Discrimination among pest species of *Bactrocera* (Diptera: Tephritidae) based on PCR-RFLP of the mitochondrial DNA

Masahiko Muraji* and Shigehito Nakahara^{1,2}

National Institute of Agrobiological Sciences, Tsukuba, Ibaraki 305–8634, Japan

¹ Yokohama Plant Protection Station, Yokohama, Kanagawa 231–0003, Japan

(Received 16 January 2002; Accepted 8 May 2002)

Abstract

A PCR-RFLP based method of species identification was considered for 18 pest species of *Bactrocera* fruit flies mainly distributed in the Asia-Pacific region. A 1.6 kb portion of the mitochondrial DNA containing portions of 16S and 12S ribosomal RNA genes was determined for eight species for which the mtDNA sequences have not been studied. Comparisons of restriction patterns expected from the sequences obtained in this and previous studies revealed that all 18 species can be discriminated by PCR-RFLP using a small number of restriction enzymes. Based on the highly conserved sequences detected among the 18 species, four sets of PCR primers were designed to amplify shorter, diagnostically informative sections within the mtDNA fragment. Given the simplicity of banding patterns and the ease of amplification of DNA fragments, such sections were considered suitable for PCR-RFLP analysis. Based on the results, a scheme for the *Bactrocera* pest species identification was proposed. The PCR-RFLP analysis using 83 individuals revealed that the scheme correctly identified most of the 18 species except for two closely related sympatric species, *Bactrocera carambolae* and *B. papayae*; the majority of individuals of the former species showed the same banding patterns as the latter species. In addition, molecular phylogenetic analyses were performed based on the nucleotide sequences in order to examine the positioning of each species among many other species of fruit flies.

Key words: Mitochondrial DNA, PCR-RFLP, species identification, *Bactrocera*, Tephritidae

INTRODUCTION

The tephritid genus *Bactrocera* is widely distributed in the Asia-Pacific region and contains a number of economically important species that cause losses in fruit and vegetable crops. Among 11 species of Japanese *Bactrocera*, the two major pest species, the oriental fruit fly *Bactrocera dorsalis* and the melon fly *B. cucurbitae*, were eradicated from the country (Yoshizawa, 1997). However, as a result of increased importation of fruits and vegetables in recent years, fruit flies of foreign origins have been frequently intercepted at quarantine inspection sites. Consequently, it has become increasingly important to take precautions against their invasion.

To that end, it is necessary to identify pest species accurately. Traditionally, they have been identified based on the morphological characteristics of adult insects (White and Hancock, 1997). However, it is sometimes difficult to identify *Bac-*

trocera species, because there are numbers of species that are not morphologically distinct. Moreover, there are many cases in which morphological criteria cannot be easily applied. For example, species identification of immature insects usually requires rearing individuals until adult emergence. Many species can be discriminated from allied species only by the characteristics of male adults. Species identification is difficult when only broken pieces or portions of insect body are available. Such situations necessitate the development of new criteria for rapid and accurate identification of *Bactrocera* species that can be applied in quarantine practices.

Recently, a number of entomologists have analyzed the nucleotide sequences of fruit flies to infer phylogenetic relationships among species and groups (Han and McPheron, 1997; Smith and Bush, 1997; Han, 2000; Muraji and Nakahara, 2001). As a result, several hundred sequences were submitted to international DNA sequence data-

* To whom correspondence should be addressed at: E-mail: mmuraji@affrc.go.jp

² Present address: Naha Plant Protection Office, Naha, Okinawa 900–0001, Japan

bases such as DDBJ, EMBL, and GenBank. These sequences can be used to design methods of species identification based on PCR-RFLP (Restriction fragment length polymorphisms in DNA fragments amplified by polymerase chain reaction), though such an analysis has not yet been performed for *Bactrocera* species.

In this study, we sequenced a 1.6 kb-long portion of mitochondrial DNA (mtDNA) using 19 individuals from eight *Bactrocera* pest species mainly distributed in the Oceanian region (i.e., *B. tryoni*, *B. frauenfeldi*, *B. musae*, *B. cucumis*, *B. neohumeralis*, *B. jarvisi*, *B. curvipennis*, and *B. psidi*). Using these and reported sequences of major pest species in the Oriental region, RFLP patterns were estimated and compared in order to develop a PCR-RFLP-based method of species identification. The utility of the method was improved by using shorter sections within the sequence amplified by PCR primers designed according to the distribution of recognition sites of diagnostic restriction enzymes. In addition, we also performed molecular phylogenetic analyses based on the sequences in order to examine the positioning of each species among many species of fruit flies.

