Random Amplified Polymorphic DNA Analysis of Japanese Isolates of *Verticillium dahliae* and *V. albo-atrum*

Masanori Koike, Assistant Professor, and **Mitsuteru Fujita,** Undergraduate Student, Department of Agro-environmental Science, Obihiro University of Agriculture & Veterinary Medicine, Obihiro, Hokkaido 080, Japan; **Hideyuki Nagao,** Assistant Professor, Laboratory of Plant Pathology, Faculty of Horticulture, Chiba University, Matsudo, Chiba, Japan; and **Satoko Ohshima,** Phytopathologist, Kimitsu Breeding Station, Sakata Seed Corp., Sodegaura, Chiba 299-02, Japan

ABSTRACT

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Japanese isolates of *Verticillium dahliae* and *V. albo-atrum* have been differentiated into four and two pathogenicity groups, respectively. Japanese isolates, 29 isolates of *V. dahliae* and 15 isolates of *V. albo-atrum,* representing different pathogenicity groups were analyzed by means of the random amplified polymorphic DNA (RAPD) method using polymerase chain reaction (PCR). *V. dahliae* could be differentiated into three subclusters and *V. albo-atrum* into two subclusters. In *V. dahliae*, the first subcluster (RAPD type I) included isolates in group A (eggplant pathotype), group C (sweet pepper pathotype), and another group with pathogenicity not determined. The second subcluster (RAPD type II) included group B isolates (tomato pathotypes). The third subcluster (RAPD type III) contained four diploid isolates (group D, brassica pathotype) and one haploid isolate (84111). In *V. albo-atrum,* the isolates were divided into two subclusters. The first subcluster (RAPD type IV) included isolates in only the alfalfa pathotype. The second subcluster (RAPD V) included one alfalfa pathotype isolate (Vaa-s02), three potato pathotype isolates, and three undetermined pathotype isolates from alfalfa. Similarities and differences in banding patterns obtained by RAPD could be a useful molecular tool in identification and phylogenetic studies of the pathogenicity groups.

Verticillium dahliae Kleb. is generally considered a pathogen with a wide host range (5). In Japan, host specificity of the pathogen has been recognized on tomato (*Lycopersicon esculentum* L.), eggplant (*Solanum melongena* L.), sweet pepper (*Capsicum annuum* L. var. *grossum*), and cruciferous plants such as Chinese cabbage (*Brassica campestris* L. spp. *pekinensis*), Japanese radish (*Raphanus sativas* L.), and turnip (*Brassica rape* L.) (4,6). Isolates were differentiated into four major groups: pathogenic on eggplant and turnip (group A, eggplant pathotype), pathogenic on eggplant, tomato, and turnip (group B, tomato pathotype), pathogenic on eggplant, sweet pepper, and turnip (group C, sweet pepper pathotype), and pathogenic on turnip only (group D, crucifer pathotype). This last group is known to be diploid (4,6). The closely related species *Verticillium albo-atrum* Reinke & Berthier is also an important pathogen, causing significant damage on alfalfa (*Medicago sativa* L.) and potato (*Solanum tuberosum* L.) in Japan. The latter fungus is differentiated into two groups (alfalfa and potato pathotypes); both pathotypes are pathogenic to their respective host but not to the other host (13). At present, it is very laborious and may take 2 to 3 months to identify these species and pathogenicity groups. However, identification of pathotypes is important for disease resistance breeding and crop selection for a particular field or greenhouse.

Recently, random amplified polymorphic DNA (RAPD) analysis has been applied in basic studies of genetic relationships between taxa (17). RAPD analysis also may be useful in identifying races and subgroups of plant-pathogenic fungi (1,3). In a previous paper, preliminary evidence suggested that *V. albo-atrum*–specific RAPD markers could be identified, and pathogenicity groups of *V. dahliae* could be tentatively classified by RAPD analysis (8). The present study included a wider range of isolates and primers to examine the usefulness of RAPD analysis in determination of pathogenicity groups in *Verticillium* species and their phylogenetic relationship.

Corresponding author: Masanori Koike
E-mail: koike@obihiro.ac.jp

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MATERIALS AND METHODS

A list of the strains used and their pathogenicity groups is given in Table 1. Strains were maintained throughout the study on potato sucrose agar (500 ml of potato broth made from 200 g of potato, 20 g of sucrose, and 20 g of agar in 1 liter) slants stored at 4°C. The fungus was cultured on potato sucrose broth for 5 to 8 days without shaking at room temperature in the dark to obtain biomass for DNA extraction.

