

Detection of environmental DNA of the Japanese orange fly, *Bactrocera tsuneonis* (Diptera: Tephritidae), from immature mandarin orange fruits

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Abstract

Early detection of insect pest outbreaks is critical for effective pest control. However, this is challenging, especially for pest species such as the Japanese orange fly, *Bactrocera tsuneonis* (Miyake) (Diptera: Tephritidae), whose larvae grow inside fruits. *B. tsuneonis* is a univoltine citrus pest, with females laying their eggs directly into the fruits during summer, and larvae develop inside the fruits until pupation in early winter. To monitor *B. tsuneonis* outbreaks, we developed and validated methods for the detection of environmental DNA (eDNA) in immature fruits collected during summer using quantitative real-time PCR. eDNA samples were prepared from individual fruits with oviposition pinholes left by gravid females during ovipositor probing and oviposition. By immersing the fruits in distilled water for 1 h to extract eDNA, *B. tsuneonis* eDNA was detected in 20% of the fruits. Additionally, *B. tsuneonis* eDNA was detected in a fruit without oviposition pinholes, albeit at a low rate, suggesting that residual eDNA on fruit surfaces during mating and searching for oviposition sites can be detected. While there remains a possibility of cross-contamination between infected and uninfected fruits due to the lack of negative controls during eDNA extraction, we conducted preliminary field examination in several orchards where *B. tsuneonis* was either visually observed or not. *B. tsuneonis* eDNA was detected even at a location where adults were not observed visually, but were captured using plastic tub traps, indicating a low population density. Thus, our method has practical applicability in various areas, regardless of *B. tsuneonis* population density.

Keywords Environmental DNA · Quantitative real-time PCR · Surveillance · Infected fruit · Detectability · Fruit fly

Introduction

Early detection of pest outbreaks is crucial for effective pest management, allowing for rapid responses, such as targeted pesticide application in infested areas. Therefore, various methods have been developed and applied to detect pest species outbreaks. The use of sex pheromones and attractants is prevalent in the surveillance of pest fruit flies (Shelly 2010; Tan et al. 2014; Ono et al. 2021). For example, male attractants, such as methyl eugenol and cue-lure, have been used successfully to monitor and control several notorious tephritid fruit flies, including the Oriental fruit fly, *Bactrocera dorsalis*, and the melon fly, *Zeugodacus cucurbitae* (Shelly 2010). These compounds exhibit a strong specificity in attracting their respective targeted species, but their utility for other species is limited.

The Japanese orange fly, *Bactrocera tsuneonis* (Miyake) (Diptera: Tephritidae), is a univoltine pest of citrus fruits, particularly mandarin oranges (Yasumatsu and Nagatomi 1959; Weems, Jr. and Fasulo 2002; Plant Health Australia 2018). In Japan, *B. tsuneonis* was initially restricted to a limited area in the southern island of Kyushu, but it has recently expanded to adjacent areas (Kanazaki et al. 2011; Mochizuki and Narahara 2022). The adults typically emerge between June and September (Narahara et al. 2007). As the adult flies mature, young fruits become mating sites for *B. tsuneonis*. After mating, gravid females exhibit ovipositor-probing behavior on immature fruits before oviposition and subsequently lay their eggs directly in appropriate fruits (Fig. S1a). The oviposition pinholes (c.a. 0.25-0.5 mm diameter) could be observed on the surface of fruits in the orchards where *B. tsuneonis* occurs (Fig. S1b, c). However, it is difficult to locate them visually in the field because of their small size. Hatched larvae feed internally until

maturation and subsequently emerge from mature fruits to pupate in the soil, mainly at the beginning of winter, from mid-October to mid-January. Although many *Bactrocera* species are attracted to male attractants such as methyl eugenol and cue-lure, males of *B. tsunoneis* are unresponsive to these known male attractants (Plant Health Australia 2018) and no sex pheromones have been identified for this species (Ono et al. 2020). Currently, *B. tsunoneis* is monitored in Japan using traps baited with a liquid protein hydrolysate that non-specifically attracts fruit flies during the summer (Mochizuki and Narahara 2022; Mochizuki et al. 2024). Monitoring traps have significantly enhanced their effectiveness but remain insufficient to reliably detect flies in areas with a low-density population of *B. tsunoneis*.

