Res. Bull. Pl. Prot. Japan No. 42: 23–34 (2006)

Morphological Characters and PCR-RFLP Markers in the Interspecific Hybrids between *Bactrocera carambolae* and *B. papayae* of the *B. dorsalis* Species Complex (Diptera: Tephritidae)

Takao Ebina and Kenji Ohto

Research Division, Yokohama Plant Protection Station, 1–16–10,
Shin-yamashita, Naka-ku, Yokohama 231–0801, Japan

Abstract: Morphological characters and PCR-RFLP markers of interspecific hybrids between *Bactrocera carambolae* and *B. papayae* (Diptera: Tephritidae) were investigated. Both species are economically important pests that belong to the *Bactrocera dorsalis* species complex. They have never been found in Japan. For the present study, test insects were imported into Japan from Malaysia and Thailand, and they were maintained in the laboratory, with permission from the relevant authority. A pair of flies, *B. carambolae* female and *B. papayae* male, were crossed. The aculeus lengths of the F_1 and F_2 hybrids were found to be intermediate between those of the two parental species. In addition, the range of morphological variation was expanded in the F_2 hybrids. Therefore, aculeus length in these two species is considered to be determined by quantitative inheritance. In all F_1 hybrids, the restriction pattern of the PCR-RFLP fragments of mitochondrial DNA (mtDNA; 16S rRNA) was the same as that of the maternal species, and that for nuclear ribosomal DNA (rDNA; 5.8S rRNA and ITS) contained the restriction fragments of both P_1 species in a heterozygous banding pattern (hybrid pattern). The frequency ratio in F_2 females of the *B. carambolae* pattern, the *B. papayae* pattern and the hybrid restriction pattern was 14 : 1 : 27. There was no correlation between the morphological characters and the rDNA markers in the F_2 hybrids. It is therefore suggested that in both species, the inconsistency between the morphological characters and the DNA markers, and the continuous variation of the aculeus length, were mainly caused by interspecific hybridization in the distribution area.
Key words: *Bactrocera carambolae*, *Bactrocera papayae*, interspecific hybrids, PCR-RFLP, *Bactrocera dorsalis* species complex

Introduction

Bactrocera carambolae Drew and Hancock and *B. papayae* Drew and Hancock (Diptera: Tephritidae) are sympatric species that are endemic to Southeast Asian countries such as Indonesia and Malaysia, and they are economically important fruit flies belonging to the *Bactrocera dorsalis* species complex (Drew and Hancock, 1994). Their larvae feed on many kinds of commercial fruits (White and Elson-Harris, 1992; Drew and Hancock, 1994).

The *B. dorsalis* complex, described by Drew (1989) and Drew and Hancock (1994), is a group of sibling species belonging to the genus *Bactrocera*, which share common morphological characters such as wings and thorax. More than 70 species have so far been reported (Drew, 1989; Drew and Hancock, 1994; Tsuruta and White, 2001; Drew *et al*., 2005). Among the species of the complex, only a small number of species are listed as being economically critical (Drew and Hancock, 1994; Drew and Romig, 1997; Clarke *et al*., 2005). Of these species, *B. dorsalis*, *B. carambolae*, *B. occipitalis*, *B. papayae* and *B. philippinensis* in particular have been intercepted by the import plant quarantine in Japan (Iwaizumi, 2004). These five pest species were once classified as one species, *B. dorsalis* (*Dacus dorsalis* and *D. dorsalis* var. *occipitalis*) (Hardy and Adachi, 1956; Hardy, 1969; White and Elson-Harris, 1992; Drew and Hancock, 1994), and they are remarkably similar with respect to biological and morphological characters. The five species have a wide host plant range, and cause a substantial amount of damage to plants in infested areas (White and Elson-Harris, 1992; Drew and Hancock, 1994; Clarke *et al*., 2005). *B. dorsalis* was eradicated from Japan (Yoshizawa, 1997), and at present, no species of the *B. dorsalis* complex, including *B. carambolae* and *B. papayae*, occurs in Japan, but there is concern about the potential for these species to invade.

Correct identification of important pestiferous fruit flies, including the *B. dorsalis* complex, to

the species level is necessary to carry out effective plant quarantine procedures. For example, it is necessary to discriminate between species for pest risk assessments, for development of the appropriate standards for plant quarantine treatment (e.g. vapor heat treatment), and for developing monitoring and controlling programs for newly introduced pests. Currently, the taxonomy of the *B. dorsalis* complex as defined by DREW and HANCOCK (1994), is generally recognized. Therefore, discrimination between *B. carambolae* and *B. papayae* is needed for plant quarantine purposes.

Although *B. carambolae* and *B. papayae* have several recognizable morphological diagnostic characteristics in adult females, for example the aculeus (the hard apical segment of the ovipositor) (DREW and HANCOCK, 1994), discrimination between the two species using traditional morphological features is sometimes difficult because specimens whose morphological characters fall within an intermediate range segregate within a population, and those that have polymorphic characters are found at nontrivial frequencies (YONG, 1995; IWAHASHI, 1999; CLARKE *et al*., 2005). In addition, the sympatric distribution of these species causes further difficulties in identification.

In some species belonging to the genus *Bactrocera*, the polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) method has been used to discriminate among species or strains (HE and HAYMER, 1997; ARMSTRONG and CAMERON, 2000; MURAJI and NAKAHARA, 2002). It has been reported, however, that in some cases discrimination between *B. carambolae* and *B. papayae* is impossible, even by using PCR-RFLP techniques on mitochondrial DNA (mtDNA) (MURAJI and NAKAHARA, 2002).

Interbreeding between *B. carambolae* and *B. papayae* in their overlapping distribution area has been assumed to be one cause of the difficulties in identifying these two species (YONG, 1995; IWAIZUMI *et al*., 1997; MURAJI and NAKAHARA, 2002). However, in interspecific hybrids of the two species, the morphological characters and PCR-RFLP markers remain unclear. Therefore, to provide more information for discrimination between the two species, we interbred *B. carambolae* and *B. papayae*, which were confirmed by assessments of morphological characters and DNA markers to be different species, and investigated the morphological characters and PCR-RFLP markers in the F_1 and F_2 hybrids.

Materials and Methods

B. carambolae and *B. papayae* were used in the present study. Neither species has been found in Japan. For use in present study, the species were imported from Malaysia (*B. carambolae*; import permit no. 3Y-968) and Thailand (*B. papayae*; import permit no. 14Y-168) with permission from the Minister of Agriculture, Forestry and Fisheries of Japan. Both species were reared artificially at a constant temperature (25±1℃), at 60% RH, and under a photoperiod of 16 h : 8 h (L : D) in the rearing facilities (Biotron) at the Yokohama Plant Protection Station, Ministry of Agriculture, Forestry and Fisheries of Japan (Kanagawa Prefecture, Japan), with permission of the relevant authority. Female adults were used for investigation in the present study. For examination of the morphological characters and PCR-RFLP markers of the two species (parental generations), 30 adult females of each species were tested.

