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Relationship Between Pathogenicity and Phylogeny Based on Restriction Fragment Length Polymorphism in *Leptosphaeria maculans*

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Isolates of *Leptosphaeria maculans* collected in widely separated geographic regions were characterized for virulence on cultivars of *Brassica napus* spp. *oleifera* and for DNA restriction fragment length polymorphisms (RFLPs). Cotyledons of the cultivars Westar, Quinta, and Glacier were inoculated with 39 isolates, and based on disease reactions, isolates were grouped as "nonaggressive" or "aggressive." Aggressive isolates were further divided into three pathogenicity groups based on differential reactions of the three cultivars. All of the aggressive isolates and none of the nonaggressive isolates produced phytotoxins (sirodesmins) *in vitro*. DNAs from 28 isolates were analyzed for RFLPs with two restriction enzymes and 42 probes of cloned nuclear DNA sequences from *L. maculans*, and the resulting RFLP data were used to construct a phylogenetic tree. For many of the probes, aggressive and nonaggressive isolates had different RFLP patterns and hybridization intensities, and they were separated into two phylogenetically distant groups. Aggressive isolates were subdivided into more closely related phylogenetic groups, which in some cases corresponded to their pathogenicity groups. These results suggest that aggressive and nonaggressive isolates may belong to different species, and that the phylogeny of aggressive isolates is partially related to their pathogenic performance.

Additional keywords: blackleg, differential hosts, host-pathogen interaction, *Phoma lingam*.

Leptosphaeria maculans (Desmaz.) Ces. & De Not., the perfect state of *Phoma lingam* (Tode:Fr.) Desmaz., is the causal organism of blackleg disease of crucifers. In the economically important genus *Brassica* L., *B. rapa* L. (syn. *B. campestris* L.), *B. oleracea* L., and *B. napus* L. are the most susceptible to the pathogen; however, susceptibility in accessions of *B. nigra* (L.) W. Koch, *B. juncea* (L.) Czernj. & Coss., and *B. carinata* A. Braun has been reported (Sjodin and Glimelius 1988). *L. maculans* is distributed worldwide. Infections of young oilseed rape plants are initiated by airborne ascospores, and the disease becomes most destructive when crown cankers develop around flowering time. Systemic spread of the pathogen from leaves to the stem has been demonstrated (Hammond *et al.* 1985). Because of the possibility of genetic analyses of sexual progenies in both the pathogen and the host, *L. maculans* in combination with brassicas has considerable potential as a model host-parasite system (Hill and Williams 1988). Differential host-parasite interactions have been reported (Cargeeg and Thurling 1980; Delwiche and Williams 1979; Hammond and Lewis 1987; Newman 1984); however, because methods of inoculation have varied considerably and different hosts and fungal isolates have been used, results from various studies have not always been comparable. Until now, an internationally recognized set of differential hosts has not been described.

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Within *L. maculans*, two groups of isolates can be distinguished readily based on pathogenicity: a strongly pathogenic, virulent, or aggressive group and a weakly pathogenic, avirulent, or nonaggressive group (Cunningham 1927; Hill *et al.* 1980; Humpherson-Jones 1986; Koch *et al.* 1989; McGee and Petrie 1978). Aggressive isolates can be differentiated from nonaggressive ones by a slower growth rate on agar media, by the absence of dark pigments in liquid culture (Bonman *et al.* 1981; Hill *et al.* 1980; Koch *et al.* 1989; McGee and Petrie 1978), by the production of sirodesmin toxins (Koch *et al.* 1989), by shorter conidial germ tubes on certain media (Petrie 1988), and by differences in electrophoretic banding patterns of malate dehydrogenase (Hill *et al.* 1980), pectic enzymes (Hanaczywskyj and Drysdale 1984), and esterases (E. Koch, unpublished data). These differences, together with reports of unsuccessful matings between aggressive and nonaggressive isolates (Bonman *et al.* 1981; Delwiche 1980), suggest that these two groups of isolates are genetically very distinct and may belong to different species. Further information on the genetic makeup of this pathogen could be very useful in determining the genetic relationship of isolates with different pathogenic performances in order to study the evolution of pathogenicity.

Analysis of restriction fragment length polymorphisms (RFLPs) is a powerful method of determining the genetic composition of organisms. RFLP analysis of nuclear and mitochondrial genomes has been used to study inter- and intraspecific variation of fungal pathogens of plants (Christiansen and Giese 1990; Forster *et al.* 1989; Hamer *et al.* 1989; Hulbert and Michelmore 1988; also see reviews by Leong and Holden 1989 and Michelmore and Hulbert

1987). In most cases, RFLPs could distinguish among species or *forma specialis* and among some isolates within species. RFLP patterns were compared to the pathogenicity of isolates in some studies; however, no clear relationships were observed (Manicom *et al.* 1987; Vilgalys and Gonzalez 1990; Jacobson and Gordon 1990).

For the study reported here, we assembled a collection of *L. maculans* isolates from Europe, North America, Australia, and South Africa. A set of *B. napus* rapeseed cultivars was identified, which readily differentiated isolates into pathogenicity groups, and the phylogenetic relationships of these isolates were determined by RFLP analysis of nuclear DNA sequences with random genomic DNA clones. These data were compared to determine if aggressive and nonaggressive isolates represent distinct phylogenetic groups, and if the pathogenicity of aggressive isolates is related to their phylogenetic groupings or genetic backgrounds.

