

1 **Development of microsatellite markers for the**
2 **Japanese orange fly, *Bactrocera tsuneonis***
3 **(Diptera: Tephritidae)**

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24 Abstract

25 To monitor and control the Japanese orange fly *Bactrocera tsuneonis*, it is
26 important to determine their dispersal ability. Therefore, to determine the genetic
27 structure of this species, we developed microsatellite markers at 17 loci for *B.*
28 *tsuneonis*. A total of 80 adult females and males randomly collected from
29 abandoned orchards in the Oita and Yamaguchi Prefectures, in the western part of
30 Japan, were used for polymorphism analysis using the microsatellite markers. The
31 average number of alleles was 2.65 for the 17 loci, in which two loci did not show
32 polymorphism. The expected heterozygosity ranged from 0 to 0.663, although the
33 observed heterozygosity was relatively low between 0–0.105. The null allele
34 frequencies varied among populations. Moreover, no polymorphism was found in
35 the three larval populations collected in the Oita Prefecture. These results
36 suggested a high degree of inbreeding for this species. Significant genetic
37 differentiation between females and males of the same population was not
38 observed, although it was observed between the Oita and Yamaguchi populations.
39 A bottleneck effect was detected in Yamaguchi but not in Oita, and this is
40 congruent with the history of an expanding distribution in this species. These
41 markers could be used for population discrimination and to expand the geographic
42 history of this species, potentially contributing to the pest management of *B.*
43 *tsuneonis*.

45 Keywords

46 *Bactrocera tsuneonis* • Japanese orange fly • quarantine pest • microsatellite
47 markers • population structure

48

49 **Introduction**

50 Fruit fly species from the family Tephritidae include many serious pests. Among
51 them, several species belonging to the Tribe Dacini, such as *Bactrocera dorsalis*,
52 *B. tryoni*, and *Zeugodacus cucurbitae*, are subject to strict quarantine controls
53 worldwide (Clarke 2019). The Japanese orange fly, *B. tsuneonis* (Miyake)
54 (Diptera: Tephritidae), is an univoltine pest of citrus fruits (Weems 1967;
55 Yasumatsu and Nagatomi 1959). Although the distribution of this species is
56 restricted to Japan, southern China, and Taiwan (Plant Health Australia 2018),
57 there is a risk of its expansion to other areas. In Japan, this species was first
58 recorded in the Oita Prefecture at the end of the 19th century (Miyake 1919).
59 Since the end of the 1990s, the damage caused by *B. tsuneonis* has become
60 serious, possibly because the number of unmanaged citrus orchards has increased
61 owing to aging orchardists (Mochizuki and Narahara 2022; Narahara et al. 2007).
62 Although the export of citrus fruits from Japan is promoted by government
63 policies, several countries require quarantine for *B. tsuneonis* and permit imports
64 only from unaffected areas. Therefore, to monitor and control this species, it is
65 important to determine its dispersal ability. For instance, if geographically isolated
66 strains exist, the control and quarantine procedures will be affected. Since
67 information about the genetic structure and geographical variability of this pest
68 species could contribute to the prediction of its mobility, the development of
69 molecular markers is crucial for the pest management of *B. tsuneonis*.

70 Microsatellite markers are highly efficient tools for genetic and population
71 analyses, particularly in species lacking any other means of genetic analysis,
72 owing to their wide genome distributions, high polymorphism, locus specificity,
73 and codominance. Microsatellites are tandemly repeated units of two to five base
74 pairs that are distributed throughout eukaryotic genomes (Hancock 1999).
75 Microsatellite markers have been developed and used for many fruit fly species
76 (Augustinos et al. 2008; Bonizzoni et al. 2001; Buahom et al. 2013; Leal et al.
77 2021; Lin et al. 2020; Qin et al. 2018; Wang et al. 2021; Yan et al. 2015).
78 Nevertheless, this technique has been applied only to a limited number of species
79 because the isolation of microsatellite markers involves complex procedures,
80 including molecular cloning, colony selection, and sequencing (Zane et al. 2002).
81 Recently, owing to the development of next-generation sequencing techniques and
82 transcriptome analysis, isolation of microsatellite markers has become easier in

some arthropod species (Cook et al. 2011; Duan et al. 2017; Ohbayashi et al. 2019; Ouyang et al. 2021; Zhu et al. 2016). Consequently, in this study, we developed and characterized microsatellite DNA markers for *B. tsuneonis* using transcriptome data and analyzed the genetic diversity of these markers in the Oita and Yamaguchi Prefectures.

