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Low level of DNA polymorphisms in isolates of *Leptosphaeria korrae* pathogenic on *Poa pratensis*

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Abstract

The genetic diversity of the turfgrass pathogen, *Leptosphaeria korrae* was examined with random amplified polymorphic DNA (RAPD) markers at the sibling, population, regional and cross-continental levels using 71 isolates. The seven RAPD primers tested showed polymorphisms within and between regions in North America. No polymorphisms were observed between sibling spores nor between twenty-one isolates from a single field. Based on differences observed with 48 polymorphic RAPD markers, only six unique but closely related haplotypes were identified among 33 geographically separated field isolates from British Columbia, Ontario, and Quebec in Canada, and Washington State in the USA. Although there was clustering of isolates of the same haplotype, some haplotypes had wide distributions across different regions. We hypothesize that few haplotypes of *L. korrae* were introduced into these regions from the centre of origin.

Introduction

Leptosphaeria korrae Walker & Smith (=*Ophiosphaerella korrae* [Walker & Smith] Shoemaker & Babcock) was first isolated from diseased patches of bermudagrass (*Cynodon dactylon* [L.] Pers.) in Australia (Walker and Smith, 1972). In North America, Worf et al. (1983) reported a disease on Kentucky bluegrass (*Poa pratensis* L) which they named necrotic ring spot (NRS). After a some controversy (Couch, 1985; Smiley, 1987), the causal agent of NRS was determined to be *L. korrae*. This fungus is a soilborne, ectotrophic, homothallic loculoascomycete which produces black runner hyphae on roots, crowns and rhizomes of infected grass plants. NRS is widely distributed through the northern half of the United States on *P. pratensis* (Jackson, 1993). In Canada, it has been found in British Columbia (MacDonald, 1990), Ontario (Hsiang et al. 1992), and Quebec (O'Gorman, 1994). *Leptosphaeria korrae* is also known as one of the causal agents of spring dead spot of *C. dactylon* and other warm-season grasses, and is widely distributed through the southern half of the United States, Australia and New Zealand (Jackson, 1993).

Symptoms of NRS first appear in lawns 1 to 3 years after establishment from sod, usually in the autumn or spring when cool, wet conditions are common; seeded lawns are also susceptible to the disease (Smith et al., 1989). As weeds and surviving grass plants recolonise the initial small chlorotic patches, the characteristic "frog eye" pattern develops with a dead ring and a live centre. These rings can reach sizes of 30 to 50 cm in diameter.

Little is known about the biology of *L. korrae*. It has no reported conidial state, and although pseudothecia can be produced *in vitro* (Chastagner & Hammer 1997), pseudothecia are seldom observed in nature (Smiley et al., 1992). The importance of ascospores in the dissemination of this pathogen is not known, but spread of root pathogens, possibly *L. korrae*, through turf plugs has been demonstrated (Pair et al., 1986). It is unknown if cultural practices such as mowing, dethatching, and coring aid in disseminating the disease.

There is some indirect evidence for genetic variation in *L. korrae*. Chastagner (1997) detailed how some fungicides were able to control NRS in some locations in the United States, but not in others. The major part of this difference in control is probably due to environmental influences since turfgrass root pathogens are difficult to control by fungicides, but there may also be variation in fungicide sensitivity between *L. korrae* isolates at different locations. Chastagner & Hammer (1997) reported that the capacity of *L. korrae* to produce pseudothecia *in vitro* varied depending on the

geographic origin on the isolates. Hsiang & Chastagner (1997) re-analysed data from field cultivars trials which tested the resistance of *P. pratensis* to *L. korrae*. They found enormous differences in the results across North America with some cultivars seemingly resistant in some trials but the same cultivars showing susceptibility in other trials. These differences may be due to environment, but there also may be an element of pathogenic variation between isolates of different locations.