The DNA extraction method used was the miniprep protocol of Lee et al. (9). Arbitrary random amplification of DNA sequences was performed with a set of 12 primers (each having 12 bases) obtained from Wako Inc. (Japan) (Table 2). A modification of the RAPD method of Williams et al. (17) was used to perform amplification. Each 25 µl of reaction contained 1 U of recombinant Taq polymerase (Takara, Japan), 0.1 M Tris-HCl (pH 8.3), 0.5 M KC1, 15 mM MgCl (Takara 10× buffer), 0.2 mM of each dNTP (Takara), 2 µM primer, and approximately 10 ng of template DNA. A DNA Thermal Cycler Astec PC-700 (Astec Inc., Japan) was programmed for initiation at 94°C for 3 min followed by 45 reaction cycles of denaturation at 94°C for 1 min, annealing at 36°C for 1 min, and primer elongation at 72°C for 2 min. The final primer elongation segment of the run was extended to 7 min. The reaction products were resolved in a 2% agarose gel (Agarose S, Wako Inc.) at 4 V cm^{-1} for 3 h. PCR products were visualized by UV-fluorescent staining with ethidium bromide.

The banding patterns produced by 12 primers on DNA from 29 isolates of *V. dahliae* and 15 isolates of *V. albo-atrum* were analyzed. From these 12 primers, eight primers (Table 2) were selected which showed several polymorphic bands. The entire experiment, from spore growth to DNA extraction and amplification, was repeated to verify the results and to test the consistency of the method. The polymorphic bands scored were clear and reproducible (ca. <1,500 bp). Banding patterns were analyzed by scoring 1 for the presence of bands and 0 for their absence, and a dendrogram was constructed from the similarity coefficient data by the group average method (16).

RESULTS AND DISCUSSION

In RAPD analyses, 36 bands were produced by eight primers that showed polymorphisms (Table 2, Fig. 1). A dendrogram with branch length proportional to the differences among isolates was constructed using group average methods (Fig. 2). *V. dahliae* and *V. albo-atrum* clearly could be distinguished as previously reported (8).

For *V. dahliae*, the isolates were differentiated into three subclusters (Fig. 1). The first subcluster (RAPD type I, from the top of dendrogram) included group A, group C, and another group of isolates whose pathogenicity is unknown. These results indicated that groups A (eggplant pathotype) and C (sweet pepper pathotype) may be phylogenetically very closely related. *Verticillium* spp. are known to expand host range (5), and it is tempting to speculate that some group A isolates may have acquired the ability to attack sweet pepper and thereby formed pathogenicity group C. Further pathogenicity studies need to be conducted with the cucumber, melon, and soybean isolates to examine their relationships to groups A and C.

The second subcluster (RAPD type II) included only group B isolates (tomato pathotype). Before Hagiwara's determination of four major groups, Iijima categorized Japanese isolates of *V. dahliae* as "tomato pathotype" and "nontomato pathotype" according to their pathogenicity on tomato (susceptible cultivar Beiju) (7). Using this latter classification, groups A and C were included in the nontomato pathotype. The data concerned with haploid *V. dahliae* strains presented here agree with Iijima's classification.

The third subcluster (RAPD type III) contained four diploid isolates (group D) and one haploid isolate (84111). This indicated that the diploid strain is phylogenetically different from haploid strains that were characterized previously. Recent molecular analysis of the ITS (internal transcribed spacer) region suggests that diploid *V. dahliae* is genetically closer to *V. albo-atrum* than haploid *V. dahliae* (10). In this experiment, however, all diploid and haploid *V. dahliae* were phylogenetically distinct from *V. albo-atrum.*

Carder and Barbara also analyzed some isolates of Japanese pathogenicity groups of *V. dahliae* with RFLPs using random genomic probes (2). However, unlike in this study using RAPDs, they concluded that none of the three Japanese haploid pathotypes (A, B, and C groups) corresponded directly with their RFLP groups, suggesting that host specificity in Japanese isolates of *V. dahliae* may have evolved independently.

