standards of known copy numbers. The amplicon was cloned using the TOPO®TA vector cloning kit (Invitrogen, Carlsbad, CA, USA) as per the manufacturer's instructions. Cloning was performed for one DNA sample from each species, and the clones containing the correct insert were selected for preparing the plasmid DNA standards. Plasmid DNA was extracted using the Wizard® Plus SV Miniprep (Promega, Madison, WI, USA). The plasmid DNA was quantified using a µDrop plate in MultiSkan GO DNA quantification system (Thermo Scientific, USA) and normalized to a concentration of 10^8 copies/ μl^{-1}. A dilution series of the plasmid from 10^7–10^0 copies was created using TE buffer (10 mM TriHCl and 1 mM EDTA, Invitrogen). Analytical sensitivity of the assay was determined using the dilution series with each concentration in triplicate per reaction. Linear regression was performed between the detection threshold (*Cq*) and the $\log_{10}$ of the copy number, measuring the fit as r^2. Amplification efficiencies for individual reactions were calculated using the formula, $E = 10^{|1/slope|}$ and converted to $E_{\%}$ by (E-1) × 100 in the R environment version 3.1.1 (R Development Core Team, 2011).

Blind panel test

A total of 26 specimens were provided to the operators with no knowledge of the sample origin and identity (table 4). The samples were tested against the real–time assay in singleplex for *B. xanthodes*, *B. paraxanthodes* and *B. neoxanthodes*, respectively. It was also tested in the multiplex for *B. xanthodes*, *B. paraxanthodes* and *B. neoxanthodes*. All the samples were tested in duplicate wells, positive and non- template controls were included.

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Table 2. Information of the non-target species tested in the real-time PCR assays.

Samples ID	Number of individuals	Life stage	Country	Host
Anastrepha sp.	1	Larva	Peru	Unknown
Anastrepha striata	1	Larva	Chile	*Psidium guajava* (guava)
B. correcta	1	Larva	Vietnam	*Psidium guajava* (guava)
B. cucumis	4	Egg and larva	Australia	*Cucumis melo* (rockmelon)
B. cucumis	2	Adult	Australia	Lab colony
B. dorsalis	3	Larva	Philippines	*Psidium guajava* (guava)
B. dorsalis	2	Adult	India	*Psidium guajava* (guava)
B. dorsalis	2	Larva	Unknown	*Litchi chinensis* (lychee)
B. dorsalis	4	Adult	Cook Islands	Trap
B. invadens	2	Adult	Sri Lanka	*Mangifera indica* (mango)
B. jarvisi	3	Larva	Australia	*Mangifera indica* (mango)
B. latifrons	1	Pupa	Thailand	*Capsicum annuum* (chili)
B. melanotus	3	Larva	Cook Islands	*Asimina triloba* (pawpaw)
Bactrocera sp.	1	Egg	Fiji	*Mangifera indica* (mango)
B. tryoni complex	2	Larva	Australia	*Pyrus* sp. (pear)
B. passiflorae	6	Egg/larva/adult	Fiji	*Mangifera indica* (mango)
B. tryoni complex	4	Larva	Australia	*Mangifera indica* (mango)
B. tryoni complex	2	Egg	Australia	*Litchi chinensis* (lychee)
B. oleae	1	Pupa	Italy	*Olea europaea* (olive)
B. zonata	1	Egg	Unknown	*Psidium guajava* (guava)
Dirioxa pornia	2	Larva	Australia	*Citrus* sp. (orange)

Results

DNA barcoding

DNA barcoding of *Bactrocera xanthodes* and *B. paraxanthodes* were obtained using Folmer's primers, LCO1490 and HCO2198 (Folmer *et al.*, 1994). For *B. neoxanthodes*, DNA barcoding was achieved by using BNX211F-345R and BNX345F-607R primer pairs which amplified two overlapping regions of the COI gene and resulted in 460 bp sequences. Sequence comparison of the three species showed that *B. xanthodes* is distant from the other two species of the complex by about 12% while *B. neoxanthodes* and *B. paraxanthodes* are more closely related, with around 5% difference in their COI sequences.

