Genetic divergence among populations of the white grub beetle, *Dasylepida ishigakiensis* (Coleoptera: Scarabaeidae), distributed in the southern part of the Ryukyu Islands of Japan, detected from mitochondrial DNA sequences

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Abstract

The white grub beetle, *Dasylepida ishigakiensis*, was originally known only in the Yaeyama region, including Ishigaki-jima and Iriomote-jima Islands, of the southern part of the Ryukyu Islands, Japan. At the end of the 1990s, a population was discovered to occur abundantly in sugarcane fields in the Miyako region, including Miyako-jima and Irabu-jima Islands, approximately 90 km northeast of the Yaeyama region. Using specimens collected from these islands, fragments of mitochondrial DNA containing portions of ribosomal RNA, cytochrome c oxidase subunit I, and cytochrome *b* genes were sequenced. Phylogenetic analyses using the sequences revealed discrete separation into three clades: two comprised exclusively of individuals from Ishigaki-jima and Iriomote-jima, respectively, and the third comprised of individuals from the Miyako region, including both Miyako-jima and Irabu-jima. Separation of the clades by rather long branches suggested that they have been geographically isolated for an evolutionarily long period and indicated that the population in the Miyako region was not established by the recent invasion of insects from the Yaeyama region.

Key words: Nucleotide sequence; mitochondrial DNA; phylogenetic analysis; *Dasylepida*; sugarcane pest

INTRODUCTION

The white grub beetle *Dasylepida ishigakiensis* (Niiijima et Kinoshita) is distributed in the southern part of the Ryukyu Islands of Japan (Fujioka, 2001). Before a population was discovered to occur abundantly in sugarcane fields in the Miyako region (Miyako-jima and Irabu-jima Islands) in 1997 (Sadoyama et al., 2001), this species was long considered to be rather rare, occurring only in the natural forests of the Yaeyama region (Ishigaki-jima and Iriomote-jima), approximately 90 km southwest of the Miyako region (Hirai, 2001). In the Miyako region, its larval stage causes considerable economic damage to the sugarcane crop

(Sadoyama et al., 2001).

In addition to their different habitats and larval food preferences, differences between the Miyako and Yaeyama populations have been detected in male body color, body length, and cuticular hydrocarbon profile (Hirai et al., 2008); however, it is not clear whether such differences are due to genetic differences between the populations or to phenotypic differences responding to environmental differences between the regions. Thus, the question of whether the Miyako population was established by insects that migrated from the Yaeyama region remains unanswered.

In recent years, entomologists have increasingly used DNA markers to analyze phylogenetic rela-

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tionships among local populations. Such analysis will surely be useful for *D. ishigakiensis*, whose populations are separated among islands. In this study, we examined nucleotide sequences of mitochondrial DNA (mtDNA) using individuals collected from the four islands, Miyako-jima, Irabu-jima, Ishigaki-jima, and Iriomote-jima. The results of phylogenetic analyses revealed that they were divided into three groups that have been genetically isolated for a long period. Based on these results,

we discuss the causes of genetic divergences and the origin of the population recently discovered in the Miyako region.

MATERIALS AND METHODS

The materials used in this study are listed in Table 1. Specimens were captured on the islands of Miyako-jima, Irabu-jima, Ishigaki-jima, and Iriomote-jima (Fig. 1) using an insect net. Template DNA was extracted from the legs of individuals using a GenomicPrep Cell & Tissue DNA Isolation Kit (Amersham Biosciences, Buckinghamshire, UK) and dissolved in 200 μ l sterilized distilled water.

PCR primers used in this study are listed in Table 2. Three sets of universal primers, 16Fa/12Rb, UMT10603F/UMT11388R, and C1-J-2195/AAMT3038R, were used for amplification and sequencing of the mtDNA fragment from the 16S ribosomal RNA gene (16S rDNA) to 12S rDNA, and portions of the cytochrome *b* gene (*cyt b*), and the cytochrome oxidase subunit I gene (*COI*), respectively. Amplification was carried out in a 20 μ l volume containing 1.0 μ l of DNA template, 1.0 μ l of each primer (5 pmol/ μ l), 2.0 μ l of 10 $\times$ PCR buffer, 1.6 μ l of dNTPs (2.5 mM each), and 0.12 units of Ex-Taq DNA polymerase (TaKaRa-Bio, Otsu, Japan). Temperature cycling was carried out in a Program Temp Control System PC-805 (Astec Co., Ltd., Fukuoka, Japan). After a heating

Fig. 1. Map showing localities where the materials were collected.