MATERIALS AND METHODS

Materials used in this study are listed in Table 1. Template DNA for sequencing and PCR-RFLP analyses was extracted from whole body or portions (mainly legs) of individual adults using a GenomicPrep™ Cells and Tissue DNA Isolation Kit (Amersham Pharmacia). Template DNAs were dissolved in 200 or 50 µl of sterilized water. Using 19 individuals of eight Oceanian species, the nucleotide sequence of the PCR amplified fragment (1.6 kb long) was analyzed. Experimental procedures for amplification, purification, labeling, and sequencing were the same as those described in Muraji and Nakahara (2001). Nucleotide sequences obtained were submitted to DDBJ/EMBL/GenBank nucleotide sequence databases (Accession numbers; AB074018–AB074029).

The sequences were aligned with those of 10 previously reported species of *Bactrocera* (described at the end of this section) and three other dipteran insects, the fruit fly *Drosophila yakuba* (X03240), the Mediterranean fruit fly *Ceratitis capitata* (AJ242872), and the Mexican fruit fly

Anastrepha ludens (AB035102), using the program TreeAlign (Hein, 1990) with default parameters (gapa=8, gapb=3). For phylogenetic analyses, ambiguously aligned portions (226 bp in total length, including gaps) were excluded from the alignment, and consistently aligned sequences (1,458 bp) were analyzed using PAUP* Version 4.0 b8 (Swofford, 1999) and MEGA version 1 (Kumar et al., 1993). In the weighted parsimony analysis using PAUP, a weighting scheme generated using the "REWEIGHT" command with an "index=RC" option, treating the consensus tree of the unweighted parsimony analysis as the starting tree, was applied. The support for each node was assessed by 1,000 replications of bootstrapping, using a 100 maxtree option.

For 43 sequences of the 18 species obtained in this and previous studies (Muraji and Nakahara, 2001), recognition sites of 50 restriction enzymes were estimated using the computer program Genetyx-Mac Version 10.1 (Software Development). Based on the distribution of diagnostic restriction sites and on the homology in the aligned sequences, four sets of PCR primers (8 primers in total) were designed to amplify diagnostically informative sections within the mtDNA fragment. PCR products obtained using these primers were used for the PCR-RFLP analyses. In order to test the efficiency of the primers, PCR was also performed using several dried specimens (*B. dorsalis* and *B. cucurbitae*) left at room temperature for more than 4 months. Amplification was carried out under the experimental conditions described in Muraji and Nakahara (2001). Two to 4 µl of the PCR product was incubated in a total reaction volume of 10 µl containing 2 U restriction enzyme, *AseI*, *DraI* (Nippon Gene), *ApoI*, or *MseI* (NEW ENGLAND BioLabs), and 1×reaction buffer for 2 h at 37°C (or 50°C for *ApoI*). Restriction fragments (5 µl of reaction mixture) were electrophoresed in 2, 4, or 4.5% MetaPhor™ agarose (BioWhittaker Molecular Applications) gel in 1×TBE buffer at 6–7 V/cm for 2 h then visualized by staining with ethidium bromide.

Species and accession numbers of the sequences used in phylogenetic and restriction fragment analyses were as follows: *B. carambolae* (AB035117, AB035118), *B. dorsalis* (AB035114, AB035115, AB035116), *B. kandiensis* (AB048737, AB048738), *B. occipitalis* (AB048741, AB048742),