Those DNA barcoding sequences are AT-rich, with average 58.8% for *B. xanthodes*, 60.1% for *B. paraxanthodes* and 58.3% for *B. neoxanthodes*. The sequences of *B. xanthodes* were around 98% similar to each other and to three *B. xanthodes* sequences in Genbank (DQ116351-DQ226353), but ~88% similar to another three sequences in Genbank, DQ116354-DQ116356 (Armstrong & Ball, 2005). The later three sequences from Armstrong & Ball 2005, were labelled as *B. paraxanthodes* in the supplemental figure and Appendix table 3 in Armstrong & Ball's publication (2005) though they are recorded as *B. xanthodes* in GenBank. Surprisingly, the sequences from *B. neoxanthodes* obtained in this study, are identical to the above three sequences (DQ116354-DQ116356) in the overlap region of the 458 bp. On the other hand, the sequences of *B. paraxanthodes* obtained in this study were quite different to those three sequences (DQ116354-DQ116356) by ~12%. Therefore the results suggested that the three sequences (DQ116354-DQ116356) in BOLD and GenBank are more likely derived from *B. neoxanthodes*, but not *B. paraxanthodes* or *B. xanthodes*.

Real-time PCR design

The *B. xanthodes* COI sequences obtained in this study were used for alignment and design of the real-time PCR assay, and the unreliable sequences were discarded from this analysis. Initially, real-time PCR assay was designed to test whether two SNPs near the 3'-end of the probe binding sites (table 3) could distinguish *B. xanthodes* and *B. neoxanthodes* (fig. 1). However, when testing the two probes (Bxand_1P vs.

Table 3. The detailed information about the real-time PCR assay for the detection the *B. xanthodes* species complex and the cycling parameters.

Primer/Probe name	Sequences	Target/PCR size (bp)	Concentration (nM)	Cycling conditions
Bxand_1F	AGGAGCCCCAGATATAGC	*B. xanthodes*,	300	95°C for 2 min
Bxand_1R	TAGTGGTGGGTATACAGTTCA	*B. neoxanthodes*,	300	
Bxand_1P or Bxand_V_1P	TCTACCTCCTTCCCTGACCCTACTG or TCTACCTCCTTCCCTGACCCTGCTA	*B. paraxanthodes*/136	250	40 cycles of
Bxand_2F	ACCCACCACTATCCTCAGTTATTGC	*B. xanthodes*/152	250	95°C for 15 s
Bxand_2P	TCATTTAGCAGGTATTTCATCAATCTTGG		200	62°C for 60 s
Bxand_2R	GTCGAAGGTGATTCCTGTAGAACG		250	Plate read
Bxand_V-2F	GGCATCCGTTGATCTTGCAATT	*B. neoxanthodes*/121	300	
Bxand_V_2P	TCACTCCATTTAGCAGGAATCTCCTCA		250	
Bxand_V_2R	CCGGTCGAATGTGATACCTGTAG		300	
Bpara_PF	GCTCACGGAGGAGCATCTGT	*B. paraxanthodes*/131	250	
Bpara_1P	TCATCTAGCAGGAATTTCCTCAATTCTAG		200	
Bpara_PR	CGGTCGAATGTGATACCTGTAGAT		250	

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Table 4. Sample information used in the blind panel test of the real-time PCR assay for the *B. xanthodes* complex.