Environmental DNA (eDNA) analysis has been developed as a biological monitoring method to detect trace amounts of DNA in environmental samples, particularly in aquatic systems (Ficetola et al. 2008; Jerde et al. 2011; Minamoto et al. 2012). Recently, several studies have reported methods for detecting the eDNA of arthropods, including herbivorous insects from terrestrial plants (Valentin et al. 2018, 2020; Thomsen and Sigsgaard 2019; Kudoh et al. 2020; Yoneya et al. 2023). Notably, by rinsing the fruits, eDNA of the target pest species was extracted and detected from the rinsed water. (Valentin et al. 2018). Similarly, the simple method of spraying water onto crops enables the extraction of arthropod eDNA from crops and subsequent metabarcoding analysis (Yoneya et al. 2023). Here, we developed a method to detect *B. tsunoneis* eDNA from immature Satsuma mandarin orange fruits (*Citrus unshiu*) in the summer using quantitative real-time PCR (qPCR). We designed PCR primers and a fluorescent probe from the sequences of mitochondrial cytochrome C oxygenase subunit 2. We developed and validated an experimental design focusing on the efficacy of DNA

extraction methods and detectability of the target DNA. Using this established method, we assessed the detectability of *B. tsuneonis* in fruits collected from orchards. While there is a possibility of cross-contamination between infected and uninfected fruits due to the lack of negative controls during eDNA extraction, we conducted preliminary field examination in several orchards where *B. tsuneonis* was either visually observed or not. We also examined whether *B. tsuneonis* eDNA could be detected from a mixture of one fruit with oviposition pinholes and four uninfected fruits collected from an orchard where *B. tsuneonis* was absent to improve survey efficiency.

Materials and methods

DNA extraction from *B. tsuneonis*, PCR amplification of the target DNA, and cloning of the PCR product

Total DNA was prepared from the whole body of male *B. tsuneonis* using a DNeasy Blood and Tissue Kit (Qiagen, Crawley, UK). The DNA of *B. tsuneonis* was amplified using species-specific primers, Bt-COII-F and Bt-COII-R (Table 1), designed from the mitochondrial cytochrome oxidase subunit 2 gene region (AB095918) as per a previous study (Nakahara et al. 2005). To validate the amplification of the targeted fragment, the PCR product was cloned into a pGEM-T vector (Promega, WI, USA), and the clone was sequenced using T7 and SP6 universal primers. To test the limit of detection for eDNA, the cloned vector was linearized with NotI and purified using gel electrophoresis. The linearized vector was quantified by UV absorbance and a dilution series was prepared.

Collection of eDNA from Satsuma mandarin orange fruits

Satsuma mandarin orange fruits were collected from several abandoned orchards at three

sites (Oita-A, Oita-B, and Oita-C) in Oita Prefecture and at one site (YM) in Yamaguchi Prefecture, Japan (Mochizuki and Narahara 2022; Opadith et al. 2022), from early to mid-August 2022 and 2024. Uninfected Satsuma mandarin orange fruits were collected from orchards in Kyoto and Shizuoka prefectures, where *B. tsuneonis* has not been found. In the abandoned orchards, fruits were inspected visually for the presence or absence of oviposition pinholes. The fruits were then covered with ziplock plastic bags (170 mm × 120 mm; UNI-PACK F-4, Seisannippon-sya Ltd., Japan) and picked by tearing them off the branches without direct hand contact. Gloves were worn during collection to prevent trace DNA contamination. The fruits were used for eDNA extraction within five days of collection.

Before eDNA extraction, all possible equipment, including plastic products and centrifuge rotors, was bleached for more than 30 min. Lab benches and the interiors of the centrifuges were wiped with DNA-OFF (Takara, Shiga, Japan), and micropipettes were cleaned with DNA-OFF after each use. The micropipettes used to handle the eDNA were kept separate from those used for other purposes. All procedures were conducted on aluminum foil placed on a laboratory bench while wearing gloves. eDNA extraction and experiments involving high concentrations *B. tsuneonis* DNA, such as DNA extraction from bio-tissue samples and purification of PCR amplicons, were conducted at different locations on the lab bench. During each extraction, no more than six samples were processed, including an uninfected fruit used as a negative control, to ensure that there was no cross-contamination, except during field examinations in several orchards. One or five fruits were immersed in 100 mL or 200 mL of distilled water, respectively, in plastic bags for 1 h, unless otherwise noted (Fig. S2a). The immersion water was filtered through Sterivex (0.45 µm-pore size, Merck Millipore, Darmstadt, Germany) using a 50-