Table 1. Specimens used in this study

Subgenus	Species ^a	Collection site ^b	Analysis performed ^c
<i>Afrodacus</i>	<i>B. jarvisi</i>	Queensland, Australia	PCR-RFLP (3) / Sequencing (2)
<i>Austrodacus</i>	<i>B. cucumis</i>	Queensland, Australia	PCR-RFLP (3) / Sequencing (3)
<i>Bactrocera</i>	<i>B. curvipennis</i>	New Caledonia	PCR-RFLP (2) / Sequencing (2)
	<i>B. frauenfeldi</i>	Queensland, Australia	PCR-RFLP (2) / Sequencing (2)
	<i>B. musae</i>	Queensland, Australia	PCR-RFLP (2) / Sequencing (2)
	<i>B. neohumeralis</i>	Queensland, Australia	PCR-RFLP (2) / Sequencing (2)
	<i>B. psidi</i>	New Caledonia	PCR-RFLP (3) / Sequencing (3)
	<i>B. tryoni</i>	Queensland, Australia	PCR-RFLP (2) / Sequencing (2)
		New Caledonia	PCR-RFLP (1) / Sequencing (1)
	<i>B. carambolae</i> *	Kuala Lumpur, Malaysia	PCR-RFLP (1)
		Pattani, Thailand	PCR-RFLP (3)
		Nakhon Si, Thailand	PCR-RFLP (2)
		Bandung, Indonesia	PCR-RFLP (3)
	<i>B. dorsalis</i> *	Amami, Kagoshima, Japan	PCR-RFLP (5)
		Naha, Okinawa, Japan	PCR-RFLP (5)
		Ogasawara, Tokyo, Japan	PCR-RFLP (5)
	<i>B. kandiensis</i> *	Katunayake, Sri Lanka	PCR-RFLP (3)
	<i>B. occipitalis</i> *	Narita Airport (Philippines)	PCR-RFLP (1)
		Nagoya Airport (Philippines)	PCR-RFLP (1)
		Cebu, Philippines	PCR-RFLP (2)
		Davao, Philippines	PCR-RFLP (3)
	<i>B. papayae</i> *	Kuala Lumpur, Malaysia	PCR-RFLP (1)
		Pattani, Thailand	PCR-RFLP (3)
		Bandung, Indonesia	PCR-RFLP (5)
	<i>B. philippinensis</i> *	Narita Airport (Philippines)	PCR-RFLP (4)
		Davao, Philippines	PCR-RFLP (3)
<i>Tetradacus</i>	<i>B. tsuneonis</i>	Oita, Japan	PCR-RFLP (3)
<i>Zeugodacus</i>	<i>B. cucurbitae</i>	Amami, Kagoshima, Japan	PCR-RFLP (1)
		Naha, Okinawa, Japan	PCR-RFLP (1)
		Sri Lanka ^d	PCR-RFLP (1)
	<i>B. depressa</i>	Nago, Okinawa, Japan	PCR-RFLP (1)
		Yokohama, Kanagawa, Japan	PCR-RFLP (1)
	<i>B. tau</i>	Kegalla, Sri Lanka	PCR-RFLP (4)

^a Species denoted by an asterisk belong to the *Bactrocera dorsalis* complex.

^b The country that exported fruits from which *Bactrocera* fruit flies were detected is shown in parentheses.

^c Numerals in parentheses refer to the number of individuals used for sequencing and PCR-RFLP analyses.

^d A laboratory strain established using insects collected at Puttalam, Negombo, and Dumbulla in Sri Lanka.

B. papayae (AB035119, AB035120), *B. philippinensis* (AB035111, AB048743, AB048744), *B. depressa* (AB035100, AB035101), *B. cucurbitae* (AB035112, AB035113, AB048754, AB048748), *B. tau* (AB048745, AB048746), and *B. tsuneonis* (AB074030).

RESULTS

Sequence alignment

Among the 18 species treated in this study, mtDNA sequences had not been previously studied for eight species distributed mainly in the Oceanian

region. For such species, we successfully determined 1,599–1,601 bp-long sequences using 19 individuals. Nucleotide composition of these sequences was biased for A and T, and showed an average A+T content of 77.6% (range: 73.5–78.4%). When the sequences were aligned with those of 10 previously reported *Bactrocera* fruit flies and the three other dipteran species, at least 17 sites of insertion/deletion mutations were detected. Due to this phenomenon, these sequences could not be aligned unambiguously in such portions. Thus, for phylogenetic analyses, 17 ambiguously aligned portions (226 bp in total length including gaps)

Fig. 1. One of the two most parsimonious trees generated by the weighted parsimony analysis based on consistently aligned 1,458 bp-long sequences. Nucleotide sequences of species denoted by an asterisk were obtained in this study.

were excluded from the alignment according to the method described by Muraji and Nakahara (2001).