Materials and Methods

Sample collection and DNA extraction

Male and female adults of *B. tsuneonis* were obtained from satsuma mandarin orange fruits randomly collected from several abandoned orchards in the Oita and Yamaguchi Prefectures, Japan. The samples emerged from fruits harvested in 2019 that were checked for infection. The adult flies were identified morphologically and by polymerase chain reaction (PCR) using species-specific primers as described below. Larvae were also collected from four abandoned orchards in three cities in the Oita Prefecture, while checking for suspected infection of fruits. All specimens were preserved in ethyl alcohol (99.5%) and stored in refrigerators at 4 °C until further molecular analysis.

Genomic DNA was extracted individually from each fruit fly using a NucleoSpin® DNA Insect Kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany) and dissolved in 200 µL TE buffer (10–0.1 mM). Species identification and DNA extraction were confirmed by PCR using species-specific primers according to the methods described by Zhang et al. (2018).

RNA extraction and sequencing

Before pupariation, the last instar larvae of *B. tsuneonis* were immediately collected from mandarin fruits harvested from abandoned orchards at the beginning of winter in Yamaguchi Prefecture. Adult flies eclosed in early June after overwintering as pupae and were staged 3–7 d later. The antennae of 35 males and 38 females were dissected and homogenized in TRIzol reagent (GIBCO-BRL, Gaithersburg, Maryland, USA). Total RNA was extracted from homogenates and purified using the NucleoSpin RNA (Macherey-Nagel). The quality of the total RNA samples was measured using an Agilent 2100

114 Bioanalyzer (Agilent Technologies, Santa Clara, California, USA). Sequence
115 libraries were prepared from 1 µg total RNA using a TruSeq RNA Sample
116 Preparation Kit v2 (Illumina, Inc., San Diego, CA, USA) according to the
117 manufacturer's protocol. RNA sequencing was performed using an Illumina
118 MiSeq system with a 600-cycle MiSeq Reagent Kit v3 (Illumina, Inc.). The
119 resulting data were deposited in the DNA Data Bank of Japan (DDBJ) Sequence
120 Read Archive under accession numbers DRR321520 and DRR321521 for the
121 male and female antennae, respectively.

122 **Design of microsatellite markers, genotyping PCR, and fragment** 123 **analysis**

124 By searching for dinucleotide motifs with more than 10 repeats using QDD ver.
125 3.1.2 (Meglécz et al. 2014) and Krait ver. 1.3.3 (Du et al. 2018) software, we
126 found and designed primer sets for 17 loci from 67,365 contigs using the software
127 Primer3 (Du et al. 2018; Untergasser 2012). PCRs were carried out in a 10.0 µL
128 reaction mixture containing 0.5 µL genomic DNA solution, 0.2 µL Gflex (Takara
129 Bio Inc., Shiga, Japan), 5.0 µL of 2 × Gflex buffer, and 0.2 µM of each primer
130 under the following cycling profile: 94 °C (1 min); 35 cycles of 98 °C (10 s),
131 60 °C (15 s), and 68 °C (30 sc); 72 °C for last-strand elongation (10 min). The
132 PCR amplification using the designed primers was confirmed using agarose gel
133 electrophoresis. Subsequently, fragment analyses using fluorescent primers were
134 conducted using an ABI 3130 Genetic Analyzer (Thermo Fisher Scientific,
135 Waltham, MA, USA) by applying 1 µL of the multiplex mixture of four PCR
136 products in a ratio of 1:1:2:2 for FAM:VIC:NED:PET dyes, 0.25 µL GeneScan™
137 500 LIZ® Size Standard (Thermo Fisher Scientific), and 10 µL Hi-Di™
138 formamide.

139 **Data analysis**

140 All data obtained using the genetic analyzer were genotyped using GeneMarker®
141 software ver. 3.0.1 (SoftGenetics, State College, Pennsylvania, USA). Genetic
142 polymorphisms within populations were estimated in terms of the number of
143 alleles, observed heterozygosity, expected heterozygosity, inbreeding coefficient
144 (F_{IS}), and null allele frequencies of the microsatellite markers in adult populations
145 of *B. tsuneonis* using GenePop ver. 4.7.5 (Rousset 2008) and FSTAT ver. 2.9.4

(Goudet 2003). To identify loci under neutral and positive selection, BayeScan ver. 2.1 (Foll and Gaggiotti 2008) was used for a fixation index (F_{ST})-based outlier test. In these analyses, males and females were treated as independent populations. The F_{ST} values between populations were calculated between each pair of populations using Arlequin ver. 3.5.22 (Excoffier and Lischer 2010). Bottleneck effects were also analyzed using BOTTLENECK ver. 1.2.02 (Piry et al. 1999), based on data in which females and males in the same prefecture were grouped together.