mL syringe. The Sterivex filters were then individually placed either on the lid or the bottom of a plastic petri dish to prevent cross-contamination. Extraction of eDNA absorbed on the Sterivex filters was performed using a DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) based on the method described in the Environmental DNA Sampling and Experiment Manual (version 2.1) (Minamoto et al. 2021), with a slight modification. Any remaining water within the filter was thoroughly removed using a vacuum pump (DTC-21, Ulvac, Japan). To prevent contamination, a disposal joint consisting of a plastic tube and tips was used to connect the pump to the filter, which was then replaced for each sample (Fig. S2b, c). After the removal of water, 440 μ L of lysis mix comprising 220 μ L PBS (137 mM NaCl, 2.68 mM KCl, 10.1 mM Na₂HPO₄, 1.76 mM KH₂PO₄; pH 7.2), 200 μ L Buffer AL (Qiagen), and 20 μ L proteinase K (Qiagen) was loaded on the Sterivex filter. The Sterivex filter was incubated with the lysis mix at 56 °C for 10 min in a ziplock plastic bag (UNI-PACK F-4) (Fig. S2d), and subsequently, connected to a 2-mL microtube using adhesive tape and placed in a 50 mL-falcon tube (Fig. S2e, f). The falcon tube was then centrifuged at 6,000 $\times$ g for 1 min. The eluate from the Sterivex filter was mixed with 440 μ L of ethanol. The mixture was loaded onto a DNeasy Mini spin column (Qiagen) and centrifuged at 6,000 $\times$ g for 1 min. Subsequently, the column was washed with buffers and the eDNA was eluted in 100 μ L of buffer AE, according to the manufacturer's protocol. The samples were stored at -20 °C until further use.

Quantitative real-time PCR analysis

The DNA of *B. tsuneonis* was amplified using a pair of primers (Bt-COII-F and Bt-COII-R) and probe (Bt-COII-probe) (Table 1). 2 μ L of DNA sample was subjected to PCR

amplification using TaKaRa Taq HS PCR Kit UNG plus (Takara) on a Thermal Cycler Dice Real Time System (Takara). The reaction condition was 25 °C for 15 min, 95 °C for 30 sec, followed by 50 cycles each of 95 °C for 5 sec and 58 °C for 30 sec. PCR experiments were designed according to the Environmental DNA Sampling and Experiment Manual (version 2.1) (Minamoto et al. 2021). Briefly, PCR was performed in triplicates. Any eDNA sample was considered positive if at least one of the three replicates was positive. Uninfected fruit samples collected from *B. tsuneonis* non-distributed areas served as negative controls for all qPCR assays, with no amplification signal observed in the three wells used for each control. Some PCR products were purified and sequenced using the primers Bt-COII-F and Bt-COII-R.

Statistical analysis

Statistical analyses were conducted using R software (version 4.2.1; <https://www.r-project.org/>). Plots based on the fitted linear model were generated using ggplot2 and ggpmisc.

Results

Development and validation of experimental design

We designed PCR primers and a fluorescent probe to specifically amplify the *B. tsuneonis* DNA. Next, we cloned the resulting PCR product in a cloning vector using the designed PCR primers, followed by linearization and purification of the vector. Thereafter, we examined the limit of detection using PCR primers and fluorescent probe in real-time PCR by targeting a dilution series of the linearized vector containing the cloned PCR product of *B. tsuneonis* DNA. The dilution series ranging from 1×10^0 to 1×10^6 copies

per 20-μL PCR reactions were tested in triplicates. Amplification was consistently observed at copy numbers ranging from 1×10^2 to 1×10^6 (Fig. S3). Additionally, ten copies showed amplification in one out of three replicates whereas one copy exhibited no amplification.