Phylogenetic analysis

Phylogenetic analyses were performed using 1,458 bp-long aligned sequences from which ambiguously aligned sections were excluded. In the case of the maximum parsimony analysis, the unweighted method generated the two most parsimonious trees (length: 577 steps, CI: 0.759) based on 145 parsimony-informative characters. Analysis using the character weighting also generated the two most parsimonious trees (length: 382 steps, CI: 0.907) (Fig. 1). Among trees, differences were observed only in the relationships between the closely related species *B. dorsalis*, *B. papayae*, and *B. carambolae* within the same clade. In these trees, many of the phylogenetic relationships were supported by bootstrap analysis. In the case of neighbor-joining analyses, topologies generated based on Jukes-Cantor, Tamura, Tamura-Nei, and Kimura 2-parameter distances were in agreement. When only nodes supported by bootstrap confidence levels of higher than 50% were considered, neighbor-

joining trees agreed well with those generated by the weighted parsimony method except in the case of the positioning of *B. tsuneonis*.

PCR-RFLP analysis

For 43 sequences of the 18 *Bactrocera* species obtained in this and previous studies (Muraji and Nakahara, 2001), recognition sites of 50 restriction enzymes were examined using the computer program Genetyx (data not shown). Restriction patterns expected from the sequences revealed that all 18 species could be discriminated among by PCR-RFLPs with *MseI* and several other restriction enzymes (*ApoI* and *AluI* or *Sau3AI*). Figure 2 shows banding patterns of representative individuals of the 18 species treated with the enzyme *MseI*. Each sample contains a considerable number of restriction bands, and there are several bands that could not be clearly resolved. Banding patterns of *ApoI*, *AseI*, and *DraI* were expected to be less variable, and they could not discriminate among several species even when results from different enzymes were combined.

In order to simplify the banding patterns to be

Fig. 2. Banding patterns detected among the 18 *Bactrocera* species using a PCR-amplified 1.6 kb-long mtDNA fragment and the restriction enzyme *Mse*I. Samples were electrophoresed in 4.5% MetaPhorTM agarose gel. Lanes: 1: *B. carambolae*, 2: *B. kandiensis*, 3: *B. jarvisi*, 4: *B. depressa*, 5: *B. tsuneonis*, 6: *B. cucumis*, 7: *B. cucurbitae*, 8: *B. tau*, 9: *B. frauenfeldi*, 10: *B. psidi*, 11: *B. curvipennis*, 12: *B. tryoni*, 13: *B. neohumeralis*, 14: *B. dorsalis*, 15: *B. papayae*, 16: *B. philippinensis*, 17: *B. occipitalis*, 18: *B. musae*, M: 25 bp-ladder.

Fig. 3. Recognition sites of representative restriction enzymes expected from 43 nucleotide sequences of the 18 *Bactrocera* species shown along the length of the mtDNA fragment. Horizontal lines indicate the mtDNA sections used in the PCR-RFLP analyses.

treated in PCR-RFLP analyses, PCR primers were designed to amplify shorter diagnostically informative sections within the mtDNA sequence. Figure 3 shows the distribution of recognition sites of the four most variable enzymes along the length of the DNA sequence. In this figure, variable sites seem to be distributed among four separate sections (bp 150–400, bp 400–900, bp 900–1,300, and bp 1,350–1,600). Based on the highly conserved sequences that flank these sections, we designed four sets of PCR primers to amplify these sections separately (Table 2). These primer sets successfully amplified DNA fragments from specimens of all 18 species that had been freshly killed and stored in 99.9% ethanol. They also worked effectively when

Table 2. PCR primers designed to amplify four different sections within the 1.6 kb-long mtDNA sequence shown in the 5'–3' direction

Section	Primer	Sequence
I	AFP2	ATCCAACATCGAGGTCGCAAAC
	ARP1	GGCTGGTATGAACGGTTGGACGAG
II	BFP2	CTCGTCCAACCGTTCATACCAGCC
	BRP2	GTTATTCGTTTATAAAGGTATCT
III	CFP2	AAACTAGATACCTTTATAAAC
	CRP1	GTAACATAGTAGATGTACCGGAA
IV	DFP1	TAATGAGAGCGACGGGCGAT
	DRP2	GGAACCTGTCCTGTAATTGATAAT

Table 3. Restriction fragment length estimated among 43 sequences of 18 *Bactrocera* species^a