Sample	Species ID	Year (Collected)	Host	Life stage	Country
BA1	*B. tryoni*	2015	*Prunus* sp. (cherry)	Larva	New Caledonia
BA2	*B. xanthodes*	2001	*Artocarpus altilis* (breadfruit)	Larva	Tonga
BA3	*B. jarvisi*	2015	*Mangifera indica* (mango)	Larva	Australia
BA4	*B. dorsalis*	2013	Methyl eug. Trap	Adult	Rarotonga/ Cook Islands
BA5	*Bactrocera* sp.	2015	*Sandoricum koetjape* (santol)	Larva	Vietnam
BA6	*B. xanthodes*	2008	*Artocarpus altilis* (breadfruit)	Egg	Samoa
BA7	*Bactrocera sp.*	2016	*Mangifera indica* (mango)	Larva	Cambodia
BA8	*B. xanthodes*	2009	*Artocarpus altilis* (breadfruit)	Egg	Samoa
BA9	*B. latifrons*	2014	*Capsicum annuum* (chili)	Pupa	Thailand
BA10	*B. tryoni* complex	2014	*Mangifera indica* (mango)	Larva	Australia
BA11	*Dirioxa pornia*	2014	*Citrus* sp. (orange)	Larva	Australia
BA12	*B. neoxanthodes*	2013	*Barringtonia edulis*	Adult	Vanuatu
BA13	*B. correcta*	2015	M. eugenol trap	Adult	Vietnam
BA14	*B. tryoni* complex	2016	*Mangifera indica* (mango)	Larva	Australia
BA15	*B. bimaculata*	2015	Cue lure	Adult	Vietnam
BA16	*B. xanthodes*	2012	*Artocarpus altilis* (breadfruit)	Egg	Fiji
BA17	*B. cucumis*	2010	*Cucumis melo* (rockmelon)	Egg	Australia
BA18	*B. xanthodes*	2011	*Artocarpus altilis* (breadfruit)	Larva	Tonga
BA19	*B. curvipennis*	2013	Lab colony	Adult	New Caledonia
BA20	*Bactrocera* sp.	2016	*Mangifera indica* (mango)	Larva	Australia
BA21	*B. neoxanthodes*	2012	Fresh Fruit	Larva	Vanuatu
BA22	*Diroxia pornia*	2016	*Citrus* sp. (orange)	Larva	Australia
BA23	*B. jarvisi*	2016	*Mangifera indica* (mango)	Larva	Australia
BA24	*B. xanthodes*	2013	*Artocarpus altilis* (breadfruit)	Egg	Tonga
BA25	*B. carambolae*	2015	unknown fruit	Larva	Indonesia
BA26	*B. xanthodes*	2011	*Artocarpus altilis* (breadfruit)	Egg	Tonga

Bxand_V_1P) using the DNA extracted from the two species, amplification curves were observed with similar *Cq* values for either one of the probes. Further tests also showed that the assay could detect *B. paraxanthodes* even though there were two SNPs in the probe and one SNP in the reverse primer binding sites, respectively (fig. 1). Therefore, this real-time PCR assay can be used as a generic analysis for the detection of the three described species of the *B. xanthodes* complex.

Since *B. xanthodes* is the most common interception from the *B. xanthodes* complex at the New Zealand border, it is more applicable if the real-time PCR assay targeted this species specifically. Thus specific probes and primers for each of the species (fig. 2) were designed to distinguish the three species. In the design, at least three SNPs in the probe with additional SNPs in the primers binding sites were selected to maximize the specificity of each assay (fig. 2). The primers and probes for each of the assays and their PCR conditions are listed in table 3.

Real-time PCR optimization

The real-time PCR assay tested with different temperatures showed that similar *Cq* values were obtained from the temperatures at 56–62°C. Higher *Cq* values were obtained for temperatures above 62°C, thus the optimal annealing/extension temperature was chosen as 62°C. The cycling condition is 95°C for 2 min, followed by 40 cycles of 95°C for 15 s and 62°C for 60 s.