In *V. albo-atrum,* the isolates were divided into two subclusters. The first subcluster (RAPD type IV) included isolates in only the alfalfa pathotype. The second subcluster (RAPD type V) included one

Table 1. Isolates of *Verticillium dahliae* and *V. albo-atrum* used in this study

Species	Strain designation	Host origin	Pathogenicity group (pathotype)	Source[a]	RAPD group
V. dahliae					
	Vdu-1	Udo	A	3	I
	84034	Eggplant	A	2	I
	2630	Soybean	A	1	I
	Vds-l	Soybean	A	1	I
	Vdt-1	Tomato	B	1	II
	Vdt-2	Tomato	B	3	II
	Vdt-3	Tomato	B	1	II
	Vdt-4	Tomato	B	1	II
	Vdt-k01	Tomato	B	1	II
	TV-103	Tomato	B	4	II
	0101	Tomato	B (race 2)	2	II
	84011	Tomato	B	2	II
	Vde-3	Eggplant	B	1	II
	Vdb-6	Chinese cabbage	B	1	II
	84023	Sweet pepper	C	2	I
	Vdp-1	Sweet pepper	C	1	I
	Vdp-2	Sweet pepper	C	1	I
	Vdp-3	Sweet pepper	C	1	I
	Vdp-4	Sweet pepper	C	3	I
	84010(2n)	Chinese cabbage	D	2	III
	Vdb-2(2n)	Chinese cabbage	D	1	III
	Vdb-4(2n)	Chinese cabbage	D	1	III
	Vdb-5(2n)	Chinese cabbage	D	1	III
	Vdc-1	Cucumber	ND[b]	1	I
	Vdc-2	Watermelon	ND	1	I
	Vdc-3	Melon	ND	1	I
	Vdc-4	Melon	ND	1	I
	Vdf-1	Butterbur	ND	1	I
	84111	Unknown	ND	1	III
V. albo-atrum					
	Vaa-k01	Alfalfa	ND	1	V
	Vaa-k03	Alfalfa	ND	1	V
	Vaa-k04	Alfalfa	ND	1	V
	Vaa-s01	Alfalfa	Alfalfa	5	IV
	Vaa-s02	Alfalfa	Alfalfa	5	V
	Vaa-s10	Alfalfa	Alfalfa	5	IV
	Vaa-s12	Alfalfa	Alfalfa	5	IV
	Vaa-920	Alfalfa	Alfalfa	5	IV
	Vaa-s22	Alfalfa	Alfalfa	5	IV
	Vaa-s24	Alfalfa	Alfalfa	5	IV
	Vaa-s26	Alfalfa	Alfalfa	5	IV
	Vaa-s011	Alfalfa	Alfalfa	5	IV
	Vap-01	Potato	Potato	5	V
	Vap-2	Potato	Potato	1	V
	Vap-3	Potato	Potato	1	V

[a] 1. Authors; 2. H. Hagiwara, National Agricultural Center (Tsukuba, Japan); 3. H. Kusakari, Osaka Prefectural Agricultural and Forestry Research Center (0saka, Japan); 4. T. Iijima, Tokyo Metropolitan Agricultural Research Station (Tokyo, Japan); 5. R. Sato, Yukijirushi Seed Coop. (Sapporo, Japan).

[b] Not determined.

Table 2. Codes and sequences of the 12 primers tested, with number of amplified and polymorphic DNA fragments obtained with each primer in random amplified polymorphic DNA (RAPD) experiments

Code	Sequence 5′ to 3′	Amplified fragments	Polymorphic fragments
A-01	TGCACTACAACA	8	7
A-02	GGCATGGCCTTT	3	0
A-03	CGACGACGACGA	2	0
A-04	ATCAGCGCACCA	4	4
A-05	AGCAGCGCCTCA	8	5
A-06	GCCAGCTGTACG	5	5
A-07	TGCCTCGCACCA	5	2
A-08	GCCCCGTTAGCA	8	4
A-09	CCGCAGTTAGAT	0	0
A-10	ACTGGCCGAGGG	3	0
A-11	GATGGATTTGGG	5	3
A-12	TTCGGACGTATG	6	6
TOTAL		56	36