	Section I	Section II	Section III	Section IV
<i>ApoI</i>	A: 149 139 47 12 B: 149 112 47 27 12 C: 148 81 58 47 12 D: 149 58 54 47 39 E: 149 58 54 47 27 12	A: 199 156 102 43 13 10 B: 163 156 102 48 43 C: 163 156 102 43 35 13 D: 163 102 86 71 48 43 E: 163 102 86 71 43 35 13	A: 261 184 B: 261 164 19 C: 164 142 119 19 D: 164 134 128 19 E: 164 119 114 29 19	A: 236 62 B: 236 47 16 C: 189 62 47 D: 189 47 47 16 E: 76 62 57 54 47
<i>DraI</i>	A: 212 135 B: 218 129 C: 179 135 33 D: 168 129 50 E: 135 129 50 33 F: 347	A: 401 83 B: 319 84 83 29 C: 319 83 79 30 D: 311 90 83 28 E: 278 123 83 29	A: 348 51 45 B: 301 98 45 C: 270 123 51 D: 250 98 51 45 E: 225 123 51 45 F: 127 123 98 51 45	A: 182 60 55 B: 309
<i>AseI</i>	A: 228 119 B: 228 96 23 C: 150 119 78 D: 150 96 78 23 E: 347	A: 369 144 B: 249 167 96 C: 249 144 119 D: 249 119 96 48	A: 337 107 B: 233 211 C: 173 160 107 D: 173 128 107 21 15 E: 149 111 107 62 15 F: 128 126 107 62 21 G: 128 126 107 84 H: 128 111 107 84 15 I: 128 111 107 62 21 15	A: 247 49 B: 297
<i>MseI</i> ^b	A: 92 78 57 44 41 B: 92 78 57 50 44 C: 92 78 44 41 33 D: 92 78 44 33 E: 88 78 77 41 F: 88 78 44 41 33 G: 88 78 44 33 H: 88 78 44 41 I: 78 73 59 57 44	A: 135 93 52 45 35 B: 109 93 52 45 35 C: 109 67 52 45 35 D: 106 93 52 45 35 E: 93 62 52 45 43 35 F: 93 60 52 48 45 G: 93 60 58 52 49 45 35 H: 84 60 52 45 41 I: 84 60 52 49 45 J: 84 58 52 50 45 35	A: 128 76 50 40 30 B: 103 59 50 46 40 C: 103 98 54 54 50 46 D: 98 59 50 46 40 36 E: 98 59 50 46 40 F: 82 71 50 46 40 G: 82 59 54 50 46 H: 82 59 50 46 40 I: 82 52 50 50 46 27 J: 82 52 50 50 46 K: 82 50 50 46 40 37 27 L: 82 50 50 46 40 M: 72 52 50 50 46 27	A: 140 55 49 43 10 B: 110 55 49 43 30 10 C: 71 55 49 43 38 30 D: 71 69 59 55 43 E: 309

^a The length of each fragment was averaged.^b Restriction fragments shorter than 25 (Sections I and III) or 30 bp (Section II) were ignored.

template DNA obtained from dried specimens of *B. dorsalis* and *B. cucurbitae* left at room temperature for more than 4 months were used (data not shown). In this study, the four sections were denoted as sections I, II, III, and IV. They corresponded to bp 12,948–13,291, 13,268–13,778, 13,751–14,192, and 14,209–14,504 of the *D. yakuba* sequence (X03240), respectively.

Table 3 shows the length of restriction fragments expected among the 43 sequences when the four DNA sections were treated separately with restriction enzymes *ApoI*, *AseI*, *DraI*, and *MseI*. Banding

patterns expected for each species are presented in Table 4. In this table, no single section could discriminate among all the species. However, when results from different sections are combined, species-specific patterns are seen in all species. Based on these results, the method for *Bactrocera* species identification was designed (Fig. 4). In this scheme, five of the 18 species are clearly identified by banding patterns generated using the sections I and *MseI*, and the remaining species are separated by one or two further analyses.