MATERIALS AND METHODS

Plant materials and seedling test. Seeds were planted in multipots (Com-pack D 812, T.O. Plastics, Minneapolis, MN) in Jiffy Mix and covered with vermiculite. Plants were kept in a growth chamber (22 ± 2° C) under continuous lighting (250 $\mu E \cdot s^{-1} \cdot m^{-2}$), watered with tap water, and watered once weekly with 50% Hoagland's solution. For inoculation, cotyledons of 5-day-old seedlings were punctured on each cotyledon half (four sites per seedling), 5-μl drops of conidial suspensions (10^6 conidia per milliliter) were deposited over the wounds, and plants were returned to the growth chamber. In the screening for host differentials, four plants were inoculated per cultivar-fungal isolate combination, and eight plants were inoculated in the characterization of isolates. To allow full expansion of cotyledons, developing true leaves were removed frequently. Disease was rated 11 days after inoculation with a 0–9 scale as described previously (Williams 1985) with minor modifications: 0 = no darkening around wounds, as in controls (inoculated with water only); 1 = limited blackening around wounds, lesion diameter 0.5–1.5 mm; 3 = dark necrotic lesions 1.5–3 mm; 5 = dark lesions 3–6 mm, brownish on lower surface; 6 = as in 5, but less necrotic; 7 = gray-green lesions of limited size or large necrotic lesions; 8 = spreading gray-green lesions with no or few pycnidia; 9 = large gray-green lesions with profuse sporulation.

Fungal cultures. Cultures of *L. maculans* isolates selected from a collection held by the Crucifer Genetics Cooperative (Williams 1985) were initiated from single conidia and maintained on V8 agar. Conidial suspensions for inoculation were prepared as described previously (Hill and Williams 1988). Isolates were cultured in Czapek's medium supplemented with 0.2% yeast extract for 28 days. The pigmentation of culture filtrates was scored, and sirodesmins were extracted with ethyl acetate, followed by thin-layer chromatography, as described previously (Koch *et al.* 1989). To obtain mycelium for DNA extraction, 1–5 ml of conidial suspensions (10^6–10^8 conidia per milliliter) was added to 200 ml of Czapek's medium (+0.2% yeast extract) in 1-L Erlenmeyer flasks. Flasks were kept on a rotary shaker at room temperature for 2–4 days.

Preparation of DNA. Fungal mycelium was harvested by vacuum filtration, washed with distilled water, kept at −70° C for several hours and lyophylized. Using a mortar and pestle, we ground with sand 400 mg of dried mycelia to a fine powder. Alternatively, 3 g of fresh mycelia was ground in liquid nitrogen. Total DNA was extracted by the method of Klich and Mullaney (1987) with the following modifications: two phenol extraction steps, followed by two chloroform extractions were employed before the DNA was finally precipitated with isopropanol, pelleted by centrifugation, and suspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8).

Construction of genomic DNA libraries. Two random genomic DNA libraries were constructed with nuclear DNAs from isolates PHW129 and PHW843. Total DNAs were prepared by the procedure described above and then separated into mitochondrial and nuclear DNA fractions with bisbenzimide-cesium chloride ultracentrifugation (Hudspeth *et al.* 1980). Samples containing 300 μg of bisbenzimide, 4.8 g of cesium chloride, and 1–2 mg of total DNA in TE were centrifuged in 5-ml quick-seal tubes in a VTi 65 rotor (Beckman Instruments, Inc., Fullerton, CA) for 24 hr at 45,000 rpm. After centrifugation, two bands were visible under UV light. The lower band containing nuclear DNA was collected and bisbenzimide was removed by three extractions with NaCl-saturated isopropanol. DNAs were precipitated with isopropanol, suspended in TE buffer, and digested with the restriction endonuclease *Pst*I (Promega, Madison, WI). After electrophoresis, 1- to 2-kb fragments were recovered from the agarose gel, ligated into the plasmid pTZ18R (Pharmacia, Inc., Piscataway, NJ) cut with *Pst*I, and recombinant plasmids were used to transform *Escherichia coli* strain DH5α.

Detection of RFLPs. For each lane of a gel, approximately 5 μg of total fungal DNA was digested with 20

Table 1. Cotyledon reactions of *Brassica* cultivars with various isolates of *Leptosphaeria maculans*[a]

		L. maculans isolates					
Species	**Cultivar**	**IIa1**	**PHW914**	**P146**	**PHW100**	**IX2**	**PHW843**
B. napus	Global	S	S	S	S	S	S
B. napus	Westar	S	S	S	S	S	S
B. napus	Marnoo	S	S	S	S	S	S
B. napus	Maluka	S	S	S	S	S	S
B. napus	Cresor	S	S	S	S	S	S
B. napus	Mikado	S	S	S	S	S	S
B. napus	Wesroona	S	S	S	S	S	S
B. napus	Rubin	S	S	S	S	S	S
B. napus	Lirabon	S	S	S	S	S	S
B. napus	Doral	S	S	S	S	S	S
B. napus	Ceres	S	S	S	S	S	S
B. napus	Topas	I	I	I	I	I	I
B. napus	Jet Neuf	S	S	S	S	S	R
B. napus	Glacier	S	S	S	S	S	R
B. napus	Bienvenue	S	S	S	S	S	R
B. napus	Quinta	S	S	I	I	I	I
B. rapa	Tobin	S	S	S	S	S	S
B. rapa	BLC 198	S	S	S	S	S	R
B. rapa	Huang	S	S	S	I	I	S
B. juncea	Cutlass	R	R	R	R	R	R

[a] Four plants, four cotyledon inoculation sites per plant. Symptoms were rated 11 days after inoculation with an interaction phenotype scale of 0–9 (see text). Reactions are classified as resistant (R: 0–3), intermediate (I: 4–6), and susceptible (S: 7–9).

units of the restriction endonuleases *Eco*RI (Promega) or *Hind*III (Bethesda Research Laboratories, Gaithersburg, MD) according to the manufacturer with the addition of 5 μg of RNase A per reaction. Digested DNAs were separated by electrophoresis in 1% agarose gels containing 40 mM Tris-acetate and 1 mM EDTA. Gels were stained with ethidium bromide and photographed on a UV transilluminator. Southern blotting, probe-labeling, and hybridization were conducted as described previously (Osborn *et al.* 1987). Plasmid DNAs used as probes were isolated by the boiling lysis method (Holmes and Quigley 1981) from bacteria containing DNA clones from each of the two nuclear DNA libraries. After hybridization, blots were washed two times in 2× SSC (0.3 M NaCl, 30 mM sodium citrate, pH 7) at room temperature for 5 min, one time in 2× SSC, 1% SDS, at 65° C for 60 min, and one time in 0.1× SSC, 0.1% SDS, at 65° C for 60 min. Blots then were exposed to X-ray film for 1–4 days at −70° C.