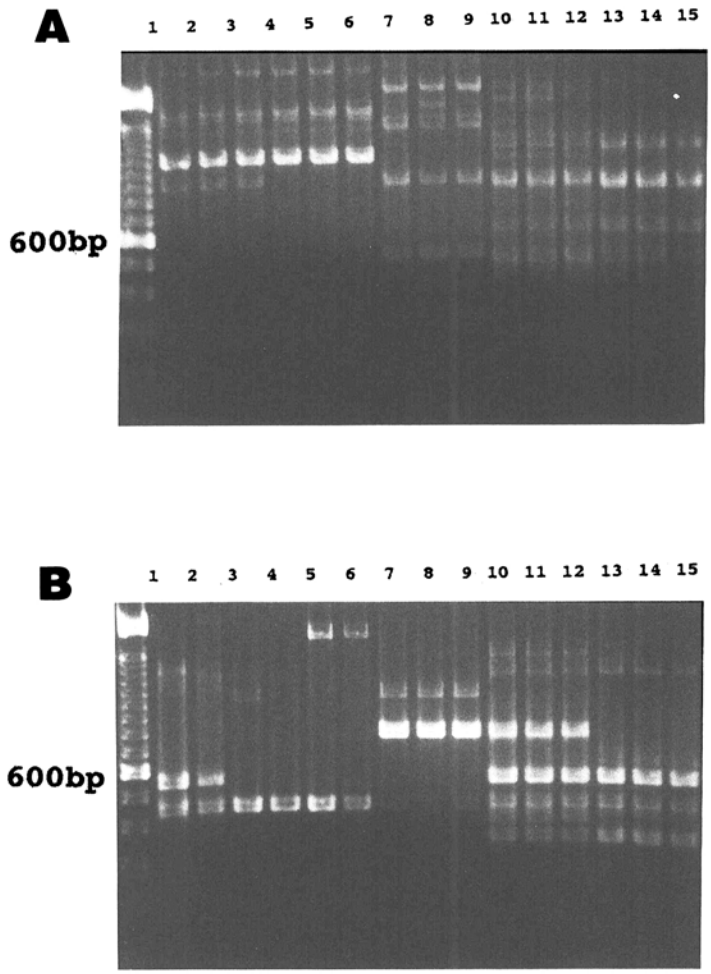

Fig. 1. Random amplified polymorphic DNA (RAPD) profiles of amplification with primer A-04 **(A)** and A-06 **(B)**. **(A)** *Verticillium dahliae* (1:84023, 2.26ED, 3:Vdp-1, RAPD Group I) (4:0101, 5:Vdb-6, 6:Vdt-4, RAPD Group II) (7:Vdb-2, 8:Vdb-6, 9:84010, RAPD Group III). *V. albo-atrum* (10:Vaa-sl2, 11:Vaa-s01, 12:Vaa-s10, RAPD Group IV), (13:Vap-01, 14:Vaa-k04, 15:Vap-3, RAPD Group V). **(B)** *V. dahliae* (1:84023, 2:84034, RAPD Group I) (3:Vdt-1, 4:Vdt-k01, 5:Vdb-6, 6:Vde-3, RAPD Group II) (7:Vdb-2, 8:Vdb-4, 9:Vdb-5, RAPD Group III). *V. albo-atrum* (10:Vaa-s26, 11:Vaa-s24, 12:Vaa-s011, RAPD Group IV) (13:Vaa-s02, 14:Vaa-k01, 15:Vaa-k03, RAPD Group V). Molecular marker: 100-bp ladder.

alfalfa pathotype isolate (Vaa-s02), three potato pathotype isolates, and three undetermined pathotype isolates from alfalfa. Recently, Okoli et al. observed that *V. albo-atrum* isolates could be divided into two RFLP types, L (lucerne) and NL (nonlucerne) (12,13). A PCR-based assay has been described, which uses primers derived from the internal transcribed spacer regions of the ribosomal RNA genes, which also is capable of distinguishing among *V. albo-atrum* subgroups and *V. dahliae* (11,14). However, it is at present unclear whether the two subclusters described in the present paper corresponded to the RFLP type and subgroups described by Okoli et al. (12) and Robb et al. (14).

Two of the 44 isolates (84111 and Vaa-s02) appeared to be phylogenetically at odds with their reported pathogenicity. At present, it is unclear why this should be the case. RAPD profiles of 84111 (RAPD type III) were similar to those of 84010 (RAPD type III), as previously reported using other primers (8). Therefore, this may suggest that haploidization initially occurred in 84111 or another diploid isolate. Vaa-s02 (RAPD type V) is an alfalfa pathotype (15); however, this isolate was similar to the potato pathotype isolates. This phenomenon could not be explained by the acquisition of pathogenicity. A conversion in pathogenicity must have occurred. Further research is needed on these two isolates as well as on the pathogenicity of the *V. albo-atrum* isolates from alfalfa that fall into RAPD type V.