Figure 5 shows banding patterns of representa-

Table 4. Banding patterns expected for the four sections of the mtDNA treated with four different restriction enzymes^{a,b}

Species	Section I				Section II				Section III				Section IV			
	<i>ApoI</i>	<i>DraI</i>	<i>AseI</i>	<i>MseI</i>	<i>ApoI</i>	<i>DraI</i>	<i>AseI</i>	<i>MseI</i>	<i>ApoI</i>	<i>DraI</i>	<i>AseI</i>	<i>MseI</i>	<i>ApoI</i>	<i>DraI</i>	<i>AseI</i>	<i>MseI</i>
<i>B. frauenfeldi</i>	E	A	A	C	B	B	C	E	C	D	F	J	E	A	A	B
<i>B. psidi</i>	E	E	A	C	B	B	C	E	C	D	F	L	E	A	A	B
<i>B. jarvisi</i>	E	C	B	D	C	A	C	C	C	D	I	F	E	A	B	B
<i>B. curvipennis</i>	E	A	D	G	B	C	C	J	E	D	G	G	E	A	A	B
<i>B. tryoni</i>	D	A	D	G	B	C	C	J	E	D	H	H	E	A	A	B
<i>B. neohumeralis</i>	E	A	D	G	B	C	C	J	E	D	H	H	E	A	A	B
<i>B. cucumis</i>	C	A	A	A	B	A	B	I	B	E	D	M	D	A	A	D
<i>B. musae</i>	E	E	C	H	C	E	C	B	C	A	E	B	E	A	A	B
<i>B. dorsalis</i>	E	E	C	H	C	E	A	A	C	D	E	E	E	A	A	A
<i>B. papayae</i>	E	E	C	H	C	E	A	A	C	D	E	E or D*	E	A	A	B
<i>B. philippinensis</i>	E	E	C	H	C	E	A	A	C	D	E	E	E	A	A	C
<i>B. occipitalis</i>	E	C	C	H	C	E	C	B	C	D	E	D	E	A	A	B
<i>B. carambolae</i>	E	E	A	F	C	E	A	A	C	D	E	E or D*	E	A	A	B
<i>B. kandiensis</i>	B	D	A	E	C	E	C	D	C	D	I	F	E	A	A	B
<i>B. cucurbitae</i>	C	A	A	A	E	A	D	H	B	F	D	I	B or D*	A	A	D
<i>B. tau</i>	C	A	A	A	E	A	D	H	B	F	D	K	B	A	A	D
<i>B. depressa</i>	A	B	E	B	D*	D*	C	F	D	C	A*	A	C	A*	A	D
	A	B	E	B	E*	A*	C	F	D	C	C*	A	C	B*	A	D
<i>B. tsuneonis</i>	A	F	E	I	A	D	C	F	A	B	B	C	A	B	A	E

^a Characters A–M refer to the restriction fragment patterns defined in Table 3.^b Asterisks refer to intraspecific variations. Fig. 4. Scheme for the *Bactrocera* species identification based on PCR-RFLP patterns estimated for the four sections of the mtDNA fragment. Characters A–M refer to the banding patterns defined in Table 3.

tive individuals of the 18 species obtained using diagnostic mtDNA sections and the restriction enzymes shown in Fig. 4. When compared with the

image shown in Fig. 2, banding patterns became simpler and are considered to be suitable for discrimination of different restriction fragments. In

Fig. 5. Banding patterns detected among the 18 *Bactrocera* species using PCR-amplified mtDNA sections and restriction enzymes indicated in Fig. 4. Samples were electrophoresed in 2 (D and F) or 4% (A, B, C, and E) MetaPhorTM agarose gel. Lanes: 1: *B. carambolae*, 2: *B. kandiensis*, 3: *B. jarvisi*, 4: *B. depressa*, 5: *B. tsuneonis*, 6: *B. cucumis*, 7: *B. cucurbitae*, 8: *B. tau*, 9: *B. frauenfeldi*, 10: *B. psidi*, 11: *B. curvipennis*, 12: *B. tryoni*, 13: *B. neohumeralis*, 14: *B. dorsalis*, 15: *B. papayae*, 16: *B. philippinensis*, 17: *B. occipitalis*, 18: *B. musae*, M: 25 bp-ladder.

this analysis, banding patterns of 83 individuals were compared and no intra-specific variation was observed except in the case of *B. carambolae* in which six of the nine specimens examined showed the same banding patterns as *B. papayae*. Thus, the analysis confirmed that the scheme proposed in Fig. 4 could be applied to almost all individuals of the species treated in this study.

