DETECTION OF *LEPTOSPHAERIA KORRAE*, THE CAUSAL AGENT OF NECROTIC RING SPOT, AND ITS OCCURRENCE IN SOUTHERN ONTARIO

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ABSTRACT

DETECTION OF *LEPTOSPHAERIA KORRAE*, THE CAUSAL AGENT OF NECROTIC RING SPOT, AND ITS OCCURRENCE IN SOUTHERN ONTARIO

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Isolations from 122 suspected necrotic ring spot (NRS) samples collected throughout southern Ontario, were used to confirm the presence of the disease in the province. Based on morphology of the sexual structures, *Leptosphaeria korrae*, the causal agent of NRS, was confirmed in 17 Ontario counties. To increase the efficiency of pathogen detection, an assay based on the polymerase chain reaction (PCR), was tested using the primer pair, LK17S/5.8SC, selected from the internal transcribed spacer region 1 of L. korrae ribosomal DNA. Specific amplification of *L. korrae* DNA was obtained, while no amplification occurred with DNA isolated from 15 other fungal species or healthy Kentucky bluegrass, the major host of NRS.

The use of PCR with RAPD (random amplified polymorphic DNA) primers, allowed detection of genetic variability in *L. korrae* single spore siblings and isolates collected from a single field site. The distribution pattern of polymorphic isolates at the field site suggests both mycelial growth and released ascospores were involved in the spread of NRS.

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This thesis is dedicated to the memory of a very special friend, the late great Lewis Snape.

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NECROTIC RING SPOT

Introduction

Patch diseases of turfgrass in North America are caused by a diverse group of pathogenic fungi. There are at least 17 different patch diseases (Couch, 1986) caused by both foliar and root infecting fungi. Several distinctly different soil-borne ectotrophic ascomycetes infect the roots of turfgrass causing diseases which have similar symptoms. These diseases include spring dead spot of *Cynodon* species (Endo et al., 1985; Wadsworth and Young, 1960; Walker and Smith, 1972), take-all patch of *Agrostis* species (Gould et al., 1961; Walker, 1981), and summer patch and necrotic ring spot (NRS) of *Poa* species (Chastagner et al., 1984; Jackson, 1984; Smiley and Craven-Fowler, 1984; Worf et al., 1986).

Necrotic ring spot is a relatively new disease of turfgrass. It has been reported to affect a number of different turfgrass species, but is especially destructive on Kentucky bluegrass (*Poa pratensis* L.) (Smiley et al., 1992; Worf et al., 1986), which is one of the most commonly used turfgrass species in the temperate regions of North America.

Necrotic ring spot was first suspected as being a new

disease in North America when the application of the fungicide triadimefon was unsuccessful in controlling a patch disease believed to be Fusarium blight (Worf, 1980). Fusarium blight, which was first reported in 1959 (Bean, 1966; Couch and Bedford, 1966), is a patch disease which occurs in mature stands of Kentucky bluegrass. It initially starts as small, pale, straw-coloured patches a few centimetres in diameter which can spread to form circular or ring-shaped patches of dead turf up to 100 cm in diameter (Couch and Bedford, 1966). *Fusarium roseum* (LK) amend. Snyd. & Hans. f.sp. *cerealis "Culmorum"* and *F. tricinctum* (Cda.) Snyd. & Hans. f.sp. *poa* were the most common foliar organisms isolated from disease samples and were, therefore, considered the causal agents of the disease (Couch and Bedford, 1966). However, *Fusarium* species could not always be isolated from diseased plants (Worf et al., 1986).

Smiley and Craven-Fowler (1984) found that a more diverse group of organisms was associated with Fusarium blight than first thought. They isolated two ectotrophic ascomycetes that were pathogenic to Kentucky bluegrass and both caused identical symptoms to that of Fusarium blight. The organisms were *Magnaporthe poae* Landschoot and Jackson, which is now known to be the causal agent of summer patch, and *Leptosphaeria korrae* Walker & Smith which is now known to be the cause of NRS. These were the first reports of these pathogens in North America.

Prior to 1984, reports of *L. korrae* had only been made in Australia (Smith, 1965) where it was known to cause spring dead spot of Bermudagrass (*Cynodon dactylon* (L) Pres.). In Australia, *L. korrae* was initially identified as *Ophiobolus herpotrichus* (Fr.) Sacc. (Smith, 1965). Several years later a second closely related species was reported to be a more common cause of spring dead spot in Australia. This second pathogen belonged to the genus *Leptosphaeria* and was identified as *L. narmari* Walker & Smith. This new information prompted the re-examination of Smith's 1965 collection and *O. herpotrichus* was proposed as a synonym of *Leptosphaeria korrae* (Walker and Smith, 1972).

Spring dead spot of Bermudagrass is also found in the United States (Endo et al., 1985; Crahay et al., 1988). It was first reported in 1960 (Wadsworth and Young, 1960) but its cause was not determined until 1985 when *L. korrae* was reported as one cause of the disease in California (Endo et al., 1985).

Biology of *Leptosphaeria korrae*

Leptosphaeria korrae is a soil-borne ectotroph, producing dark runner hyphae on the surfaces of the host roots and rhizomes. It belongs to the class Loculoascomycetes which was first recognized by Luttrell (1955). Loculoascomycetes have unilocular ascostroma (pseudothecia) which are difficult to

distinguish from the true perithecia of other ascomycota without studying the development of the ascocarp (Talbot, 1971). The most characteristic feature of the Loculoascomycetes is the bitunicate asci (Talbot, 1971). In the past it was common practice to place long-spored species such as *Gaeumannomyces graminis* (Sacc.) Von Arx and Oliver, and *L. korrae* in the genus *Ophiobolus* (Walker and Smith, 1972; Walker, 1975).

Although *L. korrae* resembles *O. herpotrichus*, some differences do exist when comparing pseudothecial walls and ascospore sizes. A major difference is that a pycnidial stage has been described for *O. herpotrichus*, while there are no reports of an anamorphic state occurring in *L. korrae*. However, some characteristics of *L. korrae* do not exactly agree with the criteria which define the genus *Leptosphaeria*. One discrepancy is the presence of paraphyses pointing upwards in the neck canal, which should exclude it from being placed in *Leptosphaeria*. Despite the fact that *L. korrae* does not completely fit into the genus *Leptosphaeria*, the classification has been accepted because of its relationship to *L. narmari* and other *Leptosphaeria* species based on the presence of scolecospores (Walker and Smith, 1972). A brief summary of its morphology as described by Walker and Smith (1972) follows:

> pseudothecia are erumpent 400-600 µm high and 300-500 µm wide, flask shaped with a globose body with a thick neck, often exhibiting a thickened pressure ridge around it. Pseudothecial walls are 80-120µm

thick at the base and upper side walls are 40-80 μm. The neck canal is 80-100 μm wide lined with upwardly pointing paraphyses. The asci are bitunicate, cylindrical to clavate narrowing towards the foot and contain eight spores. Ascospores are long and needle-like (scolecospores) with rounded ends, (120)140-170(180) x 4-5 μm, pale brown, with a multiple septation pattern (1-3)-7-(15). Hyphae on the host are dark brown, septate, branched, 2.5-5.0 μm wide and often form flattened sclerotia 50-400 μm in diameter.

The etiology of *L. korrae* is similar to *G. graminis*, the causal agent of take-all disease of cereals and turfgrass (Smiley and Craven Fowler, 1984). They both possess similar ectotrophic runner hyphae, and under artificial conditions *L. korrae* can cause severe take-all like symptoms on cereals such as wheat and oats (Walker and Smith, 1972). These pathogens can be distinguished by the fact that *G. graminis* possesses unitunicate asci and distinctly different cultural characteristics (Walker and Smith, 1972).

When grown on potato dextrose agar, *L. korrae* produces sterile colonies with slow growth rates of 2.6-5.3 mm per day at 25°C. Colonies are white initially, but as they mature, light to dark grey aerial hyphae are produced, and the agar becomes black from the production of pigments (Endo et al., 1985; Hammer, 1988; Walker and Smith, 1972).

Symptoms of Necrotic Ring Spot

Microscopic Symptoms *Leptosphaeria korrae* produces

dark ectotrophic hyphae which run along infected crowns, roots and rhizomes (Smiley et al., 1992; Walker and Smith, 1972). Emerging from these dark runner hyphae are thin hyaline hyphae which penetrate the host tissue (Smiley et al., 1992). External sclerotia are also produced on basal leaf sheaths, crowns and rhizomes (Smiley et al., 1992). On infected Bermudagrass, numerous penetration holes and long black lesions have been observed on the host tissue beneath sclerotia (Walker and Smith, 1972; Endo et al., 1985). Infected roots and rhizomes become black as hyphae colonize the tissue (Walker and Smith, 1972). Plants in advanced stages of disease development may also produce pseudothecia on infected tissue (Smiley et al., 1992).

