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Species Differentiation of Bruchid Beetles (Coleoptera: Bruchidae) Analyzed by Mitochondrial DNA Polymorphism[1]

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One of the applications of molecular biological techniques is the analysis of DNA restriction site variation or restriction fragment length polymorphism (RFLP) between taxa. An arbitrary DNA segment of single copy genes can be rapidly amplified by polymerase chain reaction (PCR) from a very small amount of material, and then subjected to RFLP analysis (SAIKI et al., 1988; ENNOS, 1992).

Bruchid beetles are pests of legume seeds and have been used as experimental organisms for ecological studies (YOSHIDA, 1990). Species are identified by adult morphological characters (SOUTHGATE, 1958), because at the larval stage they are difficult to distinguish from each other. This has complicated studies on the ecology of bruchid beetle larvae burrowing into legume seeds, particularly when the larvae fail to develop into morphologically-distinguishable adults.

In this paper, we report PCR-RFLP analysis of a mitochondrial DNA (mtDNA) region which includes cytochrome oxidase I and II genes in bruchid beetles collected from different regions of the world and maintained under laboratory conditions. We discuss the applicability of the method to species identification.

MATERIALS AND METHODS

Bruchid beetles. Four bruchid species, *Callosobruchus chinensis* (L.), *C. rhodesianus* (PIC), *C. phaseoli* (GYLLENHAL) and *Zabrotes subfasciatus* (BOH.), were collected and maintained under laboratory conditions of 30°C and 70% RH, unless otherwise noted. Four local populations of *C. chinensis* were collected at different localities in Japan; specifically, Kyoto (1936), Shizuoka (1969), Fukui (1991) and Kagoshima (1991). The Kyoto and Shizuoka populations were kept in the laboratory at the University of Kyoto and sent to our laboratory in 1985 and 1993, respectively (UTIDA, 1972 a, b). The Fukui population has been maintained at our laboratory since collection. The Kagoshima population has been maintained at a laboratory at the University of Tsukuba and samples preserved in acetone were sent to our lab. The latter three bruchids, *C. rhodesianus*, *C. phaseoli* and *Z. subfasciatus*, do not inhabit Japan and were imported with the permission of the Plant Quarantine Station. *C. rhodesianus* was collected at Harare, Zimbabwe shortly before being imported from the laboratory at the University of Zimbabwe in 1993 (D.P. GIGA, pers. comm.). *C. phaseoli* was imported to a laboratory at the University of Tsukuba from the Pest Infestation Laboratory, Slough, England in 1979 and sent to our laboratory in 1993. The collection of *C. phaseoli* is not known. Two local populations of *Z. subfasciatus* were collected in Colombia in 1986, and at Kampala, Uganda in 1980, and kept at a laboratory at the University of London at 27°C and 70% RH (CREDLAND and DENDY, 1992). These two *Z. subfasciatus* populations were sent to our lab. in 1991. The sizes of founder populations are mainly unknown, but those of Fukui and Kagoshima were more than 20 individuals. The size of the founder population from Kyoto was about 10 individuals (UTIDA, 1972 b). The laboratory stock culture of each local population has been maintained by arbitrarily selecting about 150–200 individuals every generation. The number of generations that have passed from collection to the present analysis varied from about 15 (*C. rhodesianus*) to about 900 (Kyoto population of *C. chinensis*). In the present PCR-RFLP analysis, we examined two individuals of first-instar larvae, two fourth-instar larvae, and two female adults for each local population. First-instar larvae were sampled when they were still in the egg shell attached to the surface of beans. Sampled individuals were preserved in acetone.

DNA extraction. After being blotted on filter paper to remove acetone, an individual sample was homogenized in 500 μl of lysis buffer [50 mM Tris-HCl