Table 1. Materials used in this study

Localities	Date	<i>n</i> ^a	Code	Collection
Yaeyama region				
Ishigaki-jima Island				
Takeda Rindo, Hirae, Ishigaki City	2000. III. 20–21	4 (2)	ISG a	T. Fukaishi
Takeda Rindo, Hirae, Ishigaki City	2003. III. 13	4 (3)	ISG b	K. Kohno, Y. Hirai
Takeda Rindo, Hirae, Ishigaki City	2004. III. 10	2 (1)	ISG c	K. Kohno
Iriomote-jima Island				
Komi, Taketomi Town	2004. III. 10	6 (3)	IRM a	Y. Hirai, K. Takahashi
Uehara, Taketomi Town	2004. III. 11–12	1 (0)	IRM b	Y. Hirai
Miyako region				
Miyako-jima Island				
Jimori, Hirara, Miyakojima City	2000. III. 1	3 (1)	MYK a	N. Arakaki
Nobarugoshi, Hirara, Miyakojima City	2003. II. 7–10	3 (1)	MYK b	Y. Hirai
Gusukube, Miyakojima City	2004. II. 9–14	2 (2)	MYK c	Y. Hirai
Irabu-jima Island				
Irabu, Miyakojima City	2000. III. 8	4 (3)	IRB	Y. Hirai

^a Numbers in parentheses indicate the number of individuals used for sequencing the *COI* and *cyt b* fragments.

Table 2. PCR primers used in this study shown in 5'–3' direction

16S–12S rDNA										
16Fa	CCG	GTT	TGA	ACT	CAG	ATC	ATG			(Muraji and Nakahara, 2001)
12Rb	AAC	TAG	GAT	TAG	ATA	CCC	TA			(Muraji and Nakahara, 2001)
DIMT13350F	TCA	ACT	CCT	CAT	TGG	GCA	GGC	TAG		(present study)
DIMT14111R	AAG	TTA	GTT	GTG	TGA	ATC	AGT			(present study)
cyt <i>b</i>										
UMT10603F	CTT	GAT	GAA	ATT	TTG	GAT	C			(present study)
UMT11388R	CTC	CTC	CTA	ATT	TAT	TAG	GAA	TTG		(Muraji et al., 2000)
COI										
C1-J-2195	TTG	ATT	TTT	TGG	TCA	TCC	AGA	AGT		(Simon et al., 1994)
AAMT3038R	TCC	ATT	GCA	CTA	ATC	TGC	CAT	ATT	AG	(Muraji et al., unpublished)

The sequence of UMT10603F was designed as described in Muraji et al. (2000), and that of AAMT3038R was based on reported sequences of the *Anomala* species (AB330384–AB330391). The number included in the name of the DIMT, UMT, and AAMT series of primers refers to the nucleotide position in the *Drosophila yakuba* total mtDNA sequence (Clary and Wolstenholme, 1985) corresponding to the 5' end of the primer.

step at 92°C for 2 min, samples were incubated for 35 cycles of 92°C for 1 min, 50°C for 30 s, and 72°C for 2 min. Purification, labeling, and sequencing of the amplified products were performed as described in Muraji and Nakahara (2001). Two additional primers, DIMT13350F and DIMT14111R, designed based on the sequences obtained in this study, were used for sequencing. The sequences of several individuals were submitted to DDBJ/EMBL/GenBank nucleotide sequence databases (Accession numbers: AB332100–AB332111).

Nucleotide sequences were aligned using Genetyx-Mac ver. 10 (Genetyx Co., Tokyo, Japan) and modified by manual optimization. The aligned sequences were analyzed using MEGA software ver. 3.1 (Kumar et al., 2004) to compute basic statistical data and to generate phylogenetic trees based on the neighbor-joining method. The aligned sequences were also analyzed using PAUP* ver. 4.0b10 (Swofford, 2003) to generate phylogenetic trees based on the maximum parsimony method. This analysis was performed using a heuristic search procedure with TBR swapping and 100 max tree options.

RESULTS

Mitochondrial DNA sequences, including portions of the 16S and 12S rDNA, and tRNA^{Val}, were determined for 29 *D. ishigakiensis*. Their length varied from 1,608 bp to 1,610 bp, and the nucleotide composition was biased to A and T (average A+T%=77.5). A 1,610 bp-long data set gen-

erated by aligning the sequences showed 78 polymorphic sites, 71 of which were parsimony-informative.

In parsimony analysis, two shortest trees were detected. In these trees, sequences were discretely divided into three clades that were highly supported by bootstrap analysis (Fig. 2). Of these, two clades were exclusively composed of insects from Ishigaki-jima and Iriomote-jima, respectively, while the remaining clade included insects from both Miyako-jima and Irabu-jima. Between the shortest trees, the only difference detected was the positioning of sequence ISGc1. The same topology, in terms of sequence grouping, was obtained from neighbor-joining analyses using various distance methods implemented in MEGA.

