A convenient estimation of the sources of sweet potato weevils, *Cylas formicarius* (Fabricius) (Coleoptera: Brentidae), in recently invaded areas in Japan, by random amplified polymorphic DNA technique

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Abstract

RAPD-PCR was used to clarify the genetic properties of the sweet potato weevil, *Cylas formicarius*, which was collected from several of the Southwest Islands, Ogasawara Islands, Muroto City and Yakushima Island in Japan. PCR amplification of *C. formicarius* DNA using three primer sets, i.e., T13/T13, T05/T05, and T01/T07, resulted in products of several different sizes among individuals and primer sets. The 393 wild weevils tested were classified into 27 distinctive categories by combining the three patterns detected for each weevil. The frequency of these categories varied both among islands and among collection sites within the same island. The categories confirmed for weevils from Muroto City and Yakushima Island were detected only in those from Okinawa-Hontou Island and the more northern islands of the Southwest Islands. *C. formicarius* from these islands probably invaded Muroto City in 1995 and Yakushima Island in 1997.

Key words: *Cylas formicarius*; DNA polymorphism; invasion; RAPD-PCR

INTRODUCTION

The sweet potato weevil, *Cylas formicarius* (Fabricius) (Coleoptera: Brentidae), is a serious pest of sweet potatoes in tropical and subtropical areas (International Institute of Entomology, 1993). It has poor dispersal ability (Sugimoto et al., 1994; Miyatake et al., 1997; Moriya and Hiroyoshi, 1998) and is spread around the world mainly by the transportation of infested tubers (Wolfe, 1991). In Japan, this weevil was first recorded on Okinawa Island in 1903 (Nawa, 1903) and is currently distributed throughout the Southwest Islands and the Ogasawara Islands. Recently, it has been found in other areas such as Muroto City and Yakushima Island, despite plant quarantine regulations; however, it has been thoroughly eradicated from these areas by emergent control projects directed by the national and prefectural governments (Fujimoto et al., 2000; Nishioka et al., 2000; Oogaki and Yokoyama, 2002). To facilitate these projects, a

convenient and accurate method of estimating the sources of *C. formicarius* in newly invaded areas is desired.

The random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) has been used to analyze genetic variation in insect populations and identify insect species and biotypes (Kambhampati et al., 1992; Gawel and Bartlett, 1993; Wilkerson et al., 1993; Williams et al., 1994; Cognato et al., 1995; Moya et al., 2001; Callejas et al., 2005) because it can detect extensive genomic DNA polymorphisms (Williams et al., 1990). However, RAPD-PCR has some problems as a detection technique. It requires the standardization of PCR conditions for reliable and consistent use (Black, 1993; Unruh and Woolley, 1999; Caterino et al., 2000) because the reproducibility of amplified DNA fragments is susceptible to slight changes in the PCR conditions. Another problem is that the homology of amplified DNA fragments cannot be assumed between species (Black, 1993;

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Unruh and Woolley, 1999). Although RAPD-PCR is not recommended for phylogenetic studies based on genetic distance, it is a fast and inexpensive method for the identification of insect species (Black, 1993).

Kawamura et al. (2002) detected genomic DNA polymorphisms in laboratory-reared *C. formicarius* using RAPD-PCR, established a reproducible protocol, and proposed a convenient procedure for classifying the genetic variation of *C. formicarius* more minutely by combining RAPD patterns from three primer sets. We used this procedure to examine the genetic variation of *C. formicarius* collected from various areas of Japan, and then estimated the sources of *C. formicarius* that invaded Muroto City, Kochi Prefecture, in 1995 and Yakushima Island, Kagoshima Prefecture, in 1997 (Hiramatsu, 1996; Maehara, 1998).

MATERIALS AND METHODS

Insects. Adult males *C. formicarius* were collected for one night using pheromone traps (Jansson et al., 1991) in the Southwest Islands, the Ogasawara Islands, and Muroto City, Kochi Prefecture (Fig. 1 and Table 1). Only living weevils were placed in 99.5% ethanol for preservation after collection and stored at -20°C until DNA extraction.

