Electrophoretic Karyotypes of *Sclerotinia sclerotiorum*

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Electrophoretic karyotypes (EKs) of 83 isolates were variable within agricultural and natural populations of *Sclerotinia sclerotiorum*, as well as among *S. sclerotiorum*, *Sclerotinia minor*, and *Sclerotinia trifoliorum*. Variation in EKs was not observed within six mitotic or three meiotic lineages of isolates. EKs of 8 to 10 chromosome-sized DNAs were observed. Homologous and heterologous probes hybridized to four linkage groups.

In previous studies, we described a predominantly clonal population structure in the plant pathogenic fungus *Sclerotinia sclerotiorum* on Canadian canola (4, 6, 8). All members of a clone share a unique DNA fingerprint in Southern hybridizations of *Bam*HI-digested genomic DNA to a cloned probe that contains a repeated dispersed element from *S. sclerotiorum*. Also, all members of a clone are somatically compatible with all other members of the clone but incompatible with members of other clones. From 2,876 isolates recovered from 1989 to 1992, 659 genotypes have been identified by DNA fingerprinting. Clone frequency profiles have shown both a small group of clones sampled frequently and over a wide area and a large group of genotypes sampled once or only a few times. We term these common clones and rare genotypes or clones, respectively. Clonality arises by asexual reproduction via sclerotia, melanized propagules capable of perennation in soil, plant debris, and perhaps among infested seed, and by seasonally occurring, self-fertilized sexual reproduction. Both asexual and sexual reproduction are easily observed under laboratory conditions. In addition to canola, *S. sclerotiorum* infects many cultivated and wild plants (1), particularly broad-leaved plants in temperate climates. Preliminary study of the fungus from two populations of a wild woodland plant, *Ranunculus ficaria*, in Norway indicated patterns of inbreeding against which clonality could not be detected (7). Samples from Norwegian canola and potato, however, were clonal. Mitochondrial haplotyping indicated that agricultural populations may have spread from a common source and that they are distinct from the wild populations associated with *R. ficaria* in Norway (5).

Chromosome polymorphisms are common in fungal species, and it is postulated that their occurrence and extent may be inversely correlated with the frequency of meiosis (3). However, karyotype variability was observed among meiotic progeny (3, 10, 12), as well as in serial transfers of mitotic progeny (9, 16). Could karyotypic plasticity inhibit outcrossing in *S. sclerotiorum* on any of several hierarchical levels? The objectives of our study were (i) to compare electrophoretic karyotypes within several frames, clone, population, agricultural and natural populations, and species; and (ii) to determine whether karyotypes were stably maintained over six mitotic and three meiotic generations.

All isolates used in this study are listed in Table 1. Clones were identified according to previously described protocols (6, 8). To study mitotic stability, strain F2-29-L1 was serially transferred weekly, over a 6-month period. At 4-week intervals, cells

were harvested for protoplast production and karyotyping. Isolates F2-111-4-1P (clone 2) and F2-90-L3 (clone 36) were induced to undergo self-fertilized sexual reproduction in the laboratory, and 90 single ascospore isolations were made from each apothecium. Cultures from three consecutive meiotic generations of ascospores were used for the production of protoplasts and karyotyping.

For protoplast production, each isolate was grown on potato dextrose agar (Difco Laboratories, Detroit, Mich.) for 2 days in the dark. Then 12 1-mm plugs of mycelium from the colony margin were inoculated in 15 ml of liquid complete yeast medium (6) and incubated on a shaker (120 rpm) at 22 to 23°C for 72 h. The mycelium was harvested with 15 ml of complete yeast medium, was homogenized in a Waring blender, and was then inoculated into 100 ml of fresh complete yeast medium and incubated on a shaker (120 rpm) at 22 to 23°C for 48 h. The mycelium was filtered through Miracloth (Calbiochem-Novabiochem, La Jolla, Calif.) and immediately used for protoplast preparation. Protoplasts were prepared according to the method of Royer et al. (13) with the following modifications. Mycelium was collected by filtration through a layer of Miracloth, washed in freshly made protoplasting buffer (0.8 M $\text{MgSO}_4 \cdot 0.2$ M sodium citrate, pH 5.5), and digested in 20 ml of protoplast buffer with 0.2% (wt/vol) Novozyme 234 (Novo Industries, Wilton, Conn.) for 1.5 h at 22 to 23°C on a shaker.