All breeding experiments were carried out with one pair of flies. F_1 hybrids ($n=18$) were obtained from mating one *B. carambolae* female and one *B. papayae* male. F_2 hybrids ($n=42$) were obtained by interbreeding a pair of the F_1 hybrids. Adult female F_2 hybrids were used for examinations. In addition, reciprocal crosses (*B. papayae* female×*B. carambolae* male) were performed for reference purposes, and adult female F_1 hybrids ($n=5$) were examined. Backcrosses between F_1 female (*B. carambolae* female×*B. papayae* male) and P_1 male (*B. papayae*) were performed for reference purposes. Adult female BC_1 hybrids were examined.

The report by DREW and HANCOCK (1994) on the morphological characteristics of the two fruit fly species was used for reference regarding to for discrimination between them. The specimens used for examinations were dissected and the body parts needed for observation and measurement were extracted. The aculeus length in female adults was measured as a diagnostic charac-

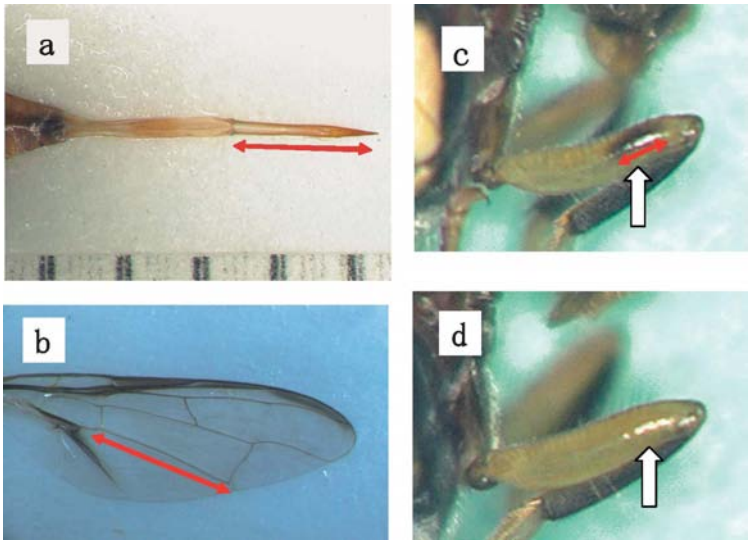

Fig. 1. Morphological characters examined.
(a) Ovipositor. Length of aculeus (the hard apical segment of the ovipositor). (b) Wing. Length of wing cell dm along vein CuA1, as an index of body size. (c) Longitudinal length of the black spot on outer surface of fore femora of *B. carambolae*. (d) Fore femora of *B. papayae* without a black spot.

ter (Fig. 1a). The length of wing cell dm along vein CuA1 (cell dm length) was measured as an indicator of body size (Fig. 1 b). For reference, the longitudinal length of the black spot on the outer surface of the fore femora (black spot length) was measured (Fig. 1c). Although presence/absence of the black spot on the fore femora is not useful for discrimination between the two species (DREW and HANCOCK, 1994), it was used for genetic research. Aculeus length, cell dm length and black spot length were measured using a digital microscope (Keyence).

Base sequences from mtDNA (16S ribosomal RNA gene) and nuclear ribosomal DNA (rDNA; 5.8S ribosomal RNA gene and internal transcribed spacer [ITS]) were used for the development of DNA markers to distinguish the two species. Information on these base sequences was downloaded for reference from a nucleotide sequence database (e.g. DDBJ, EMBL or GenBank) via the Internet. The accession numbers of the base sequences were AB035117 (*B. carambolae*) and AB 035119 (*B. papayae*) for the mtDNA, and AF121144 (*B. carambolae*) and AF121157 (*B. papayae*) for the rDNA. A 347-bp partial sequence of the 16S rRNA gene, which was designated "Section I" in a report by MURAJI and NAKAHARA (2002), was used for the mtDNA marker. For rDNA, sequences registered on DDBJ were referred to. Complete sequences of the 5.8S rRNA gene and partial sequences of the ITS-1 and ITS-2 in the rDNA (*B. carambolae*, 595 bp: *B. papayae*, 558 bp) were used.

DNA samples were extracted from the legs of each specimen. A GenomicPrep™ Cell and Tissue DNA Isolation Kit (Amersham Pharmacia) was used for DNA extraction. Each DNA sample extracted was dissolved in 200 μl of sterile distilled water (this was the DNA template) and frozen for storage. The sequence-specific PCR primers were 5′-ATCCAACATCGAGGTCGCAAAC-3′ (forward primer) and 5′-GGCTGGTATGAACGGTTGGACGAG-3′ (reverse primer) (MURAJI and NAKAHARA, 2002) for the mtDNA, and 5′-GAATTTCGCATACATTGTAT-3′ (forward primer) and 5′-ATGTGTCCTGCAGTTCACA-3′ (reverse primer) (submitted to the DDBJ/EMBL/GenBank by ARMSTRONG and CAMERON, 1999) for the rDNA. PCR reaction mixtures consisted of 4 mM $MgCl_2$, Gene PCR buffer II, 10 pmol/μl of each oligonucleotide primer, dNTPs, 2 U AmpliTaq Gold® polymerase, the DNA template and sterile distilled water in a final volume of 50 μl. The optimized thermal cycling (PCR) profile consisted of a one-time initial denaturation for 9 min at 95℃, followed by 35 cycles consisting of 30 s denaturation at 94℃, 30 s annealing at 50℃, and 90 s extension at 72℃. Amplification was performed using a Program Temp Control System (Astec; Model PC-707). PCR products were digested with restriction enzymes. The known base sequences cited above were analyzed by the computer program Genetix® Version 7.0.1 (Software Development). Restriction enzymes that produced the most distinct differences in DNA restriction pat-

Table 1. Aculeus length (mm) of P_1 generation, and F_1 and F_2 generation hybrids between two *Bactrocera* species.

Generation	*n*	Aculeus length				Regression*1		
		Mean	*t* test*2	SD	Range	Regression equation	*t* test*3	*r*
B. carambolae; P_1	30	1.525		0.031	1.457–1.573	$y=1.579-0.024x$	d	0.045
B. papayae; P_1	30	1.928		0.055	1.808–2.037	$y=0.675+0.524x$	e	0.507
B. carambolae ♀ × *B. papayae* ♂; F_1	18	1.813	a	0.059	1.718–1.952	$y=0.411+0.582x$	e	0.665
B. carambolae ♀ × *B. papayae* ♂; F_2	42	1.763	a,b	0.102	1.532–1.956	$y=0.122+0.706x$	e	0.742
B. papayae ♀ × *B. carambolae* ♂; F_1	5	1.745	b	0.076	1.659–1.860	$y=0.833+0.395x$	d	0.521

*1 Regression between aculeus length (y) and cell dm length (x).
*2 Mean values with the same letter are not significantly different at the 0.05 levels.
*3 d, $p>0.05$; e, $p<0.05$

Table 2. Longitudinal length of the black spot on fore femora (mm) and the length of wing cell dm (along with vein CuA1) (mm) of P_1 generation , and F_1 and F_2 generation hybrids between two *Bactrocera* species.