Results and Discussion

We developed 17 microsatellite markers based on RNA sequences (Table 1). Generally, microsatellite DNA loci are less polymorphic (Table 2). The average number of alleles was 2.65 for the 17 loci, and two loci did not show polymorphism in the adult populations. The observed heterozygosity was 0–0.105, whereas the expected heterozygosity ranged between 0–0.663. The null allele frequencies ranged between 0.149–0.975. The F_{IS} values were quite high for each locus in each population, showing significant deviation from zero. No clear difference between the females and males in the same population was detected, suggesting that these markers were positioned on autosomes. Regarding outliers analyses, BayeScan, which defines a q-value that is a false discovery rate analogue of the p value, did not detect any loci subject to selection (q-values range: 0.893–0.918). Notably, however, that the null allele frequencies and polymorphic loci varied among populations; therefore, loci must be carefully selected when used for population structure analysis.

We analyzed the larvae populations using ten microsatellite markers (BtMS025, 041, 048, 050, 053, 058, 060, 081, 082, and 252), for most of which polymorphisms were detected in the adult population of the Oita Prefecture. However, no polymorphisms were found in or among the three populations (Table 3). The marker BtMS050 failed to amplify completely in the larval populations, although null allele frequencies were moderate in the adult populations from Oita, showing that the presence of null alleles varied among populations.

Next, we analyzed population differentiation among adult populations from the two prefectures using the 15 microsatellite markers (all markers except BtMS252 and 304) for which the polymorphisms were detected above. The F_{ST}

179 values were significantly deviated from zero between the geographically different
180 populations, Oita and Yamaguchi, but not between the sexes in the same
181 population (Table 4).

182 For adult flies, although we found polymorphisms in most loci,
183 heterozygotes were rarely found. The adults obtained in this study emerged from
184 fruits collected from several orchards; however, the geographic origins of the
185 samples were not known. High homozygosity suggested that this species has a
186 strong inbreeding structure, and high null allele frequencies may be overestimated
187 because of this high homozygosity. Adults of *B. tsuneonis* can only migrate less
188 than 1 km within 3–6 days, and have only about two weeks of longevity in
189 adulthood (Miyake 1919). Therefore, *B. tsuneonis* is thought to inhabit a very
190 narrow area, consistent with the high homozygosity observed in these populations.
191 Furthermore, the number of alleles detected in this study was not very high,
192 especially in the Yamaguchi Prefecture. The average number of alleles in the Oita
193 populations was 1.853 and higher than the average (1.382) in Yamaguchi
194 (Wilcoxon rank sum test; $p = 0.044$). Additionally, a bottleneck effect was
195 detected in the Yamaguchi population under the two-phase model (Wilcoxon's
196 signed rank test; $p = 0.002$) but not in Oita ($p = 0.695$). This is consistent with
197 historical observations that this species was first found in the Oita Prefecture and
198 its distribution had expanded.

199 In summary, we developed 15 polymorphic microsatellite markers for *B.*
200 *tsuneonis*. These markers were developed based on RNA sequencing data,
201 possibly related to functional genes; however, a neutrality test indicated these
202 markers can be used for population genetic analysis, although the extent of the
203 polymorphism was not high. Genetic differentiation between the Oita and
204 Yamaguchi Prefectures was detected, and a bottleneck effect was detected in the
205 latter prefecture. Therefore, these markers could be used for population
206 discrimination and for analysis of an expanding distribution in this species.
207 Further investigations using a larger number of populations are required in the
208 future.

209

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309 **Table 1** Primer sequences of 17 microsatellite markers developed for *Bactrocera tsuneonis*.