To examine the detectability of the eDNA using PCR primers and a fluorescent probe, mandarin fruits with oviposition pinholes of *B. tsuneonis* were collected from an unmanaged citrus orchard where adults were visually observed in YM. The fruits were immersed in water for 1 h or 18 h to extract eDNA. We detected the *B. tsuneonis* eDNA in 20% ($n = 15$) and 33% ($n = 15$) of individual fruits immersed in water for 1 h and 18 h, respectively (Table 2). The amplification rates varied among the samples, with Ct values ranging from 30.2 to 40.8, and the calculated copy numbers based on the Ct values ranging from 89.8 to 2.62×10^4 . *B. tsuneonis* eDNA was more frequently detected in fruits immersed in water for 18 h, but there was no significant difference in the detection rate between the different immersion times (Fisher's exact test, $p = 0.682$). Therefore, we considered that immersion for 1 h was sufficient to extract eDNA from the fruit surface.

Detection of *B. tsuneonis* eDNA in individual mandarin fruits without observable oviposition pinholes

B. tsuneonis females frequently lick the surface of mandarin fruits during their search for suitable oviposition sites but sometimes leave without ovipositing in the field. Therefore, we investigated the possibility of detecting eDNA in individual mandarin fruits in which oviposition pinholes were not observed. We detected *B. tsuneonis* eDNA in one of 10 fruits (amplification rate: 1/3; Ct value: 41.9) collected from unmanaged citrus orchards where *B. tsuneonis* had been visually observed in YM. Although the detection rate (10%)

was low, it indicated the detectability of eDNA on the fruit surface, even in cases where *B. tsuneonis* had not oviposited.

Detection of *B. tsuneonis* eDNA from a mixture of infected and uninfected fruits

To improve survey efficiency during the inspection of fruits infected by *B. tsuneonis*, we considered it advantageous to process multiple fruits collected from a field together to determine whether they contain any infected fruit. Therefore, one fruit with oviposition pinholes was placed along with four uninfected fruits in a plastic bag. DNA samples were prepared after immersing the fruits in water. *B. tsuneonis* eDNA was detected in one of the 14 fruits (amplification rate: 1/3; Ct value: 40.9).

Preliminary field examinations in several orchards

We examined whether eDNA could be detected in individual mandarin fruits with oviposition pinholes collected from fields with either high or low population densities. It is important to note that uninfected fruit was not included as a negative control during the eDNA extraction process in these experiments. Therefore, there remains a possibility that cross-contamination may have occurred between infected and uninfected fruits.

Mandarin fruits were collected from four unmanaged citrus orchards. Adult *B. tsuneonis* were visually observed in three sites (Oita-A, Oita-B, and YM), indicating a high population density of the fruit fly. Adults were not visually observed during collection at the Oita-M location, however, they were captured using plastic tub traps baited with protein hydrolysate, thus indicating a low population density of *B. tsuneonis* in that area. DNA samples were prepared within 3 days of collection. We detected *B. tsuneonis* eDNA in fruits collected from all the orchards. The detection of *B. tsuneonis*

eDNA ranged from 60% to 80% of fruits in each orchard, with an average detection rate of 65.7% ($n = 35$) (Table 3). In the Oita-A and Oita-B locations, the detection rates of *B. tsuneonis* were 70% and 60%, respectively. Among the fruits in which DNA was detected, nine out of 13 exhibited PCR amplification in all three replicates. The highest detection rate of 80% was observed at the YM location, but only one out of the three replicates exhibited PCR amplification in half of the detected samples. At the Oita-M location, where adults were not visually observed, *B. tsuneonis* eDNA was detected in six out of 10 samples, with only one out of three replicates showing PCR amplification in half of the detected samples. It should be noted that the high detection rates may be partially derived from cross-contamination. However, since we extracted eDNA simultaneously from fruits collected at the same locations and did not include those from different locations, any potential cross-contamination would likely be limited to samples collected within the same location. Thus, these results still indicate that *B. tsuneonis* eDNA could be detected in fruits with oviposition pinholes collected from the fields, regardless of whether the adults were visually observed.

Sequence analysis of the PCR amplicons

For samples in which *B. tsuneonis* eDNA was detected, one of the PCR amplicons from the three replicates was sequenced and analyzed. The amplicon sequences, excluding the primer regions, matched with the reference sequence (AB05918) at identities of 100 or 98.5%. A nucleotide BLAST search on the corresponding reference sequence with a threshold of $1e-10$ identified *B. tsuneonis* sequences in the top seven hits, with identities ranging from 100% to 97.1%. Additionally, sequences from three other dipteran species, *Cochliomyia hominivorax*, *Rhinomorinia sarcophagina*, and *Bactrocera unistriata*,

showed identities from 90.8% to 87.9%; however, to our knowledge, none of these species have been reported in Japan. We confirmed that the amplified sequences did not align with those of related species within the Tribe Dacini distributed in Japan and/or China (Fig. S4).