Generation	*n*	Black spot length				Cell dm length			
		Mean	*t* test*	SD	Range	Mean	*t* test*	SD	Range
B. carambolae; P_1	30	0.416		0.032	0.361–0.500	2.328	a	0.059	2.223–2.441
B. papayae; P_1	30	0.000				2.393	c	0.054	2.298–2.511
B. carambolae ♀ × *B. papayae* ♂; F_1	18	0.148	a	0.091	0.000–0.281	2.411	c	0.068	2.315–2.541
B. carambolae ♀ × *B. papayae* ♂; F_2	42	0.181	a	0.144	0.000–0.448	2.325	a	0.107	2.058–2.539
B. papayae ♀ × *B. carambolae* ♂; F_1	5	0.321		0.055	0.229–0.372	2.309	a	0.100	2.173–2.444

*Mean values with the same letter in each column are not significantly different at the 0.05 level.

terns between the two species were selected, with *Ase*I and *Mse*I used for the mtDNA and rDNA, respectively. *Ase*I recognizes the sequence 5′-ATTAAT-3′, and *Mse*I recognizes the sequence 5′-TTAA-3′. The PCR product (3.0–3.5 μl) was incubated in a final volume of 10–12 μl, containing two units of enzymes and buffer, for 2 h at 37℃. After digestion with restriction enzymes, the restricted fragments of DNA were resolved by electrophoresis (100 V, 90 min) alongside a molecular weight marker (25 bp ladder, Promega Corporation) in 12% polyacrylamide gel (Bio-Rad). The electrophoresed gel was then stained with ethidium bromide, and the restriction patterns were visualized with ultraviolet radiation. The restriction patterns detected were used as DNA markers for discriminating between the two fruit fly species.

Results and Discussion

Morphological characters

The results of the investigations on morphological characters are shown in Tables 1 and 2. The aculeus lengths (mean ± SD) in the parental species were 1.525 ± 0.031 mm in *B. carambolae* and 1.928 ± 0.055 mm in *B. papayae*. In addition, it was confirmed that there were significant differences in aculeus length, and presence/absence of the black spot on the fore femora between the two species (Tables 1 and 2). The morphological characters observed in the two species were consistent with those described by DREW and HANCOCK (1994).

Figure 2 shows the distribution of the aculeus lengths in each generation in the breeding experiment (*B. carambolae* female × *B. papayae* male). The aculeus lengths of the F_1 and F_2 hybrids were distributed in an approximately intermediate zone between the two parental species (Fig. 2). The aculeus lengths of the F_1 hybrids were found to be half those of the parental species in the re-

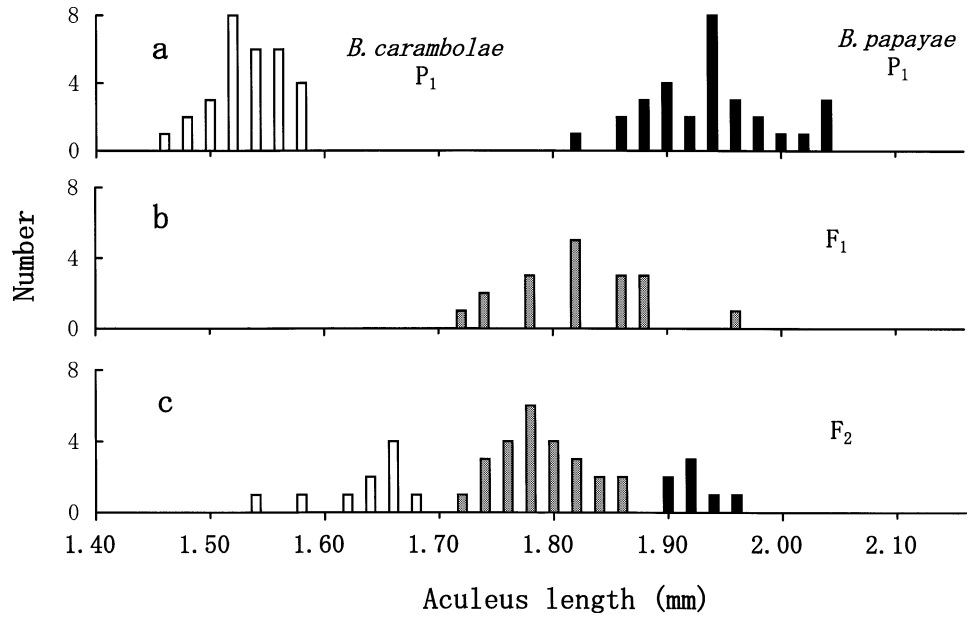

Fig. 2. Distribution of aculeus length of P_1 generation, and F_1 and F_2 generation hybrids between *Bactrocera carambolae* females and *B. papayae* males.
(a) P_1 generation; *Bactrocera carambolae* females (open column) and *B. papayae* females (closed column). (b) F_1 hybrids. (c) F_2 hybrids. The distribution of aculeus length in the F_2 generation was separated into three groups: the shorter aculeus group (open column), the intermediate aculeus group (gray column) and the longer aculeus group (closed column).

ciprocal crosses (*B. papayae* female×*B. carambolae* male, Table 1). Similar trends were found for the black spot lengths (although non-additive effects were also observed, Table 2; Fig. 3). These results indicate that if there were regions where frequent crossings between *B. carambolae* and *B. papayae* occurred so as to constantly produce F_1 progenies, individuals that were intermediate with respect to these characters would inevitably occur. The same results were reported in a similar breeding experiment among species in *Anastrepha fraterculus* (Diptera: Tephritidae), *A. sororcula* and *A. obliqua* (SANTOS *et al*., 2001). In that study, morphological characters, such as aculeus tips, wing-band patterns, and creamy white longitudinal strips on the mesoscutum, had forms that were intermediate between those of the parental species (SANTOS *et al*., 2001).

In addition, it was confirmed that the range of variation of the aculeus lengths in the F_2 hybrids as wider compared with that in the parental species or the F_1 hybrids (Table 1; Fig. 2). Figure 2 shows that the aculeus lengths in the F_2 hybrids ranged between 1.532 and 1.956 mm, with a median value of approximately 1.80 mm. Thus, Figure 2 demonstrates that continuous variation in this morphological trait readily occurs as a result of interspecific hybridization. This finding is consistent with these traits being quantitatively inherited (CROW, 1983). Therefore, aculeus length in these two species is presumed to be determined by a mechanism of quantitative inheritance (e.g. polygenes). Fruit flies with a wide range of aculeus lengths, including intermediate forms between the two focal species, have been detected among specimens collected within the distribution area. The results of the present study indicate that the polymorphism in the aculeus lengths of these two species of fruit fly can be attributed to interspecific crosses between *B. carambolae* and *B. papayae*. Similarly, results suggesting quantitative inheritance have been found during interspecific breeding experiments in the genus *Anastrepha* (SANTOS *et al*., 2001).