Using 16 individuals representing the four islands, 788 and 721 bp-long sequences containing portions of COI and cyt *b*, respectively, were determined. The sequences were more variable than the rDNAs, and 76 variable sites were detected in each fragment. Neighbor-joining and parsimony analyses of the two data sets produced the same topology as shown in Fig. 2, in terms of sequence grouping (data not shown). The grouping in these analyses was supported by bootstrap values higher than 99%.

By combining the sequences of the three mtDNA regions obtained from the same individuals, 3,123 bp-long sequences were obtained for 16 insects. The phylogenetic trees generated using these sequences evidenced the same topology as shown in Fig. 2, and their topology was strongly

Fig. 2. One of the two most parsimonious trees obtained from maximum parsimony analysis of the 1,610 bp-long mtDNA sequence from the 16S to 12S rDNA (length: 87; CI: 0.920; RI: 0.987; RC: 0.908). Bootstrap values higher than 50% calculated based on 1,000 replications are shown near the branches. The values were calculated using the maximum parsimony method (left) and the neighbor-joining method based on the Jukes-Cantor distances (right).

supported by high bootstrap values (Fig. 3).

DISCUSSION

In the phylogenetic trees shown in Figs. 2 and 3, individuals are divided into three clades. Because the clades are separated by rather long branches, local populations of *D. ishigakiensis* might have been geographically separated for a long period. When the mitochondrial molecular evolutionary clock, 2.3% per one million years (Brower, 1994), was applied to the combined sequences, a divergence time of 1.5–2.1 million years was estimated from nucleotide substitution rates of 3.6–4.9%, among the three clades. The suitability of applying such a molecular clock, calculated from different insect groups using various molecular data, to *D. ishigakiensis* is obviously questionable. Nevertheless, the estimation indicates that the population

Fig. 3. One of the two most parsimonious trees obtained by maximum parsimony analysis of the 3,123 bp-long combined sequence (length: 260; CI: 0.912; RI: 0.970; RC: 0.885). Bootstrap values were calculated and are shown as in Fig. 2.

in the Miyako region was not established by the recent invasion of insects from the Yaeyama region and suggests that they have been genetically isolated during their evolutionary history.

The causes of genetic divergences of terrestrial animals in the Ryukyu Islands have been discussed in relation to paleogeography (Ota, 1998); however, in the case of *D. ishigakiensis*, genetic divergence cannot be fully explained by the paleo-land configuration postulated in this area. Comprehensive hypotheses on paleogeography suggest the emergence of large paleo-islands connecting the islands between the Yaeyama and Miyako regions (Kimura, 1996, 2002) or connecting islands within each region (Kizaki and Oshiro, 1980; Ota, 1998) during the late Pleistocene. It seems difficult for insects to have attained rather high nucleotide substitution rates (3.6–4.9%) within the short period after the last land connection. Therefore, the present study suggests that the putative land connection had no influence on the intermingling of local populations of *D. ishigakiensis*, especially between Iriomote-jima and Ishigaki-jima Islands within the Yaeyama region.

One possible explanation for this phenomenon concerns the paleo-climate and vegetation. Studies on pollen assemblages (Kuroda and Ozawa, 1996) suggested diminishing forests of broadleaf evergreen trees, the original habitat of *D. ishigakiensis* supposed from the Ishigaki-jima and Iriomote-jima populations (Hirai, 2001), due to the arid climate in

the Ryukyu Islands during the last glacial period. Wide development of ancient dunes has also been postulated in this period (Kuroda and Ozawa, 1996). Under such situations, habitats of *D. ishigakiensis* might be separated into different paleo-regions. Because the flight activity of this species is restricted both temporally and spatially (Arakaki et al., 2004), the local populations might not have been connected even during periods of land connection.

Although the present study showed relationships among local populations of *D. ishigakiensis*, two major questions about this species remain unanswered: 1) Why did the species become a sugarcane pest only in the Miyako region? and 2) Why was the species not recorded in the Miyako region before the 1990s when the population was first recognized as a sugarcane pest? Before performing a series of studies to answer these questions, it is inevitable to examine the possibility that the population might have diverged in other regions and then been introduced into the Miyako region by human activity. Because the genus *Dasylepida* includes six other species distributed mainly in tropical and subtropical Asia, such as China, Vietnam, and Taiwan (Fujioka, 2001), future studies must examine the phylogenetic positioning of the three clades among these other *Dasylepida* species. In addition, detailed studies on morphology are also needed to reconfirm the taxonomic status of the insular populations of this species.

The present study indicated that the mtDNA of the Miyako population is different from those of the Yaeyama region; however, because mtDNA represents only the maternal lineage, further analyses using nuclear DNA markers are needed to examine whether the male adults dispersed between the regions. Results of these studies could also be used to predict the future status of the Yaeyama populations as pests of the sugarcane crop, which is extensively cultivated over a large area in both the Miyako and Yaeyama regions. At present, the populations have not caused damage to the sugarcane crop in the Yaeyama region.

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