Phylogenetic analysis. Because we did not have genetic data on allelism of nuclear RFLPs observed in this study,

Table 2. Characterization of *Leptosphaeria maculans* isolates by *in vitro* production of sirodesmin and pathogenicity on the cotyledons of *Brassica napus* rapeseed cultivars

Isolate	Origin[a]	Host	Toxin[b]	Disease rating on cultivars[c]			PG[d]	RFLP[e]
				Westar	Quinta	Glacier		
PHW851	A	*B. oleracea*	−	1.0	1.2	1.3	PG1	−
PHW853	A	*B. oleracea*	−	0.2	0.6	0.9	PG1	−
PHW863	A	*B. oleracea*	−	1.8	1.4	2.2	PG1	+
PHW865	A	*B. juncea*	−	1.3	1.5	1.6	PG1	−
PHW790	C	*B. rapa*	−	1.6	1.5	2.4	PG1	+
Unity	C	*B. napus*	−	1.7	1.7	2.1	PG1	+
IIIa5	G	*B. napus*	−	1.3	1.3	1.4	PG1	+
VIII2	G	*B. napus*	−	0.5	0.7	1.2	PG1	+
PHW841	NZ	*B. oleracea*	−	1.0	1.3	1.2	PG1	+
PHW850	SA	*B. oleracea*	−	1.2	1.8	2.4	PG1	+
PHW126	US	*B. oleracea*	−	1.0	0.4	1.0	PG1	+
PHW129	US	*B. oleracea*	−	0.3	0.5	0.1	PG1	+
Mean				1.1 ± 0.5	1.2 ± 0.5	1.5 ± 0.17		
PHW843	A	*B. oleracea*	+	8.3	6.8	1.1*	PG2	+
PL87-5	C	*B. napus*	+	9.0	4.9	1.2	PG2	−
Leroy	C	*B. napus*	+	9.0	6.8	1.5	PG2	+
PHW620	F	*B. oleracea*	+	8.5	7.9	2.1*	PG2	+
Lm2	UK	*B. napus*	+	9.0	5.6	1.0	PG2	+
CBS275.63	Unknown	Unknown	+	8.5	7.3	2.0*	PG2	−
PHW839	SA	*B. oleracea*	+	7.0	3.2	1.0	PG2	−
PHW128	US	*B. oleracea*	+	9.0	8.3	2.3	PG2	−
Mean				8.5 ± 0.7	6.4 ± 1.7	1.5 ± 0.5		
PHW888	A	*B. napus*	+	8.9	5.4*	7.8	PG3	+
PHW478	F	*B. napus*	+	8.9	5.9*	8.2	PG3	+
PHW479	F	*B. napus*	+	8.9	5.5*	8.1	PG3	+
PHW437	G	*B. napus*	+	9.0	5.6	8.2	PG3	+
IX2	G	*B. napus*	+	9.0	6.0*	9.0	PG3	+
MIX7	G	*B. napus*	+	8.8	6.0	8.4	PG3	+
P146	UK	*B. napus*	+	8.9	4.7	8.4	PG3	−
PHW423	N	*B. napus*	+	8.2	5.9	8.0	PG3	+
PHW100	US	*B. napus*	+	8.9	5.5*	7.5	PG3	+
Mean				8.8 ± 0.3	5.6 ± 0.4	8.2 ± 0.4		
PHW914	A	*B. rapa*	+	9.0	8.3	7.6	PG4	+
PL87-111	A	*B. napus*	+	8.8	8.8	7.8	PG4	+
PL87-114	A	*B. napus*	+	8.9	8.9	8.2	PG4	−
PL87-2	C	*B. napus*	+	8.6	9.0	8.0	PG4	−
PHW431	G	*B. napus*	+	8.5	8.7	7.5	PG4	+
PHW433	G	*B. napus*	+	9.0	8.8	8.4	PG4	+
IIa1	G	*B. napus*	+	9.0	8.9	8.3	PG4	+
V14	G	*B. napus*	+	9.0	9.0	8.0	PG4	+
41A4	UK	*B. napus*	+	8.8	8.8	8.4	PG4	−
Lm1	UK	*B. napus*	+	8.2	8.1	7.7	PG4	+
Mean				8.8 ± 0.3	8.7 ± 0.3	8.0 ± 0.3		

[a] Geographic origins: A = Australia; C = Canada; F = France; G = Germany; NZ = New Zealand; SA = South Africa; UK = United Kingdom; US = United States.

[b] Sirodesmin presence (+) or absence (−).

[c] Eight plants, four inoculation sites per plant. Values marked * are based on less than eight plants (see text). Symptoms were rated 11 days after inoculation with a 0–9 scale.

[d] PG = pathogenicity group; PG1 = nonaggressive group; PG2 = aggressive group differentiated by Glacier; PG3 = aggressive group differentiated by Quinta; PG4 = most aggressive group, sporulated on all three differential hosts.

[e] Isolates used for RFLP analysis (+) or not used (−).

each restriction fragment that showed detectable hybridization to a probe was treated as a unit character and scored as present or absent across all isolates (Song *et al.* 1990). General information on genetic relationships among isolates was obtained by conducting a principal coordinates analysis for RFLP data with the computer program package NTSYS-pc (Rolhf 1989). The Wanger parsimony method (PAUP 3.0b, David Swofford, Illinois Natural History Survey, Champaign) was applied to determine the phylogenetic relationships among the isolates.