Furthermore, Figure 2 demonstrates that the distribution of the character in the F_2 hybrids can be divided into three groups. In the F_2 hybrids, the observed ratio of shorter aculeus groups (1.621 ± 0.042 mm, mean±SD), longer aculeus groups (1.915 ± 0.022 mm) and intermediate aculeus groups (1.777 ± 0.039 mm) was 10 : 7 : 25 (Fig. 2c). This ratio is consistent with the theoretical Mendelian ratio for a trait produced by a single locus (1 : 1 : 2; $\chi^2=1.952$, $p=0.376$). However, this trait is not believed to result from a single qualitative gene, because a convergence in morphological distribution to a mean value was observed in all F_2 hybrids. Although a pair of alleles function-

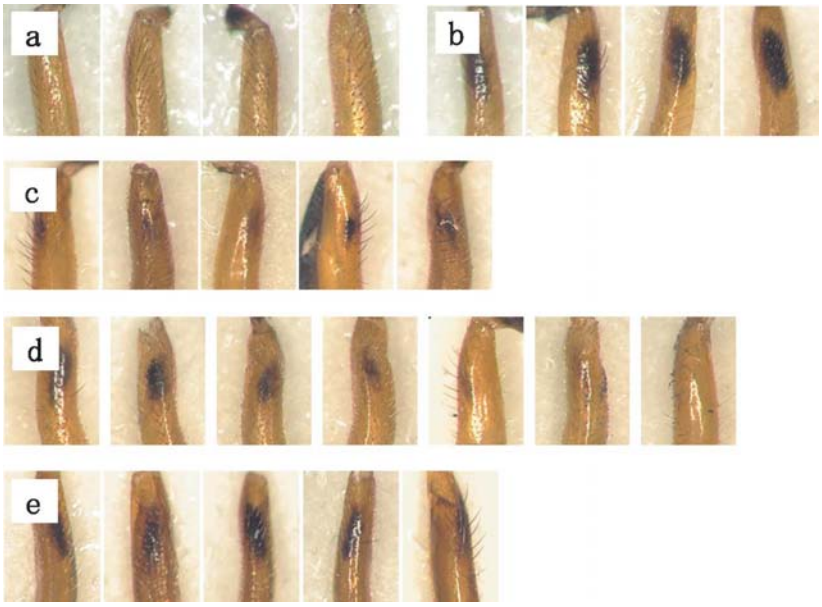

Fig. 3. Black spot on the outer surface of fore femora of P_1 generation, and F_1 and F_2 hybrids between *Bactrocera carambolae* and *B. papayae*.
(a) *B. papayae* P_1 females, (b) *B. carambolae* P_1 females, (c) F_1 hybrids (*B. carambolae* female × *B. papayae* male), (d) F_2 hybrids (*B. carambolae* female × *B. papayae* male), (e) F_1 hybrids of a reciprocal cross (*B. papayae* female × *B. carambolae* male) .

ing as a major gene is assumed to cause the three separate zones in the distribution of the morphological characters of the F_2 females, further research may be required to elucidate this point.

Correlations between the aculeus lengths and the cell dm lengths, as well as between the aculeus lengths and the black spot lengths, are shown in Fig. 4. It is clear that the aculeus lengths have no correlation with cell dm lengths or black spot lengths in the F_2 hybrids. This result suggests that the loci controlling these morphological characters are not linked. It is important to avoid confusing relationships caused by species isolation with those caused by gene linkage when multiple diagnostic characters are observed. However, there is a strong possibility that individuals are hybrids between *B. carambolae* and *B. papayae* when multiple morphological diagnostic characters are inconsistent with the corresponding characters in *B. carambolae* or *B. papayae*.

Although some heterosis was observed in the aculeus lengths among the F_1 hybrids (*B. carambolae* female × *B. papayae* male; average cell dm length in the F_1 hybrids, 2.411 mm, Table 2), the average length in the F_1 or F_2 hybrids approached that in the paternal species of *B. papayae*. On the other hand, in the F_1 hybrids obtained from the reciprocal crosses, the aculeus lengths became shorter, approaching the morphology of the paternal generation of *B. carambolae*. In addition, it was observed that the average black spot length (0.148 mm) in the F_1 hybrids (*B. carambolae* female × *B. papayae* male) was relatively short, whereas the average length (0.321 mm) in the F_1 hybrids from the reciprocal crosses (the maternal species was *B. papayae*) was relatively long (Fig. 3). In other words, it was revealed that the morphological characters of the F_1 hybrids are somewhat similar to those of the paternal species. These results suggest that non-additive factors are involved in the inheritance of aculeus length and black spot length in an interspecific cross between the two species. Based on these findings, it is likely that multiple factors operate on the inheritance of morphological characters, including the aculeus length, of both species.

DNA markers

The mtDNA and rDNA markers indicated different restriction patterns for *B. carambolae* and *B. papayae* (Figs. 5 and 6). These markers could be used to clearly discriminate between the two species. The DNA of these species was reliably amplified by the PCR method. This was due to the relatively short base sequences (347, 558 or 595 bp) of each marker region. As a result, the restric-

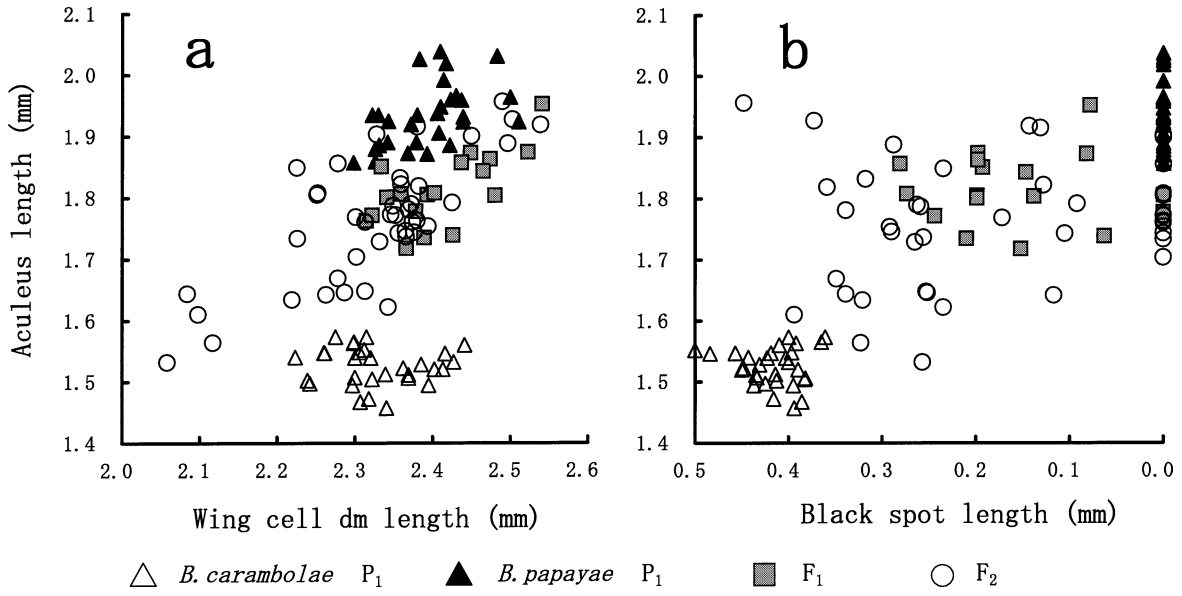

Fig. 4. Correlation between aculeus length and wing cell dm length (a), and between aculeus length and vertical length of the black spot on fore femora (b), in each generation.

