The Philippines is a possible source of the Bactrocera dorsalis complex species (Diptera, Tephritidae) occasionally collected in the Ryukyu Islands of Japan; analyses of mitochondrial DNA

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Abstract
The Oriental fruit fly, Bactrocera dorsalis (Hendel), was eradicated from all Japanese territories in 1986; however, because neighboring countries remain infested by this or closely related species of the B. dorsalis complex, a small number of flies occasionally fly into the Ryukyu Islands of southwestern Japan. In order to examine the geographic origins of these flies, we compared restriction-banding patterns of the PCR-amplified mitochondrial 12S ribosomal RNA gene (rDNA) fragment (300 bp-long) among 455 specimens collected in the Ryukyu Islands, Taiwan, and the Philippines, and detected from fruits imported from the Philippines and Continental China and intercepted at Narita International Airport. Of five banding patterns detected using the restriction enzyme MseI, one pattern was observed only from insects originating in the Philippines and the Sakishima region in the southwestern part of the Ryukyu Islands. In phylogenetic analysis based on the sequence obtained in this and previous studies (1,138 bp-long mitochondrial DNA fragment from 16S to 12S rDNA), insects showing this banding pattern were tightly grouped with B. philippinensis Drew and Hancock endemic to the Philippines. Based on the results, we discussed the possibility that the fruit flies might have flown into the Sakishima region directly from the Philippines.

Key words: PCR-RFLP; phylogenetic analysis; phytophagous pests; B. philippinensis; invasive insect

INTRODUCTION
The Bactrocera dorsalis complex contains 75 species of fruit flies and includes several polyphagous invasive pest species of international significance, i.e., Bactrocera dorsalis (Hendel), B. papayae Drew and Hancock, B. carambolae Drew and Hancock, and B. philippinensis Drew and Hancock (Drew and Hancock, 1994; Clarke et al., 2005). One of these, B. dorsalis, invaded the Ryukyu Islands and the Ogasawara Islands of Japan during the early 20th century (Nawa, 1919; Yoshizawa, 1997) and was eradicated from all Japanese territories in 1986 (Yoshizawa, 1997). However, because neighboring countries are still infested, precautions need to be taken to prevent the colonization of migrant insects. A small number of flies are trapped every year by the field monitoring system set in the Ryukyu Islands (Ueno, 1998; Kobashigawa, 2000). Although the population has not settled in recent years (Oga, 2006; Aoki, 2007), invasions occur continuously in this area.

Determining the origin of invaded flies has crucial importance in consideration of measures to prevent the establishment of their population. To do this, Nakahara et al. (2008) compared the genetic composition of populations collected from Asian countries using PCR-RFLP of the mitochondrial AT-rich control region; however, they could
not specify the origin of the insects trapped in the Ryukyu Islands due to the extremely high mutation rate of the mtDNA region and to the lack of a distinct geographic structure among populations. This suggested the need for further analyses of the genetic structure using many other DNA markers, including those less polymorphic than the AT-rich control region.

In this study, we further analyzed PCR-RFLP patterns of the mitochondrial 12S ribosomal RNA gene (rDNA) using 343 specimens collected from eight localities within and around the Ryukyu Islands, and 112 specimens detected from imported fruits intercepted at Narita International Airport in Japan. As a result, we found that one haplotype detected from the Sakishima region in the southern part of the Ryukyu Islands was the same as that of B. philippinensis endemic to the Philippines. Phylogenetic analysis based on the longer mitochondrial DNA (mtDNA) sequences indicated the close relationship between flies from the Philippines and the Sakishima region. Based on the results, we discussed the possibility that the fruit flies might have flown into the Sakishima region directly from the Philippines.