Locus	Dye	Primer sequences (Forward)	Primer sequences (Reverse)	bp*	Repeat motif	Accession No.
BtMS025	NED	TCGCATCATATTCAGCTGCA	CAAGCGGGCGATGAAATCAT	136	(GC) ₄ (GT) ₁₀	LC667453
BtMS041	FAM	GCTAACAGTGCGTACATTCATACA	TGTAAGAGCTGTTATGGACTTGGT	129	(TG) ₁₀	LC667454
BtMS048	PET	CTCACACCCTCCAACCTGCAT	TTGTATTTTCGCACACGCTGT	104	(AC) ₁₀ AT(CA) ₄	LC667455
BtMS050	NED	AGCGCAAGACAAAATGTGTGAC	CCTGTCGTAGTCAGTATACCAGG	133	(AT) ₁₁	LC667456
BtMS053	PET	TCAGGAGTGTTTCAGCTGTGG	TGCGCTCTCTGCAACTTCAT	105	(AC) ₁₀ AT(CA) ₄	LC667457
BtMS058	VIC	CTGTATTTGCCTCGAGCCGA	TGTTGATAATGATCTTAGTTCCCCA	142	(TA) ₁₀	LC667458
BtMS060	VIC	GCAGTTCAGCAGCGTTTGT	CTGCCAGGCAAACCCATAGA	140	(CA) ₁₀	LC667459
BtMS079	PET	TGTGCTACATTTTCGTCGTCG	GGAGCTAAGCGAATGCAACG	176	(TG) ₁₀	LC667461
BtMS081	FAM	CCTCGGCGCTTGTCTTTAAAC	ACGAGCGCAATTGAAACGAA	167	(GA) ₁₀	LC667462
BtMS082	FAM	CAAGGGTAGTGGCTTCTGCA	TGCTTTTCATACAGATGAGCTAGC	96	(AG) ₁₀	LC667463
BtMS221	PET	TGAGATTGTTGTTGTGGCTGC	TTGAAGAAATGTTACACTTCCGC	130	(GA) ₁₁ AA(GA) ₄	LC667465
BtMS252	FAM	AGAAAAATGTGTGTATGTAACCTAAGG	GGGTACCGTTTGTCTTTCCG	197	(TG) ₁₀	LC667466
BtMS265	NED	TGTGTGACAAATAGTTGAAACATGG	CCTGTCGTAGTCAGTATACCAGG	120	(AT) ₁₁	LC667467
BtMS294	PET	CAAACGAGCGCAATTGAAACG	GGCGCTTGCTTTTAACTTTAGC	166	(TC) ₁₀	LC667469
BtMS304	VIC	TCTGTATTTGCCTCGAGCCG	GGTTGTTGATAATGATCTTAGTTCCCC	146	(TA) ₁₀	LC667470
BtMS328	VIC	AGCAAATGTCTGTAGTTCAAGTTTCG	TCCTAAATGTCAAATGATGGGTTTACC	94	(CA) ₁₀	LC667471
BtMS332	PET	TTTTGGTGCGTTGGATAGCG	AAGGAAGGAAAGTGAAGCTGG	88	(AT) ₁₀	LC667472

310

311 * Length of amplified fragments estimated from RNA sequence data.

312

Table 2 The number of individuals which were successfully genotyped (n), observed and expected heterozygosity (H_O and H_E), inbreeding coefficient (F_{IS}), null allele frequencies (Null), and number of alleles detected (Na) of 17 microsatellite markers in two populations of *Bactrocera tsuneonis*. Twenty adult females and males were used for each population.