Several biochemical methods exist to detect genetic variation in fungi including the use of isozymes (Micales et al, 1986) and restriction fragment length polymorphisms (RFLP) and other molecular markers (Correll, 1992; Karp et al., 1996). A simple yet powerful technique which has been used increasingly, involves random amplified polymorphic DNA (RAPD) (Williams et al., 1990, 1993). Although the RAPD technique has been criticized for several undesirable features such as non-codominant inheritance, anonymous nature (Backeljau et al. 1995), lack of positional homology (van de Zande and Bijlsma 1995) and especially inconsistent reproducibility (Staub et al. 1996), RAPD data can detect genetic diversity between related species (Harvey and Botha 1996) and within species (van Oppen et al. 1996), and is capable of detecting differences between intersterility groups (Crowhurst et al., 1991; Garbelotto et al., 1993) and individuals (Aufauvre-Brown et al. 1992), including siblings (Doudrick et al., 1993; Doudrick et al., 1995). Because of its ease of use and low cost, the technique has profoundly affected our ability to explore genetic variation in organisms. The technique has been applied recently to examine genetic variation in several turfgrass pathogens (Huff et al., 1994; McCann et al., 1994; Mahuku et al., in press; Raina et al., 1997). The purpose of this study was to use RAPD markers to examine and quantify genetic variation in *L. korrae* from *P. pratensis* using at the sibling, population, regional and cross-continental levels.

Materials and Methods

To examine genetic variation within a population, 21 isolates were obtained from a single field. Turf samples were collected from the outside edge of 17 individual necrotic rings or small patches within a 2.6 m radius plot in a *P. pratensis* field at a commercial sod farm in Cambridge, Ontario. Four more samples were obtained from necrotic rings 100, 200, 300 and 400 m from this plot centre. The field had been seeded 6 years previously with a mixture of Midnight, Glade, and Ram I cultivars. The current turf was regrowth from remnant roots of a sod removed three years previous, and disease was prevalent throughout this field.

To isolate the pathogen, samples of diseased roots and crowns were washed overnight in running tap water. Segments with black runner hyphae were surface sterilized in 1% (w/v) silver nitrate for 30 s, transferred to 5% (w/v) sodium chloride for 30 s to precipitate the silver, and then rinsed twice in sterile distilled water. Plant tissue in 1-cm pieces were placed on one-fifth strength potato-dextrose media containing 2% agar and 30 ppm streptomycin. Slow-growing white hyphae were transferred to potato dextrose agar after seven days.

Additional isolates of *L. korrae* were obtained from Quebec and Ontario (O'Gorman, 1994), British Columbia, Canada (courtesy of L. MacDonald, B.C. Ministry of Agriculture, Fisheries, and Food), and Washington State, USA (courtesy of G. Chastagner, Washington State University). For multiple samples from particular cities, each sample was collected from a different lawn. Single-spore progenies from one Washington isolate and from one Ontario isolate were obtained by homothallic fruiting on hard red wheat in the greenhouse (O'Gorman, 1994). Eighteen progeny isolates and 53 field isolates including 21 isolates from a single field were used in this study. The number and origin of the isolates are given in Table 1.

For DNA extraction, cultures were grown on 1.5% malt extract agar on nitrocellulose membranes (Flexel Sales, Covington, Indiana, USA) for 14 days. The original plug was removed and the mycelium was scraped off each membrane. Total genomic DNA was then extracted using a

microwave miniprep method (Goodwin & Lee, 1993), with slight modifications. Briefly, mycelial samples were placed into lysis buffer (containing 50 mM Tris-HCl at pH 7.2, 50 mM EDTA, 3% sodium dodecyl sulfate, 1% 2-mercaptoethanol), and heated in an microwave on full power for 1 min 30 s. Immediately after microwaving, more lysis buffer was added with incubation at 80°C for 10 min. After phenol-chloroform extraction, isopropanol precipitation and resuspension in buffer, the DNA samples were electrophoresed through 0.8% agarose gels alongside lambda DNA-*Hind* III digest markers (Promega, Unionville, ON, Canada) and stained with ethidium bromide to estimate DNA concentration.