FIG. 1. Graphic representation of karyotypes of 75 isolates of *S. sclerotiorum*. Isolate numbering corresponds to that in Table 1. The two size standards used were *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* (not shown).

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TABLE 1. Characteristics of the isolates used in this study

Sp. and no.	Isolate ^{a,b}	Clone ^c	Origin		Occurrence ^d
			Yr	Location	
<i>Sclerotinia sclerotiorum</i>					
1	LMK-200	1	1989	Ontario	Common
2	LMK-198	2	1989	Ontario	Common
3	LMK-211	2	1989	Ontario	Common
4	RM-172	2	1990	Saskatchewan	Common
5	F2-24-L1	2	1991	Saskatchewan	Common
6	F4-21-4P(A)	2	1992	Alberta	Common
7	F4-25-5P	2	1992	Alberta	Common
8	F5-134-2-5L	2	1992	Alberta	Common
9	F5-16-3-L3	2	1992	Alberta	Common
10	LMK-205	3	1989	Ontario	Common
11	RM-176	33	1990	Saskatchewan	Common
12	F2-21-2P	33	1991	Saskatchewan	Common
13	F2-111-4-2P	33	1991	Saskatchewan	Common
14	F2-111-4-2P-A1-13	33	1991	Saskatchewan	Common
15	F2-111-4-2P-A1-14	33	1991	Saskatchewan	Common
16	F2-111-4-2P-A1-20	33	1991	Saskatchewan	Common
17	F2-111-4-2P-A1-20-1	33	1991	Saskatchewan	Common
18	F2-111-4-2P-A1-20-3	33	1991	Saskatchewan	Common
19	F2-6-L3	33	1991	Saskatchewan	Common
20	F2-27-L3	33	1991	Saskatchewan	Common
21	F2-38-L2	33	1991	Saskatchewan	Common
22	RM-24	34	1990	Alberta	Common
23	F2-2-L2	34	1991	Saskatchewan	Common
24	F2-29-L1	34	1991	Saskatchewan	Common
25	F2-29-L1-1	34	1991	Saskatchewan	Common
26	F2-29-L1-2	34	1991	Saskatchewan	Common
27	F2-29-L1-3	34	1991	Saskatchewan	Common
28	F2-29-L1-4	34	1991	Saskatchewan	Common
29	F2-32-2P	36	1991	Saskatchewan	Common
30	F2-5-4P	36	1991	Saskatchewan	Common
31	F2-70-1P	36	1991	Saskatchewan	Common
32	F2-1-L3	36	1991	Saskatchewan	Common
33	F2-19-L1	36	1991	Saskatchewan	Common
34	F2-59-L2	36	1991	Saskatchewan	Common
35	F2-55-5-L1	36	1991	Saskatchewan	Common
36	F2-90-L3	36	1991	Saskatchewan	Common
37	F2-90-L3-A1-12	36	1991	Saskatchewan	Common
38	F2-90-L3-A1-13	36	1991	Saskatchewan	Common
39	F2-90-L3-A1-15	36	1991	Saskatchewan	Common
40	F2-90-L3-A1-15-1	36	1991	Saskatchewan	Common
41	F2-90-L3-A1-15-2	36	1991	Saskatchewan	Common
42	F2-90-L3-A1-15-3	36	1991	Saskatchewan	Common
43	RM-26	38	1990	Alberta	Common
44	F2-14-1-L1	38	1991	Saskatchewan	Common
45	F2-1-L1	38	1991	Saskatchewan	Common
46	F2-57-L1	39	1991	Saskatchewan	Common
47	RM-2	67	1990	Alberta	Common
48	F2-38-L1	67	1991	Saskatchewan	Common
49	F2-55-3-L2	67	1991	Saskatchewan	Common
50	F2-40-L3	72	1991	Saskatchewan	Common
51	F2-54-L1	72	1991	Saskatchewan	Common
52	F2-40-L2	80	1991	Saskatchewan	Rare
53	F2-40-L1	279	1991	Saskatchewan	Rare
54	F2-43-L1	280	1991	Saskatchewan	Rare
55	F4-47-L1	321	1992	Alberta	Common
56	F4-107-L3	321	1992	Alberta	Common
57	F4-18-1P	322	1992	Alberta	Common
58	F4-21-2P	322	1992	Alberta	Common
59	F4-90-5P	322	1992	Alberta	Common
60	NOR-2-3	NA ^e	1993	Norway	NA
61	NOR-3-1	NA	1993	Norway	NA
62	NOR-4-2	NA	1993	Norway	NA
63	NOR-5-1	NA	1993	Norway	NA
64	S4-A2-5	NA	1993	Norway	NA
65	S3-A2-1	NA	1993	Norway	NA