DISCUSSION

Phylogeny

In the molecular phylogenetic analyses, topologies generated by the neighbor-joining and maximum parsimony methods agreed very well. Although most of the phylogenetic relationships were supported by relatively high bootstrap confidence levels, molecular phylogenetic trees disagreed in

part with the currently accepted morphological classification (Hardy and Foote, 1989; White and Hancock, 1997). Among five subgenera included in the analyses, monophylies of subgenera *Bactrocera* and *Zeugodacus* were revealed to be questionable. The monophyly of the subgenus *Zeugodacus* was violated by a close relationship between *B. (Austrodacus) cucumis* and *B. (Zeugodacus) cucurbitae*+*B. (Z.) tau*. In all the phylogenetic trees obtained in this study, *B. (Afrodacus) jarvisi* was always positioned within the clade including *B. (Bactrocera)* species. Unexpected relationships were also detected between *B. musae* and species of the *B. dorsalis* complex. The *B. dorsalis* complex species, *B. dorsalis*, *B. papayae*, *B. carambolae*, *B. philippinensis*, and *B. occipitalis*, always showed a closer relationship with *B. musae* than with the other members of the complex, *B. kandi-*

ensis.

As in many other genera belonging to the subfamily Dacinae, classification of *Bactrocera* taxa has long been complicated. Before Drew (1989) proposed the current status of taxa within the genus, taxonomic positions of this and related groups had been changed many times. Our results are considered to reflect such a situation and to support the view of Drew (1989) that the supraspecific classification of the subfamily is unsettled. The present study strongly demonstrated the necessity of closer analyses of morphological characteristics and of new criteria, such as nucleotide sequences, to test the morphologically based classification.

Species identification

In this study, we considered a PCR-RFLP-based method of *Bactrocera* species identification based on nucleotide sequences of the mtDNA. Using the spacer (ITS) region of the nuclear ribosomal DNA, Armstrong et al. (1997) also analyzed PCR-RFLPs of fruit fly species of quarantine importance in New Zealand. However, because they used PCR-primers designed to amplify a relatively long DNA fragment (3–3.5 kb), their primers were sometimes difficult to use for DNA samples obtained from specimens left under protracted bad conditions (Muraji, unpublished). In addition, because nucleotide sequences of ITS and the flanking regions were known for only a small number of species of this insect group, it has been difficult to redesign PCR primers to analyze shorter sections within the DNA fragment. In the present study, we determined diagnostically useful PCR primers and restriction enzymes based on nucleotide sequences of a number of *Bactrocera* species. However, we could not confirm the applicability of the method for a wide variety of *Bactrocera* species. As discussed below, the applicability of such a method is determined by several aspects included in the experimental procedure. The method proposed in this study was devised to deal with such restrictions.

For analyses performed in quarantine practices, the PCR primers must work with samples stored or left under a variety of conditions and must be able to amplify DNA fragments from a variety of species. Thus, we designed the primers to amplify short sections within the sequence, because PCR usually works effectively when a short DNA frag-

ment is amplified. We designed the primers based on highly conserved sequences detected among various *Bactrocera* taxa. The results of PCR reflected these considerations, and indicated that these primers could work well with DNA template extracted from various *Bactrocera* species and from dried specimens left under protracted room conditions. Because our primers also work with Dipteran flies of several other groups, such as the *Anastrepha* and *Drosophila* (data not shown), they are expected to be applicable for a wide variety of *Bactrocera* species.

For rapid analysis of restriction fragments, banding patterns should be as simple as possible. Because the resolution of different-size DNA fragments depends on the concentration of agarose gel (Sambrook et al., 1989), it is often difficult to determine the size of various bands simultaneously. Because we used relatively short sections of the mtDNA (296–515 bp) in restriction analyses, this problem was to some extent avoided. On the other hand, these mtDNA sections frequently generated very short restriction fragments, which could not be separated by usual agarose gel electrophoresis. However, we confirmed that a 3 bp difference between DNA bands of about 40 bp in length was clearly detected by agarose gel electrophoresis using a special kind of agarose developed for separation of small DNA fragments (see Materials and Methods).

In order to assure the applicability of the method for a wide variety of *Bactrocera* species, banding patterns should be as variable as possible. However, because mtDNA sequences are available only for a relatively small number of species, we could not, at this point, confirm the usefulness of the method. However, given that all the sequences used were submitted to international DNA databases accessible on the World Wide Web, researchers can easily test and redesign the scheme for species identification in the event that they obtain the sequence of *Bactrocera* not included in this study.

