Use of Random Amplified Polymorphic DNA (RAPD) to Characterize Race 2 of *Fusarium oxysporum* f. sp. *pisi*

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ABSTRACT

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Genetic variability within four races of *Fusarium oxysporum* f. sp. *pisi* was assessed by random amplified polymorphic DNA (RAPD). Banding patterns generated from isolates of race 2 by 14 selected primers were uniform relative to patterns generated from races 1, 5, and 6. Isolates of races 1, 5, and 6 showed much greater variability within each race than did isolates of race 2. The high level of genetic variability among and within races of *F. o. pisi* allowed for fingerprinting of most of the isolates by examining patterns produced by several primers. Generalized race-specific patterns were not found for races 1, 5, and 6. The results suggested that races 1, 5, and 6 be grouped separately from race 2. Similarities and differences in banding patterns obtained by RAPD could be a useful molecular tool in evolutionary studies of the origins of different races.

Additional keywords: Fusarium wilt of peas.

Fusarium is a heterogeneous genus with a worldwide distribution that includes many species. A common characteristic of *Fusarium* spp. is the production of distinctive sickle-shaped macroconidia (27). A sexual stage has been identified for some *Fusarium* spp. Other than morphological and pathological traits, very little is known about the genus and, more specifically, about the species *Fusarium oxysporum*.

The high host specificity found among plant-pathogenic strains of *Fusarium* led Snyder and Hansen (19) to create a new taxa of the species based on this specificity. Isolates that cause disease in members of a particular family, genus, or species are forma specialis. Race characterizes isolates by resistance genes found in particular cultivars of a specific host species.

Identifying *Fusarium* at the species, forma specialis, and race levels is difficult (12) because many variables must be considered (27). Different methods have been used to assess genetic variability in *Fusarium* spp. and to identify markers at the species, forma specialis, and race levels. Studies on genetic relationships and phylogeny among *Fusarium* spp. have been conducted at the protein (15,16) and DNA levels (9,10,12,17,20).

Another approach to identifying different formae specialis and races resulted from studies of vegetative incompatibility (18). Puhalla (18) found a relationship between vegetative compatibility groups (VCGs) and formae specialis in *F. oxysporum*. Puhalla stated that without sexual reproduction, genes that determine vegetative compatibility and genes that determine pathogenicity and host specificity became fixed together because the only possible genetic exchange was through heterokaryon production among strains with the same *vic* genes (8,18). Although clear relationships between pathogenicity and VCGs have been found in several instances (1,4,8,10), more complex associations were found in other instances (2,5,7). No relationship was found between VCGs and pathogenicity in other instances (6). Even in instances in which clear associations between VCGs and pathogenicity existed, there were several limitations to broad application of the technique (2,3).

Random amplified polymorphic DNA (RAPD) (26) offers several advantages that may be useful in studying formae specialis and races of *F. oxysporum*, to identify RAPD markers for forma specialis and race identification. RAPD reduces the time needed for race identification in diseased plants, and provides genetic information on isolates studied, allowing for fingerprinting of isolates (24,25). RAPD has several advantages over other polymorphic DNA-detecting techniques, including RFLP (restriction fragment length polymorphism). These advantages include quickness, small amounts of template DNA needed, no requirement of DNA sequence information, and use of fluorescence (22). Applications of RAPD include development of genetic maps, targeting genetic markers, pooling techniques (13), and phylogenic information (21).

This research was conducted to study different isolates and races of *Fusarium oxysporum* Schlechtend.:Fr. f. sp. *pisi* (J.C. Hall) W.C. Snyder & H.N. Hans. present in Washington, where Fusarium wilt of peas is a predominant disease, by means of RAPD. RAPD markers were found that identify race 2 isolates from isolates of races 1, 5, and 6. RAPD markers have been used to evaluate genetic diversity among isolates of races 1, 2, 5, and 6 of *F. o. pisi* and to fingerprint different isolates.

MATERIAL AND METHODS

Isolates of *F. o. pisi* were maintained as chlamydospores in autoclaved soil (11). The isolates of race 1 used in this study were taken from three areas of Washington; isolates of race 2 were taken from two areas of Washington and one isolate was taken from England. The isolates of race 5 were found in three areas of Washington, and the isolates of race 6 came only from the western part of Washington. All isolates used in this study are readily available through F. J. Muehlbauer (Pullman, WA), J. M. Kraft (Prosser, WA), and W. H. Haglund (Mt. Vernon, WA).