RESULTS

Screening for differential hosts. To select a set of differential host genotypes, 16 *B. napus* cultivars were initially screened with six isolates representing different pathovars of *L. maculans* (Table 1). Eleven of the 16 *B. napus* cultivars were susceptible to all *L. maculans* isolates. Cultivars Jet Neuf, Glacier, and Bienvenue were susceptible to all isolates except PHW843. Quinta expressed full susceptibility to only two isolates, whereas Topas always showed an intermediate reaction. *B. rapa* cultivars BLC-198 and Huang also showed differential responses; however, reactions on individual plants varied considerably, suggesting genetic heterogeneity within these accessions. Within the *Brassica* cultivars tested, seedlings showed relatively uniform reactions to inoculation with each of the six different isolates of *L. maculans*. Based on these observations, the three *B. napus* cultivars Westar, Quinta, and Glacier were selected as differential hosts.

Pathogenicity of isolates and toxin production. Interaction phenotypes of *L. maculans* isolates on the differential hosts provided the basis for distinguishing nonaggressive and aggressive isolates and for the further division of aggressive isolates into three pathogenicity groups (Table 2). Twelve nonaggressive isolates (pathogenicity group 1 or PG1) caused only small necrotic lesions on all three hosts (Fig. 1A), and none of them produced sirodesmins in culture. In contrast, all of the aggressive isolates examined produced sirodesmins and were able to form sporulating lesions on Westar (Fig. 1B). Culture filtrates from the aggressive strains never showed brown pigmentation, and only a few of the nonaggressive isolates produced yellow-brown pigments.

Isolates in PG4 were characterized by their ability to sporulate on Westar, Quinta, and Glacier. Isolates in PG3 sporulated on Westar and Glacier, and caused brown, nonsporulating lesions on Quinta (Fig. 1C). In some cases, only five to seven of the Quinta plants showed this type of reaction, whereas the rest expressed full susceptibility. Because the difference between these two interaction phenotypes was always clear-cut, the seed of Quinta that we used may not have been homogeneous for this trait. Plants showing the PG3-type reaction were self-pollinated for pure-

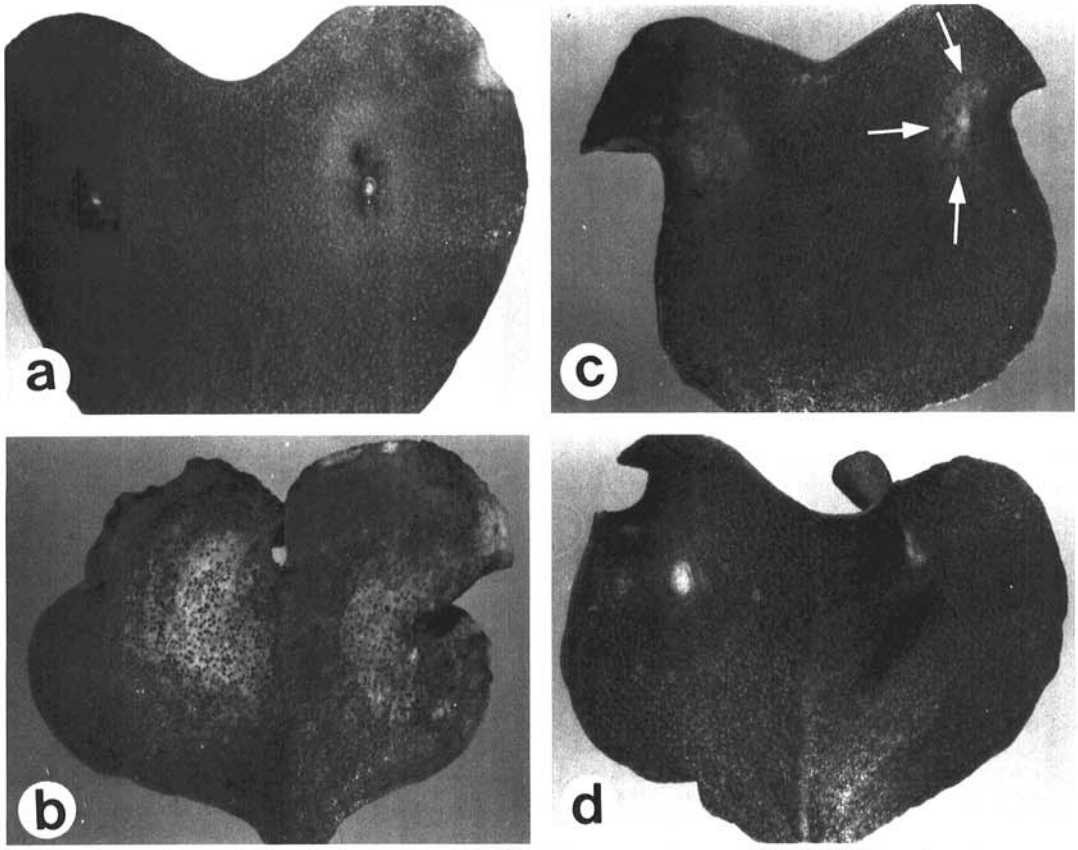

Fig. 1. Interaction phenotypes on cotyledons of *Brassica napus* observed 11 days after inoculation with different isolates of *Leptosphaeria maculans*. **A,** Nonaggressive pathogenicity group (PG) 1 isolate on cultivar Westar (small necrotic lesions). **B–D** are the aggressive isolates. **B,** PG4 isolate on cultivar Westar (large, gray-green, sporulating lesions). **C,** PG3 isolate on cultivar Quinta (nonsporulating, medium-size lesions as shown by arrows). **D,** PG2 isolate on cultivar Glacier (small, nonsporulating lesions, less necrotic than in A).

line development.

Isolates in PG2 sporulated on Westar but caused varying interaction phenotypes on Quinta. The lesions on Glacier produced by PG2 isolates were similar in size to those caused by nonaggressive isolates, but showed less necrosis around the inoculation point (Fig. 1D). Four of 64 Glacier plants inoculated with PG2 isolates were susceptible, suggesting some heterogeneity in the seed lot of this cultivar.