RAPD primer set #2 of the Biotechnology Laboratory (University of British Columbia, Vancouver, British Columbia) was used to screen isolates for polymorphisms. Isolates from Washington, British Columbia and some from Ontario and Quebec (32 in total) were tested with three primers from this set which had earlier revealed significant polymorphisms in a related pathogen, *Leptosphaeria maculans* (Mahuku et al., 1997). Based on this initial screening, one Ontario, one British Columbia and two Washington isolates were chosen to screen the remaining 87 primers. Primers that produced polymorphic fragments were then used on all 53 field isolates and the 18 single-spore isolates at least twice more to verify presence of the polymorphisms. The seven primers chosen for this study were: #116 (5'-TACGATGACG-3'), #122 (5'-GTAGACGAGC-3'), #145 (5'-TGTCGGTTGC-3'), #146 (5'-ATGTGTTGCG-3'), #169 (5'-ACGACGTAGG-3'), #181 (5'-ATGACGACGG-3'), and #186 (5'-GTGCGTCGCT-3'). Reaction volume was 12.5 µl and contained 50 mM KCl, 10 mM Tris HCl, 0.1% Triton X-100, 200 µM of each dNTP, 0.2 µM primer, 0.5 units of *Taq* DNA polymerase (Sangon, Scarborough, Ontario, Canada) and approximately 15 ng DNA. The concentration of $MgCl_2$ in the reactions was 2.0 mM, except for primer #181 (2.5 mM) and primer #169 (3.0 mM). Reaction mixtures were overlaid with light mineral oil (Fisher, Toronto, Ontario, Canada). All amplifications were carried out in a BioOven or BioOven III (BioTherm Corporation, Arlington, Virginia, USA). An initial denaturing step of 1 minute at 94°C was followed by 40 cycles of 1 minute at 94°C, 1 minute at 37°C and 2 minutes at 72°C with a final extension time of 10 minutes at 72°C. Amplification products were separated by gel electrophoresis in a 1.5% agarose gel containing ethidium bromide (5 µg/ml) with 1X TBE buffer (0.89 M Tris base, 0.89 M boric acid, 0.002 M EDTA). Products in agarose gels were visualized using UV light (305 nm).

The presence or absence of reproducible amplified DNA bands was determined for all individuals, and scored as 1 or 0, respectively. Positional homology of amplified fragments was assumed, and only polymorphic bands were considered. Similarity coefficients (S) were calculated between isolates across bands for all primers using the formula $S = 2N_{xy}/(N_x+N_y)$, where N_x and N_y are the number of fragments amplified in isolates X and Y respectively, and N_{xy} is the number of bands shared by the two isolates (Nei and Li, 1979). Similarity coefficients were converted to genetic distance using the equation: $D = (1/S)-1$ (Swofford and Olsen, 1990). A genetic distance matrix of isolate by isolate was generated using the entire haplotypic dataset and then used to construct a dendrogram with the UPGMA method in the Neighbor algorithm of the software package PHYLIP (version 3.55c, available via anonymous FTP from the website evolution.genetics.washington.edu).

Results

Out of 90 primers screened, 67 produced amplification products, and seven were chosen for testing of all isolates. RAPD fragment patterns for the seven primers were reproduced consistently in at least 3 replicate runs, and only intense bands were used in the analyses. Of 81 bands scored, only the 48 polymorphic bands were retained for analysis (Table 2). Primer #181 distinguished two groups; primer #116, three groups; primers #146 and #186, four groups each; primers #122 and #169, five groups each; and primer #145, six groups (Table 2). After combining data from all primers (Table 2), the analysis showed only 6 haplotypes present among the 71 isolates (Table 1). In practical terms, primer #145 alone was able to distinguish all six haplotypes.

The isolates from British Columbia were collected from five different lawns in the same city in central British Columbia, and all were of haplotype 1 (Table 1). The isolate from Quebec and one of the Washington isolates were also of haplotype 1. Ontario isolates were mostly of haplotypes 2 and 3 with a single Ontario isolate representing haplotype 6. Aside from the Ontario isolates, one isolate from Washington State was also of haplotype 3. Most isolates from Washington were of haplotype 4, and a single Washington State isolate represented haplotype 5.

The single haplotype 6 isolate was very similar to the haplotype 2 isolates (Table 3, Figure 1); out of 48 possible bands, there were only five band differences between these two haplotypes (Table 2). Similarly, the single haplotype 5 isolate was very close to the haplotype 4 isolates; there were 6 band differences between these two haplotypes (Table 2). Haplotypes 2 and 6 found only among Ontario isolates were closer to haplotype 1 which was represented by one Quebec, one Washington and all the British Columbia isolates. Haplotype 3 which was represented by the remaining Ontario isolates plus one Washington isolate diverged the most from all the other haplotypes (Table 3, Figure 1).