Macroscopic Symptoms Damage from necrotic ring spot initially occurs on 2-4 year old stands of Kentucky bluegrass which were established from sod, but seeded lawns can also be affected (Chastagner and Byther, 1985; Smiley et al., 1992). Symptoms first appear in the spring or early summer as chlorotic patches 5-10 cm in diameter. Decomposition of dead plants can create sunken bare patches in the turf (Couch, 1986). More commonly, symptoms will develop a "frog eye" appearance when brown arcs or rings form as weeds and surviving grass plants recolonize the centres of the patches (Chastagner and Byther, 1985). These rings can reach a size of 50 cm in diameter but are normally 30 cm or less (Smiley et al., 1992). Symptoms of necrotic ring spot can occur

throughout the growing season with the advancing margins of actively growing patches exhibiting a light reddish-brown colour (Chastagner, 1986). Symptoms are normally most prominent when the pathogen is active during periods of cool wet weather in the spring and fall (Worf et al., 1986), and heavy outbreaks have been reported after long periods of heavy rain (Couch, 1986). Temperatures greater than 30 °C inhibit the growth of the pathogen (Smiley et al., 1992) and can cause symptoms to temporarily subside, only to reoccur in autumn or the following spring (Hammer, 1988; Worf et al., 1986). However, since the pathogen has a higher tolerance to environmental extremes than its host, infected plants suffering from heat or drought stress may show pronounced symptoms (Smiley et al., 1992).

Distribution

There has been a considerable number of reports of NRS from American states which border Canada, such as New York (Smiley and Craven Fowler, 1984), Wisconsin (Worf et al., 1986) and Washington (Chastagner et al., 1984). In Canada, the only confirmed reports of NRS have been from British Columbia (MacDonald, 1990; MacDonald et al., 1991). Little is known about the occurrence and distribution of NRS in Ontario and the rest of Canada. This is principally due to difficulty in making positive identification based on physical symptoms.

Because "frog eye" patch disease symptoms are commonly observed on Kentucky bluegrass in Ontario, the presence of necrotic ring spot is suspected, but has not yet been confirmed.

Identification and Detection

A prompt and accurate diagnosis is essential for an effective disease management program. The fungi which cause patch diseases are difficult to identify, and the positive identification of NRS and *L. korrae* is no exception. There are at least 9 patch diseases capable of producing the "frog eye" symptom pattern exhibited by NRS (Couch, 1986). Because of the similarity of the foliar symptoms, NRS can easily be confused with other patch diseases during the initial field diagnosis (Smiley et al., 1992).

Morphological Techniques The presence of ectotrophic hyphae on the roots cannot be used for identification since these symptoms are also common to both summer patch and take-all patch (Smiley and Craven Fowler, 1984). The use of colony morphology and growth rate to distinguish *L. korrae* from other patch disease pathogens is valuable but can only be considered as tentative identification at best (Nameth et al., 1990; Smiley and Craven Fowler, 1984; Walker and Smith, 1972). Positive identification of *L. korrae* requires the production of pseudothecia and ascospores. These sexual structures

however, are found only occasionally in nature (Smiley et al., 1992) and are difficult to induce in culture (Hammer, 1988).

Molecular Techniques Molecular approaches which have recently been applied to the identification of *L. korrae* include isoelectric focusing of fungal proteins to detect *L. korrae* (Hawkes and Harding, 1985); the development of monoclonal antibodies specific to *Leptosphaeria* spp. (Nameth et al., 1990); and hybridization with cloned DNA probes for identification of *L. korrae* (Tisserat et al., 1991). The detection limits for both the monoclonal antibody method [2 μg/ml of lyophilized homogenate] (Nameth et al., 1990) and the slot blot hybridization test [1 μg of lyophilized mycelium] (Tisserat et al., 1991) were reported as being equivalent (Tisserat, et al., 1991). The most common reported detection limit using a hybridization-based assay is 1 ng of fungal DNA (Goodwin and Nassuth, 1993). In contrast, Dong et al. (1992) found the sensitivity of the polymerase chain reaction in the detection of a plant pathogen to be one or two orders of magnitude more sensitive than the slot blot hybridization technique.

The Polymerase Chain Reaction The polymerase chain reaction (PCR) has been found to provide a fast, sensitive and reliable alternative to other molecular and morphological identification techniques of plant pathogens (Dong et al., 1992; Goodwin and Annis, 1991; Xue et al., 1992; Henson et al., 1993; Rollo et al., 1990). The reaction is based on the

extension of two oligonucleotide primers, which anneal to complementary strands of a DNA molecule. The DNA sequence which is amplified, lies between and includes the sequences of the flanking primers. The DNA duplex is denatured at high temperature (~93° C), and the primers anneal to the single DNA strands as the temperature is lowered (35-65°C). Extension of both primers is initiated from the 3' hydroxyl end at a third temperature (~72°C) and is directed along the template strand towards the position of the second primer on the complementary strand (Erlich et al., 1991). Extension of the primers is driven by a thermostable DNA polymerase, such as Taq DNA polymerase which was isolated from the thermophilic bacterium *Thermus aquaticus* (Saiki et al., 1988). The thermostable nature of the polymerase allows the reaction to repetitively cycle through the denaturation, annealing and extension temperatures and still remain enzymatically active (Erlich et al., 1988).

The extension product includes both the complementary and original primer sequence, allowing it to act as the template for the subsequent amplification cycle. After each cycle the target DNA concentration is doubled, and thus, amplification occurs in an exponential manner (Erlich et al., 1988). Therefore, target DNA, useful for analysis or further genetic applications, can be amplified from picogram amounts of DNA (Maniatis, 1989).

Primers developed from sequence analysis of ribosomal DNA

have been used with PCR technology to identify and differentiate pathotypes of the plant pathogen *Leptosphaeria maculans* (Xue et al., 1992). The sensitivity of PCR enables pathogens to be detected in DNA samples isolated from infected plant tissue, for diseases such as take-all of wheat (*G. graminis*) (Henson et al., 1993), wilt disease of lemon (*Phoma tracheiphila*) (Rollo et al., 1990) and Verticillium wilts (*Verticillium albo-atrum* and *V. dahliae*) (Nazar et al., 1991). Therefore, it should also be possible to develop a PCR-based assay for the detection of turfgrass patch disease pathogens.

Random Amplified Polymorphic DNA A DNA polymorphism assay based on PCR amplification of random DNA segments with primers of arbitrary sequence was developed by Williams et al. (1990) and Welsh and McClelland (1990). Genetic polymorphisms were detected between individual samples of human DNA, between DNA from segregating F2 soybean progeny, between DNA samples from individual fungal isolates (Williams et al., 1990) and between DNA from individual strains of bacteria (Welsh and McClelland, 1990). The generated polymorphisms were named RAPD markers for Random Amplified Polymorphic DNA. These RAPD markers have proven useful in the identification of genetic polymorphism within species (Plummer and Howlett, 1993), between varieties (Strongman and Mackay, 1993), pathotypes (Goodwin and Annis, 1991) or races (Grajal-Martin et al., 1993) which exhibit few morphological differences.

The use of RAPD markers is based on unique DNA sequences which exist in the genome of the organism under study. Differences in the DNA sequence can shift, delete or add new primer binding sites, generating amplification products that give variable banding patterns when visualized on electrophoretic gels (Hedrick, 1992). These polymorphisms can be used to distinguish between slightly divergent strains within a species (Welsh et al., 1991). One of the merits of using RAPD primers is that it requires no prior DNA sequence information (Welsh & McClelland, 1990).

Disease Management

Disease Triangle Plant disease development requires three essential components, (1) the presence of the pathogen, (2) a susceptible host, and (3) favourable environmental conditions. These three components comprise the disease triangle (Agrios, 1988). If one of the components is lacking, then significant disease development will not occur. Management strategies can be designed to ensure the removal or alteration of any or all of these three essential components. Host presence is a matter of user preference and therefore not easily altered. Environmental conditions may be altered to a limited extent. Pathogen presence is the factor we least understand in the NRS disease cycle. The mode of disease transmission is not well understood, and exclusion of the

pathogen from a site will remain difficult until methods are developed that can quickly and reliably identify sources of this disease.

Cultural Control Management of NRS should be based on cultural practices which enhance the growth of turfgrass, while creating conditions less favourable for pathogen growth. Practices that maximize good growing conditions for turf and promote deep root development in the spring and the fall may minimise the damage caused by NRS (Chastagner and Byther, 1985).