Each pathogenicity group included isolates that had been collected in widely separated geographic regions (Table 2), and thus pathogenicity of the isolates tested did not appear to be related to their geographic origin. However, only a few isolates from any one country were analyzed, and a relationship between pathogenicity and geographic origin might be revealed by analyzing more isolates from various regions.

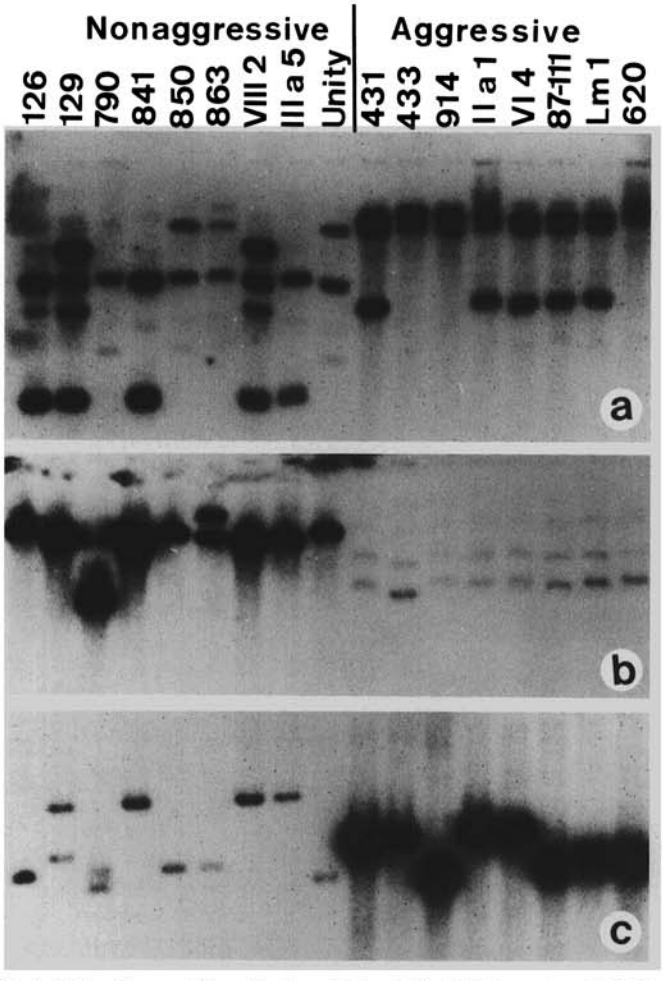

Fig. 2. Autoradiograph from Southern blots of *Hin*dIII-digested total DNAs of *Leptosphaeria maculans* isolates probed with different nuclear DNA clones. Each lane containing DNAs from a different isolate is labeled with the isolate number (see Table 2; three-digit numbers are PHW isolates). **A,** RFLP patterns from hybridization with clone 129A5 from the nonaggressive isolate 129 library, showing variation between the nonaggressive and aggressive isolates, among the nonaggressive isolates, and among the aggressive isolates. Hybridization intensities between aggressive and nonaggressive isolates are equivalent, but the nonaggressive isolates are more variable. **B,** RFLP patterns from hybridization with clone 129A6. The nonaggressive isolates show a much stronger hybridization intensity than the aggressive isolates. **C,** RFLP patterns from hybridization with clone 843B2 from the aggressive isolate 843 library. The aggressive isolates have a much stronger hybridization intensity than the nonaggressive isolates.

RFLP analysis and phylogeny of isolates. In a preliminary analysis, 95 random genomic DNA clones (15 from the isolate PHW129 library and 80 from the isolate PHW843 library) were screened for low-copy DNA clones that hybridized to polymorphic fragments. DNAs of nine isolates were digested with *Eco*RI and *Hin*dIII and were probed with the 95 genomic DNA clones. A high percentage of probes hybridized to fragments that were polymorphic between the nonaggressive and aggressive groups (91/95 or 96%) and within nonaggressive groups (49/95 or 52%), but a low percentage of probes hybridized to fragments that were polymorphic within the aggressive group (16/95 or 17%).

Forty-two of the 95 probes (six from the 129 library and 36 from the 843 library) were selected and used to identify RFLPs among 28 isolates representing the different pathogenicity groups (Table 2). Total DNAs of the 28 isolates were digested with either *Eco*RI or *Hin*dIII, depending on which enzyme revealed the most polymorphism in the initial screening. Polymorphic fragments were detected between the nonaggressive and aggressive isolates for all 42 probes, among isolates within the nonaggressive group for 18 probes, and among isolates within the aggressive group for 16 probes (Fig. 2A). Two probes from the 129 library showed strong hybridization to all of the isolates from the nonaggressive group, but gave very weak signals or no signals for isolates from the aggressive group (Fig. 2B). The reverse situation was observed for eight probes from the 843 library (Fig. 2C). The nonaggressive and aggressive groups also could be distinguished easily by visualizing gels after ethidium-bromide staining. For instance, a distinct 1.7-kb fragment was observed in *Eco*RI digests of all 19 aggressive isolates, but not in the nonaggressive isolates (Fig. 3). Similar differences were observed in *Hin*dIII digests of DNAs (data not shown).

Results from the principal coordinates analysis were projected into a three-dimensional configuration (Fig. 4).

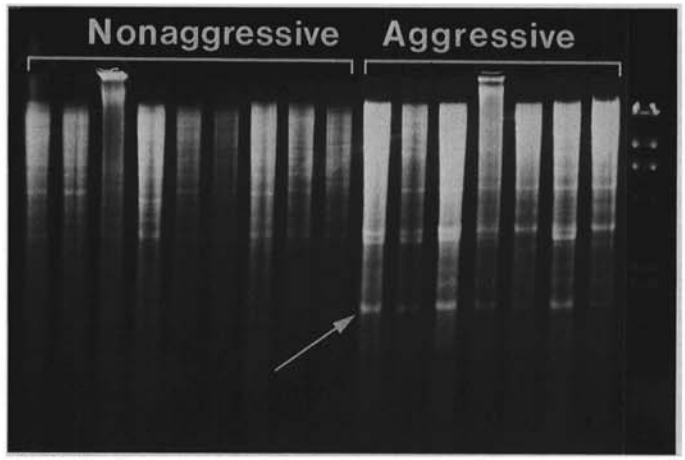

Fig. 3. Agarose-gel electrophoresis of *Eco*RI-digested total DNAs from *Leptosphaeria maculans* isolates. Aggressive isolates have a distinct 1.7-kb fragment (arrow), which is missing in nonaggressive isolates. Designations of isolates are the same as in Figure 2, except the last lane, which is a *Hin*dIII-digest of λ DNA.