Discussion

The use of PCR to identify or distinguish closely related species has been reported in multiple tephritid fruit flies, mainly as a diagnostic tool for quarantine applications (Armstrong et al. 1997; Yu et al. 2004, 2005; Jiang et al. 2014; Koohkhezade et al. 2018; Li et al. 2019; Zheng et al. 2019). However, no challenges using PCR techniques have been reported for the early detection of outbreaks in difficult-to-control tephritid fruit flies. In the present study, we attempted to detect *B. tsuneonis* eDNA using quantitative qPCR in fruits collected from the fields where the distribution of *B. tsuneonis* had been confirmed. We found that the target DNA with a copy number of 1×10^2 could be consistently detected using the designed primers and probe. Additionally, 10 copies were detected in one of the three replicates. The remarkable sensitivity of qPCR was in agreement with a previous study, indicating that the threshold for detecting target DNA in tephritid fruit flies can be as low as 10 copies (Li et al. 2019). Since eDNA samples extracted from fruits may contain inhibitors, the efficiency of PCR amplification is expected to be lower than that observed in experiments using plasmid samples. Nevertheless, *B. tsuneonis* eDNA was detected in fruits collected from orchards, where *B. tsuneonis* had been both visually observed and not observed, indicating the practicality of our method.

Because gravid females insert their ovipositors to lay eggs, thereby leaving the

oviposition pinholes in the fruits, we initially predicted that *B. tsuneonis* eDNA could be extracted from the oviposition pinholes and eggs. However, *B. tsuneonis* eDNA was detected in fruits even without oviposition pinholes, although at a relatively low detection rate. Notably, the young fruit serves as a mating site for *B. tsuneonis*. The detailed behavioral patterns have been reported in a closely related species, *B. minax* (Dong et al. 2014). Furthermore, *B. tsuneonis* adults often lick the surface of mandarin fruits. Considering these behaviors of *B. tsuneonis* on fruits, we inferred that *B. tsuneonis* eDNA was primarily extracted from the fruit surfaces rather than from the oviposition pinholes and eggs.

Initially, the fruits were immersed in water for 18 h to ensure a thorough extraction of *B. tsuneonis* eDNA. However, we found that immersing the fruits for 1 h was sufficient to detect *B. tsuneonis* eDNA. Notably, previous studies have shown that a simple rinse with water is sufficient for eDNA extraction from terrestrial plant materials such as fruits, trees, and vegetables (Valentin et al. 2018, 2020; Yoneya et al. 2023). Given our inference that *B. tsuneonis* eDNA can be extracted from fruit surfaces, we propose that a shorter extraction time, including a simple rinse, may be sufficient to extract the DNA.

B. tsuneonis eDNA was detected in some, but not all, fruits with oviposition holes. Previous studies have revealed that environmental factors, such as DNA degradation by UV solar radiation and DNA elution caused by rainfall, can affect the persistence of eDNA on the surface of terrestrial plants (Valentin et al. 2021). Furthermore, eDNA on the surface of fruits may degrade over the days following contact with the flies. Thus, various environmental and biological factors in the fields may reduce the detectability of *B. tsuneonis*. Therefore, it is necessary to collect fruits from various

locations within the field for surveillance. We further found that *B. tsuneonis* eDNA could be detected in a mixture of fruits, consisting of one infected and four uninfected fruits. The possibility of eDNA detection from multiple fruits indicates an improvement in survey efficiency, however, it also raises technical challenges, such as the risk of filter clogging due to the increased volume of immersion water. To accommodate the simultaneous treatment of a higher number of fruits, it is essential to consider experimental conditions, such as allocating a portion of the immersion water for DNA preparation.