Chemical Control Work done in the Pacific Northwest has shown the systemic fungicides with sterol demethylation inhibiting mode of action such as: fenarimol, propiconazole, and diniconazole, or thiophanate which inhibits DNA synthesis in fungal mitosis, to be effective in the control of NRS (Chastagner et al., 1984; Chastagner and Hammer, 1987). However, contact fungicides have not proven effective in the control of NRS (Smiley et al., 1992).

Although fungicides may be considered the most efficacious means of controlling turfgrass diseases, there are no registered chemicals for the control of NRS in Canada. Therefore, additional studies of pathogen biology, ecology and disease epidemiology are required for further development of NRS management strategies.

Research Objectives

This study examines three integral aspects of NRS which are discussed in separate chapters. Each chapter focuses on one of the three major research objectives.

(1) The confirmation of the presence of NRS in Ontario and the positive identification of *L. korrae* as the causal agent. Information gathered in this study will also permit the distribution of NRS to be mapped in southern Ontario.

(2) The development of a protocol using a PCR based assay with species-specific primers capable of detecting *L. korrae* DNA. The sensitivity and specificity of the assay for the detection of the pathogen directly from infected plant tissue will be determined.

(3) The utilization of PCR with RAPD primers to examine the genetic diversity within a population of *L. korrae* isolates collected from a single field site. Genetic polymorphisms which potentially exist between neighbouring *L. korrae* isolates may give insight into the role played by ascospores in the spread of NRS.

CHAPTER ONE

THE OCCURRENCE OF NECROTIC RING SPOT IN SOUTHERN ONTARIO

Introduction

Necrotic ring spot (NRS) is a destructive disease of turfgrass which severely affects Kentucky bluegrass (Smiley et al., 1992; Worf et al., 1986). The causal agent, *Leptosphaeria korrae* was first identified in Australia as the incident of spring dead spot, a disease of Bermudagrass (Walker and Smith, 1972). In North America *L. korrae* was positively identified in 1984 as one of the causal agents of a disease called Fusarium blight complex of Kentucky bluegrass (Smiley and Craven Fowler, 1984).

Kentucky bluegrass exhibits a wide variety of horticultural characteristics which permit it to be used in many environmentally distinct landscapes and recreational sites. Consequently, Kentucky bluegrass is one of the most commonly used turfgrass species in temperate regions of North America (Smiley and Craven Fowler, 1984). Necrotic ring spot of Kentucky bluegrass has been reported in many areas of North America, particularly in the Pacific Northwest (Chastagner et al., 1984) and the Great Lake states (Smiley and Craven

Fowler, 1984; Worf et al., 1986). Although there have been numerous reports from the bordering American states, the only mycologically confirmed reports of NRS in Canada have been from British Columbia (MacDonald, 1990; MacDonald et al., 1991). Consequently, there is little known about the occurrence of NRS in the rest of Canada. In the province of Ontario, the presence of NRS was suspected but was not confirmed.

Patch diseases share a number of common symptoms. Dead or dying plants, forming sunken bare patches, arcs or rings of up to 0.5 m can be produced under similar environmental conditions by at least 9 different patch disease pathogens (Couch, 1986). Therefore a positive diagnosis based on physical symptoms can be difficult.

Tentative identification of *L. korrae* is based on colony characteristics which typify the fungus when it is grown on potato dextrose agar. Positive identification of*L. korrae* is based on the morphology of its sexual structures. The spore producing structures (pseudothecia) of *L. korrae*, however, are infrequently observed in nature (Smiley et al., 1992). Confirmation of *L. korrae* as the cause of NRS in Ontario, will therefore require the following: (1) isolation of the pathogen from suspected NRS samples; (2) tentative identification of the isolates based on cultural morphology; (3) examination of the pathogenic nature of the isolates; and (4) induction of pseudothecia and ascospores for positive identification.

Methods and Materials

Fungal Isolates Numerous isolates of *L. korrae* were collected from sites within Wellington, Waterloo, Durham, and York counties in southern Ontario. In addition, a survey distributed in 1991 and 1992 to members of the turfgrass industry requested suspected NRS samples to be sent to the University of Guelph. These samples were examined for microscopic symptoms of NRS, and fungal isolations were then performed.

Six isolates of *L. korrae* received from Leslie MacDonald, B.C. Ministry of Agriculture and Fisheries, were used for comparison with Ontario isolates. Three of these isolates (90-447-1, 90-447-2, 89-570) were positively identified as *L. korrae* by Dr. Gary Chastagner, Washington State University, on the basis of ascospore production. The three remaining isolates (90-794, 90-796, 90-797) were only tentatively identified using morphological techniques.

Pathogen Isolation Fungal isolations from 3 x 5 x 5 cm plugs of turfgrass from the advancing edge of patch disease samples were performed using methods similar to those described by Hammer (1988). Turf plugs were placed in 250 ml beakers covered with a fine nylon mesh and washed under running water for 3-4 h. If additional washing was required, the beakers were occasionally agitated to remove remaining

soil from the roots and were left under running water for up to 24 h.

Washed samples were examined microscopically, and segments of roots and crowns which were black in colour, or possessed dark runner hyphae, were removed. These root pieces were surface sterilized in a 1% solution of $AgNO_3$ for 30-60 sec. The silver was then removed from the roots by precipitation in a 5% NaCl solution for 30 sec, followed by a rinse in sterile distilled H_2O. Roots were placed on autoclaved paper towelling and then dried for 20 min in a laminar flow bench.

Dried root pieces were cut into 1 cm lengths and placed on 1/5 strength potato dextrose agar (8.3 g Difco PDA, 6.7 g Fisher agar in 1000 ml H_2O) amended with 30 μg/l streptomycin. After 7-10 days, developing colonies were transferred to full strength PDA plates.

Culture Conditions After the initial isolation, hyphal tips were subsequently transferred to new PDA plates at least twice to ensure purity of the cultures. Stock cultures were transferred to PDA slants in 15 ml screw-top vials and grown under fluorescent light for 16 h alternating with 8 h of darkness, and at 22-24°C for 2 weeks before long-term storage at 4°C. Actively growing colonies were also maintained on PDA Petri plates under the same light and temperature conditions.

Isolate Morphology The colony colour, its mycelial growth patterns and growth rates were compared with

descriptions in the literature (Walker and Smith, 1972; Worf et al., 1986) and with the confirmed B.C. isolates of *L. korrae*.

Agar plugs (1 cm diameter) of six *L. korrae* isolates from British Columbia and 19 tentatively identified isolates from Ontario were transferred to the centre of 9 cm diameter Petri plates containing 10-15 ml of PDA. Two replicates of each isolate were used and plates were incubated under total darkness at 24°C (Crahay et al., 1988). After 4 days, radial growth was measured every 48 h for 2 weeks or until the colonies covered the diameter of the plate.

Production of Sexual Structures Table A.1 (Appendix A) lists the tentatively identified *L. korrae* isolates which were used in trials to produce mature pseudothecia and ascospore production. Inoculum production and inoculations followed methods similar to those described by Landschoot and Jackson (1990). The trials were conducted in the greenhouse on Baron Kentucky bluegrass and hard red winter wheat seedlings grown in cone-shaped containers (4-cm-diameter by 16-cm-deep) filled with turface (Aimcore, Deerfield IL).

Inoculum was prepared in 15 ml vials containing wheat seeds pre-soaked for 4 h in water, autoclaved twice 24 h apart and then inoculated with mycelial plugs from colonies on PDA plates. The inoculum was incubated for 3-4 weeks. Approximately 0.5 g of inoculum was placed in sod within shallow incision made in the four-wk-old Kentucky bluegrass

turf. For wheat plants, the inoculum was placed in partially filled cone containers and covered with 2-3 cm of turface before seeding. Heat-killed inoculum used in the controls for both the wheat and Kentucky bluegrass trials was produced by autoclaving colonized wheat seeds. Seedlings were watered daily and fertilized weekly (20-8-20) with a hand-held spray bottle. Six weeks after inoculation, whole plants were removed on a weekly basis, washed under tap water and examined microscopically for pseudothecia on the roots and crowns.

Measurement of Ascospores Pseudothecia were removed from the roots and crowns of inoculated plants with forceps while under a dissecting microscope. Individual pseudothecia were then placed in a drop of water on a microscope slide. A needle was used to crush the pseudothecia in order to release ascospores. Intact asci were examined for bitunicate cell wall structures. The length, width and number of septa were recorded for 10 randomly selected ascospores from each pseudothecium and compared with values from taxonomic descriptions (Smiley & Craven Fowler, 1984; Walker and Smith, 1972; Worf et al., 1986). The ascospore characteristics for the Ontario and B.C. isolates were also contrasted.