A fertile isolate from Washington State, LK57 (Table 1), was of the same haplotype as all 10 of its single-ascospore progeny isolates (haplotype 4). Similarly, 8 single-ascospore sibling progeny of isolate LK25 from Ontario shared identical RAPD patterns and all were of haplotype 3. For 17 isolates intensively sampled from one site in Ontario, all had identical RAPD patterns and were indistinguishable from isolates sampled 100, 200, 300, and 400 metres from this site. All 21 isolates were of haplotype 3 (Table 1).

Discussion

A low level of polymorphism was found among the *L. korrae* isolates tested, but the actual level of genetic variation may even be lower since selection of RAPD primers for polymorphic results exaggerates the magnitude of calculated genetic variation (Harvey and Botha, 1996). The level of genetic variation was further exaggerated when monomorphic bands were excluded from analyses. For example, among the 81 bands scored across the seven RAPD primers tested, only the 48 bands which showed polymorphism were used in this analysis, and this resulted in an average genetic similarity of 70.7% among the six distinct haplotypes. If monomorphic bands were included in the analysis, the average genetic similarity among the haplotypes would be 88.4%.

At the population level, 21 isolates from a single stand of *P. pratensis* were of the same haplotype. In general, isolates from the same cities such as Kelowna, Richland, Renton or Spokane were of the same haplotype. Some haplotypes such as type 4 could be found across regional levels (Washington State).

Out of several hundred observable markers among all the RAPD primers tested and with the 48 informative genetic markers of the seven RAPD primers chosen, the occurrence of only six unique haplotypes in 33 geographically distinct isolates in 19 different cities indicates a low level of genetic variation in this species in northern North America. Haplotype 3 diverged the most from the other haplotypes, but was found in both Ontario and Washington. Due to their similarities, we suspect that the single isolate in haplotype 6 diverged from haplotype 2, and the single isolate in haplotype 5 diverged from haplotype 4.

No genetic variation was detected between isolate LK57 and its ten single-ascospore progeny, nor between eight sibling progeny of isolate LK25. One explanation is the homothallic nature of *L. korrae* which results in homogeneous progeny. In other fungi, RAPD markers have been used successfully to differentiate sibling single-spore progeny from heterothallic basidiomycetes (Doudrick et al., 1993; Doudrick et al., 1995) and the putatively homothallic ascomycete *Hypoxylon truncatum* (Yoon & Glawe, 1993). With self-fertile haploid organisms such as *L. korrae*, the parasexual cycle and mutations may then be the major means of generating detectable variability between isolates.

Another possible explanation for the low level of molecular variation, in conjunction with the homothallic nature of the organism, may be that only a limited number of genotypes were introduced to and spread across this continent. There is no data on the centre of origin of *L. korrae*, although speculatively it would have come to North America with its major hosts. The distribution pattern of the six haplotypes supports this hypothesis with haplotype 4 found only in Washington, haplotype 2 exclusively in Ontario, and most haplotype 1 isolates in British Columbia. The six haplotypes could represent six different genets or they could be the result of accumulated mutations or mitotic recombinations of a single founding genet. Such a situation has been documented in the potato late blight pathogen *Phytophthora infestans* (Goodwin et al., 1994). Using mating types, allozymes, and RFLP fingerprinting, the authors were able to document that a single clonal lineage of the pathogen has been distributed world-wide. Differences between the isolates were small, such as a change from presence to absence of a single band or heterozygosity to homozygosity at one of the allozyme loci. Similarly, Trigiano et al. (1995) speculated that *Discula destructiva*, cause of dogwood anthracnose, was recently introduced into North America after detecting very little variation in the species from across North America. Delye et al. (1995) found a low level of polymorphism *Uncinula necator* in Europe and speculated that a low number of genotypes was introduced over a short period. Other cases where RAPD or RAPD-like markers have detected very little intra-specific variation in fungi include *Colletotrichum fragariae* (Freeman and Rodriguez, 1995) and *Hirsutella longicolla* subspecies (Strongman and MacKay, 1993), and the European race of *Gremmeniella abietina* in North America (Hamelin et al., 1993).