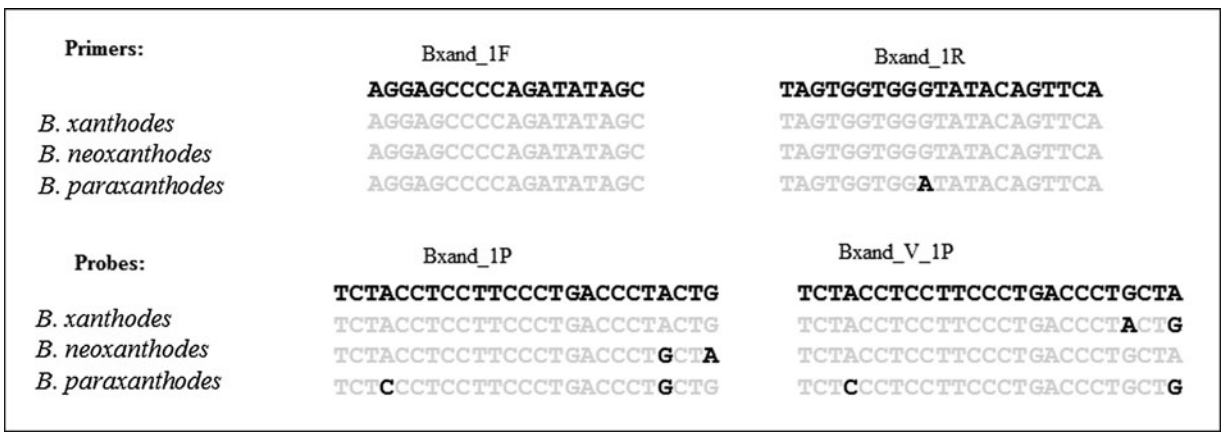

Fig. 1. Alignment of the primers and probe regions for the real-time PCR assay targeting the *Bactrocera xanthodes*, *B. neoxanthodes* and *B. paraxanthodes*. The bold black letters indicate the SNPs in the species to the listed primers/probe.

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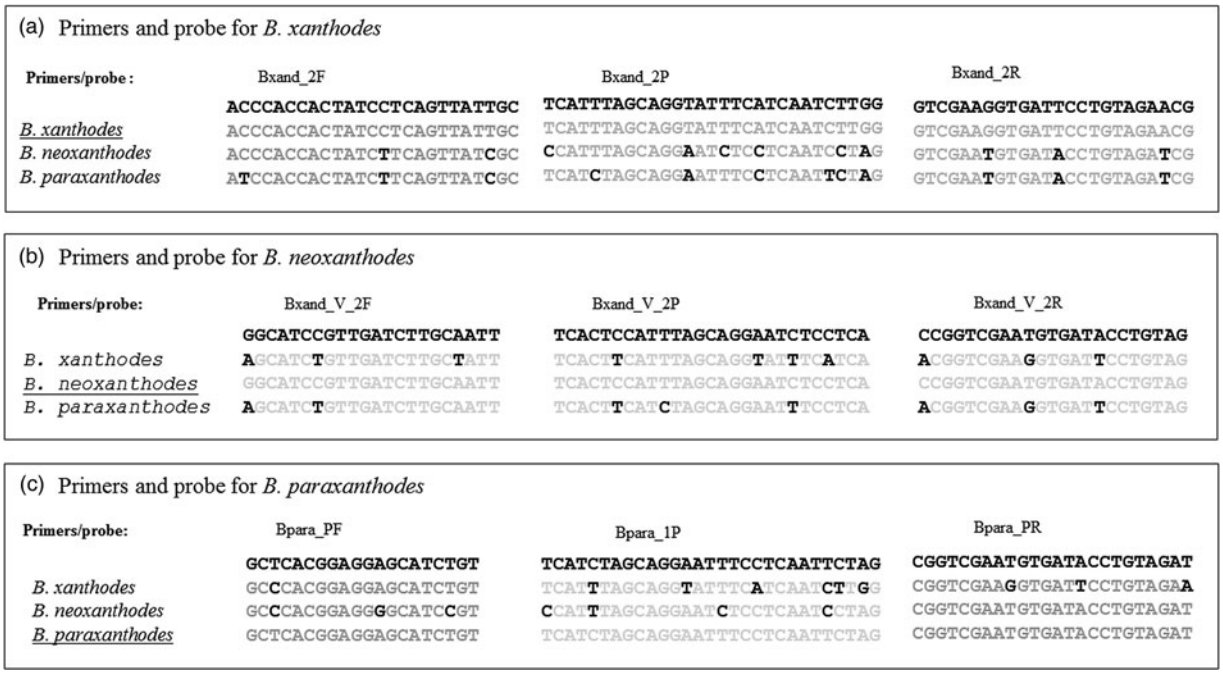

Fig. 2. Alignment of the primers and probe regions of COI sequences from the *Bactrocera xanthodes* complex. The alignment listed all three real-time PCR assays, (a) *B. xanthodes*; (b) *B. neoxanthodes* and (c) *B. paraxanthodes*. The bold black letters indicate the SNPs in the species to the listed primers/probe.