tion patterns showed strong reproducibility. It would be difficult and impractical to determine the base sequences of all the species belonging to the genus *Bactrocera*. However, species belonging to the genus *Bactrocera* detected in plant quarantine inspections were limited; therefore, an investigation of species of high importance and the use of short base sequences may be applied to plant inspection in the future.

In all F_1 hybrids (*B. carambolae* female × *B. papayae* male), the restriction pattern of the PCR-amplified fragments of mtDNA was the same as that in the maternal species (*B. carambolae*, Fig. 5a), and that for rDNA contained the restriction fragments of both parent species in the heterozygous banding pattern (hybrid pattern, Fig. 6a). In the reciprocal crosses (*B. papayae* female × *B. carambolae* male), the same results were obtained (Figs. 5b and 6b). Below is a discussion of the reasons for these results.

MtDNA is known to be strictly inherited through the maternal line in almost all animals (GYLLENSTEN *et al*., 1985). Although there are a few reported examples in some animal species of interspecific hybridization in which mtDNA is inherited paternally, for example, a cross between *Drosophila simulans* and *D. mauritiana* (Diptera: Drosophilidae), it has been reported that the likelihood of such an event occurring is extremely low (KONDO *et al*., 1990; KANEDA *et al*., 1995). In the present study, the results obtained indicate maternal inheritance of the mtDNA of *B. papayae* and *B. carambolae*.

On the other hand, F_1 hybrids always have rDNA from both parents (i.e. both species), because nuclear genes are inherited from both parents. Thus, the F_1 hybrids probably had hybrid patterns as a result of the mixing of parental rDNA fragments amplified through PCR. In a similar study, it was reported that F_1 progeny exhibited a hybrid PCR-RFLP pattern as a result of interbreeding between two strains belonging to the Pine wood nematode, *Bursaphelenchus xylophilus* (Nematoda: Aphelenchoididae), which have different PCR-RFLP patterns in their rDNA (AIKAWA *et al*., 2003). These results from a study on nematodes are considered to be consistent with the results of the present study.

The rDNA restriction patterns of the F_2 hybrids (*B. carambolae* female × *B. papayae* male) could be divided into three types: the same pattern as *B. carambolae* (*B. carambolae* pattern), the same pattern as *B. papayae* (*B. papayae* pattern), and a hybrid pattern (Fig. 7). The observed ratio among the *B. carambolae* pattern, the *B. papayae* pattern and the hybrid pattern was 14 : 1 : 27. This ratio did not statistically agree with the theoretical Mendelian ratio for a trait produced by a single locus (1 : 1 : 2; $\chi^2 = 11.476$, $p = 0.003$). In the present study, a significant imbalance was observed in the ratios of the three rDNA markers in the F_2 hybrids: The incidence of the *B. papayae*

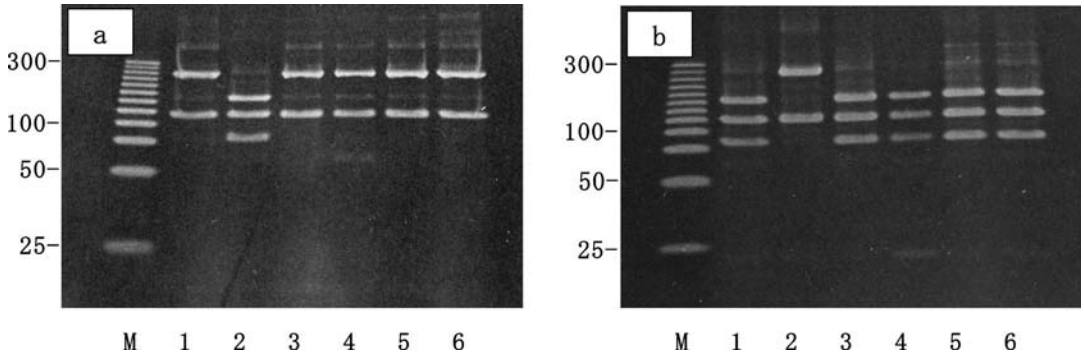

Fig. 5. DNA markers (*Ase*I-digested PCR-RFLP pattern) of mtDNA of P_1 and F_1 hybrids between two *Bactrocera* species.
(a) *B. carambolae* female×*B. papayae* male. Lane numbers: 1, *B. carambolae* P_1 female; 2, *B. papayae* P_1 male; 3–6, F_1 hybrids. (b) *B. papayae* female×*B. carambolae* male (as reciprocal cross). Lane numbers: 1, *B. papayae* P_1 female; 2, *B. carambolae* P_1 male; 3–6, F_1 hybrids. M, Molecular weight markers (25 bp DNA ladder).

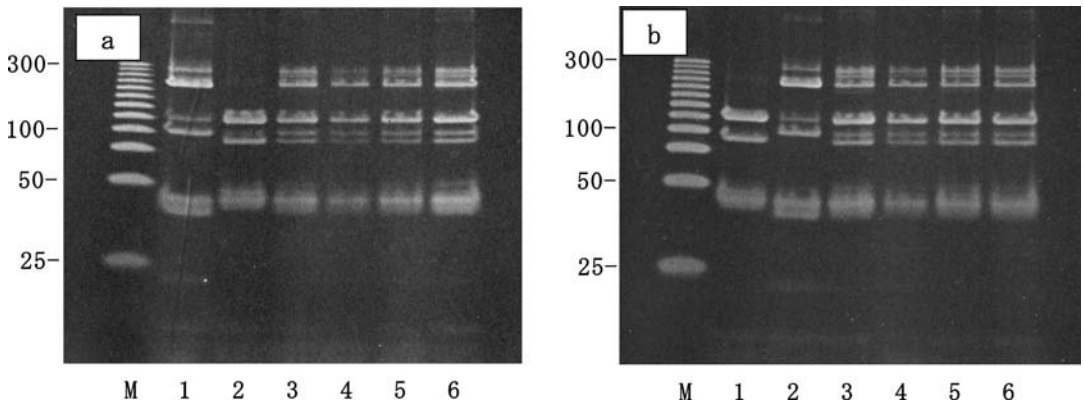

Fig. 6. DNA markers (*Mse*I-digested PCR-RFLP pattern) of rDNA of P_1 and F_1 hybrids between two *Bactrocera* species.
(a) *B. carambolae* female×*B. papayae* male. Lane numbers: 1, *B. carambolae* P_1 female; 2, *B. papayae* P_1 male; 3–6, F_1 hybrids. (b) *B. papayae* female×*B. carambolae* male (as reciprocal cross). Lane numbers: 1: *B. papayae* P_1 female; 2, *B. carambolae* P_1 male; 3–6, F_1 hybrids. M, Molecular weight marker (25bp DNA ladder).