Proof of Pathogenicity Koch's postulates (Agrios, 1988) were tested on six week old Kentucky bluegrass plants grown in the greenhouse in 12.5-cm-diameter pots containing Turface. Ten Ontario and two B.C. isolates positively identified as *L. korrae* were tested. Hard red winter wheat

seeds were used to generate inoculum as previously described. Approximately 0.5 g of inoculum was placed in a 2-3 cm deep slit made in the turf at the centre of each pot. There were four replicates for each isolate plus a control which consisted of heat-killed inoculum. After 8 weeks, fungal isolations were carried out, as described earlier, on plants removed from the outer edge of developing patches.

Results

Isolate Morphology A total of 122 suspected NRS samples of Kentucky bluegrass were collected from 17 counties throughout southern Ontario. Out of 122 isolates, 84 were tentatively identified as *L. korrae* on the basis of colony morphology (Fig 1.1 & Table A.1). Overall colony colour, production of aerial mycelium, as well as colour and growth pattern of hyphae at the colony margin were considered in the tentative identification of each isolate.

On PDA, typical *L. korrae* colonies initially produced abundant white aerial mycelium which turned grey after several days (Fig 1.1). Agar directly beneath the colonies became black as colonies matured. In some isolates a "halo" of white hyphae appeared around the outer edge of the colony. The "halo" appearance was more pronounced on darker coloured colonies, and was apparently produced as the older mycelium at the colony centre darkened leaving the younger white mycelium at the outer margin.

Radial growth of typical *L. korrae* colonies ranged from 2.7 - 3.1 mm/day for the B.C. isolates, 2.8-3.6 mm/day for the sporulating Ontario isolates and 1.7-4.9 mm/day for the non-sporulating isolates. The mean radial growth values (data collected over a two week period) are presented in Table 1.1.

Some isolates initially produced typical *L. korrae* colony

morphology, but later developed appressed green/brown mycelium with hyphal tips that curled back towards the colony. These atypical isolates were kept in the collection but were judged not to be *L. korrae*.

Production of Sexual Structures Fifty isolates tentatively identified as *L. korrae* were used in experiments on infected host plants to produce sexual structures for positive identification. After several weeks, inoculated plants developed small chlorotic areas characteristic of patch disease. Plants in these patches were colonized by black runner hyphae and possessed darkened roots. Production of sexual structures was not observed prior to 8 weeks after inoculation, and sampling ended after 12 weeks to avoid the possibility of cross contamination from released ascospores.

Thirty-three of the 50 isolates tested were positively identified as *L. korrae* based on the production of mature pseudothecia with ascospores (Table 1.2, & A.1). Black erumpent pseudothecia were found firmly attached to the surface of larger roots and rhizomes of host plants (Fig 1.2). Intact asci were not found for all isolates. Those samples containing intact asci however, were bitunicate. Ascospores were tan-coloured, long, needle-shaped or slightly curved and were often found in loosely twisted bundles (Fig 1.3). Ascospores ranged in length from 80-160 μm with a mean length of 132 μm, and a width of 4-5 μm (Table 1.2). Septa ranged from 3 to 7 per ascospore. Of the 33 positive

identifications, six isolates were from British Columbia and 27 isolates were from Ontario originating from within 17 southern Ontario counties (Fig 1.4 & Table A.1).

Proof of Pathogenicity Koch's postulates were satisfied for the ten Ontario and two B.C. *L. korrae* isolates tested (Table 1.3). Characteristic patch disease symptoms developed in the potted turfgrass. Patch development varied between isolates and replicates, but was present in all cases except for the controls which remained green and healthy. Re-isolations from infected tissue produced characteristic *L. korrae* colonies for all ten test isolates but not for the controls. Many of the test isolates produced pseudothecia on rotting plant material in the centre of the patches.

Table 1.1 Mean daily radial growth (mm/day) of both tentatively and positively identified *Leptosphaeria korrae* isolates. Isolates are placed in one of three columns depending on their source (B.C. or Ontario) or identification category (positively identified as *L. korrae* = sporulating, and tentatively identified as *L. korrae* = non-sporulating).

British Columbia		Ontario			
Sporulating isolates	Mean radial growth (mm/day)	Sporulating isolates	Mean radial growth (mm/day)	Non-Sporulating isolates	Mean radial growth (mm/day)
90-794	2.8	OLC-2	3.2	K1	2.1
90-796	2.7	PPO91-12	3.2	K2	2.9
90-797	3.1	PPO91-22	3.6	PPO91-2	1.9
89-570	2.9	PPO91-24	3.0	PPO91-3	
	2.7				
90-447-1	3.0	PPO91-22	3.6	PPO91-7	
	2.8				
90-477-2	3.0	PPO91-25	3.3	PPO91-11	
	1.7				
		PPO91-26	2.9	PPO91-18	
	3.2				
		PPO91-29	2.8	PPO91-19	
	3.2				
		PPO91-32	3.3	PPO91-21	
	3.3				
		PPO91-34	3.3	PPO91-38	

4.9

Fig 1.1 Typical colony morphology of *Leptosphaeria korrae* isolates from Ontario (LK01) and British Columbia (LK09).

Fig 1.2 Ontario *Leptosphaeria korrae* isolate producing pseudothecia on the roots of inoculated wheat plants grown in cone containers under greenhouse conditions.

Fig 1.3 Ascospores of *Leptosphaeria korrae* released from mature pseudothecia produced *in vitro*.

Table 1.2 Characteristics of *Leptosphaeria korrae* ascospores produced on inoculated Kentucky bluegrass seedlings grown in the greenhouse.

Isolate	Length (µm)		Width (µm)	Number of septa
	range	mean		
90-79	89 - 129	100	4 - 5	3
90-797	99 - 129	123	4 - 5	3
90-447-2	80 - 109	95	4 - 5	3
90-794	120 - 150	139	4 - 5	5
89-570	105 - 145	128	4 - 5	5
OLC-2	105 - 145	140	4 - 5	7
PPO91-12	80 - 109	95	4 - 5	3
PPO91-22	150 - 158	148	4 - 5	7
PPO91-24	142 - 160	150	4 - 5	7
PPO91-27	126 - 140	135	4 - 5	5
PPO91-29	147 - 156	151	4 - 5	7
PPO91-32	95 - 125	108	4 - 5	3
PPO91-34	133 - 159	150	4 - 5	7
PPO91-43	138 - 156	148	4 - 5	7
PPO91-44	98 - 130	118	4 - 5	3
PPO91-46	118 - 145	139	4 - 5	3
PPO91-47	129 - 146	135	4 - 5	7
PPO91-50	141 - 150	144	4 - 5	7
PPO91-52	133 - 145	139	4 - 5	5
PPO91-53	144 - 160	153	4 - 5	7

Table 1.3 Pathogenicity testing of *Leptosphaeria korrae* isolates on Kentucky bluegrass grown in the greenhouse.

	Isolate No.	Re-isolation of pathogen[1]
Ontario isolates	OLC-2	+
	PPO91-12	+
	PPO91-24	+
	PPO91-26	+
	PPO91-29	+
	PPO91-34	+
	PPO91-46	+
	PPO91-50	+
B.C. isolates	89-570	+
	90-447-2	+
	Control[2]	-

[1] Isolates satisfying Koch's postulates are identified by a "+" symbol.