DNA extraction and PCR amplification. DNA was extracted from the whole body of each individual using a method reported previously (Kawamura et al., 2002) and dissolved in $200\ \mu\text{l}$ of TE buffer. After the DNA concentration was estimated using a spectrophotometer (DU 640, Beckman, CA, USA), the solution was diluted to $10\ \text{ng}/\mu\text{l}$ and stored at 4°C . The sample used for PCR was frozen at -20°C . We used three primer sets, i.e., T13/T13, T05/T05, and T01/T07 (Operon Technologies, CA, USA), which amplify DNA from *C. formicarius* reproducibly and provide RAPD patterns (Kawamura et al., 2002). PCR amplification was carried out under the same conditions described in Kawamura et al. (2002), and $10\ \mu\text{l}$ of the PCR products was electrophoresed in 1.5% agarose gel in TAE buffer. The gel was stained with $10\ \mu\text{g}/\text{ml}$ ethidium bromide solution for 10 min, visualized on a UV transilluminator at a wavelength of 312 nm (FV1-20M, Funakoshi, Tokyo, Japan), and imaged with a Kodak DC120 Zoom digital camera using the Electrophoresis Documentation

Fig. 1. Islands where *C. formicarius* specimens were collected in this study.

and Analysis System 120, Limited Edition (Kodak Digital Science, NY, USA), according to the manufacturer's instructions. PCR with each primer set was repeated at least three times for each specimen to assess the reproducibility of the amplified DNA fragments.

Categorization of RAPD patterns. Based upon reproducible DNA fragments, the RAPD patterns of wild *C. formicarius* amplified using the three primer sets T13/T13, T05/T05, and T01/T07 are referred to as types A, B, and C RAPD patterns, respectively. Because there were multiple RAPD patterns for each type, each individual was given a triplet notation by combining the RAPD patterns of the three types detected for that individual, following the method of Kawamura et al. (2002).

RESULTS AND DISCUSSION

The RAPD patterns of the 393 weevils examined were classified into patterns A_1 to A_5 , B_1 to B_5 , and C_1 to C_8 (Fig. 2). However, because some DNA fragments amplified by T01/T07 (approximately

Table 1. Collection records of *C. formicarius* specimens used in this study

Collection sites (No. of sites)	Collection years	Collectors and Providers	Note
Shikoku Island			
Muroto City, Kochi Prefecture (1)	1995	M. Takai and I. Yamashita	Invaded but eradicated areas of <i>C. formicarius</i>
Muroto City, Kochi Prefecture (1)	2000	M. Takai and I. Yamashita	
Southwest Islands			
Yakushima Island, Kagoshima Prefecture (1)	1997	S. Kawasaki	Infested areas of <i>C. formicarius</i>
Takarajima Island, Kagoshima Prefecture (1)	2003	H. Kamiwada	
Amami-Oshima Island, Kagoshima Prefecture (4) ^a	2001–2003	S. Hayashikawa and K. Kawamura	
Okinawa-Hontou Island, Okinawa Prefecture (4) ^b	2002–2004	H. Saragai and K. Kawamura	
Miyakojima Island, Okinawa Prefecture (4) ^c	2002–2003	K. Kawamura	
Ishigakijima Island, Okinawa Prefecture (4) ^d	2002–2003	K. Kawamura	
Iriomotejima Island, Okinawa Prefecture (3) ^e	2003	K. Kawamura	
Haterumajima Island, Okinawa Prefecture (1)	2003	K. Kawamura	
Yonagunijima Island, Okinawa Prefecture (1)	2003	H. Saragai	
Ogasawara Islands			
Chichijima Island, Tokyo Metropolis (1)	2004	S. Sato	Invaded but eradicated areas of <i>C. formicarius</i>
Otoutojima Island, Tokyo Metropolis (1)	2004	S. Sato	
Nakoudojima Island, Tokyo Metropolis (1)	2004	S. Sato	

^a Collected from Tatsugou Town, Yamato Village, Sumiyou Village and Setouchi Town in Amami-Oshima Island.