A small amount of infested soil was sprinkled onto petri dishes containing Nash medium (1.5% Difco peptone, 0.1% KH_2PO_4, 0.5% $MgSO_4 \cdot 7H_2O$, 2% agar, and 0.1% 75% wettable powder Terraclor, pH adjusted to 5.5–6.5, that was autoclaved for 20 min, when the medium cooled, 0.01% streptomycin and 0.0012% neomycin were added) (12,14). The cultures were incubated for

5 days at 25 C in light to produce colonies (23). Resultant cultures were aseptically transferred to flasks of potato-dextrose broth. The flasks were placed on a rotary shaker (125 cycles per minute) and given 24 h of artificial light per day for 5 days at room temperature. The mycelia were filtered from the liquid medium through four cheesecloth layers. DNA was extracted from 1 g of mycelia by a slightly modified CTAB method (9).

Arbitrary random amplification of DNA sequences was performed with a set of 40 primers (each having 10 bases) obtained from Genosys Biotechnologies, Inc. (The Woodlands, TX). A modification of the RAPD method of Williams et al (26) was used to perform the amplification. Each 25 μl of reaction contained 1 U of *Taq* polymerase (Promega, Madison, WI); 50 mM KCl; 10 mM Tris-HCl, pH 8.8; 1.5 mM $MgCl_2$; 0.1% Triton X-100 (Promega 10× buffer); 0.1mM of each dNTP (Promega); 2 μM primer; and approximately 50 ng of template DNA.

Forty amplification cycles were performed on a Perkin-Elmer/Cetus Gene Amp PCR system 9600 (Norwalk, CT). Each cycle consisted of denaturation at 94 C for 20 s, annealing at 36 C for 1 min, followed by a 3-min rise to 72 C and primer elongation at 72 C for 1 min. The final primer-elongation segment of the run was extended to 9 min. The reaction product was resolved in a 2% agarose gel (1% Seakem and 1% NuSieve [FMC Bioproducts, Rockland, ME]) at 4 V/cm for 3 h. PCR product was visualized by UV-fluorescent staining with ethidium bromide.

The banding patterns produced by 40 primers on DNA from 23 isolates of *F. o. pisi* were analyzed. From these 40 primers, we selected the 14 primers that showed a greater number of polymorphisms (Table 1). 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. Banding patterns were analyzed by scoring 1 for the presence of major bands and 0 for their absence.

The discrete-data set generated was analyzed by the MIX program of PHYLIP 3.4 (developed by J. Felsenstein in 1991), using the Wagner parsimony method (changes from 0 to 1 are equally probable to changes from 1 to 0). This program generates an unrooted most parsimonious tree with the fewest possible stepwise changes.

RESULTS AND DISCUSSION

Many primers in this study clearly distinguished race 2 from races 1, 5, and 6 (Fig. 1). Isolate 167 was received as race 1 but yielded a banding pattern similar to that of race 2 isolates. Pathogenicity tests with a set of standard cultivars revealed that isolate 167 was a race 2 isolate.

All race 2 isolates tested showed a highly conserved and characteristic banding pattern (Fig. 1). The isolates of races 1, 5, and 6 presented more genetic variability than was apparent in race 2 isolates (Fig. 1).

TABLE 1. Source and sequence of the primers used in this study[a]

Primer identification	Sequence	Source
CS12	GCGACGCCTA	Genosys
CS14	GTCACCCGGA	Genosys
CS15	AACACATGCC	Genosys
CS16	CGTTGGATGC	Genosys
CS19	TACGGCTGGC	Genosys
CS22	CGTCGTGGAA	Genosys
CS24	GCGGCATTGT	Genosys
CS27	AGTGGTCGCG	Genosys
CS29	CCAGACAAGC	Genosys
CS30	GCGTAGAGAC	Genosys
CS31	CTCGACACTG	Genosys
CS34	GATAGCCGAC	Genosys
CS42	CCCAGAACAC	Genosys
CS43	CGTACGCGTT	Genosys

[a] Primers are listed 5′ to 3′ end, from left to right.