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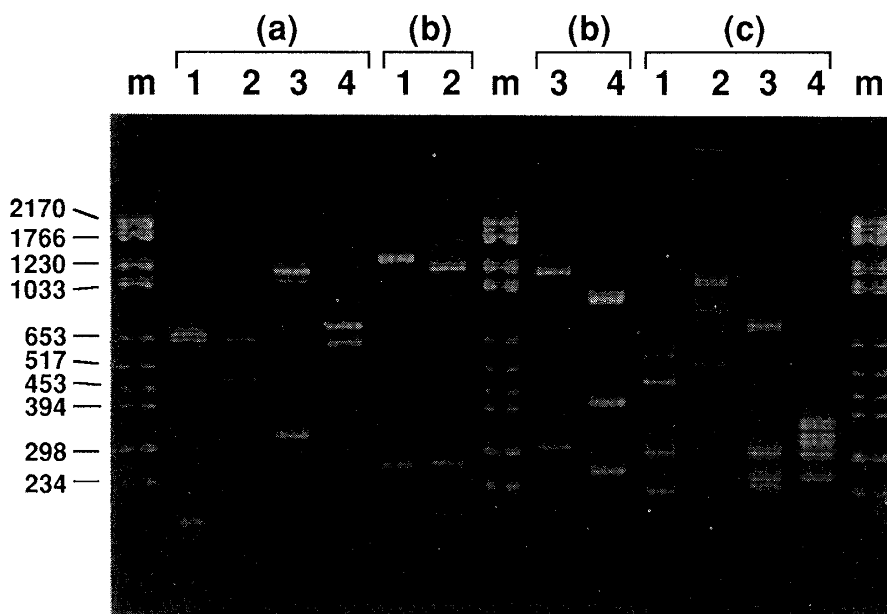

Fig. 1. RFLP patterns produced by (a) *Taq*I; (b) *Rsa*I; and (c) *Sau*3AI: 1. *C. chinensis*; 2. *C. rhodesianus*; 3. *Z. subfasciatus*; 4. *C. phaseoli*; and m, size markers in bp.

buffer (pH 8.0), 10 mM EDTA, 0.5% SDS, 400 μg/ml Proteinase K, 100 μg/ml RNase A] and kept at 55°C for 3 h. Then, the lysate was extracted twice with phenol and once with phenol-chloroform. After 40 μl of 2 M sodium acetate and 1 μl of glycogen were added to the lysate, DNA was precipitated by addition of 700 μl of isopropanol. The precipitate was pelleted by centrifugation, washed twice with chilled 70% ethanol and dissolved in 20 μl of TE buffer [10 mM Tris-HCl buffer (pH 8.0), 0.1 mM EDTA]. For PCR amplification, the DNAs from fourth-instar larvae and adults were diluted with TE buffer to approximately 50 μg/ml, while those from first-instar larvae were used as they were.

Primers. Primer COI2-1 5′-CTTTATCAACATTTATTTTGATTTTTT-3′ and primer COII1-2 5′-ATTTATACCACAAATTTCTGAACATTG-3′ were used for PCR amplification. The annealing site of primer COI2-1 is in the coding region of the cytochrome oxidase I gene, while that of primer COII1-2 is in the cytochrome oxidase II gene.

PCR-RFLP analysis. In 0.5 ml plastic tubes, 1 μl of template DNA solution was added to 30 μl of reaction mixture [10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 1 mM each of dATP, dGTP, dCTP and dTTP, 200 nM of each primer, 25 U/ml Taq DNA polymerase], overlaid with mineral oil and subjected to 30 cycles of 94°C 30 s, 45°C 1 min and 60°C 3 min in a thermal cycler.

PCR products were divided into 3 μl aliquots, and each aliquot was completely digested with *Taq*I, *Rsa*I, *Sau*3AI, *Bfa*I, *Dde*I or *Hin*fI, electrophoresed in 3% agarose gels and visualized by ethidium bromide staining. Approximate sizes of the resultant DNA fragments were estimated on the gels in comparison with the mobility of DNA size markers (DNA molecular weight marker VI, Boehringer Mannheim; see also Fig. 1).

RESULTS AND DISCUSSION

PCR amplification

DNAs of high molecular mass were successfully extracted from all individuals, including quite tiny first-instar larvae. When the DNAs were subjected to PCR, a DNA fragment of approximately 1.6 kb was amplified in *C. chinensis*, *C. rhodesianus*, *C. phaseoli* and *Z. subfasciatus*. The fragment was consistently amplified in all individuals, including first- and fourth- instar larvae and adults, of different local populations.

The primers were designed, based on honeybee and fruit fly sequences, to amplify a segment of mtDNA including cytochrome oxidase genes whose size is 1,746 bp in the honeybee (Hymenoptera) and 1,517 bp in the fruit fly (Diptera) (CROZIER et al., 1989; DE BRUIJN, 1983). The size obtained from

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Table 1. Approximate sizes of DNA fragments produced by each of the six restriction endonucleases

	Approx. size of fragments					
	*Taq*I	*Rsa*I	*Sau*3AI	*Bfa*I	*Dde*I	*Hin*fI
C. chinensis	670	1350	550	1150	1000	1150
	650	260	470	400	450	180
	160		300		150	180
			220			
C. rhodesianus	660	1200	1050	1600	620	900
	470	270	520		620	460
	160	190			250	180
	150					
Z. subfasciatus	1150	1150	730	1600	800	900
	330	310	300		700	520
		180	250		100	180
			240			
C. phaseoli	710	880	370	760	800	840
	610	420	340	410	700	460
	120	260	330	240	90	140
			310	140		
			250			

bruchid beetles (Coleoptera), 1.6 kb, is consistent with these values, suggesting that the region of the bruchids is successfully amplified with the primers.