[2] Control = heat-killed inoculum

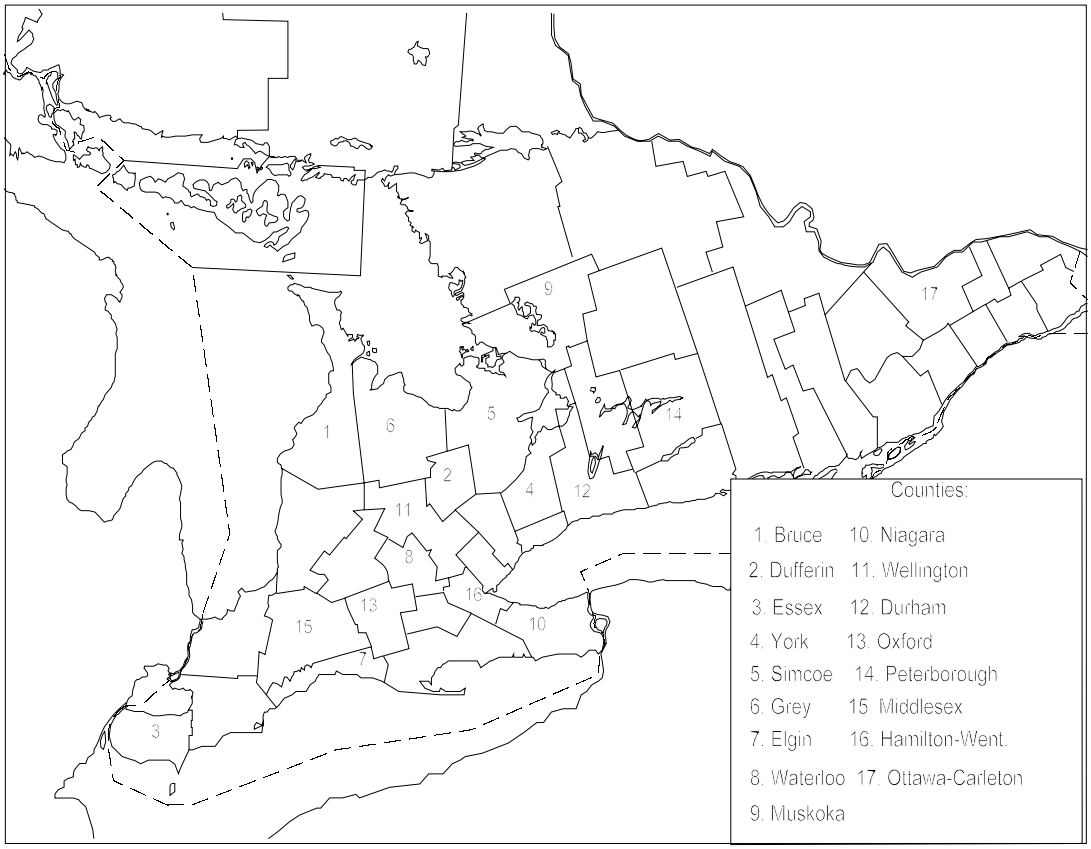

Fig 1.4 Distribution of necrotic ring spot caused by *Leptosphaeria korrae* in southern Ontario counties.

Discussion

This study focused on the use of established methods to confirm that *L. korrae* was the causal agent of NRS in southern Ontario. Determining the identity of the 122 isolates and their association with NRS required morphological studies, evaluation of isolate pathogenicity, and the *in vitro* production of sexual structures.

Sixty-nine percent of Ontario isolates collected fit the morphological description of *L. korrae* , as described in literature (Nameth et al., 1990; Smiley and Craven Fowler,1984; Walker and Smith, 1972; Worf et al., 1986), with only minor colour variations, and were also morphologically similar to the *L. korrae* isolates from B.C. The atypical isolates which produced appressed olive-green/brown hyphae that curled back towards the colony, have characteristics similar to *Gaeumannomyces graminis* and *Magnaporthe poae* (Smiley and Craven Fowler, 1984). However, since *G. graminis* generally shows a greater specificity for bentgrass (Walker and Craven-Fowler, 1984), these atypical isolates may possibly be *M. poae* the cause of summer patch, which can also occur on Kentucky bluegrass.

Growth rates for positively identified Ontario and B.C. isolates ranged from 2.8-3.6 mm/day and 2.7-3.1 mm/day respectively. These values were similar to those from New York and Wisconsin reported as 3 mm/day (25°C) (Smiley et al.,

1985) and 2.6-4.3 mm/day (24°C) (Worf et al., 1986) respectively. Growth rate values as high as 4.5-5.3 mm/day (25°C) are reported from the Pacific Northwest (Hammer, 1988) and 4-5 mm/day from Australia (Walker and Smith, 1972). These differences in growth rates may be influenced by the age and source of the cultures. Smiley et al. (1985) report higher growth rates for *L. korrae* from single spore cultures than hyphal tip cultures. In contrast to *L. korrae*, growth rates of other patch disease pathogens such as *G. graminis* and *M. poae* are reported at 8-12 mm/day (20-25°C) and 4-6 mm/day (28-30°C) respectively (Smiley et al., 1985).

Other non-sporulating isolates exhibited growth rates below the range of the reported values. However, there were some non-sporulating isolates which possessed the colony morphology of *L. korrae* and also had growth rates that were within the accepted range. Nameth et al. (1990) found approximately 50% of the isolates in their collection which possessed *L. korrae* colony morphology did not produce pseudothecia, but still elicited a strong I-ELISA reaction with the *L. korrae* specific monoclonal antibody LKc50. This illustrates the tentative nature of identification based on disease symptoms, colony morphology and growth rate characteristics.

Sixty-six percent of the isolates tested in the fruiting study produced fertile pseudothecia and were therefore positively identified as *L. korrae*. Black erumpent

pseudothecia were often found in clusters along larger roots and rhizomes of inoculated plants. Compression ridges around the mid section of the pseudothecia were common but not always present. These ridges are thought to form as embedded pseudothecia grow up through layers of plant tissue (Walker and Smith, 1972).

Ascospores produced in bitunicate asci were observed and ranged from 80-160 μm x 4-5 μm. These values are comparable to those reported from New York and Wisconsin (Smiley and Craven Fowler, 1984; Worf et al., 1986), but are shorter than *L. korrae* isolated from bermudagrass, which measured 100-230 μm in length (Crahay, 1988). Differences in size may be attributed to ascospore maturity. The shorter spores observed in this study may have been released before maturation when examined by crushing pseudothecia. Ascospores were observed having 3, 5 or 7 septa per spore. This is not completely consistent with the septation pattern reported for *L. korrae* ascospores, which develop septa in a ordered series of 1, 3, 7 and then 15 as they mature. The Ontario and British Columbia isolates show a similarity to *L. narmari* which exhibits a septation pattern of 1, 3, 5, 7 and 14. It should be noted that variations in both of these patterns are common (Walker and Smith, 1972).

Final confirmation of *L. korrae* as the pathogen responsible for NRS in southern Ontario was obtained by satisfying Koch's postulates. In addition, the positive

identification of the pathogen, based on its sexual structures, has confirmed the presence of NRS in 17 counties ranging from Essex to Ottawa-Carleton. This demonstrates the disease is widespread in southern Ontario. The technique for the induction of sexual structures in *L. korrae* works in over 60% of the isolates, and ultimately provides a means of positively identifying the fungus; however, the time requirement is prohibitive and a quicker technique would be desirable for diagnostic and research purposes.

CHAPTER TWO

DETECTION OF LEPTOSPHAERIA KORRAE WITH PCR AND PRIMERS FROM THE RIBOSOMAL INTERNAL TRANSCRIBED SPACERS

Introduction

Accurate diagnosis of the turfgrass patch disease, necrotic ring spot (NRS), is difficult. Diagnosis of patch diseases in general is complicated by a number of factors including the variability of primary symptom patterns which can be dictated by environmental conditions and management practices (Couch, 1986). The characteristic "frog eye" pattern exhibited by NRS is commonly produced by a number of patch diseases such as take-all patch, summer patch, fusarium patch, yellow patch, pythium blight, rhizoctonia blight, and sclerotium blight (Couch, 1986). To compound the problem, fungi which cause patch disease are difficult to identify due to their similar morphology and appearance on the host.

Tentative confirmation of NRS can be made from microscopic investigation of roots taken from active margins of NRS samples. Necrotic ring spot activity is evidenced by the presence of dark ectotrophic hyphae growing along the crowns and roots of the infected grass plants (Smiley and Craven Fowler, 1984). Colonized crowns and roots eventually become blackened, and under severe conditions the crowns

collapse causing plant death (Worf et al., 1986). However, the diagnostic value of these symptoms is limited, because similar structures and effects can be produced by a number of turf pathogens and saprophytes (Smiley et al., 1985).

Although, tentative identification can also be made based on growth rates and colony morphology (Worf et al., 1986), sexual fruiting structures (pseudothecia) and ascospores are required for positive identification. Unfortunately, pseudothecia of *L. korrae* are only occasionally found in nature (Smiley et al., 1992) and are difficult to induce in culture. The production of pseudothecia induced under artificial conditions can take several months (Crahay, 1988; Hammer, 1988; Smiley and Craven Fowler, 1984; Worf et al., 1986), and even then not all isolates will produce the sexual structures (Hammer, 1988; Nameth et al., 1990). There remains, therefore, a need for more rapid pathogen diagnostic techniques in turfgrass pathology.

Rapid and accurate identification of the causal agent is very important for effective strategies of disease control, and several diagnostic methods have been developed for identifying turfgrass pathogens. These include DNA restriction fragment length polymorphisms (Kohn et al., 1988; Tisserat, 1988), polyclonal and monoclonal antibodies (Nameth et al., 1990; Novak and Kohn, 1991), isoelectric focusing (Hawkes and Harding, 1985) and cloned DNA probes (Tisserat et al., 1991).