We scored 52 bands produced by 14 primers that showed polymorphism, in all isolates and in both repetitions. The same banding patterns were found 98.5% of the time; when a different result was observed in both repetitions, it was scored as "?".

A phylogeny tree with branch lengths proportional to the differences among isolates was constructed (Fig. 2). *F. o. pisi* race 2 isolates were discrete from the other isolates, forming a separate and homogeneous group. Race 5 isolates 34 and 758 formed a subgroup separate from the other isolates of races 1, 5, and 6. The other isolates of race 5 were closely related to the isolates of races 1 and 6, and no race-specific grouping of the isolates of the latter three races was detected (Fig. 2).

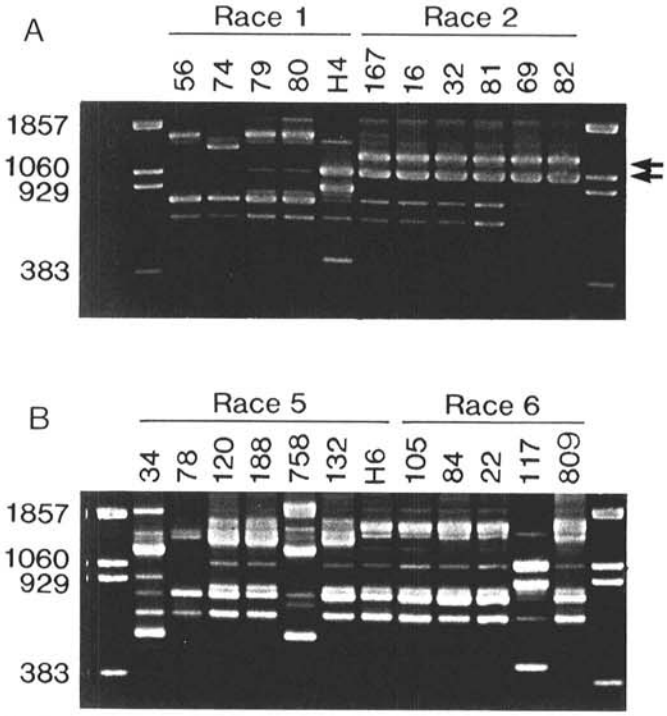

Fig. 1. Banding pattern of 23 *Fusarium oxysporum* f. sp. *pisi* isolates determined by primer CS31 (5′CTCGACACTG 3′). Isolates of race 2 showed a highly conserved banding pattern, indicated by the arrows.

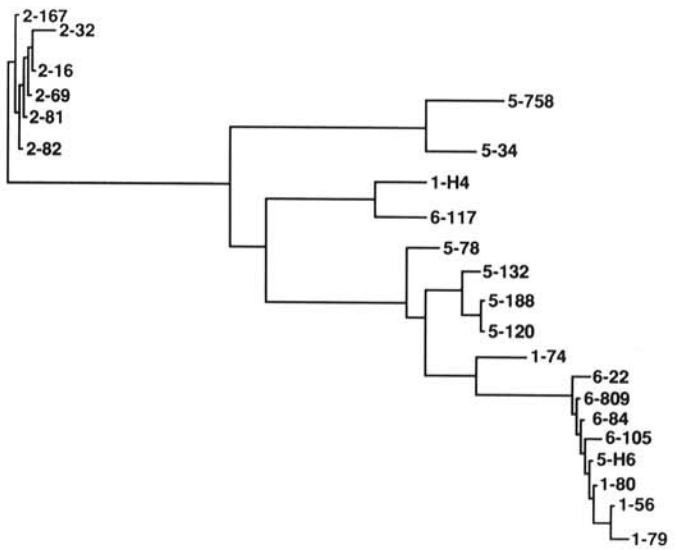

Fig. 2. Relationships among *Fusarium oxysporum* f. sp. *pisi* isolates as estimated by random amplified polymorphic DNA analysis. Isolates are identified by a number indicating the race and are separated by identification of particular isolates. The length of the branches is proportionate to the number of changes found between isolates.

CS12 (A,B,C,D), CS14 (A,B,C,D,E,F,G,H), CS15 (A,B,C,D,E), CS16 (A,B,C,D), CS19 (A,B,C), CS22 (A), CS29 (A,B), CS24 (A,B,C), CS30 (A,B,C,D,E,F), CS31 (A,B,C), CS34 (A,B,C,D), CS42 (A,B,C), CS43 (A,B,C,D,E,F).