In Japan, the distribution of *B. tsuneonis* is limited to areas within the Oita Prefecture on Kyushu Island. However, the distribution of *B. tsuneonis* has now expanded to areas where it was previously absent (Kanazaki et al. 2011; Mochizuki and Narahara 2022; Mochizuki et al. 2024). To survey *B. tsuneonis* infection, a common and useful practice is to cut mature fruits to search for larvae (Kanazaki et al. 2011; Mochizuki et al. 2024), but this method is laborious and time-consuming. Moreover, it only detects damage close to the harvest season when the larvae have reached a certain size, making early detection of outbreaks difficult. Currently, trap surveys are employed during the summer to monitor *B. tsuneonis* outbreaks (Mochizuki and Narahara 2022; Mochizuki et al. 2024). Ideally, this monitoring method using traps should reliably detect *B. tsuneonis* even in areas with a low population density. However, this has not been the case due to the lack of a strong attractant for this species. Therefore, a combination of trap surveys and eDNA detection would ensure the detection of *B. tsuneonis* outbreaks. Field-testing for eDNA detection in areas with varying population densities, whether high, low, or unknown, will provide insights into its practicality. Moreover, a comparison of the results from trap surveys and eDNA detection will provide valuable information regarding *B.*

tsuneonis populations in the fields.

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Data availability Datasets generated during the current study are available from the corresponding author on reasonable request.

Conflict of interest The authors declare no conflicts of interest or competing interests.

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432

1 **Table 1** Primers and TaqMan probe used for quantitative RT-PCR

2	Bt-COII-F	5'-AGTCCCAACAAATGAACTACCA-3'
3	Bt-COII-R	5'-CGTCTGCGGCTCTTACTAAG-3'
4	Bt-COII-probe	5'-FAM-TCCTACCACTAAACTCACAAATTCGA-BHQ-3'

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Table 2 Amplification rates and Ct values of samples following 1 h or 18 h of DNA extraction.

Extraction time	Sample No.	Amplification rate	Ct $\pm$ SD
1 h	10	3/3	40.4 $\pm$ 1.46
	11	3/3	36.6 $\pm$ 0.12
	14	3/3	37.3 $\pm$ 0.13
18 h	4	1/3	40.7
	9	2/3	40.8 (40.6; 41.0)
	10	3/3	40.3 $\pm$ 1.04
	11	1/3	40.0
	12	3/3	30.2 $\pm$ 0.18

Samples in which PCR amplification was observed in triplicates are indicated ($n = 15$ for 1 h extraction; $n = 15$ for 18 h extraction). Average Ct values are shown. In cases where amplification was observed in all three replicates, SD values are presented. In cases where amplification was observed in two out of three replicates, both Ct values are shown in parentheses.

Table 3 Amplification rates and Ct values of samples collected from several orchards.

Location	Sample No.	Amplification rate	Ct $\pm$ SD
Oita-A (+)	1	3/3	37.8 $\pm$ 0.29
	2	3/3	43.1 $\pm$ 2.04
	4	3/3	37.8 $\pm$ 0.61
	5	3/3	36.6 $\pm$ 0.42
	6	3/3	36.3 $\pm$ 0.14
	7	1/3	36.5
	10	2/3	41.8 (40.7; 43.0)
Oita-B (+)	2	3/3	39.9 $\pm$ 0.70
	3	3/3	33.7 $\pm$ 0.13
	6	3/3	39.6 $\pm$ 0.31
	7	2/3	40.6 (40.8; 39.4)
	8	2/3	40.0 (40.4; 40.6)
	9	3/3	37.7 $\pm$ 0.28
	10	1/3	39.5
Oita-M (–)	3	3/3	39.4 $\pm$ 0.24
	5	2/3	39.0 (39.8; 38.3)
	6	1/3	40.0
	7	3/3	39.2 $\pm$ 0.35
	9	1/3	40.9
	10	1/3	39.5
	10	1/3	39.5
YM (+)	1	3/3	39.6 $\pm$ 0.65
	2	1/3	41.6
	3	2/3	39.2 $\pm$ 0.58
	4	1/3	39.9

In the locations, the signs, ‘+’ or ‘–’, in parentheses indicate whether *B. tsuneonis* was visually observed or not. Samples in which PCR amplification was observed in triplicates are indicated ($n = 10$ for Oita-A; $n = 10$ for Oita-B; $n = 10$ for Oita-M; $n = 5$ for YM). Average Ct values are shown. In cases where amplification was observed in all three replicates, SD values are presented. In cases where amplification was observed in two out of three replicates, both Ct values are shown in parentheses.