Continued on following page

TABLE 1—Continued

Sp. and no.	Isolate ^{a,b}	Clone ^c	Origin		Occurrence ^d
			Yr	Location	
66	S3-A4-3	NA	1993	Norway	NA
67	S3-A4-4	NA	1993	Norway	NA
68	S3-A5-4	NA	1993	Norway	NA
69	S6-A3-4	NA	1993	Norway	NA
70	S1-A3-2	NA	1993	Norway	NA
71	SI-A3-3	NA	1993	Norway	NA
72	W1-A1-2	NA	1993	Norway	NA
73	W1-A1-3	NA	1993	Norway	NA
74	W2-A5-1	NA	1993	Norway	NA
75	W2-A5-2	NA	1993	Norway	NA
76	W3-A1-4	NA	1993	Norway	NA
77	W3-A4-1	NA	1993	Norway	NA
<i>Sclerotinia minor</i>					
77	HM-1	NA	1993	Oklahoma	NA
78	HM-2	NA	1993	Oklahoma	NA
79	HM-3	NA	1993	Oklahoma	NA
80	HM-4	NA	1993	Oklahoma	NA
<i>Sclerotinia trifoliorum</i>					
81	628	NA	1992	Georgia	NA
82	636	NA	1992	Georgia	NA

^a Isolates within a clone are mycelially compatible and share a unique fingerprint when *Bam*HI digested and probed with pLK44.20.

^b Isolates of *S. sclerotiorum* were collected from hosts as follows: 1 to 59 from canola in Canada, 60 to 63 from canola in Norway, and 64 to 77 from *R. ficaria* in Norway.

^c Clones were identified by DNA fingerprinting with pLK44.20 and mycelial compatibility grouping (8).

^d Occurrence: common, clones found in a high frequency over a wide geographic area; rare, clones found once.

^e NA, not applicable.

Protoplasts were harvested by filtration through glass wool, and 30 ml of 0.6 M KCl was added to the filtrate. The suspension was centrifuged at setting 3 ($1,300 \times g$) for 15 min on a clinical centrifuge. Resulting pellets were washed in 10 ml of ST (1.0 M Sorbitol and 0.05 M EDTA), centrifuged again, and resuspended in ST at a concentration of 10^9 ml^{-1} . Protoplasts were embedded in 0.6% (wt/vol) low-melting-point agarose (Bio-Rad Laboratories, Richmond, Calif.). Agarose plugs containing protoplasts were then incubated in 1.0% (wt/vol) *N*-laurylsarcosine–0.2% proteinase K–0.05 M EDTA at 50°C for 24 h. Plugs were washed three times and stored in 0.05 M EDTA at 4°C. High numbers of protoplasts (10^{10} ml^{-1} versus 10^8 ml^{-1}) were produced when the culture was enriched with fresh cells compared with nonenriched cultures.