Finally, these studies show that RAPD analysis may be a useful molecular tool in detection and phylogenetic studies of Japanese pathogenicity groups of *Verticillium* spp. Two primers (A-04 and A-06) can readily detect the five RAPD groups (Fig. 1). This approach may be an efficient method for the identification of pathogenicity groups of Japanese *Verticillium* isolates. Further research would be required to determine its applicability to isolates from other countries.

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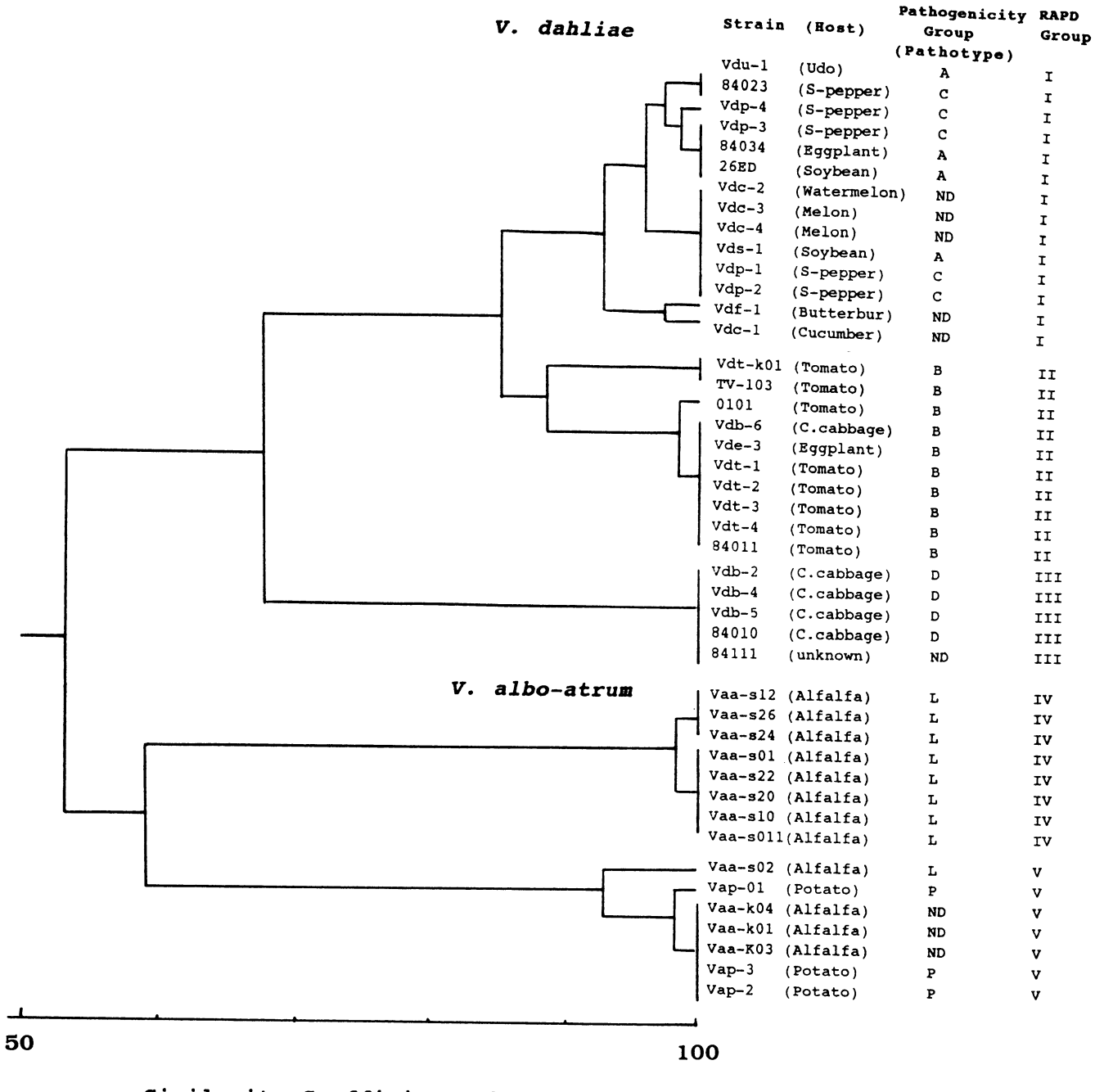

Fig. 2. Relationships among different pathogenicity groups of *Verticillium dahliae* and *V. albo-atrum*. The dendrogram was constructed from the similarity coefficient data. Alfalfa and potato pathotypes were designated as L (lucerne) and P, respectively.

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