MATERIALS AND METHODS

The specimens of B. dorsalis complex collected at monitoring sites in the Ryukyu Islands between 1999 and 2003 were divided into two groups according to geographic areas (Table 1; Fig. 1); SAKISHIMA includes specimens collected on the islands of Shimoji-shima, Ishigaki-jima, Taketomi-jima, Iriomote-jima, and Hateruma-jima, and OKINAWA includes those of the central Ryukyu Islands, Okinawa-jima and Kume-jima islands. These specimens, as well as those of KEELUNG, TAIPEI, TAITUNG, KAOHSIUNG, TAITUNG, and DAVAO, were collected using traps with host plants or the male attractant methyl eugenol. CHINA and PHILIPPINES include insects detected from imported fruits intercepted at Narita International Airport. Specimens were stored in 99.9% ethanol until DNA extraction.

Discrimination of the B. dorsalis complex species from other Bactrocera species was based on external morphology (Drew and Hancock, 1994). The complex contains a number of species that are morphologically closely related. It has been reported that field-collected samples sometimes showed intermediate states of morphological diagnostic traits between different species (Yong, 1995; Iwaizumi et al., 1997; Nakahara et al., 2002; Clarke et al., 2005; Ebina and Ohto, 2006). Thus, in this study, we did not determine the species of individual specimens.

Template DNA for PCR was extracted from individual insects as described in Muraji and Nakahara (2002). Amplification of mitochondrial 12S
rDNA was performed using two primers, DFP1 and DRP2, according to the method of Muraji and Nakahara (2002) with a slight modification in annealing (51°C) and extension temperatures (65°C). The amplified products were digested with the restriction enzyme MseI (New England BioLabs, Beverly, MA). Three microliters of PCR product was treated with 10 units of the enzyme in a total volume of 10 μl at 37°C for 3.0 h. They were electrophoresed on 4.0% MetaPhor™ agarose gels (Cambrex Bio Science Rockland, Rockland, ME) using 1×TBE buffer at 5.5 V/cm for 2.5 h, and then visualized by staining with ethidium bromide. A 20 bp-ladder (Gibco BRL, Rockville, MD) was used as a molecular size marker.

In order to verify the results obtained above, the mtDNA section from 16S to 12S rDNAs were sequenced using representative specimens according to the methods described in Muraji and Nakahara (2002). In this study, two primers MT16500 (TC-CAACCGTTCATACCAGCCTCA) and DRP2 were used for sequencing. The former primer was designed as described in Muraji and Nakahara (2001). The sequences obtained were aligned with previously reported sequences of several B. dorsalis complex species, B. dorsalis (AB035114–AB035116), B. papayae (AB035119 and AB035120), B. carambolae (AB035117 and AB035118), B. philippinensis (AB035111, AB048743, and AB048744), B. occipitalis (Bezzi) (AB048741 and AB048742), and B. kandensis Drew and Hancock (AB048737 and AB048738). Phylogenetic analyses of the sequences were performed as described in Muraji and Nakahara (2002) using computer programs MEGA ver. 3.1 (Kumar et al., 2004) and PAUP* ver. 4.0b10 (Swofford, 2003).

RESULTS

Among 455 specimens from ten populations, five different banding patterns were detected (Fig. 3).
Comparisons of the frequency of banding patterns revealed two major haplotypes (Table 3). Of these, type A was detected in all populations. This haplotype was found in more than 95% of individuals in populations except for SAKISHIMA, PHILIPPINES, and DAVAO. Type E was detected only in SAKISHIMA and the Philippines populations (PHILIPPINES and DAVAO). No other population contained mtDNA of this type.

PCR-amplified fragment of the mtDNA from 16S to 12S rDNA was sequenced using 23 specimens. In the data set of 1,138 bp-long generated using these and previously reported sequences of B. dorsalis, B. papayae, B. carambolae, B. philippinensis, B. occipitalis and B. kandiensis, 58 polymorphic sites and 34 parsimony informative sites were detected.