RACE10056 00101100011001011 0010100001001011111 1000000000110010
RACE10074 00101100011001011 0010100001001011111 1000000000110010
RACE10079 00101100010001100 0110001011001010011 1000000000110010
RACE10080 00101100010001110 0110000011001010011 1000000000110010
RACE100H4 10011100010001001 0010000010000000011 0010000000000001
RACE20167 11001111010011100 1010110100110101001 0111000100101100
RACE20016 11001111010011100 1010110100110101001 0111000100101100
RACE20032 01001111010011100 1010110100110101001 0111000100101100
RACE20081 11001111010011100 1010110100110101001 0111000100101100
RACE20069 11001111010011100 1010110100110101001 0111000100101100
RACE20082 11001111010011100 1010110100110101001 0111000100101100
RACE50034 0000000010010000 0001000011000007000 0100111000000000
RACE50078 0000110001100101 0010101001001017111 0000000000010010
RACE50120 1?101100011001010 0010101011001017111 0000000000010010
RACE50188 1010110001100101 0010101011001017111 0000000000010010
RACE50758 0000000010010001 0001100000000007000 0100111010000010
RACE50132 1010110001100101 0010101?100101?111 0000000000010000
RACE500H6 0010110001000111 0110000011001010011 1000000000110010
RACE60105 0010110001000111 0110000011001010011 1000000001110010
RACE60084 0010110001000111 0110000011001010011 1000000000110010
RACE60022 0010110001000100 0110000011001010011 1000000000110010
RACE60117 1001110001000100 0010000011000010011 0011000000007?01
RACE60809 0010110001000111 0110000011001010011 1000000000110010

Fig. 3. Isolates used in the study and polymorphisms scored with different primers. Polymorphisms are indicated with the primer identification followed by different letters that identify the scored bands.

Most isolates used in this study could be fingerprinted with the use of primers that differentiate the banding pattern of a particular isolate from other isolates (Fig. 3). This is of interest for culture storage and might be useful in studies of the evolution of new races.

Pathogenicity tests are the only means of determining the pathological classification of fungal strains present in diseased plants or in soil samples. RFLP and RAPD applied to fungal studies would be useful in providing markers for identification purposes, such as the RAPD markers found for race 2 isolates in this study. These techniques can reduce the time and greenhouse space needed for pathological classification. RFLP and RAPD also could provide an additional means of studying the genetic characteristics and evolution of *Fusarium* spp., formae specialis, and races. In this study, we found that *F. o. pisi* race 2 clearly differs from the other races.