In this study, PCR-RFLP analyses could not discriminate between *B. papayae* and the majority of individuals of *B. carambolae*. However, such individuals showed banding patterns specific to *B. carambolae* when the spacer region of the nuclear ribosomal DNA was analyzed (Muraji, unpublished). Thus, such individuals may be hybrids or interbreeding descendants between the closely re-

lated sympatric species within the *B. dorsalis* complex. In fact, several researchers have suggested the possibility of interbreeding and introgression among sibling species within this insect group (Yong, 1995; Iwaizumi et al., 1997; McInnis et al., 1999). In order to confirm the genetic interrelationship among the *B. dorsalis* complex species, analyses of field populations using many other genetic markers are needed. Such information could help confirm the reliability and accuracy of the PCR-RFLP-based method of species identification of this insect group.

ACKNOWLEDGEMENTS

We thank Mr. Robert Corcoran of the Queensland Horticulture Institute, Australia, and Dr. David J. Rogers of the Horticulture and Food Research Institute of New Zealand Ltd., New Zealand, for supplying materials sequenced in this study. Mr. M. Narahara of the Oita Prefectural Citrus Experiment Station, Mr. Y. Kobashigawa and Mr. M. Sasaki of the Naha Plant Protection Office, Mr. T. Kawashita and Mr. R. Iwaizumi of the Yokohama Plant Protection Station, and Dr. O. Iwahashi of University of the Ryukyus kindly provided specimens used in PCR-RFLP analyses. Mr. T. Sugimoto and Mr. M. Kaneda of the Yokohama Plant Protection Station gave us useful suggestions. This work is part of a project supported by the Budget for Nuclear Research of the Ministry of Education, Culture, Sports, Science and Technology, Japan.

REFERENCES

- Armstrong, K. F., C. M. Cameron and E. R. Frampton (1997) Fruit fly (Diptera: Tephritidae) species identification: a rapid molecular diagnostic technique for quarantine application. *Bull. Entomol. Res.* 87: 111–118.
- Drew, R. A. I. (1989) The tropical fruit flies (Diptera: Tephritidae: Dacinae) of the Australian and Oceanian regions. *Mem. Queensl. Mus.* 26: 1–521.
- Han, H.-Y. (2000) Molecular phylogenetic study of the tribe Trypetini (Diptera: Tephritidae) using mitochondrial 16S ribosomal DNA sequences. *Biochem. Syst. Ecol.* 28: 501–513.
- Han, H.-Y. and B. A. McPherson (1997) Molecular phylogenetic study of Tephritidae (Insecta: Diptera) using partial sequences of the mitochondrial 16S ribosomal DNA. *Mol. Phylogenet. Evol.* 7: 17–32.
- Hardy, D. E. and R. H. Foote (1989) Family Tephritidae. In *Catalog of the Diptera of the Australasian and Oceanian Regions* (N. L. Evenhuis ed.). Bishop Museum Press, Honolulu, pp. 502–531.
- Hein, J. J. (1990) A unified approach to alignment and phylogenies. *Methods Enzymol.* 183: 626–645.
- 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: 664–666.
- Kumar, S., K. Tamura and M. Nei (1993) *MEGA: Molecular Evolutionary Genetics Analysis, version 1.01*. The Pennsylvania State University, University Park, Pennsylvania.
- McInnis, D. O., P. Rendon, E. Jang, A. van Sauers-Muller, R. Sugiyama 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: 758–765.
- Muraji, M. and S. Nakahara (2001) Phylogenetic relationships among fruit flies, *Bactrocera* (Diptera, Tephritidae), based on the mitochondrial rDNA sequences. *Insect. Mol. Biol.* 10: 549–559.
- Sambrook, J., E. F. Fritsch and T. Maniatis (1989) *Molecular Cloning: A Laboratory Manual*. 2nd ed., Vol. 1. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York. 787 pp.
- Smith, J. J. and G. L. Bush (1997) Phylogeny of the genus *Rhagoletis* (Diptera: Tephritidae) inferred from DNA sequences of mitochondrial cytochrome oxidase II. *Mol. Phylogenet. Evol.* 7: 33–43.
- Swofford, D. L. (1999) *PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods), Version 4*. Sinauer Associates, Sunderland, Massachusetts.
- White, I. M. and D. L. Hancock (1997) *CABIKEY to the Dacini (Diptera, Tephritidae) of the Asian, Pacific, and Australasian Regions*. CAB International, Wallingford, Oxfordshire.
- 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.