All of the aggressive isolates clearly constituted a large, compact group distinct from all nonaggressive isolates. Isolates within this group were closely related and no further subgrouping was detected. Within the nonaggressive group three subgroups were observed: NA1 consisted of isolates PHW129, VIII2, PHW841, and IIIa5; NA2 included isolates PHW790, PHW850, PHW863, and Unity; and NA3 consisted of isolate PHW126, which seemed to be a unique pathovar belonging to this subgroup (Fig. 4).

For Wanger parsimony analysis, 185 informative fragments (fragments shared by at least two isolates but not by all isolates) were used to construct phylogenetic trees for the 28 isolates. The nine isolates in the nonaggressive group were used as the outgroup. The PAUP program generated 1,215 of the shortest trees from which one consensus tree was developed (Fig. 5). A bootstrapping test with 100 repetitions was done and confidence levels greater than 50% were added to the consensus tree (Fig. 5). In this phylogenetic tree, the nonaggressive and aggressive isolates were separated into two distinct groups with a unit distance of 91 and a confidence level of 100%. Unit distances between the most distant isolates within groups were 40 for nonaggressive isolates (PHW129–PHW126) and 24 for aggressive isolates (IIa1–VI4). The numbers of different restriction fragments between pairs of isolates were two to 38 within the nonaggressive group, and four to 30 within the aggressive group. However, 105–123 different restriction fragments were observed between isolates from the two groups (Table 3). These observations indicated that much greater genetic diversity exists between the aggressive and nonaggressive groups than within the groups. Within the nonaggressive group, isolates were separated into three subgroups (NA1, NA2, and NA3), which had identical composition as those from the principal coordinates analysis. Phylogenetic relationships of the nonaggressive isolates revealed by the tree (Fig. 5) seemed to be unrelated to their geographic origins (Table 2).

Because little genetic diversity was observed among the aggressive isolates, it is difficult to make conclusive statements about groupings for these isolates. However, some useful information on phylogenetic relationships among

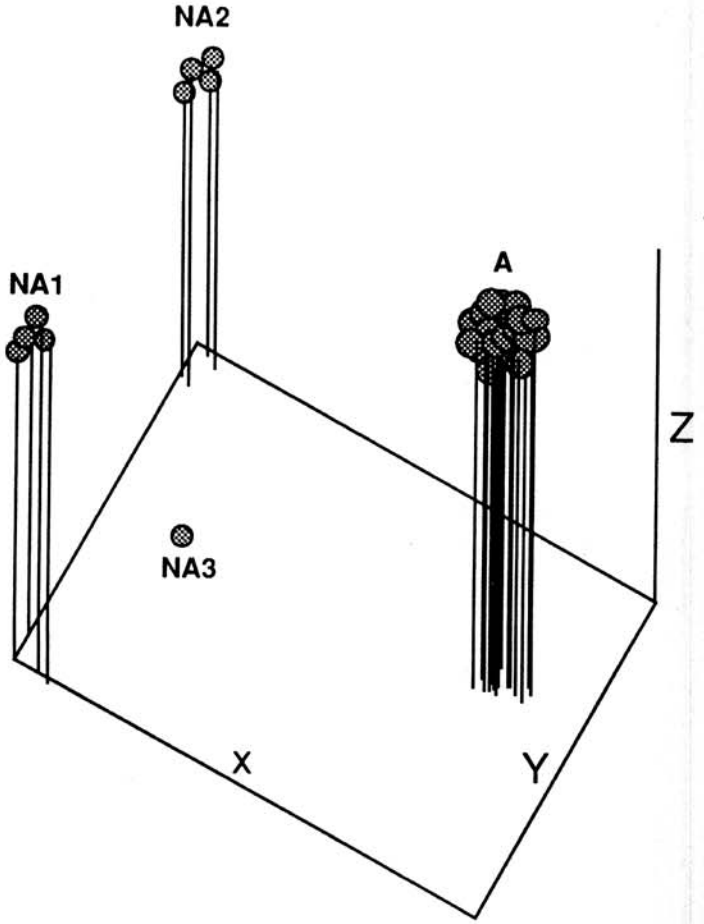

Fig. 4. A three-dimensional configuration generated by principal coordinates analysis. **A,** All of the 19 aggressive isolates used in RFLP analysis (Table 2). NA1: subgroup in the nonaggressive group containing isolates PHW129, VIII2, PHW841, and IIIa5; NA2: subgroup in the nonaggressive group including isolates PHW790, PHW850, PHW863, and Unity; NA3: subgroup in the nonaggressive group represented by isolate PHW126.