LITERATURE CITED

1. Bosland, P. W., and Williams, P. H. 1987. An evaluation of *Fusarium oxysporum* from crucifers based on pathogenicity, isozyme polymorphism, vegetative compatibility, and geographic origin. Can. J. Bot. 65:2067-2073.
2. Correll, J. C. 1991. The relationship between formae specialis, races, and vegetative compatibility groups in *Fusarium oxysporum*. Phytopathology 81:1061-1063.
3. Correll, J. C., Klittich, C. J. R., and Leslie, J. F. 1989. Heterokaryon self-incompatibility in *Gibberella fujikuroi* (*Fusarium monoliforme*). Mycol. Res. 93:21-27.
4. Correll, J. C., Puhalla, J. E., and Schneider, R. W. 1986. Identification of *Fusarium oxysporum* f. sp. *apii* on the basis of colony size, virulence, and vegetative compatibility. Phytopathology 76:396-400.
5. Elias, K. S., and Schneider, R. W. 1991. Vegetative compatibility groups in *Fusarium oxysporum* f. sp. *lycopersici*. Phytopathology 81:159-162.
6. Elmer, W. H., and Stephens, C. T. 1989. Classification of *Fusarium oxysporum* f. sp. *asparagi* into vegetative compatibility groups. Phytopathology 79:88-93.
7. Jacobson, D. J., and Gordon, T. R. 1988. Vegetative compatibility and self-incompatibility within *Fusarium oxysporum* f. sp. *melonis*. Phytopathology 78:668-672.
8. Katan, T., and Katan, J. 1988. Vegetative-compatibility grouping of *Fusarium oxysporum* f. sp. *vasinfectum* from tissue and the rhizosphere of cotton plants. Phytopathology 78:852-855.
9. Kim, D. H., Martyn, R. D., and Magill, C. W. 1992. Restriction fragment length polymorphism groups and physical map of mitochondrial DNA from *Fusarium oxysporum* f. sp. *niveum*. Phytopathology 82:346-353.
10. Kistler, H. C., Bosland, P. W., Benny, U., Leong, S., and Williams, P. H. 1987. Relatedness of strains of *Fusarium oxysporum* from crucifers measured by examination of mitochondrial and ribosomal DNA. Phytopathology 77:1289-1293.
11. Kraft, J. M. and Haglund, W. A. 1978 . A reappraisal of the race classification of *Fusarium oxysporum* f. sp. *pisi*. Phytopathology 68:273-275.
12. Manicom, B. Q., Bar-Joseph, M., Kotze, J. M., and Becker, M. M. 1990. A restriction fragment length polymorphism probe relating vegetative compatibility groups and pathogenicity in *Fusarium oxysporum* f. sp. *dianthi*. Phytopathology 80:336-339.
13. Michelmore, R. W., Paran, R. W. I., and Kesseli, R. V. 1991. Identification of markers linked to disease resistance genes by bulked segregant analysis: A rapid method to detect markers in specific genomic regions using segregating populations. Proc. Natl. Acad. Sci. USA 88:9828-9832.
14. Nash, S. M., and Snyder, W. C. 1965. Quantitative estimations by plate counts of propagules of the bean root rot Fusarium in field soils. Phytopathology 52:567-572.
15. Partridge, J. E. 1991. Specific gene product of *Fusarium*: Analysis of ribosomal proteins. Phytopathology 81:1055-1058.
16. Partridge, J. E., Nelson, P. E., and Toussoun, T. A. 1984. Ribosomal proteins in the genus *Fusarium*. Mycologia 76:533-544.
17. Peterson, S. W. 1991. Phylogenetic analysis of *Fusarium* species using ribosomal RNA sequence comparisons. Phytopathology 81:1051-1054.
18. Puhalla, J. E. 1985. Classification of strains of *Fusarium oxysporum* on the basis of vegetative compatibility. Can. J. Bot. 63:179-183.
19. Snyder, W. C., and Hansen, H. N. 1940. The species concept in *Fusarium*. Am. J. Bot. 27:64-67.
20. Szecsi, A., and Dobrovolszky, A. 1985. Phylogenetic relationships among *Fusarium* species measured by DNA reassociation. Mycopathologia 89:89-94.
21. Thormann, C. E., and Osborn, T. C. 1992. Use of RAPD and RFLP markers of germplasm evaluation. Pages 9-11 in: Applications of RAPD Technology to Plant Breeding. Crop Sci. Soc. Am., Am. Soc. Hortic. Sci., and Am. Genet. Assoc., Minneapolis, MN.
22. Tingey, S. V., Rafalski, J. A., and Williams, J. G. K. 1992. Genetic analysis with RAPD markers. Pages 3-8 in: Applications of RAPD Technology to Plant Breeding. Crop Sci. Soc. Am., Am. Soc. Hortic. Sci., and Am. Genet. Assoc., Minneapolis, MN.
23. Toussoun, T. A., and Nelson, P. E. 1976. Pictorial guide for the identification of *Fusarium* species. The Penn. State Univ. Pr., University Park. 43 pp.
24. Weising, K., Kaemmer, D., Epplen, J. T., Weigand, F., Saxena, M., and Kahl, G. 1991. DNA fingerprinting *Ascochyta rabiei* with synthetic oligodeoxynucleotides. Curr. Genet. 19:483-489.
25. Welsh, J., and McClelland, M. 1990. Fingerprinting genome using PCR with arbitrary primers. Nucleic Acids Res. 18:7213-7218.
26. Williams, J. G. K., Kubelik, A. R., Livak, K. J., Rafalski, J. A., and Tingey, S. V. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res. 18:6531-6535.
27. Windels, C. E. 1991. Current status of *Fusarium* taxonomy. Phytopathology 81:1048-1051.