RFLP analysis

The four bruchid species, *C. chinensis*, *C. rhodesianus*, *C. phaseoli* and *Z. subfasciatus*, were clearly differentiated by the RFLP patterns (Fig. 1, Table 1). Particularly, *Taq*I, *Rsa*I and *Sau*3AI were effective in that all four species were distinguished by a single enzyme digestion (Fig. 1). The sizes of restriction fragments were estimated and are listed in Table 1. By contrast, there was no within-species difference in the RFLP patterns. The patterns were always identical irrespective of developmental stages and of local populations at different collection sites. In practice, PCR enabled analysis of first-instar larvae just before hatching. Further, 100 samples can be analyzed in just 2 days. These results indicate that the PCR-RFLP system established in this study will be a useful and reliable method for species identification of bruchid beetles in general, although the possibility should be considered that intraspecific variations of the RFLP will be found within and between some populations in future studies. The method will be applicable to bruchid beetles at a very early larval stage as well as at later developmental stages.

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Aphicidal Effects of Silwet L-77, Organosilicone Nonionic Surfactant[1]

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Insecticidal effects of surfactants have been known for many years (CORY and LANGFORD, 1935; DOZIER, 1937; SALEM and SALEM, 1983), however, surfactants have seldom been put to practical use, perhaps because of their lower efficacy when compared with general synthetic insecticides. Soap, an anionic surfactant, is sometimes used as an insecticide, which makes it an exception among such surfactants. However, soap inevitably gives rise to an insoluble curdy precipitate which depresses its insecticidal activity when diluted in hard water (GINSBURG, 1935). If a nonionic surfactant could be used as an insecticide, it would have more practical value than soap. Recently, high aphicidity of nonionic surfactants was observed under laboratory conditions and a close correlation between aphicidity and surface tension was revealed (IMAI et al., 1994). To evaluate the effectiveness of Silwet L-77, a nonionic surfactant solution which has the lowest surface tension of all commercial surfactants, as an insecticide, we observed its aphicidity under three different humidity conditions. We then assessed the adjuvant effects of humectants on aphicidity which had already been proven for an insecticidal soap (IMAI et al., 1995), under both laboratory and field conditions.

MATERIALS AND METHODS

Aphids. The green peach aphid, *Myzus persicae* (SULZER), used for laboratory tests was from the same culture as described previously (IMAI et al., 1994). Field tests were carried out on natural populations of the cotton aphid, *Aphis gossypii* GLOVER, infesting *Hibiscus mutabilis* L. that were planted in a garden of the Applied Plant Research Laboratory, Yokohama Center, Japan Tobacco Inc., and had never been treated with any pesticides.

Chemicals. A commercial sample of Silwet L-77, a nonionic surfactant, was kindly supplied by Nihon Unicar, Co. Ltd. Reagent grade sodium carboxymethyl cellulose, calcium chloride dihydrate, glycerin, and magnesium chloride hexahydrate were obtained from Kanto Chemical Co., Inc. Sumithion® garden insecticide (50% fenitrothion, MEP) was obtained from Sankyo Co., Ltd. Silwet L-77, sodium carboxymethyl cellulose, calcium chloride, and glycerin were individually dissolved in distilled water at twice the density of final concentrations, and 684 ppm hard water was prepared by dissolving 803.8 mg/l calcium chloride dihydrate and 277.8 mg/l magnesium chloride hexahydrate into distilled water. Just before spraying, solutions were combined or diluted with an equal amount of distilled water to make final sample solutions.

Laboratory bioassay. Laboratory bioassay methods were the same as described previously (IMAI et al., 1995): test solutions were sprayed with a glass atomizer on *Raphanus sativus* L. (1–2 true-leaf-stage) on which more than 30 aphids (*Myzus persicae*) were feeding. The treated plants were kept under conditions of 25°C and 60% RH for 16 h. Aphids were inspected by microscopy and the numbers of living and dead insects were counted.

Field evaluation. Sample solutions were gently

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