The real-time assay was demonstrated to perform consistently well using SsoAdvanced™ Universal Probes Supermix (BioRad) and PerfeCTa® qPCR ToughMix® (Quanta Bioscience). Similar *Cq* were also obtained for each sample tested although slight intensity increases were observed in the Mastermixes when additional $MgCl_2$ was added. No significant difference in the *Cq* values were observed when the assay was run in singleplex with different concentrations of primers. To maximize the real-time PCR efficiency for each real-time PCR assay, the recommended concentrations for each primer and probe are listed in table 3 and the assay can be run as a singleplex or as a multiplex formats.

Sensitivity of the real-time PCR assay

The linear dynamic range for the assay was tested on three plasmid DNAs containing the COI inserts and extended from 10^7 to 10^0 copies μl^{-1}, respectively. The 95% confidence limits of the linear dynamic range are plotted in fig. 3, with a strong correlation coefficient ($r^2 = 0.997–0.998$). The limit of detection (LOD) for the assay was estimated to be ten copies of the target DNA. A template concentration of one copy μl^{-1} was sporadically detected in all the three assays with an average *Cq* value around 35 cycles. The calibration curves shown in fig. 3 were able to detect 100% of the samples at the ten copies μl^{-1}.

Specificity of the real-time PCR

All the Tephritidae samples listed in table 1 were successfully amplified by the real-time PCR assays and no amplifications were observed in the non-target species (table 2). The assays were able to accurately identify *B. xanthodes*, *B. neoxanthodes* and *B. paraxanthodes* samples, respectively. No cross-reaction was observed among the three target species and their closely related species, *B. passiflorare* from Fiji, *B. melanotus* from the Cook Islands and other *Bactrocera* species from other Pacific countries (tables 2 and 4). No cross-reaction was observed from other commonly intercepted fruit fly species (tables 2 and 4), including Tephritidae species from genera *Bactrocera*: *B. correcta*, *B. cucumis*, *B. dorsalis*, *B. jarvisi*, *B. neohumeralis*, *B. oleae*, *B. tryoni*, *B. zonata*; *Anastrepha*: *Anastrepha* sp. and *Anastrepha striata*; and *Dirioxa*: *Dirioxa pornia*.

Blind panel test

All the 26 samples in the blind panel test were tested in the real-time PCR protocols. All the target samples were tested positive with *Cq* values <30. No amplification was observed in all the non-target species (table 4).

Discussion

DNA barcoding of B. xanthodes *complex*

This study provided essential DNA barcode information for *B. xanthodes*, *B. neoxanthodes* and *B. paraxanthodes*, which will assist in the identification and differentiation of the three species. Up to now, there are only a handful of sequences labelled as *B. xanthodes* in BOLD and GenBank. However, sequence comparison among these sequences shows around 12% nucleotide difference. Three such '*B. xanthodes*' sequences in GenBank and BOLD are listed as *B. paraxanthodes* in the supplemental materials in the publication of Armstrong & Ball (2005) and the specimens were from Vanuatu. This information has raised the suspicion of the true identities of these sequences because *B. paraxanthodes* is only described from

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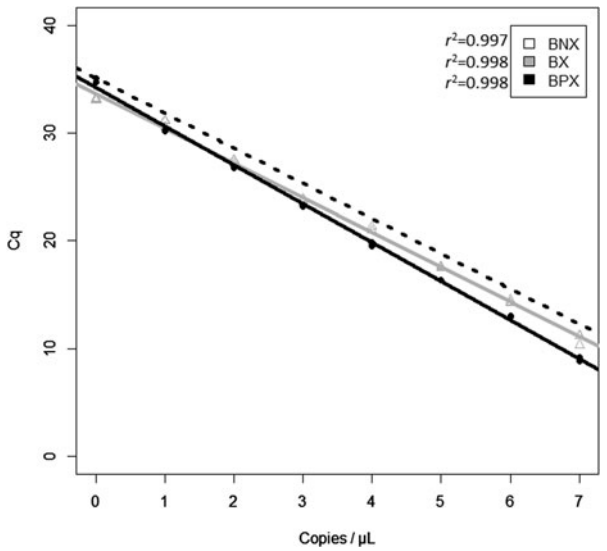