Supplementary material for:

Detection of environmental DNA of the Japanese orange fly, *Bactrocera tsuneonis* (Diptera: Tephritidae), from immature mandarin orange fruits

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Supplementary Figure Captions

Fig. S1 *Bactrocera tsuneonis* female and oviposition pinhole. **a** A female inserting its ovipositor into an immature mandarin fruit. **b** A mandarin fruit with an oviposition pinhole. The arrow indicates the pinhole. **c** An oviposition pinhole. Scale bar: 1 mm. Each fruit in the pictures is different.

Fig. S2 Pictures showing the eDNA extraction procedure. **a** Extraction of eDNA from individual fruits. **b,c** Connection of the membrane filter to a vacuum pump to remove

residual water from the filter. In practice, the membrane filter was placed on a disposal plastic dish to prevent any contamination. **b** A disposal joint consisting of a plastic tube and tips. **c** Connection of membrane filter to the vacuum pump. **d** A Sterivex filter placed on a disposal plastic dish inside a ziplock plastic bag after removal of water. **e** A Sterivex filter connected to a 2-mL microtube using adhesive tape. **f** A Sterivex filter connected to a 2-mL microtube placed inside a 50 mL-falcon tube.

Fig. S3 The standard linear regression line of the quantitative real-time PCR to detect eDNA of *Bactrocera tsuneonis*. Plasmid containing the partial sequence of mitochondrial cytochrome C oxygenase subunit 2 was 10 times serial diluted and tested in each quantitative real-time PCR experiment. The standard curve built from Ct values against the log copy number (range = 10^6 - 10^2 copies / 2 μ l) of the plasmid ($n = 3$). Based on statistics, $R^2 = 0.98$; slope = -4.3 ; y-int = 49.2.

Fig. S4 Alignment of the mitochondrial cytochrome oxidase subunit 2 gene region among *Bactrocera tsuneonis* and related species within the Tribe Dacini distributed in Japan and/or China. Categories include: 1) Species distributed in Japan, 2) Species distributed in China but not in Japan, 3) Species not established in Japan but occasional incursions, and 4) Recently invasive species now established in Okinawa Prefecture, Japan. Underlines indicate the PCR primer sites. GenBank accession numbers are as follows: NC_014402 for *B. minax*, NC_008748 for *B. dorsalis*, NC_029466 for *B. latifrons*, AB192454 for *Zeugodacus depressa*, NC_027254 for *Z. scutellate*, and NC_016056 for *Z. cucurbitae*.

Fig. S1

(a)



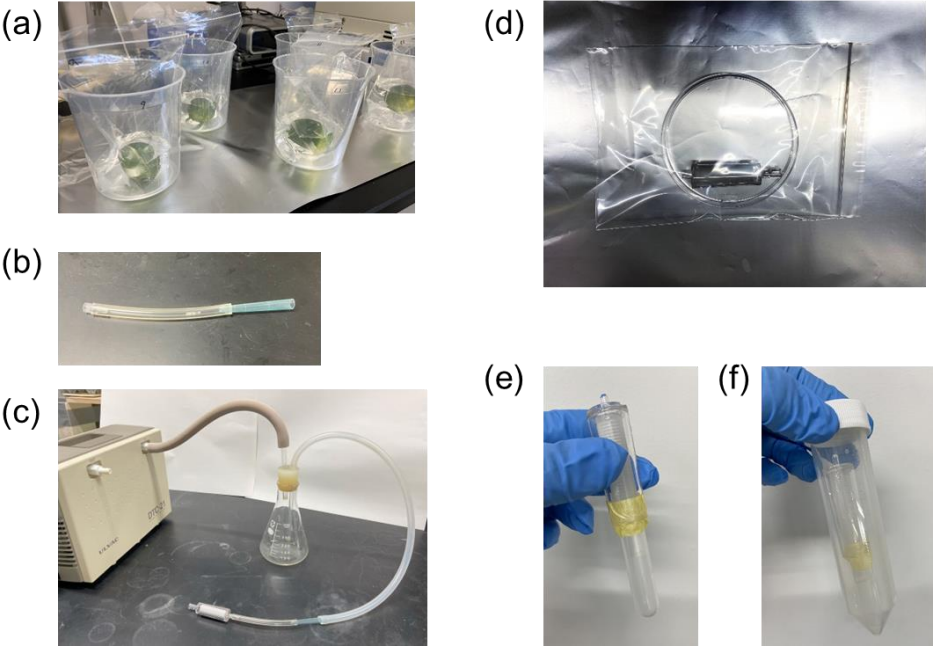
(b)