^b Collected from Nakijin Village, Nago City, Yomitan Village and Itoman City in Okinawa-Hontou Island.

^c Collected from four sites of Miyakojima City in Miyakojima Island.

^d Collected from four sites of Ishigaki City in Ishigakijima Island.

^e Collected from three sites of Taketomi Town in Iriomotejima Island.

Fig. 2. RAPD patterns of DNA of wild *C. formicarius* amplified by the three primer sets. This study referred to RAPD patterns as A, B and C types by primer sets of T13/T13 (5'-AGGACTGCCA-3'), T05/T05 (5'-GGGTTTGGCA-3') and T01/T07 (5'-GGGCCACTCA-3', 5'-GGCAGGCTGT-3'), respectively. Lanes 1 to 5 in A, 1 to 5 in B, and 1 to 8 in C represent RAPD patterns found in all weevils tested using three primer sets, A, B and C, respectively. Approximately 560 bp in C₁ and C₂, 650 bp in C₄ and C₅, and 750 bp in C₅ were excluded due to a lack of reproducibility in this study. Lane M represents a 100-bp DNA ladder size-marker.

560 bp in C₁ and C₂, 650 bp in C₄ and C₅, and 750 bp in C₅) were not reproducible, they were excluded from the detected RAPD patterns of type C.

Combining the three types of RAPD patterns of three types detected for each weevil resulted in 27 distinctive categories (Table 2). The number of

Table 2. Geographical distribution of categories in *C. formicarius*

	Collection sites	Number of weevils tested	Number of weevils belonging to various categories expressed with triplet notation identification														
			$A_1 B_1 C_1$	$A_1 B_1 C_2$	$A_1 B_1 C_3$	$A_1 B_1 C_4$	$A_1 B_1 C_5$	$A_1 B_2 C_1$	$A_1 B_2 C_2$	$A_1 B_2 C_3$	$A_1 B_2 C_4$	$A_1 B_2 C_5$	$A_1 B_3 C_3$	$A_1 B_3 C_4$	$A_1 B_3 C_7$		
Shikoku Island																	
Kochi, 1995	Muroto City	2	1	1	—	—	—	—	—	—	—	—	—	—	—	—	—
Kochi, 2000	Muroto City	6	6	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Southwest Islands																	
Yakushima	Kamiyaku Town	2	2	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Takarajima		17	6	7	2	2	—	—	—	—	—	—	—	—	—	—	—
Amami-Ohshima	Akaogi, Tatsugou Town	17	5	—	8	—	—	—	—	—	—	—	2	1	—	—	—
	Toen, Yamato Village	12	7	—	—	3	—	1	1	—	—	—	—	—	—	—	—
	Misato, Sumiyou Village	18	2	3	—	5	—	2	1	—	5	—	—	—	—	—	—
	Koniya, Setouchi Town	10	—	6	—	2	—	2	—	—	—	—	—	—	—	—	—
	Total	57	14	9	8	10	—	5	2	—	5	—	2	1	—	—	—
Okinawa-Hontou	Nakijin Village	12	3	1	—	4	2	—	1	1	—	—	—	—	—	—	—
	Yabu, Nago City	11	1	—	—	3	6	—	—	—	1	—	—	—	—	—	—
	Kina, Yomitan Village	18	4	4	—	—	9	—	—	—	—	—	—	—	—	—	—
	Nashiro, Itoan City	17	—	1	—	3	13	—	—	—	—	—	—	—	—	—	—
	Total	58	8	6	—	10	30	—	1	1	1	—	—	—	—	—	—
Miyakojima	Karimata, Miyakojima City	12	—	1	1	—	1	4	3	—	2	—	—	—	—	—	—
	Higashinakazonoe, Miyakojima City	18	—	—	—	1	—	—	1	—	14	—	—	—	—	—	—
	Hukuzato, Miyakojima City	17	—	—	—	3	—	—	—	1	12	—	—	—	—	—	—
	Sugama, Miyakojima City	18	—	—	—	—	—	—	—	—	6	—	—	—	—	—	—
	Total	65	—	1	1	4	1	4	4	1	34	—	—	—	—	—	—
Ishigakijima	Inoda, Ishigaki City	16	—	15	1	—	—	—	—	—	—	—	—	—	—	—	—
	Miwa, Ishigaki City	17	—	3	2	5	—	3	3	1	—	—	—	—	—	—	—
	Ohkawa, Ishigaki City	12	—	4	1	—	—	—	2	—	—	—	—	—	—	—	—
	Husaki, Ishigaki City	18	—	11	—	2	—	—	1	—	1	—	—	—	—	1	—
	Total	63	—	33	4	7	—	3	6	1	1	—	—	—	—	1	—
Iriomotejima	Hunaura, Taketomi Town	12	—	3	—	5	—	—	2	—	—	—	—	—	—	—	—
	Komi, Taketomi Town	10	—	6	—	—	—	—	4	—	—	—	—	—	—	—	—
	Ohhara, Taketomi Town	16	—	6	1	4	1	—	1	—	2	1	—	—	—	—	—
	Total	38	—	15	1	9	1	—	7	—	2	1	—	—	—	—	—
Haterumajima	Huka, Taketomi Town	21	—	8	—	—	—	—	—	—	2	—	—	—	—	—	—
Yonagunijima	Sonai, Yonaguni Town	18	—	10	—	6	—	—	1	—	1	—	—	—	—	—	—
Ogasawara Islands																	
Chichijima		20	—	7	—	—	—	3	2	—	3	—	—	—	—	—	—
Ootoujima		18	—	6	—	—	—	3	5	—	3	—	—	—	—	—	—
Nakoudojima		8	—	2	—	—	—	—	3	—	3	—	—	—	—	—	—