Fig. 3. Sensitivity test of the real-time PCR assay for the identification of the *Bactrocera xanthodes* complex. Plasmid containing COI insert of *B. xanthodes*, *B. neoxanthodes* and *B. paraxanthodes* were series diluted, respectively and tested in each real-time PCR assay separately. The concentrations of the plasmid DNA and the *Cq* values were used to create calibration curves for sensitivity calculations. The standard curve built from *Cq* values against the log copy number (range = 10^7–10^0 copies) of COI insert ($n = 3$). The 95% confidence intervals of the slopes were plotted with a gray line for BX (*B. xanthodes*), a white line with dots for BNX (*B. neoxanthodes*) and a black line for BPX (*B. paraxanthodes*). The $r^2 = 0.997$ for BNX, 0.998 for BX and BPX were obtained for each assay, respectively.

New Caledonia (Drew *et al.*, 1997) but *B. neoxanthodes* from Vanuatu (Drew & Romig 2001). Besides the divergences of the COI sequences submitted as *B. xanthodes*, there are errors in some of the submitted *B. xanthodes* sequences in GenBank. The sequences listed under accession numbers KT864778-KT864780 (Dhami *et al.*, 2016) in Genbank are recorded as *B. xanthodes*, but comprehensive analysis reveals that only KT864778 is *B. xanthodes*, the other two (KT864779 and KT864780) are sequences of *B. passiflorae*. On the other hand, KT864761-KT864763 deposited as *B. passiflorae* shared 100% sequence identities with *B. xanthodes* (Dhami *et al.*, 2016). Since the six sequences were submitted by the same submitters, there is the possibility of mislabelling of the sequences. In addition, sequence KT595001, submitted as *B. xanthodes* for voucher ms0896 (Leblanc *et al.*, 2015), has only ~40% sequence identity to *B. xanthodes*. The specimen was collected from Rurutu (French Polynesia) (Leblanc *et al.*, 2015), which, to our knowledge, has no record of *B. xanthodes* though it had been detected in Raivavae (French Polynesia) but subsequently eradicated (Leblanc *et al.*, 2012; Plant Health Australia, 2016). A BLAST search of this COI sequence (KT595001) showed that the closet matches are 87% to several *Bactrocera* species, thus the true identity for this specimen needs to be determined. Therefore, this study provided the reference DNA barcode sequences for *B. xanthodes* from Fiji, Samoa and Tonga, *B. neoxanthodes* from Vanuatu and *B. paraxanthodes* from New Caledonia, which further clarify the COI sequence difference among the three species.

The barcode library submitted to BOLD, including 44 *B. xanthodes*, 3 *B. neoxanthodes* and 2 *B. paraxanthodes* sequences, which further enriched the DNA barcode sequences for the species complex. Most of the *B. xanthodes* specimens were intercepted from breadfruit except two larvae which were detected in watermelon (*Citrullus lanatus*) from Tonga (table 1). Watermelon as a host for *B. xanthodes* was first reported at the New Zealand border in 1985 (Cowley *et al.*, 1991) and was questioned at the time. Although the two larvae were intercepted from the watermelon recently, whether watermelon is the host needs further investigation. In addition, this study has provided the DNA barcodes for *B. paraxanthodes* from New Caledonia and *B. neoxanthodes* species from Vanuatu. Therefore this study extended the DNA barcode capability for the identifications of *B. xanthodes* species complex. Unfortunately, no specimen/s and further study on the undescribed *B.* sp. No 1 (Drew *et al.*, 1997) were available, therefore we are unable to provide any further information about this potential species. Further research on *B.* sp. No 1 (Drew *et al.*, 1997) will be conducted if the specimen/s is available, which will reveal the relatedness of this species in the *B. xanthodes* complex.