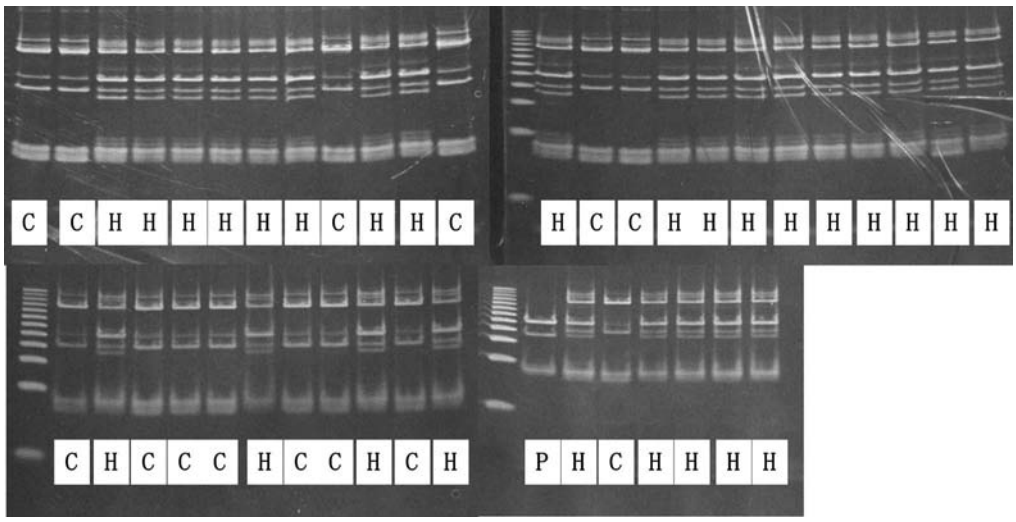

Fig. 7. DNA markers (*Mse*I-digested PCR-RFLP pattern) of rDNA of F_2 hybrids between *Bactrocera carambolae* female and *Bactrocera papayae* male.
There were three DNA marker patterns: C, *B. carambolae* pattern; P, *B. papayae* pattern; H, hybrid pattern.

pattern was extremely low. The reason for this imbalance has not been elucidated, and further research may be required to do so.

In the present study, we succeeded in identifying the rDNA markers that are characteristic of interspecific hybrids between *B. carambolae* and *B. papayae*. If a specimen displaying a hybrid rDNA pattern were to be collected in the distribution area, it would clearly demonstrate the presence of interspecific hybridization, because a hybrid pattern directly indicates that the genes of two different species are contained in a single individual.

Backcrossing

We performed backcross experiments between an F_1 female (*B. carambolae* female × *B. papayae* male) and a *B. papayae* P_1 male. However, results necessary to discuss the morphological characters and DNA markers of the progeny were not obtained, due to an insufficient number of BC_1 hybrids. Nevertheless, we observed that introgression occurred in at least two BC_1 females. In these BC_1 hybrids ($n = 2$), the restriction pattern of the mtDNA was identical to that of *B. carambolae* (the maternal P_1 species), and the restriction pattern of the rDNA was identical to that of *B. papayae* (the paternal P_1 species). In addition, the aculeus lengths of these two BC_1 females were 1.833 and 1.790 mm, respectively, and the black spots on the fore femora had disappeared. Thus, these BC_1 females had the same morphological characters as *B. papayae*. The cause of these inheritance patterns may be as follows: Half of BC_1 hybrids have paternal nuclear genes, because nuclear genes (rDNA) were inherited from the parents (F_1 female and *B. papayae* male), whereas all the BC_1 hybrids have maternal mitochondrial genes, because mtDNA is inherited only through the maternal line. This introgression of mtDNA could be readily achieved by intercrossing and subsequent backcrossing.

MURAJI and NAKAHARA (2002) reported that identification of *B. carambolae* and *B. papayae* by PCR-RFLP analyses based on mtDNA fragments was sometimes difficult, because some individuals of *B. carambolae* had the same banding patterns as *B. papayae*. We believe this phenomenon to be partly due to the introgression of mtDNA through interspecific crossing and backcrossing, as shown in the present study.

An inconsistency between mtDNA markers and morphological characters was also reported in the Carabidae (Coleoptera) (SOTA, 2003), and in the *Drosophila melanogaster* complex (Diptera: Drosophilidae) (SOLIGNAC, 2004). In these reports, introgression of mtDNA via interspecific hybridization was suggested to be the cause of the inconsistency between mtDNA markers and morphological characters. In the present study it was found that the morphological characters were consistent with the RFLP patterns of the rDNA fragments. This consistency probably occurred because the genes associated with morphological characters were present in the nuclear DNA of both species. SOTA (2003) reported that classification using nuclear genes was relatively consistent with morphological taxonomy. Therefore, not only mtDNA-RFLP analysis but also nuclear DNA-RFLP analysis would be more desirable for discriminating between *B. carambolae* and *B. papayae*. In addition, the detection of individuals demonstrating an inconsistency between mtDNA markers and morphological characters would clearly be an indication that interbreeding has occurred between *B. carambolae* and *B. papayae* in the distribution area, because the two species can be interspecifically hybridized.

Relationship between morphological characters and DNA markers

The relationship between morphological characters and RFLP marker patterns for the rDNA fragments in the F_2 hybrids is shown in Figure 8. The scatter diagram in Figure 8 shows that the marker patterns are scattered and independent of the morphological characters. For this reason, an individual with the rDNA marker patterns of *B. papayae* does not always exhibit the morphological characters of *B. papayae*. The present study showed that the DNA marker loci are not linked to the loci controlling morphological traits.