Table 2. (Continued)

[illegible]

categories varied widely among islands. Some categories were found commonly on most islands, whereas several were found on only a few islands. This was also the case among different collection sites within the same island. It is notable that category $A_1B_1C_1$ was common only on Okinawa-Hontou Island and the more northern islands of the Southwest Islands, but was not present on other islands (Table 2). Individuals from Muroto City and Yakushima Island were classified into either $A_1B_1C_1$ or $A_1B_1C_2$ (Table 2).

Mendel et al. (1994) compared the RAPD patterns of Israeli pine bast scale, *Matsucoccus josephi*, individuals and estimated that its invasion pathway occurred from Israel to Cyprus. We used the RAPD pattern-based method proposed by Kawamura et al. (2002) to estimate the invasion sources of *C. formicarius* to two areas of Japan. If we assume that the sources of the weevils that invaded Muroto City and Yakushima Island were within Japan, the characteristic geographical distribution of $A_1B_1C_1$ described above suggests that the weevils collected on Yakushima Island originated from Okinawa-Hontou Island or the more northern islands of the Southwest Islands. For the Muroto invasion in 1995, we estimated that individuals categorized either as $A_1B_1C_1$ or $A_1B_1C_2$ were transported together from the same source as described above.

More reliable estimation methods of genetic distances such as those achieved through DNA sequencing are more expensive and time-consuming than RAPD-PCR (Unruh and Woolley, 1999). In plant quarantine projects, the estimation of the source of newly invaded pests requires the quick identification of many individual weevils. In such a case, RAPD-PCR is preferable, despite its limitations, because of its relatively low cost and time requirements, if the procedure and the interpretation of its results are preformed carefully.

The ability to make accurate identifications using markers increases with the number of diagnostic markers (Campton, 1987). Using the triplet notation identification method proposed by Kawamura et al. (2002), we showed that there were common triplet notation categories in the populations of Okinawa-Hontou Island and the northern islands and estimated the source of the recently invaded weevils in Muroto City and Yakushima Island. Hence, this triplet notation method is useful in

classifying insect populations using RAPD markers. For a more precise estimation of weevil invasion sources using this method, it will be necessary to assess and classify in triplet notation the RAPD patterns of more weevils from both Japan and its neighboring countries.

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