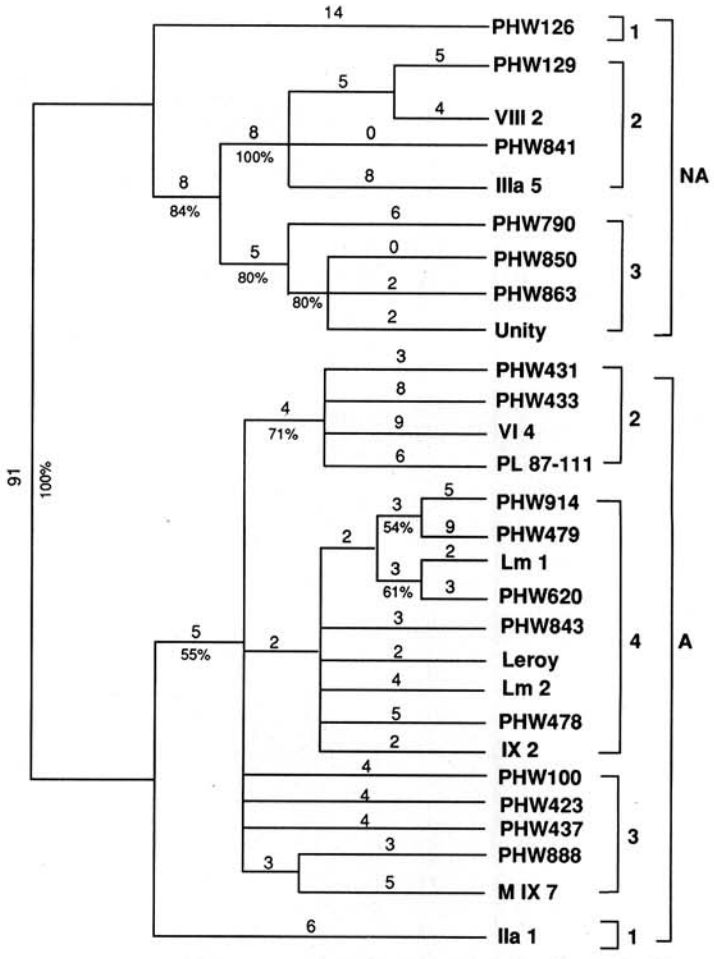

Fig. 5. A phylogenetic tree constructed with RFLP data by using the PAUP microcomputer program (PAUP 3.0b; David Swofford, Illinois Natural History Survey, Champaign, IL) with functions "hold = 10, swap = global and mulpars." Designation of isolates are at the termini of branches. The numbers on branches indicate the unit distances (minimum mutation steps) between isolates; and the numbers under branches indicate the confidence levels determined by bootstrapping with 100 repetitions (only those larger than 50% are shown). NA represents the nonaggressive group, and A represents the aggressive group. The numbers 1, 2, 3, and 4 designate subgroups within either nonaggressive or aggressive group (see text for details).

aggressive isolates can be obtained from the consensus tree (Fig. 5). Basically, the aggressive isolates could be divided into four subgroups. Subgroup A1 was represented by the isolate IIa1, which was distinct from all other aggressive isolates; A2 included isolates PHW431, PHW433, VI4, and PL87-111; A3 contained isolates PHW100, PHW423, PHW437, PHW888, and MIX7; and A4 consisted of the nine remaining aggressive isolates. Two clusters were observed in A4: one included isolates PHW914, PHW479, Lm1, and PHW620, and another included isolates PHW843, Leroy, Lm2, PHW478, and IX2. All four isolates in A2 belonged to PG4, and all of the five isolates in A3 belonged to PG3 (Table 2). The subgroup A2 had a confidence level of 71% from the bootstrap analysis, which was the highest among aggressive isolates. All four isolates in this subgroup were collected on *B. napus*, and three of them came from Germany (Table 2). The subgroup A3 was less well-defined by the analysis, and although all five isolates were collected on *B. napus*, they came from three different continents (Table 2). Isolates in A4 belonged to more than one pathogenicity group, and these isolates were collected on three different *Brassica* species and from several different geographic regions (Table 2).

DISCUSSION

Based on cotyledon reactions of the three *B. napus* rapeseed cultivars Westar, Quinta, and Glacier, *L. maculans* isolates of widely separated geographic origins were classified as "nonaggressive" (PG1) and "aggressive" (PG2, 3, or 4). The aggressive strains could be divided into three subgroups. PG4 isolates sporulated on all three cultivars, but Glacier and Quinta showed resistance to PG2 and PG3 isolates, respectively. The capacity of Quinta to differentiate among isolates was demonstrated by Delwiche (1980) based on cotyledon reactions and by Newman (1984) who scored stem cankers on 35-day-old seedlings. Disease reactions on cotyledons of Quinta inoculated with isolates 41A4 (PG4) and P146 (PG3) observed in our study agreed with Newman's results. A previously unreported finding was the *L. maculans* isolates grouped as PG2 in this study, to which cotyledons of Glacier expressed a strong resistance. Although specificity in the *B. napus-L. maculans* interaction has been reported previously, the set of differential hosts reported here can distinguish isolates into four pathogenicity groups.

The various interaction phenotypes observed in this study suggest the existence of virulence or avirulence genes in *L. maculans* isolates and resistance genes in the host, which together condition the differential interactions of the fungus with the host. Whether these specific interactions are operative in other plant organs and at later stages of development is not known. Although disease reactions of seedlings in the greenhouse have been reported to be correlated with those of adult plants in the field (Cargeeg and Thurling 1979; Newman and Bailey 1987), exceptions have been reported. In spite of these considerations, the specific interactions described offer a potential model to study the underlying host-parasite relationships. Disease reactions appear only 10–12 days after inoculation, and expression of interaction phenotypes on cotyledons is generally unambiguous and easier to score than stem infections. Transferring the resistance of Quinta and Glacier into rapid-cycling *B. napus* populations (Williams and Hill 1986) could facilitate the genetic analysis of resistance in the host. Crosses between *L. maculans* isolates belonging to different pathogenicity groups and tetrad analysis of the progeny would provide information on the genetic control of pathogenicity.

Aggressive and nonaggressive isolates could be differentiated not only on the basis of pathogenicity, but also by their ability to produce phytotoxic sirodesmins *in vitro*.