In the phylogenetic tree based on the neighbor-joining method (Fig. 3), individuals showing type E formed a clade with B. philippinensis. Individuals showing type A originating in the Philippines also formed a clade with B. occipitalis. These clades were supported by bootstrap confidence levels higher than 80%. All other individuals, excluding the outgroup taxa B. kandiensis, were positioned between the clades of B. philippinensis and B. occipitalis. Although they were closely related to B. dorsalis, B. papayae, and B. carambolae, no individual formed a monophyletic clade with these.

### Table 2. Length of restriction DNA bands included in the five banding patterns shown in Fig. 2

<table>
<thead>
<tr>
<th>Banding pattern</th>
<th>Fragment lengths (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>111, 54, 52, 43, 30</td>
</tr>
<tr>
<td>B</td>
<td>111, 54, 52, 43, 30</td>
</tr>
<tr>
<td>C</td>
<td>111, 52, 44, 43, 30</td>
</tr>
<tr>
<td>D</td>
<td>75, 54, 52, 43, 36, 30</td>
</tr>
<tr>
<td>E</td>
<td>73, 54, 52, 43, 38, 30</td>
</tr>
</tbody>
</table>

*The values were estimated based on the previously reported sequences (AB035111 and AB048742) and those obtained in this study.*

### Table 3. Compositions of banding patterns in the ten populations

<table>
<thead>
<tr>
<th>Banding pattern</th>
<th>OKINAWA</th>
<th>SAKISHIMA</th>
<th>CHINA</th>
<th>KEELUNG</th>
<th>TAIPEI</th>
<th>TAICHUNG</th>
<th>TAITUNG</th>
<th>KOAOHSIUNG</th>
<th>DAVAO</th>
<th>PHILIPPINES</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>41</td>
<td>5</td>
<td>38</td>
<td>13</td>
<td>84</td>
<td>19</td>
<td>50</td>
<td>50</td>
<td>27</td>
<td>25</td>
<td>352</td>
</tr>
<tr>
<td>B</td>
<td></td>
<td>10</td>
<td>1</td>
<td></td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td>42</td>
<td>48</td>
<td>1</td>
</tr>
<tr>
<td>C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>D</td>
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<td>1</td>
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<tr>
<td>E</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>100</td>
</tr>
<tr>
<td>Total</td>
<td>41</td>
<td>15</td>
<td>39</td>
<td>13</td>
<td>86</td>
<td>19</td>
<td>50</td>
<td>50</td>
<td>69</td>
<td>73</td>
<td>455</td>
</tr>
</tbody>
</table>

*Banding patterns A–E correspond to those defined in Fig. 2.*
species.

Unweighted parsimony analysis produced the same topology as Fig. 3 in terms of the relationships supported by bootstrap confidence levels higher than 50%. In this case, the 22 most parsimonious trees (length: 67, CI: 0.906, RI: 0.961, RC: 0.871) were obtained. In these, phylogenetic positioning of specimens was not clearly determined except for those originating in the Philippines and those of type E collected in the Sakishima region.

As in neighbor-joining analysis (Fig. 3), individuals from SAKISHIMA were divided into two groups. Those showing type E were positioned in the clade of *B. philippinensis*, and others (type A) were positioned between the clades of *B. philippinensis* and *B. occipitalis*.

**DISCUSSION**

In this study, a close relationship was detected between fruit flies collected in the Philippines and those in the Sakishima region of the Ryukyu Islands (Table 3). Phylogenetic analysis (Fig. 3) suggested that individuals showing the common haplotype (type E) were *B. philippinensis*, endemic to the Philippines (Drew and Hancock, 1994). This species is also known to have invaded the Northern Territory of Australia (Allwood et al., 2002), which is distant from the original range of this species. In this case, its introduction must have occurred with human activities.