Electrophoretic separation of DNA was carried out on a contour-clamped homogeneous electric field system (CHEF DRII; Bio-Rad Laboratories). Electrophoretic karyotypes of *S. sclerotiorum* were resolved with 0.5× Tris-borate-EDTA at 6°C. Agarose plugs containing protoplasts were loaded in each lane of a 0.6% (wt/vol) chromosome-grade agarose (Bio-Rad Laboratories) gel. Chromosome size standards were *Saccharomyces cerevisiae* YNN 295 and *Schizosaccharomyces pombe* 972 (Bio-Rad Laboratories). Condition set A was used to separate large chromosome-sized DNAs: 72 h, 55 V, 3,600 to 2,700 s; 48 h, 62 V, 1,500 to 1,100 s; 24 h, 70 V, 1,500 to 800 s; 24 h, 80 V, 800 to 500 s. Set B separated medium and large chromosomes: 72 h, 55 V, 2,700 to 1,500 s; 48 h, 62 V, 1,500 to 1,100 s; 24 h, 70 V, 1,500 to 800 s; 24 h, 80 V, 800 to 500 s. Set C separated medium and small chromosomes: 72 h, 80 V, 500 to 800 s; 48 h, 70 V, 800 to 1,100 s; 24 h, 60 V, 1,100 to 1,500 s; 24 h, 55 V, 1,500 to 2,700 s. Electrophoretic conditions B and C were used routinely. Low voltage and longer switching times were necessary for good resolution of chromosome-sized DNAs. Karyotypes were reproducible under different conditions and from different batches of protoplasts. Gels were

stained with ethidium bromide and then visualized and photographed under UV illumination at 312 nm. Southern hybridizations were performed according to standard protocols (14, 15).

Each isolate had 8 to 10 chromosome-sized DNAs, ranging in size from approximately 900 to 4,600 kb. The genome size of *S. sclerotiorum* isolates, as estimated from different electrophoretic runs, was 17.9 to 25.7 Mb. This was probably a low estimate because it was made on the assumption that each band represents a single chromosome.

Analysis of electrophoretic karyotypes of *S. sclerotiorum* isolates collected from canola in Canada indicated the following. Karyotypes were stable through both mitotic and meiotic nu-

FIG. 2. Electrophoretic karyotypes of isolates of *S. sclerotiorum* (lanes 1 and 2), *S. minor* (lanes 3 to 6), and *S. trifoliorum* (lanes 7 and 8). Lanes contain DNA from the following isolates: 1, F4-90-5P; 2, RM-172; 3, HM-1; 4, HM-2; 5, HM-3; 6, HM-4; 7, LMK-628; 8, LMK-636; 9, *Saccharomyces cerevisiae*; 10, *Schizosaccharomyces pombe*. DNA size standards are indicated on the right in megabases.

FIG. 3. Southern hybridizations of electrophoretic karyotypes of *S. sclerotiorum* with cloned single and multicopy DNAs. (A) Ethidium bromide-stained gel. Loading order of isolates, from left to right, is F4-90-5P (clone 322); F4-18-1P (clone 322); F4-21-2P (clone 322); F4-107-L3 (clone 321); F4-47-L3 (clone 321); F2-70-1P (clone 36); F2-32-2P (clone 36); F2-1-L3 (clone 36); F2-59-L2 (clone 36); F2-55-5-L1 (clone 36); F2-90-L3 (clone 36; parent); F2-90-L3-13 (clone 36; F1 progeny), and F2-90-L3-13-1 (clone 36; F2 progeny) (B to H) The Southern blot, made from the gel in panel A, was stripped and reprobed with single and multicopy cloned probe DNAs. pLK probes were random fragments cloned from *S. sclerotiorum*. (B) pLK44.6 hybridized to a single chromosome. (C) pMO63, which includes the β -tubulin gene of *Neurospora crassa*, hybridized to a single chromosome. (D) pMF2, which includes most of the rRNA gene repeat of *N. crassa*, hybridized to a single chromosome in most isolates. (E) pLK44.15 hybridized to a single chromosome in most isolates. (F) pLK44.9 hybridized to a single chromosome. (G) pLK44.2 hybridized to two chromosomes. (H) pLK44.7 hybridized to all chromosomes.