Locus	Oita												Yamaguchi												Total Number of Alleles
	Female						Male						Female						Male						
	n	H _O	H _E	F _{IS}	Null	N _a	n	H _O	H _E	F _{IS}	Null	N _a	n	H _O	H _E	F _{IS}	Null	N _a	n	H _O	H _E	F _{IS}	Null	N _a	
BtMS025	18	0	0.457	1.000***	0.371	4	19	0	0.194	1.000**	0.208	2	20	0	0.000	—	—	1	20	0	0.097	1.000*	0.149	2	5
BtMS041	18	0	0.387	1.000***	0.349	4	17	0	0.399	1.000***	0.402	3	15	0	0.000	—	—	1	20	0	0.000	—	—	1	4
BtMS048	19	0	0.273	1.000***	0.397	2	14	0	0.254	1.000**	0.378	2	11	0	0.173	1.000*	0.195	2	11	0	0.173	1.000*	0.195	2	3
BtMS050	20	0	0.328	1.000***	0.447	2	18	0	0.356	1.000***	0.471	2	20	0	0.097	1.000*	0.149	2	20	0	0.000	—	—	1	3
BtMS053	8	0	0.000	—	—	1	12	0	0.000	—	—	1	16	0	0.226	1.000**	0.354	2	19	0	0.000	—	—	1	2
BtMS058	13	0	0.000	—	—	1	16	0	0.226	1.000**	0.935	2	19	0.105	0.199	0.478**	0.895	3	19	0	0.000	—	—	1	4
BtMS060	14	0	0.254	1.000**	0.378	2	14	0	0.138	1.000*	0.267	2	11	0	0.000	—	—	1	13	0	0.000	—	—	1	2
BtMS079	17	0	0.663	1.000***	0.712	5	17	0	0.642	1.000***	0.628	3	20	0	0.000	—	—	1	18	0	0.000	—	—	1	5
BtMS081	16	0	0.226	1.000**	0.935	2	19	0	0.273	1.000***	0.918	2	20	0	0.000	—	—	1	14	0	0.000	—	—	1	2
BtMS082	15	0	0.239	1.000**	0.365	2	16	0	0.226	1.000**	0.354	2	19	0	0.000	—	—	1	20	0	0.000	—	—	1	2
BtMS221	10	0	0.484	1.000***	0.484	3	10	0	0.442	1.000**	0.548	2	19	0	0.194	1.000*	0.208	2	19	0	0.273	1.000***	0.246	2	3
BtMS252	7	0	0.000	—	—	1	8	0	0.000	—	—	1	20	0	0.000	—	—	1	20	0	0.000	—	—	1	1
BtMS265	12	0	0.000	—	—	1	12	0	0.000	—	—	1	20	0	0.097	1.000*	0.975	2	20	0	0.000	—	—	1	2
BtMS294	13	0	0.000	—	—	1	12	0	0.000	—	—	1	18	0	0.108	1.000*	0.972	2	20	0	0.000	—	—	1	2
BtMS304	12	0	0.000	—	—	1	13	0	0.000	—	—	1	19	0	0.000	—	—	1	20	0	0.000	—	—	1	1
BtMS328	12	0	0.000	—	—	1	12	0	0.000	—	—	1	17	0	0.114	1.000*	0.970	2	19	0	0.000	—	—	1	2
BtMS332	7	0	0.000	—	—	1	9	0	0.000	—	—	1	19	0	0.102	1.000*	0.229	2	18	0	0.000	—	—	1	2

Asterisks after F_{IS} indicate significant deviation from 0. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$

Table 3 The number of alleles (Na) and proportion of genotyped individuals (Pg) of ten microsatellite markers from three field populations of *B. tsuneonis* larvae collected in the Oita Prefecture.

Population	Oita* (n=13) 33.19606°N, 131.70582°E 33.15431°N, 131.68392°E		Tsukumi (n=15) 33.07192°N, 131.87285°E		Usukine (n=19) 33.15285°N, 131.81933°E		Total number of alleles
Locus	Na	Pg	Na	Pg	Na	Pg	
BtMS025	1	1.000	1	1.000	1	1.000	1
BtMS041	1	0.692	1	0.867	1	0.737	1
BtMS048	1	0.923	1	0.933	1	1.000	1
BtMS050	0	0.000	0	0.000	1	0.053	1
BtMS053	1	1.000	1	0.933	1	1.000	1
BtMS058	1	0.462	1	0.267	1	0.421	1
BtMS060	1	0.462	1	0.933	1	0.474	1
BtMS081	1	0.462	1	0.933	1	0.684	1
BtMS082	1	1.000	1	1.000	1	1.000	1
BtMS252	1	1.000	1	0.933	1	0.947	1

* The Oita population was collected from two orchards.

325 **Table 4** The pairwise genetic differentiation (F_{ST}) among four adult populations of *Bactrocera tsuneonis* calculated from microsatellite markers.
 326 The F_{ST} values and p values after 10,100 permutations are shown in the upper and the lower diagonals, respectively. Abbreviations: F and M in
 327 parentheses indicate females and males, respectively.

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Population	Oita (F)	Oita (M)	Yamaguchi (F)	Yamaguchi (M)
Oita (F)		0.002	0.305	0.359
Oita (M)	0.910		0.247	0.307
Yamaguchi (F)	<0.001	<0.001		0.061
Yamaguchi (M)	<0.001	<0.001	0.064	

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