Polymerase chain reaction (PCR) can provide an alternative to other detection methods. PCR requires less time to perform and is also more sensitive than other molecular detection techniques (Dong et al., 1992; Rollo et al., 1990). Recently PCR has been used to detect and differentiate closely related species of plant pathogens (Henson, 1992; Henson et al., 1993; Nazar et al., 1991; Schesser et al., 1991; Xue et al., 1992). To develop pathogen specific primers for PCR, sequence information from ribosomal DNA (rDNA) has been used (Nazar et al., 1991; Xue et al., 1992). The nucleotide sequence of RNA genes is highly conserved because of pressure to retain functionality of gene products. The transcribed and non-transcribed spacer sequences between RNA genes, however, are not translated into gene products, and consequently, are poorly conserved and can contain large sequence differences. These differences make it possible to discriminate between species with rDNA hybridization probes (Chambers et al., 1986; Gobel et al., 1987; Nazar et al., 1991).

The objective of this study was to test a potentially species-specific oligonucleotide primer, developed from sequence information of the internal transcribed spacer 1 (ITS 1) region, for specific amplification of *L. korrae* rDNA.

Methods and Materials

Fungal Isolates Isolates used in this study are listed in Table 2.1. *Leptosphaeria korrae* isolates 89-570, 90-447-1, 90-447-2, 90-794, 90-796 and 90-797 were received from L. MacDonald, B.C. Ministry of Agriculture; *M. poae* isolates 73-1 and 73-15 were received from P. Landschoot, Pennsylvania State University; *R. solani* isolate RS1/T(AG1) was received from E.E. Butler, University of California, Davis. All other fungal isolates were from the University of Guelph. Stock cultures were maintained on slants of potato dextrose agar (PDA) stored at 4 °C or as inoculated chicken scratch (mixed grains) stored at -10°C. All stock cultures were subcultured onto PDA plates and grown at 22°C, except for *Typhula incarnata* Fr. which was grown at 15°C.

Plant Material Field samples of Kentucky bluegrass showing symptoms of NRS were collected by removing a plug of turf 3 x 5 x 5 cm from the outer edge of necrotic patches. Isolations were made by placing surface-sterilized root tips on 1/5 strength potato dextrose agar [8.3 g PDA (Difco) and 6.7 g of agar (Fisher) per litre of H_2O] amended with 30 μg/l streptomycin (Hammer, 1988). After 10 days, colonies were transferred to full strength PDA. The remaining roots from each sample were washed free of soil under tap water, frozen in liquid nitrogen and stored at -20°C for DNA

extraction.

Kentucky bluegrass was grown in the greenhouse and inoculated with the *L. korrae* isolates listed in Table 2.1. Inoculum consisted of hard red spring wheat kernels infected with *L. korrae.* This was placed in sod within a 2 cm deep incision made at the centre of a 12-cm-diameter pot. After 2 months, symptoms were obvious, and small turf plugs (2-cm-diameter) were removed from the advancing edge of the patch. The plugs were washed under tap water, examined for characteristic runner hyphae, and frozen in liquid nitrogen for DNA extraction.

DNA Extraction DNA was isolated from fungal cultures growing on a 7 cm x 7 cm sterile cellulose membrane sheet (Flexel Inc., Atlanta, GA) overlaying 2% malt agar. The fungal mycelium was scraped from the cellulose membranes and placed into 1.5 ml microfuge tubes.

DNA extractions were carried out by one of two methods. In the first method, plant and fungal DNA were extracted using the method of Rogers and Bendich (1985) with minor modifications. Plant tissue or fungal mycelium was mixed with sterile sand and liquid nitrogen and ground to a fine powder using a mortar and pestle. Approximately 300 mg of ground tissue were transferred to a 1.5 ml microfuge tube containing 500 µl of CTAB extraction buffer [2% CTAB (w/v)(hexadecyltrimethylammonium bromide, Sigma), 100 mM Tris-HCl (pH-8.0), 20 mM EDTA (pH 8.0), 1.4 M NaCl and 1%

Polyvinylpyrolidone (PVP, Sigma)]. Tissue was suspended by gentle vortexing and incubated for 30 min at 65°C. The suspension was extracted with an equal volume phenol/chloroform/isoamyl alcohol (25:24:1) followed by chloroform/isoamyl alcohol (24:1) until the interface was clear. The aqueous phase was transferred, and the DNA precipitated with an equal volume of 3 M NaOAc/isopropanol (1:10) (Yoon et al., 1991). After centrifugation, the pellet was resuspended in buffer [10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0), 1 M NaCl], and the DNA was then precipitated with two volumes of 95% ethanol. The resuspended DNA was stored in 0.1 x TE buffer [1 mM Tris-HCl (pH 8.0), 0.1 mM EDTA (pH 8.0)].

For the second DNA extraction method, mycelium from colonies grown on cellulose membranes overlaying 2% malt agar was placed in 1.5 ml microfuge tubes and washed first with 1 ml of 1 M NaCl and then distilled water. Sterile sand was added and the tissue was ground with a disposable pellet pestle (Kontes, Vinland, NJ). Following grinding, 750 µl of 10 mM Tris-HCl (pH 8.0) was added and the samples were boiled for 20-30 minutes (Henson, 1992). The samples were then centrifuged for 5 min at 8000 *g*. The supernatant was transferred to a new tube and stored at -20°C.

PCR and Pathogen Detection Primers used for initial amplification of *L. korrae* DNA were identified from conserved sequences of the 17S and 5.8S ribosomal DNA (rDNA) of

Neurospora crassa and *Saccharomyces carlsbergensis* (Chambers et al., 1986;) and *Thermomyces lanuginasus* (Nazar et al., 1987). The sequence of the 17S primer in the 17S rDNA was 5'-TCCCCGTTGGTGAACCAGCGG-3', and the anti-sense 5.8SC primer sequence in the 5.8S rDNA was 5'- GCTGCGTTCTTCATCGATGC-3'. The DNA fragment flanked by the two primers contained the entire ITS 1 region including a portion of the 17S and 5.8S rDNA (Appendix B, Fig B.1). Based on sequence differences found in the *L. korrae* ITS 1 region, a primer, LK17S, was designed (O'Gorman et al., 1994). The LK17S primer is a 17-mer with the sequence, 5'-ACAAACTGCATGGGCGG-3' (Appendix B; Fig B.1., bp 63-79). LK17S was paired with the 5.8SC primer (Appendix B; Fig B.1.,bp 235-255), in order to test for specific amplification of *L. korrae*.

PCR amplifications were carried out in 0.5 ml microfuge tubes with 25 μl reaction mixtures. The mixture contained either 1 ng of DNA extracted by the modified Rogers and Bendich method (21) or 3 μl of DNA extracted by boiling in Tris-HCl (pH 8.0), 200 μM of each dNTP, 0.5 μM of primers, 0.5 units of Vent$_R$® DNA polymerase (New England Biolabs, Beverly, MA), 100 μg/ml BSA and DNA polymerase buffer [10 mM KCL, 10 mM $(NH_4)_2SO_4$, 20 mM Tris-HCl (pH 8.8), 2 mM or 4 mM $MgSO_4$, and 0.1 % Triton X-100). The reactions were overlaid with light mineral oil (Fisher) and amplifications were performed in a COY Tempcycler (Ann Arbor, Michigan) programmed for an initial cycle of 3 min at 94 °C, 2 min at 57°C, and 2

min at 72°C. This was followed by 35-40 cycles of 30 s at 94°C, 50 s at 57°C, 15 s at 72°C, and a final extension cycle for 10 min at 72°C. Amplification products were separated by gel electrophoresis in 2% agarose in 0.5 x TBE (45 mM Tris-borate and 1 mM EDTA). Gels were then stained with ethidium bromide and viewed under 260 nm UV light.

Results

Assay Specificity and Sensitivity Amplification with the LK17S/5.8SC primer pair showed that the assay was specific for *L. korrae*. DNA from pure cultures of 19 *L. korrae* isolates showed the specific LK17S/5.8SC amplification product, while 19 isolates chosen from 15 species of other common turfgrass pathogens, turfgrass saprophytes or related *Leptosphaeria* species, did not amplify. Only DNA from the *L. korrae* isolates was successfully amplified, and there was no amplification from the DNA of any of the other 15 species of fungi tested (Table 2.1 and Fig 2.1). The single amplification product generated using the LK17S/5.8SC primer pair was a 193 bp fragment as predicted from the sequence (Fig 2.1; Appendix B, Fig B.1). In Fig 2.1, the stained smears originating at the gel well for samples lacking the target sequence was a result of primer artifacts due to the low DNA K_m value which is a characteristic of $Vent_R$® DNA polymerase (Anonymous, 1992). To ensure that the DNA from the other 15 fungal species could be amplified by PCR, amplification of the DNA from these isolates were also tested using the conserved 17S/5.8SC primers (Table 2.1).