Table 3. Numbers of different restriction fragments detected among pairs of isolates examined using 42 genomic DNA clones as probes

	Nonaggressive isolates									Aggressive isolates																	
	126	**129**	**790**	**841**	**850**	**863**	**VIII2**	**IIIa5**	**Unity**	**431**	**433**	**914**	**IIa1**	**VI4**	**87-111**	**Lm1**	**620**	**843**	**Leroy**	**Lm2**	**100**	**423**	**437**	**478**	**479**	**888**	**IX2**	**MIX7**
126	...																										
129	38	...																									
790	29	31	...																								
841	32	10	23	...																							
850	26	30	9	22	...																						
863	28	32	11	24	2	...																					
VIII2	31	9	28	9	25	27	...																				
IIIa5	32	18	27	8	24	26	11	...																			
Unity	26	28	11	20	2	4	23	22	...																		
431	113	117	114	115	113	115	116	119	111	...																	
433	114	118	113	114	112	114	117	118	110	11	...																
914	111	117	106	111	107	109	114	115	105	18	21	...															
IIa1	109	115	112	113	111	113	114	117	109	20	23	24	...														
VI4	113	119	116	117	113	115	118	121	111	12	15	26	26	...													
87-111	112	120	113	116	112	114	117	120	110	7	12	17	23	11	...												
Lm1	108	116	109	112	108	110	113	116	106	15	14	13	19	19	14	...											
620	111	119	110	113	109	111	116	117	107	18	17	14	16	22	17	5	...										
843	109	117	108	111	107	109	114	115	105	14	13	14	16	20	13	7	8	...									
Leory	110	118	109	112	108	110	115	116	106	11	14	11	17	17	10	8	9	3	...								
Lm2	110	118	109	112	108	110	115	116	106	13	14	11	17	19	12	10	11	7	4	...							
100	110	118	111	114	110	112	115	118	108	9	14	13	17	15	8	8	11	7	4	6	...						
423	112	116	113	114	112	114	115	118	110	11	12	13	17	17	14	8	11	11	10	12	8	...					
437	110	114	111	112	110	112	113	116	108	11	12	15	13	17	14	6	11	7	8	8	8	8	...				
478	111	121	112	115	111	113	118	117	109	16	17	14	18	22	15	11	12	8	7	5	9	13	9	...			
479	111	123	112	117	113	115	120	119	111	24	25	14	24	30	21	17	16	18	15	15	17	19	19	14	...		
888	110	114	111	112	110	112	113	116	108	9	16	13	15	17	12	12	15	11	8	8	8	10	6	11	19	...	
IX2	110	118	109	112	108	110	115	116	106	13	14	11	15	19	12	8	9	5	4	4	4	10	6	5	15	8	...
MIX7	110	114	111	112	110	112	113	116	108	13	18	17	17	19	14	14	17	13	10	10	10	12	8	13	19	4	10	...

This finding, also reported previously for a large number of isolates (Koch *et al.* 1989), is universally observed for *L. maculans*, supporting the hypothesis that sirodesmins contribute to pathogenicity. However, these observations, with our RFLP results discussed below, raise questions about assigning these two groups to the same biological species.

Previous studies have reported that different species of fungi could be distinguished by banding patterns in electrophoresed digests of total DNAs (Klich and Mullaney 1987; Magee *et al.* 1987). The prominent bands in digested total DNAs are usually attributed to either mitochondrial DNA or nuclear ribosomal DNA because of their high copy number (Klich and Mullaney 1987; Magee *et al.* 1987). In our study, restriction enzyme digestion of total DNAs from aggressive and nonaggressive isolates resulted in different banding patterns that clearly distinguished the two groups. Hybridization with cloned nuclear DNA sequences also showed distinct differences in RFLP patterns between aggressive and nonaggressive groups. With nearly every probe used, aggressive isolates could be distinguished from nonaggressive ones, and differences occurred not only in hybridization patterns but in signal intensity. Although differences in hybridization intensity could be attributable to differences in copy number, they more likely reflect a different degree of homology between the hybridizing DNA sequences in the two groups of isolates. In the phylogenetic analysis, all of the nonaggressive isolates were separated clearly from aggressive isolates by a large unit distance, and there were no overlaps between groups. These results suggest that considerable genetic divergence exists between the nonaggressive and aggressive groups. These RFLP data are consistent with results obtained from our phytotoxin test and with reports on differences in pathogenicity and biochemical characteristics between isolates of *L. maculans* (Hill and Williams 1988). Phytotoxin production, RFLP patterns, and phylogenetic analysis provide evidence that aggressive and nonaggressive isolates of *L. maculans* used in our study may belong to different species.

RFLPs also were found to be useful in classification of isolates within the two groups. All of the 28 isolates could be distinguished from each other based on one or a few probe-enzyme combinations. More polymorphism was found within the nonaggressive group than within the aggressive group, suggesting greater genetic diversity within the nonaggressive group. The classification of the nonaggressive isolates into a single pathogenicity group may be attributable to the inability to distinguish additional interaction phenotypes with the cultivars and methods reported in this study.

An important question related to our RFLP study is whether there is any association between the pathogenicity of an isolate and its genetic background. Within the aggressive group, we found that some phylogenetically related isolates had the same pathogenicity characteristics. For example, subgroups A2 and A3 included isolates only from PG4 and PG3, respectively. A direct relationship between phylogenetic and pathogenicity groupings might be expected if pathogenicity is controlled by many genes distributed throughout the fungal genome. If pathogenicity is controlled by a few genes, an indirect relationship might still result from geographic or mating isolation of isolates having the same pathogenicity type. Although some isolates within subgroups A2 and A3 came from geographically distant areas, they may have been recently introduced to these regions and may have originated in restricted areas of Europe where they coevolved with specific hosts. A relationship between pathogenicity and genetic background was not observed for subgroup A4, which included isolates from three pathogenicity groups. This observation could be due to insufficient RFLP data to accurately identify the genetic relationships of all isolates in A4, and/or to intermating between isolates of different pathogenicity types within this subgroup. If lack of correspondence for A4 is because of insufficient RFLP data, then it may be possible to establish such a relationship by using a larger number of DNA probes. DNA probes also will be useful for dissecting the genetic control of host-pathogen interactions in this system by analyzing segregating progenies of *B. napus* and *L. maculans* for RFLPs and for interaction phenotypes.

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