Our finding of six haplotypes in the North American isolates is a higher level of diversity than found in previous studies on *L. korrae*. Digestion of total genomic DNA with *Eco*RI followed by gel separation and ethidium bromide staining gave identical fragment patterns for 37 *L. korrae* isolates from the USA and one from Australia (Tisserat et al., 1991). Amplification of the ITS region of 29 isolates of *L. korrae* from the USA gave one of two fragment sizes (Tisserat et al., 1994). The major objective of both of these studies was to find primers or markers that were specific for *L. korrae*, rather than determining genetic variation within *L. korrae*. Furthermore, both methods targeted conserved regions within the species, and were less likely to reveal the full extent of genetic variation. Our study is the first one to specifically examine molecular variation within *L. korrae*.

A closely related pathogen, *Ophiosphaerella herpotricha* (Tisserat et al., 1994), which is one of the causes of spring dead spot (SDS) of bermudagrass, was shown with RAPD markers to be identical within patches and groups of patches, but quite polymorphic across its range (McCann et al., 1994). *Magnaporthe poae*, the causal agent of summer patch of turfgrass, has also been shown to be very diverse with 23 of 35 individual isolates having unique RAPD patterns (Huff et al., 1994). When compared to these two other turfgrass patch disease fungi, *L. korrae* is the least diverse of the group with only a single haplotype in twenty-one rings in the same field and only six unique haplotypes in 33 geographically separated isolates.

The low level of variation among isolates of *L. korrae* causing necrotic ring spot in *P. pratensis* across temperate North America, as well as between siblings, makes it impossible to determine if local spread of this disease is clonal through mycelia or via dissemination of spores. Whether through mycelium or spores, possible routes for distance transport are via infested seed or infected sod. If *L. korrae* were seedborne, this could help account for the widespread distribution of particular haplotypes across the continent. Using the methods of Chigogora and Hall (1995), and after testing several thousand seeds of different cultivars of *P. pratensis*, we were unable to detect the presence of the pathogen (unpublished data). Pair et al. (1986) demonstrated that root pathogens, possibly *L. korrae*, can be spread through infected turf plugs; however, there has been no published evidence of disease transfer from a sod farm to sodded lawns. Unfortunately, if there is very low genetic variation between isolates of a widespread pathogen, then it cannot be easily tracked on a local scale nor then can infection sources be precisely pinpointed. Although we were unable to shed further light on the ecology of *L. korrae* and the epidemiology of NRS, this preliminary finding of low genetic variation between *L. korrae* isolates of wide geographic origin could prove useful in cultivar and fungicide trials, as well as future studies on pathogenicity and biology. More work could be done with a larger

and a world-wide collection of *Leptosphaeria korrae* to look for genetic variation, recombination events and host specificity, and to determine its centre of origin.

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Table 1. Isolate and origin of *Leptosphaeria korrae* from *Poa pratensis* in Canada and Washington, U.S.A. grouped by haplotype.

Isolate	Origin	Haplotype
LK05	Kelowna, British Columbia	1
LK06	Kelowna, British Columbia	1
LK07	Kelowna, British Columbia	1
LK08	Kelowna, British Columbia	1
LK09	Kelowna, British Columbia	1
LK10	Kelowna, British Columbia	1
92/102	Pincourt, Quebec	1
LK50	Puyallup, Washington	1
LK02	Kitchener, Ontario	2
LK04	Orangeville, Ontario	2
LK11	Guelph, Ontario	2
LK13	Ottawa, Ontario	2
SF1 to SF21[*]	Cambridge, Ontario	3
LK21	Thornhill, Ontario	3
LK23	Barrie, Ontario	3
LK25a-h[+]	Markham, Ontario	3
LK34	Stouffville, Ontario	3
91/21	Woodbridge, Ontario	3
LK52	Puyallup, Washington	3
LK54	Woodinville, Washington	4
LK55	Richland, Washington	4
LK56	Richland, Washington	4
LK57[§], LK57a-j	Richland, Washington	4
LK58	Renton, Washington	4
LK59	Renton, Washington	4
LK62	Spokane, Washington	4
LK63	Spokane, Washington	4
LK64	Spokane, Washington	4
LK65	Spokane, Washington	4
LK66	Spokane, Washington	4
LK67	Spokane, Washington	4
LK69	Coulee Dam, Washington	5
91/106	Bracebridge, Ontario	6

[*] SF1-SF21 represent 21 isolates from the same field in Cambridge, Ontario

[+] LK25a-h are eight sibling single-ascospore isolates. The parent, LK25, was not used in this study.