It is generally known that most cells contain multiple copies of mtDNA and rDNA molecules (LONG and DAWID, 1980; KONDO *et al*., 1990), so there is a considerable amount of mtDNA and rDNA present in cells. Therefore, PCR-RFLP markers using mtDNA and rDNA are highly sensi-

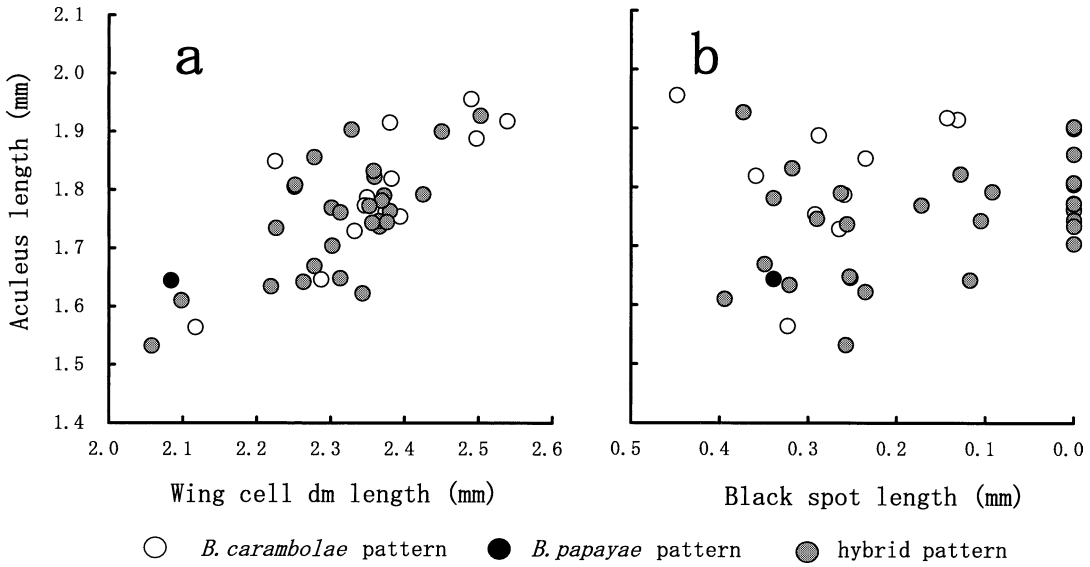

Fig. 8. Relationship between morphological characters and DNA markers in F_2 hybrids between *Bactrocera carambolae* female and *B. papayae* male. Correlation between aculeus length and wing cell dm length (a), and between aculeus length and longitudinal length of the black spot on fore femora (b).
There were three DNA marker patterns: *B. carambolae* pattern (open circle), *B. papayae* pattern (closed circle) and hybrid pattern (gray circle).

tive. Consequently, both mtDNA and rDNA are frequently used as DNA markers for taxonomic classification (SOTA, 2003; SMITH *et al*., 2002). However, as discussed above, the DNA sections used as markers were unrelated to the genes associated with the morphological characteristics. This was probably because the combination between the DNA markers and the morphological traits was broken as a result of interspecific hybridization. Thus, in the identification of species using DNA markers, the combination between DNA markers and morphological characters is likely to be important, and would be significantly influenced by the frequency of natural hybridization.

It has been observed that differences in host range (WHITE, 2000; IWAIZUMI, 2004) or habitat preference (OOI, 1991; WHITE, 2000) may isolate two species from each other. In addition, it is known that there are differences in reproductive behavior between closely related species in the genus *Bactrocera* (MCINNIS *et al*., 1999; PIKE and MEATS, 2002). However, SANTOS *et al*. (2001) stated that such factors as human-mediated population flow increase opportunities for occurrence of natural hybridization events. Thus, it is very likely that human effects may cause an increased frequency of interbreeding between *B. carambolae* and *B. papayae*, which had previously been isolated from each other by environmental conditions.

CLARKE *et al*. (2005) argued that a complete phylogeny developed from a combination of molecular and morphological data is necessary. Therefore, combining data on morphological characters and DNA markers, not just examining them independently, will be important for future research, as was shown in the present study. Further, CLARKE *et al*. (2005) claimed that if hybridization between species within a complex is found to be common, species limits need to be redefined. If a high frequency of crossings between *B. carambolae* and *B. papayae* is likely to occur to the extent that species discrimination becomes difficult, continued efforts to review the classification of the *B. dorsalis* complex, including both *B. carambolae* and *B. papayae*, will become essential.

Acknowledgements

We thank Dr. Koichi GOKA, Satomi NIWA, and other staff of the National Institute for Environmental Studies for technical direction and advice regarding the PCR method, and Dr. Masahiko MURAJI (National Institute of Agrobiological Science) for technical advice regarding the

PCR. We also thank Ren IWAIZUMI (Yokohama Plant Protection Station [YPPS], Ministry of Agriculture, Forestry and Fisheries) for technical advice regarding the identification of the *Bactrocera* species, and the staff of the YPPS for their useful suggestions.

References

AIKAWA, T., T. KIKUCHI and H. KOSAKA (2003) Demonstration of interbreeding between virulent and avirulent populations of *Bursaphelenchus xylophilus* (Nematoda: Aphelenchoididae) by PCR-RFLP method. *Appl. Entomol. Zool.* 38(4): 565–569.

ARMSTRONG, K. F. and C. M. CAMERON (2000) Species identification of tephritids across a broad taxonomic range using ribosomal DNA. In *Area-Wide Control of Fruit Flies and Other Insect Pests* (K. H. TAN ed.). Penang, Malaysia, CABI, pp. 703–710.

CLARKE, A. R., K. F. ARMSTRONG, A. E. CARMICHAEL, J. R. MILNE, S. RAGHU, G. K. RODERICK and D. K. YEATES (2005) Invasive phytophagous pests arising through a recent tropical evolutionary radiation: the *Bactrocera dorsalis* complex of fruit flies. *Annu. Rev. Entomol.* 50: 293–319.

CROW, J. F. (1983) *Genetics Notes, An Introduction to Genetics, eighth edition.* Macmillan Publishing Company. New York, 303pp.

DREW, R. A. I. (1989) The tropical fruit flies (Diptera: Tephritidae: Dacinae) of the Australasian and Oceanian regions. *Mem. Queensl. Mus.* 26: 1–521.

DREW, R. A. I. and D. L. HANCOCK (1994) The *Bactrocera dorsalis* complex of fruit flies (Diptera: Tephritidae: Dacinae) in Asia. *Bull. Entomol. Res. Suppl.* 2, 68 pp.

DREW, R. A. I. and M. C. ROMIG (1997) Overview–Tephritidae in the Pacific and Southeast Asia. In *Management of fruit flies in the Pacific. ACIAR Proceedings* 76 (A. J. ALLWOOD and R. A. I. DREW eds.). Australian centre for international agricultural research, Canberra, pp. 46–53.

DREW, R. A. I., K. TSURUTA and I. M. WHITE (2005) A new species of pest fruit fly (Diptera: Tephritidae: Dacinae) from Sri Lanka and Africa. *African Entomol.* 13(1): 149–154.

GYLLENSTEN, U., D.WHARTON and A. C. WILSON (1985) Maternal inheritance of mitochondrial DNA during back-crossing of two species of mice. *J. Hered.* 76: 321–324.

HARDY, D. E. (1969) Taxonomy and distribution of the oriental fruit fly and related species (Tephritidae-Diptera). *Proceedings of the Hawaiian Entomological Society* 20: 395–428.

HARDY, D. E. and M. ADACHI (1956) Insects of micronesia Diptera: Tephritidae. *Insects of Micronesia* 14(1): 1–28.