The simplicity of preparing DNA samples by boiling in Tris-HCl (pH 8.0) permitted fungal DNA to be prepared for PCR in less than one hour. This method also allowed DNA to be easily extracted from fungal species such as *Sclerotinia*

homoeocarpa which apparently produces large amounts of polysaccharides that remain with the DNA following extraction by the modified Rogers and Bendich method. Sensitivity of the PCR-based assay using the LK17S/5.8SC primers was tested using 10-fold serial dilutions of *L. korrae* DNA. Amplification from *L. korrae* DNA was obvious with as little as 10 pg of DNA and was also observed with 1 pg of DNA. However, the reactions containing 1 pg of *L. korrae* DNA had only faint bands that are not readily apparent in Figure 2.2. There was no observable amplification detected at 0.1 pg of DNA (Fig 2.2).

Detection of Pathogen from Plant Tissue. Positive PCR results for *L. korrae* were achieved for both naturally infected and inoculated turfgrass samples. However, the PCR products amplified from infected plant tissue were usually not as strong as that from pure culture (Fig 2.3).

Table 2.1 Identity of fungal isolates, and PCR results using universal 17S and specific LK17S primers.

Species	Isolate	PCR	
		UN[a]	LK[b]
Leptosphaeria korrae	LK-01	+	+
Leptosphaeria korrae	LK-02	+	+
Leptosphaeria korrae	LK-03	nt	+
Leptosphaeria korrae	LK-04	+	+
Leptosphaeria korrae	89-570	nt	+
Leptosphaeria korrae	90-447-1	nt	+
Leptosphaeria korrae	90-447-2	nt	+
Leptosphaeria korrae	90-794	nt	+
Leptosphaeria korrae	90-796	+	+
Leptosphaeria korrae	90-797	nt	+
Leptosphaeria korrae	LK-16	nt	+
Leptosphaeria korrae	LK-19	nt	+
Leptosphaeria korrae	LK-23	nt	+
Leptosphaeria korrae	LK-25	nt	+
Leptosphaeria korrae	LK-27	nt	+
Leptosphaeria korrae	LK-28	nt	+
Leptosphaeria korrae	LK-55	nt	+
Leptosphaeria korrae	LK-57	nt	+
Leptosphaeria korrae	LK-58	nt	+
Leptosphaeria maculans	Unity	+	
	-		
Leptosphaeria maculans	Unity	+	-
Magnaporthe poae	73-1	+	-

Magnaporthe poae	73-15	+	-
Gaeumannomyces graminis	Gg-1	+	-
Gaeumannomyces graminis	Gg-2	+	-
Gaeumannomyces graminis	Gg-3	+	-
Cladosporium sp.	Csp-40	+	
	-		
Cephalosporium sp.	C.sp-40	+	-
Drechslera poae	Dp-1	+	-
Pythium sp.	P.sp 20	+	-
Fusarium nivale	FN-30	+	-
Fusarium sp.	Fsp-02	+	-
Sclerotinia homoeocarpa	SH-01	+	-
Rhizoctonia solani	RS1/T(AG1)	+	-
Typhula incarnata	TI-17	+	-
Trichothecium roseum	TR-01	+	-
Leucoagaricus naucina	SM45b	+	-
Marasmius oreades	SM27b	+	-
Poa pratensis			
NRS field sample	LK-15	nt	+
NRS field sample	LK-24	nt	+
inoculated	LK-14	nt	+
inoculated	LK-16	nt	+
inoculated	LK-19	nt	+
inoculated	LK-20	nt	+
inoculated	LK-2	nt	+
inoculated	LK-23	nt	+
inoculated	LK-30	nt	+
inoculated	LK-34	nt	+

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[a]UN = universal 17S/5.8SC primers
[b]LK = LK 17S/5.8SC primers
"nt"= not tested
"+" = positive PCR amplification
"-" = No PCR amplification

Fig 2.1 Specific detection of *Leptosphaeria korrae* DNA using PCR with LK17S and 5.8SC primers. The species names corresponding to the identification numbers listed below are found in Table 2.1. Lane 1, 123 bp ladder (DNA marker); lane 2, LK-4; lane 3, LK-7; lane 4, LK-10; lane 5, blank (no DNA); lane 6, LM Unity; lane 7, 73-1; lane 8, GG-1; lane 9, FN-30; lane 10, RS1/T; lane 11, SM27b; lane 12, SM45b. DNA extracted using the boiling Tris-HCl method, and PCR amplification was performed through 35 cycles.

Fig 2.2 Sensitivity of detection by PCR using the LK17S and 5.8SC primers for the ITS-1 region of *Leptosphaeria korrae* DNA. Lane 1, 123 bp ladder (DNA marker); lane 2, 25 ng of *L. korrae* DNA; lane 3, 10 ng; lane 4, 1 ng; lane 5, 100 pg; lane 6, 10 pg; lane 7, 1 pg, and lane 8, 0.1 pg. DNA was extracted by the modified Rogers and Bendich method, and PCR amplification was performed through 35 cycles.

Fig 2.3 Detection of *Leptosphaeria korrae* from naturally infected field samples and artificially inoculated Kentucky bluegrass samples. Lane 1, 123 bp ladder (DNA marker); lane 2, LK-3; lane 3, LK-4; lane 4, LK-7; lane 5, healthy turfgrass sample; lane 6, LK-14 (inoculated turfgrass sample); lane 7, LK-15 (natural NRS infection); lane 8, LK-23 (inoculated turfgrass sample); lane 9, LK-24 (natural NRS infection). DNA was extracted by the modified Rogers and Bendich method, and PCR amplification was performed through 40 cycles.

Discussion

The polymerase chain reaction, when used with apparently species-specific primers, was able to detect *L. korrae* and differentiate it from other turfgrass pathogens. The primers which were based on DNA sequence differences found in the internal transcribed spacer 1 (ITS 1) region of *L. korrae* rDNA, were developed by Drs. B. Xue and P.H. Goodwin at the University of Guelph (O'Gorman et al., 1994).

The specificity of this PCR assay is based on divergent sequences of the ITS-region of rRNA genes which exhibit considerable sequence differences between fungal species (Chambers, et al., 1986; Nazar, et al., 1987; Xue et al., 1992). The use of variable ITS regions to select primer sequences for differentiation of closely related fungal plant pathogens has proven successful (Nazar et al., 1991; Xue et al., 1992). Comparison of the ITS 1 sequence between *L. korrae* isolates revealed a very high degree of similarity. The sequenced ITS 1 regions shared 94.8% similarity between the *L. korrae* isolates, and as low as 50% similarity between *L. korrae* and ITS 1 sequences of the other fungal isolates (Fig B.1, Appendix B). Comparison of fungal ITS 1 sequences showed a higher degree of similarity between the closely related species *Gaeumannomyces graminis* and *Magnaporthe poae* than with *L. korrae* (O'Gorman et al., 1994). These major differences in ITS 1 sequences facilitated the design of a unique set of primers that specifically annealed to *L. korrae* DNA.

As predicted from sequence information (Fig B.1, Appendix B), the species-specific primer pair, LK17S/5.8SC, consistently amplified a 193 bp PCR product from DNA of *L. korrae*, and did not amplify DNA from related species or any of the tested fungi which commonly inhabit turfgrass. High sensitivity of the PCR assay also allowed direct detection of *L. korrae* from infected plant tissue. However, the concentration of the 193 bp PCR product was consistently lower from infected plant tissue than DNA from pure fungal culture.

The lower intensity of the bands may have been due to lower DNA concentrations or the presence of PCR-inhibiting substances such as phenolics, tannins or terpenoids released from plant cells upon lysis. These compounds bind to the DNA and cannot be removed by conventional extraction techniques (Maliyakal, 1992). The presence of inhibitors was demonstrated here by improved amplification following dilution of the DNA extracts prior to PCR. Other DNA purification techniques which remove possible PCR-inhibitors, may increase the intensity of the PCR products obtained from infected plant tissue samples. Techniques such as spin columns (Tsai and Olson, 1992), alkaline extraction buffers containing PVP (Maliyakal, 1992), and electrophoretic gels containing PVP (Young et al., 1993) have met with some success in removing contaminants from DNA.

The use of PCR to specifically detect *L. korrae* permits reliable diagnosis of suspected NRS samples within several hours. Previously, the time required to make a positive identification of this disease with conventional morphological techniques required 8 weeks or more. The application of PCR as

a diagnostic tool for NRS should prove beneficial in disease management as well as accelerating further studies of the epidemiology of this disease.