[§] LK57 is the parent of 10 single-ascospore progeny (LK57a to LK57j)

Table 2: Haplotypic dataset of RAPD bands of *Leptosphaeria korrae* tested with seven primers.

Haplo-type	Presence or absence of polymorphic RAPD bands by primer						
	#116 (5)*	#122 (12)	#145 (5)	#146 (13)	#169 (15)	#181 (20)	#186 (11)
1	1 0	0 0 0 0 0 0 0 0 0 0	0 0 0 0 1	1 1 1 1 0	1 1 1 0 0 1 0	1 1 1 1 0 1 1 1 1 1 1 1 1	1 1 0 0 1 0 0
2	0 1	0 1 1 0 1 1 1 0 0	1 1 0 1 0	1 1 1 1 0	1 1 1 0 0 1 0	1 1 1 1 0 1 1 1 1 1 1 1 1	1 1 0 0 1 0 0
3	0 1	1 1 1 0 1 1 1 1 1	1 1 1 1 0	1 1 1 1 1	0 0 0 1 0 1 0	0 0 0 0 1 0 0 0 0 0 0 0 0	0 0 1 1 0 0 0
4	0 1	0 0 1 1 1 1 1 0 0	0 0 0 1 1	0 0 0 0 0	1 1 1 1 1 0 1	1 1 1 1 0 1 1 1 1 1 1 1 1	0 0 1 1 0 0 0
5	1 0	0 1 1 0 1 1 1 0 0	0 0 0 1 0	1 0 1 0 0	1 1 1 1 0 1 1	1 1 1 1 0 1 1 1 1 1 1 1 1	1 0 1 1 0 0 0
6	0 1	1 1 1 0 1 1 1 0 0	1 1 0 0 1	1 1 1 1 0	1 1 1 1 0 1 0	1 1 1 1 0 1 1 1 1 1 1 1 1	1 1 0 0 1 1 1

* Total number of bands, polymorphic and monomorphic, per primer

Table 3. Coefficients of genetic similarity and genetic distance between six haplotypes of *Leptosphaeria korrae* from *Poa pratensis* in Canada and Washington, USA.

Haplotype	Haplotype					
	1	2	3	4	5	6
1		0.81*	0.21	0.60	0.73	0.79
2	0.24		0.51	0.70	0.81	0.91
3	3.80	0.96		0.35	0.45	0.51
4	0.66	0.43	1.83		0.83	0.69
5	0.38	0.24	1.21	0.21		0.76
6	0.27	0.10	0.97	0.45	0.32	

* The upper right triangle gives the genetic similarity coefficients calculated from RAPD data using Nei & Li (1979), and the lower left triangle gives distance coefficients calculated using Swofford & Olsen (1990).

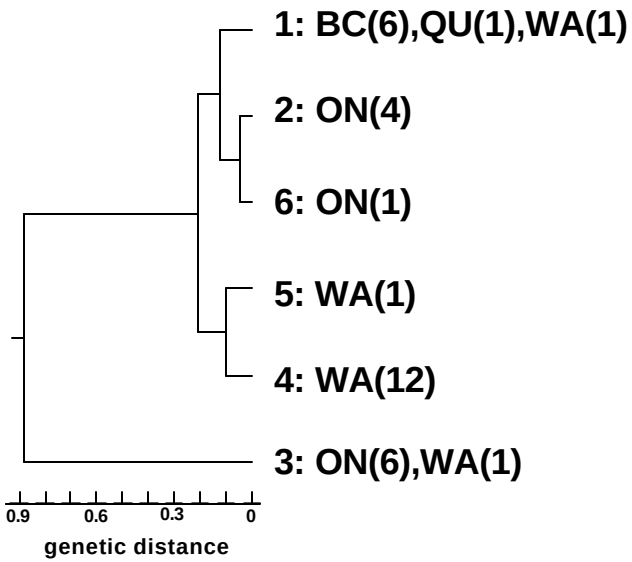

Figure 1. Relationship among *Leptosphaeria korrae* haplotypes revealed by UPGMA cluster analysis in the program PHYLIP using genetic distances derived from data of seven RAPD primers. Haplotype numbers are followed by the origin of isolates in that haplotype: BC = British Columbia, ON = Ontario, QU = Quebec, and WA = Washington, and in parenthesis, the number of isolates of that haplotype in that location.