(c)

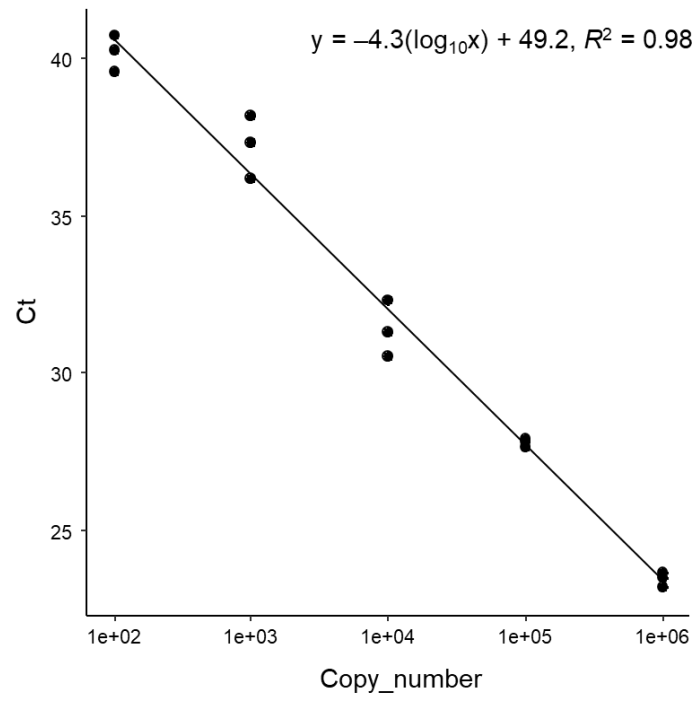


Fig. S2



50
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Fig. S3



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Fig. S4

Bactrocera tsuneonis ¹⁾	<u>AGTCCCAACAAATGA</u> ACTACCAACAGATGGATTTCGTTTACTAGACGTTGATAAT
Bactrocera minax ²⁾	AATTCCAACCAATGAATTACCAACAGACGGATTTCGACTACTAGACGTTGATAAT
Bactrocera dorsalis ³⁾	AGTTCCAACCTAATGAATTAGCAACAGACGGATTCCGACTTCTAGATGTTGACAAC
Bactrocera latifrons ⁴⁾	AGTGCCAACAAATGAATTAACCGCAGATGGATTCCGATTACTTGACGTTGACAAT
Zeugodacus depressa ¹⁾	AATTCCAACCAACGAATTAGCCGCAGACGGGTTTCGCCTACTAGATGTAGATAAT
Zeugodacus scutellata ¹⁾	AATTCCAACCTAACGAATTAGCTACAGACGGCTTCGACTATTAGATGTAGACAAC
Zeugodacus cucurbitae ³⁾	AATCCCTACTAATGAATTAGCTACAGATGGATTTCGTTTATTAGACGTAGATAAC
	* *
Bactrocera tsuneonis	CGAGTAATCCTACCACTAAACTCACAAATTCGAATC <u>TTAGTAAGAGCCGCAGACG</u>
Bactrocera minax	CGAGTAATCTTGCCCTTAAACTCACAAATCCGAATCCTAGTGACAGCTGCAGATG
Bactrocera dorsalis	CGCGTAGTTCTCCCTATAAATTCACAAATTCGAATTTTAGTAACGGCTGCAGATG
Bactrocera latifrons	CGTGTTATTTTGCCCTATAAATTCACAAATTCGAATTTTAGTCACAGCAGCAGACG
Zeugodacus depressa	CGGGTAATTTTACCTATAAATTCACAAATCCGAATTCCTAGTAACAGCCGCAGACG
Zeugodacus scutellata	CGAGTGATCCTACCTATAAATTCACAAATTCGAATTCCTAGTAACAGCTGCAGATG
Zeugodacus cucurbitae	CGAGTAGTTTACCTATAAATTCACAAATTCGAATTTTAGTAACAGCCGCAGATG
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