In the Sakishima region of the Ryukyu Islands, there is no direct international transport with the Philippines. Thus, type E might have been introduced from other areas that have been infested by this species. Taiwan could be a possible source of this haplotype because this island is positioned between the Philippines and the Sakishima region (Fig. 1) and has frequent transport between both areas; however, *B. philippinensis* has not been
recorded on this island. The fact that type E was not detected from 218 specimens collected in Taiwan (Table 3) indicates that the haplotype is absent or quite rare. This phenomenon was also confirmed by Nakahara et al. (2001) who showed that PCR-RFLP patterns of the mitochondrial control region of the B. dorsalis complex species were different between Taiwan and the Philippines; thus, this hypothesis seems unlikely.

Another possibility is that the species might fly directly from the Philippines to the Sakishima region. Because the Oriental fruit fly (which includes B. dorsalis and its close relatives) can fly across open sea (Christensen and Foote, 1960) and B. dorsalis can be dispersed by the wind for several hundred kilometers (Shi et al., 2005), its close relative B. philippinensis could also be dispersed for a long distance between the Philippines and the Sakishima region (approximately 650 km).

This hypothesis seems to be supported by meteorological records. For example, typhoons occurring in the western Pacific region sometimes move from the Philippines to the Ryukyu Islands (Japan Meteorological Agency, 2003). Shoji (1995) recognized that strong southerly winds occurring when typhoons are moving in the northwest of the Ryukyu Islands are an important factor carrying butterflies directly from the Philippines to the southwestern Ryukyu Islands. Similarly, strong southerly winds blow in the early summer when the Baiu front (a seasonal rain front) stagnates north of the Islands (Japan Weather Association Okinawa Branch, 1989). These phenomena could promote wind-borne dispersal of insects.

If this hypothesis is correct, why has type E (or B. philippinensis) not been detected in Taiwan even though the island is positioned between the two areas? One possible reason for this phenomenon is that Taiwan has a large population of B. dorsalis. Once migrants are mixed with a large majority of native flies, it is difficult to detect a haplotype specific to the migrants. Iwaizumi et al. (1997) noted that B. philippinensis can hybridize with other B. dorsalis complex species, such as B. papayae and B. carambolae, and produce fertile progenies under laboratory conditions. If this phenomenon occurred between B. philippinensis and B. dorsalis, the presence of the migrants would be obscured by hybridization and successive back crosses. In contrast, because no B. dorsalis complex species is distributed in the Sakishima region, the haplotype can be easily detected from a small number of specimens collected in this area. Further studies on morphology are needed to confirm the hypothesis that flies might enter the Sakishima region directly from the Philippines. To do this, we are accumulating specimens trapped in the latter region. Data on the relationship between meteorological factors and haplotypes of trapped insects are also needed. These data can be also used to develop a method to predict fly invasion into the Ryukyu Islands.

In addition to type E, another major haplotype, type A, was detected from the Ryukyu Islands, including the Sakishima region (Table 3). Because sequences of this haplotype were different from those of the same haplotype originating in the Philippines (Fig. 3), the B. dorsalis complex species trapped in the Ryukyu Islands must also have a geographic origin other than the Philippines.

Concerning type A detected in Continental China, Taiwan, and the Ryukyu Islands, phylogenetic analysis revealed that they did not form a monophyletic clade and were positioned ambiguously between the clades of B. occipitalis and B. philippinensis. They did not form a clade with the three major invasive species of the B. dorsalis complex, B. dorsalis, B. papayae, and B. carambolae. Thus, the specimens might include many minor species not considered in this study; however, such species are mainly distributed in southeastern Asia and are not recorded from either Continental China or Taiwan (Drew and Hancock, 1994). On the other hand, species of this group are known to crossbreed in the laboratory (Iwaizumi et al., 1997) and hybridize even under natural conditions (Yong, 1995). Thus, it seems possible that hybrids occurring in their native ranges are expanding into Taiwan, Continental China, and the Ryukyu Islands. In order to confirm this hypothesis, molecular and morphological data should be accumulated for a variety of B. dorsalis complex species.

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