clear divisions. DNA karyotypes were identical for each of the six serial transfers of strain F2-29-L1. Karyotypes were also stably maintained through three consecutive meiotic generations of F2-111-4-1P and F2-90-L3. Karyotypes were variable within all frameworks: clone, population, agricultural and natural populations, and species (Fig. 1). Twenty-four karyotypes were observed among 59 isolates collected from canola in Canada. Within each of some of the common clones, 33, 34, 38,

39, 67, 72, 321, and 322, isolates had a common karyotype. However, several karyotypes were observed in each of two other commonly sampled clones, 2 and 36.

Among the isolates collected from Norway, seven different karyotypes were observed among 14 isolates subsampled from two wild populations of *R. ficaria* and two different karyotypes were observed among four Norwegian agricultural isolates (NOR-2-3, NOR-3-1, NOR-4-2, and NOR-5-1). Karyotypes

differed among three species, *S. sclerotiorum*, *Sclerotinia minor*, and *Sclerotinia trifoliorum*. Probably because of a low concentration of protoplasts, the karyotype of HM-3 of *S. minor* in lane 6 of Fig. 2 was very faint. When the protocol was subsequently repeated, the karyotype of HM-3 was found to be similar to karyotypes of the other three isolates of *S. minor*. While there were differences among the *S. trifoliorum* isolates, isolates of *S. minor*, all associated with peanut plants in Oklahoma, shared one karyotype (Fig. 2).

Figure 3 shows Southern hybridizations of DNA fragments separated by pulsed-field gel electrophoresis of 13 isolates of *S. sclerotiorum* to 10 different homologous or heterologous DNA probes. Because of a low concentration of protoplasts, some of the karyotypes in lanes A and H in Fig. 3 were faint. Subsequent repetition of experiments with these isolates showed better resolution of karyotypes. Two of the four heterologous probes, pMF2 (rRNA gene) (2) and pMO63 (β -tubulin) (11), hybridized to the DNA blots of contour-clamped homogeneous electric field gels; the other two tested did not produce a signal under these conditions. All homologous, randomly cloned, nuclear DNA fragments from a reference isolate of *S. sclerotiorum*, LMK 44 (pLK44.2, pLK44.6, pLK44.7, pLK44.8, pLK44.9, pLK44.15, pLK44.17, and pLK44.20), hybridized to all *S. sclerotiorum* isolates. Hybridization of probes pMF2 (Fig. 3D) and pLK44.15 (Fig. 3E) to the electrophoretic linkage group indicates that this linkage group contains rRNA genes and LK44.15 DNA. In agreement with a previous report (8), pLK44.20, which includes the repeated dispersed element used for our DNA fingerprinting, hybridized to all or most of the electrophoretic linkage groups.

Karyotype variation was observed at all levels examined, except within mitotic and meiotic lineages of laboratory strains. We can conclude only that changes in karyotype occur over more generations of nuclear division than we observed in this experiment. Perhaps change is more rapid in the field. Whether karyotype plasticity is a barrier to outbreeding remains to be tested. Efforts to make crosses between isolates marked by all three different criteria, DNA fingerprints, mycelial compatibility affinities, and electrophoretic karyotypes, have not been successful, apparently because of self-fertilization. We have not attempted crosses between isolates that are

marked by different electrophoretic karyotypes but are mycelially compatible and possess identical fingerprints.

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