HE, M. and D. S. HAYMER (1997) Polymorphic intron sequences detected within and between populations of the oriental fruit fly (Diptera: Tephritidae). *Ann. Entomol. Soc. Am.* 90(6): 825–831.

IWAHASHI, O. (1999) Distinguishing between the two sympatric species *Bactrocera carambolae* and *B. papayae* (Diptera: Tephritidae) based on aedeagal length. *Ann. Entomol. Soc. Am.* 92(5): 639–643.

IWAIZUMI, R. (2004) Species and host record of the *Bactrocera dorsalis* complex (Diptera: Tephritidae) detected by the plant quarantine of Japan. *Appl. Entomol. Zool.* 39(2): 327–333.

IWAIZUMI, R., M. KANEDA and O. IWAHASHI (1997) Correlation of length of terminalia of males and females among nine species of *Bactrocera* (Diptera: Tephritidae) and differences among sympatric species of *B. dorsalis* complex. *Ann. Entomol. Soc. Am.* 90(5): 664–666.

KANEDA, H., J. HAYASHI, S. TAKAHAMA, C. TAYA, K. F. LINDAHL and H. YONEKAWA (1995) Elimination of paternal mitochondrial DNA in intraspecific crosses during early mouse embryogenesis. *Proc. Natl. Acad. Sci. USA* 92: 4542–4546.

KONDO, R., Y. SATTA, E. T. MATSUURA, H. ISHIWA, N. TAKAHATA and S. I. CHIGUSA (1990) Incomplete maternal transmission of mitochondrial DNA in *Drosophila. Genetics* 126: 657–663.

LONG, E. O. and I. B. DAWID (1980) Repeated genes in eukaryotes. *Ann. Rev. Biochem.* 49: 727–764.

MCINNIS, D. O., P. RENDON, E. JANG, A. SAUERS-MULLER, R. SUGAYAMA and A. MALAVASI (1999) Interspecific mating of introduced, sterile *Bactrocera dorsalis* with wild *B. carambolae* (Diptera: Tephritidae) in Suriname: a potential case for cross-species sterile insect technique. *Ann. Entomol. Soc. Am.* 92(5): 758–765.

MURAJI, M. and S. NAKAHARA (2002) Discrimination among pest species of *Bactrocera* (Diptera: Tephritidae) based on PCR-RFLP of the mitochondrial DNA. *Appl. Entomol. Zool.* 37(3): 437–446.

OOI, C. S. (1991) Genetic variation in populations of two sympatric taxa in the *Dacus dorsalis* complex and their relative infestation levels in various fruit hosts. In *Fruit Flies in the Tropics. Proc. of First Int. Symp., 14–16 March 1988, Kuala Lumpur, Malaysia* (S. VIJAYSEGARAN and A. G. IBRAHIM eds.). Kuala Lumpur, Malays. Agric. Res. Dev. Inst. pp. 71–80.

PIKE, N. and A. MEATS (2002) Potential for mating between *Bactrocera tryoni* (FROGGATT) and *Bactrocera neohumeralis* (HARDY) (Diptera: Tephritidae). *Aust. J. Entomol.* 41: 70–74.

SANTOS, P., K. URAMOTO and S. R. MATIOLI (2001) Experimental hybridization among *Anastrepha* species (Diptera: Tephritidae): production and morphological characterization of F_1 hybrids. *Ann. Entomol. Soc. Am.* 94(5): 717–725.

SMITH, P. T., B. A. MCPHERON and S. KAMBHAMPATI (2002) Phylogenetic analysis of mitochondrial DNA supports the monophyly of Dacini fruit flies (Diptera: Tephritidae). *Ann. Entomol. Soc. Am.* 95(6): 658–664.

SOLIGNAC, M. (2004) Mitochondrial DNA in the *Drosophila melanogaster* complex. *Genetica* 120: 41–50.

SOTA, T. (2003) Phylogeographic study of the Carabid beetles *Ohomopterus* in West Japan based on analysis of mitochondrial DNA sequences. *Bull. Hosizaki Green Found.* 6: 153–166 (in Japanese with English abstract).

TSURUTA, K. and I. M. WHITE (2001) Eleven new species of the genus *Bactrocera* MACQUART (Diptera: Tephritidae) from Sri Lanka. *Entomol. Sci.* 4: 69–87.

WHITE, I. M. (2000) Morphological features of the tribe Dacini (Dacinae): their significance to behavior and classification. In *Fruit flies* (*Tephritidae*): *phylogeny and evolution of behavior* (M. ALUJA and A. L. NORRBOM eds.). Boca Raton, FL: CRC Press, pp. 505–533.

WHITE, I. M. and M. M. ELSON-HARRIS (1992) *Fruit Flies of Economic Significance: Their Identification and Bionomics.* Wallingford, U.K, CAB International, 601pp.

YONG, H. S. (1995) Genetic differentiation and relationships in five taxa of the *Bactrocera dorsalis* complex (Insecta: Diptera: Tephritidae). *Bull. Entomol. Res.* 85: 431–435.

YOSHIZAWA, O. (1997) Successful eradication programs on fruit flies in Japan. *Res. Bull. Pl. Prot. Jpn.* 33 (Suppl.): 1–10.

和 文 摘 要

ミカンコミバエ種群 (*Bactrocera dorsalis* species complex) に属する *Bactrocera carambolae* と *B. papayae*（ハエ目：ミバエ科）との種間交雑個体の外部形態及び PCR-RFLP マーカー

海老名崇生・大戸謙二
横浜植物防疫所調査研究部

Bactrocera carambolae と *B. papayae* との間の種間交雑個体の外部形態及び PCR-RFLP マーカー (DNA マーカー) の調査を行った。両種は共にミカンコミバエ種群に属する日本未発生の重要害虫である。供試虫は農林水産大臣の許可条件の上でマレーシア及びタイから輸入され、継代飼育された。実験では *B. carambolae* 雌と *B. papayae* 雄が一対で交雑された。その結果、外部形態の調査では、F_1 及び F_2 個体の aculeus（産卵管の硬化部）の長さは両親の中間的な形態を示した。さらに F_2 個体において、aculeus の長さの変異の幅は拡がることが観察された。このため、両種の aculeus の長さは量的遺伝により作用されているものと推測された。全ての F_1 個体において、ミトコンドリア DNA (mtDNA; 16S rRNA) の DNA マーカーは母親と同じ制限パターンを示し、核リボゾーム DNA（rDNA; 5.8S rRNA 及び ITS）のマーカーは両親のパターンを重ね合わせたもの (hybrid pattern) を示した。また、F_2 個体における rDNA のマーカーの比率は、*B. carambolae* のパターン、*B. papayae* のパターン及び hybrid pattern で 14 : 1 : 27 となった。また、F_2 個体において、外部形態と rDNA のマーカーとの間に特定の関係がないことが確認された。以上のことから、検疫で発見される両種の標本において、外部形態と DNA マーカーとの不一致、あるいは aculeus の長さの連続的な変異が観察されることは、分布地域における両種の交雑が一因であるものと示唆された。