The optimization and application of the real-time PCR assays

There are real-time PCR assays developed for rapid detection of a number of fruit fly species, however, no real-time PCR protocol for *B. xanthodes* complex have been reported. For the first time, this study developed and validated the real-time TaqMan PCR assays for the detection of the *B. xanthodes* complex and for the each of the three described species of the complex. Real-time PCR assays for the detection of *B. xanthodes*, *B. neoxanthodes* and *B. paraxanthodes* and to distinguish each species individually were developed and validated. The assay targeting the three species can be used if suspecting either of them, whereas the assay targeting each can be used separately. Tephritidae samples are regularly intercepted at the New Zealand border every year and all are immature life stages, mainly eggs and larvae, and cannot be identified to genus or species level morphologically. Most of *B. xanthodes* interceptions were from cooked breadfruit, thus the immature stage samples could not be reared to adult for morphological identification. Molecular identification is needed to identify the samples, which typically consists of PCR, gel electrophoresis and sequencing, and is time consuming. The real-time PCR assays developed in this study provide a rapid means for identifying the interceptions, are conformant with the MIQE guidelines for qualitative assays (Bustin *et al.*, 2009). High specificity and sensitivity are much desired in border diagnostics and the assays showed high efficiency and sensitivity in detecting the target species. In the specificity test, the closely related species and the common interceptions of *Bactrocera* species (tables 1 and 2) were used for validation of the real-time PCR assays and no cross-reactions were observed. The assays also demonstrated their sensitivities by being able to detect ten copies μl^{-1} of target DNA (fig. 1).

The application of molecular identification for the diagnostics of *Bactrocera* species has increased in recent years (Armstrong *et al.*, 1997; Jamnongluk *et al.*, 2003; Armstrong & Ball, 2005; Chua *et al.*, 2010; Asokan *et al.*, 2011; Blacket *et al.*, 2012; Jiang *et al.*, 2013, 2014; Meeyen *et al.*, 2014; Dhami *et al.*, 2016; Kunprom & Pramual, 2016). Among those molecular diagnostics, PCR-RFLP have been used for the detection of a number of fruit fly species (Armstrong *et al.*, 1997; Chua *et al.*,

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2010) and could be used for *B. xanthodes* species (Armstrong *et al*., 1997). However, the PCR-RFLP tests can be vulnerable to false negatives if not all population-level variation had been accounted for, and also false positives if the sequences for closely related species are not available during the method development. For the PCR-RFLP assay for *B. xanthodes*, no sequences were available for the closely related species, *B. neoxanthodes* and *B. paraxanthodes* when the assays were developed, thus further validation is needed to avoid false positives. Furthermore, DNA barcoding can provide an accurate identification for *B xanthodes* to species level, however, this approach is time-consuming and typically takes up to 4 days to receive the results and often depends on an external facility to provide sequence data. In addition, species-specific PCR was also used for the identification of *Bactrocera* species, such as *B. tau*, *zonata* and *correcta* (Asokan *et al*., 2011; Jiang *et al*., 2013), but no *B. xanthodes*-specific PCR has been reported. This method normally targets smaller PCR fragment, which will effectively amplify degraded DNA, however it involves the post PCR steps, such as gel electrophoresis. In comparison, the real-time PCR protocol can provide rapid identification and no post PCR steps are needed, thus it can provide a rapid identification. Therefore the assay developed for targeting *B. xanthodes* complex in this study will allow for rapid detection and greatly assist the morphological diagnostics at New Zealand borders.

Conclusion

Here, we report the DNA barcoding for the fruit fly species, *B. xanthodes*, *B. neoxanthodes* and *B. paraxanthodes*. This is the first DNA barcoding sequences of the COI gene for *B. neoxanthodes* and *B. paraxanthodes*, which enriches the reference sequences for *Bactrocera* species in BOLD and GenBank databases. The real-time PCR assays developed for *B. xanthodes* species complex in this study are suitable for routine application by diagnostic and research agencies, for facilitating exports and imports. This assay has been fully optimized for immediate deployment at New Zealand border and has the potential to be applied in the detection of the target species in other countries around the world.

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