CHAPTER THREE

GENETIC VARIABILITY IN LEPTOSPHAERIA KORRAE, ESTIMATED BY RANDOM AMPLIFIED POLYMORPHIC DNA ANALYSIS

Introduction

There is limited information available concerning the life cycle of *Leptosphaeria korrae*, the causal agent of necrotic ring spot (NRS) (Chastagner and Byther, 1985). *Leptosphaeria korrae*, like other ectotrophic root infecting-fungi, has the ability to survive on the surface of roots or in cortical cells of turfgrass for extended periods of time without causing visible damage. If conditions which favour disease development occur, then the pathogen may invade the roots causing extensive damage to the vascular tissues and eventual plant death (Landschoot et al., 1993).

The mechanism by which NRS spreads is not known. Pair et al. (1986), however, showed that spring dead spot of Bermudagrass could spread to healthy turf from turf plugs infected with *L. korrae*. *Leptosphaeria korrae* is thought to survive unfavourable conditions as sclerotia or mycelium in plant debris (Smiley et al., 1992). This is one reason why infected sod and some types of mechanical equipment such as core aerators and dethatching machines, are presumed to play a role in the transmission of the disease (Landschoot et al., 1993). The sexual structures (pseudothecia) of *L. korrae* are

sporadically observed in nature, but the importance of ascospores in the spread and development of the disease has not been determined (Chastagner and Byther, 1985; Landschoot et al., 1993). If ascospores of *L. korrae* are ejected into the air and are capable of infecting turfgrass, then they may also play an important role in the spread of NRS (Landschoot et al., 1993).

The ability of single ascospore cultures of *L. korrae* to produce pseudothecia *in vitro* (Smiley and Craven Fowler, 1984) demonstrates the homothallic nature of this species. Homothallism is described as the ability of an individual to carry out self-fertilization during sexual reproduction, thereby eliminating the need for compatible mating types (Nauta and Hoekstra, 1992). As a sexual mechanism it is very common among all groups of fungi with the exception of basidiomycetes (Raper, 1966).

The extent of genetic polymorphisms in isolates of a homothallic species may be limited due to self-fertilization during sexual reproduction. In some homothallic fungi however, genetic recombination has been observed in progeny produced via heterokaryosis (Pontecorvo, 1956). The process of genetic recombination in a heterokaryotic state is called the parasexual cycle (Pontecorvo, 1956), and involves the following steps: fusion of vegetative hyphae; formation of heterokaryons; fusion of the unlike nuclei; and mitotic recombination through crossing over events and chromosomal rearrangement (Roper, 1966). Genetic polymorphism resulting from the parasexual cycle (MacDonald and Martinez, 1990) or sexual reproduction in heterothallic (Plummer and Howlett,

1993) and homothallic fungi (Francis and St. Clair, 1993) has been observed.

By examining genetic polymorphisms, it may be possible to "fingerprint" the source of inoculum and distinguish whether ascospores or mycelia act as the inoculum for adjacent infection centres. A DNA polymorphism assay based on polymerase chain reaction (PCR) technology was developed in 1990 (Williams et al., 1990; Welsh and McClelland, 1990). This type of assay has been used to detect genetic variations within plants (Vierling and Nguyen, 1992; Kresovich et al., 1992), animals (Williams et al., 1990), bacteria (Welsh and McClelland, 1990), and fungi (Goodwin and Annis, 1991; Yoon and Glawe, 1993) at various taxonomic levels. An example of its use with fungi is the differentiation of single ascospore isolates of *Hypoxylon truncatum* (Schw.: Fr.) Mill. *sensu* Miller originating from the same stroma (Yoon and Glawe, 1993). With this technique, genetic differences in the F2 progeny of the homothallic oomycete *Pythium ultimum* were also detected (Francis and St. Clair, 1993).

The genetic polymorphisms detected by this assay are known as RAPD (random amplified polymorphic DNA) markers (Williams et al., 1990). The size of the amplification products (RAPD markers) is governed by alterations in the DNA sequences. RAPD-PCR may be able to distinguish even a single nucleotide insertion, deletion or substitution (Ellsworth et al., 1993). Such changes in the DNA sequence can alter the RAPD primer binding sites, leading to loss of a RAPD marker or generation of new ones. When run on electrophoretic gels, the polymorphisms can be observed as variation in banding patterns

between various DNA samples (Hedrick, 1992). Therefore, by using a RAPD-PCR assay it may possible to detect genetic variability between isolates of the homothallic *L. korrae.*

The objective of this study was to use a RAPD-PCR to examine the genetic diversity within single ascospore progeny from a single pseudothecium and from a population of *L. korrae* isolates from a single field site. The results were used to elucidate the role of ascospores in the spread of NRS.

Methods and Materials

Fungal Isolates Samples of turfgrass suspected of containing *L. korrae* were collected from a single field site in Guelph Ontario. Fungal isolations from infected grass tissue taken at the margins of NRS patches followed a method similar to that of Hammer (1988) as described in Chapter One. A pure culture of each isolate was obtained by consecutive hyphal tip transfers. Identification of the isolates was confirmed on the basis of sexual structures or with the PCR-based assay using the *L. korrae* specific primers LK17S/5.8SC described in Chapter Two.

Single ascospore progeny from the *L. korrae* isolate LK30 (collected in Markham Ont.) were also studied (Table 3.1). A pseudothecium was crushed with a needle on a sterile microscope slide. The released ascospores were washed with autoclaved H_2O onto 2% water agar (Fisher) amended with 30 μg/l streptomycin or tetracycline. The dispersed ascospores were then observed under a dissecting microscope, and individual ascospores were transferred with a fine needle (26 gauge) to potato dextrose agar plates.

DNA Isolation and Amplification Mycelium was harvested from 14-day-old colonies grown on a cellulose membrane sheet (Flexel Inc., Atlanta, GA) (Dunn, 1992) overlaying 2% malt agar. The mycelium was scraped off the membranes and 0.2-0.5 g were placed in a microfuge tube for DNA extraction procedures. DNA was extracted following a method similar to Rogers and Bendich (1985) as described in Chapter Two. The DNA concentration was estimated by

comparison with the lambda DNA-Hind III digest marker (Pharmacia) on an agarose gel stained with ethidium bromide. DNA solutions were diluted in 0.1 x TE buffer [1 mM Tris-HCl (pH 8.0), 0.1 mM EDTA (pH 8.0)] to a final concentration of approximately 25 ng/µl.

PCR reaction solutions were prepared as 25 µl volumes in 0.5 ml microfuge tubes. Reaction solutions contained approximately 25 ng of genomic DNA, 0.5 µM oligonucleotide primer (10-mer) (RAPD primer synthesis project - set #2, Dr. John E. Carlson, Biotechnology Laboratory, University of British Columbia), 200 µM of each dNTP, 0.25 units of Vent$_R$® DNA polymerase (New England Biolabs, Beverly, MA), 100 µg/ml BSA and 1 x DNA polymerase buffer [10 mM KCL, 10 mM $(NH_4)_2SO_4$, 20 mM Tris-HCl (pH 8.8), 2 mM or 4 mM $MgSO_4$, and 0.1 % Triton X-100). The reactions were overlaid with light mineral oil and amplifications were conducted in a BioOven thermal cycler (BioTherm, Arlington, VA) programmed for an initial cycle of 5 min at 94°C, 2 min at 35°C, and 2 min at 72°C. This was followed by 44 cycles of 30 s at 94°C, 60 s at 35°C, 90 s at 72°C, and a final extension cycle for 10 min at 72°C. Amplification products were separated by gel electrophoresis in 1.5% agarose in 0.5 x TBE (45 mM Tris-borate and 1 mM EDTA). Gels were then stained in ethidium bromide and viewed under 260 nm UV light.

Primer Selection and Data Analysis Samples of *L. korrae* DNA from two Ontario isolates and one British Columbia isolate were used to screen 100 RAPD primers for polymorphisms in *L. korrae* DNA. Primers that generated strong patterns of 10 bands or fewer, and showed polymorphism between the three

test isolates, were then used to test the 17 *L. korrae* field isolates. Two of these primers were also tested on the eight single ascospore isolates. All amplifications were repeated three times and only bands which were reproducible (recorded as present or absent) in all three replications were considered for data analysis.

Variation in the banding patterns between all pairs of isolates was analyzed using similarity coefficients based on the equation of Nei and Li (1979)

$$\text{Similarity } (S) = (2N_{xy})/(N_x + N_y)$$

were N_{xy} = number of shared bands between isolates "x" and "y"
N_x = number of bands produced by isolate "x"
N_y = number of bands produced by isolate "y"

Similarity coefficients were converted to genetic distances using the equation

$$D = 1/S-1$$

(Swofford and Olsen, 1990). Genetic distances were subjected to cluster analysis (SAS®) with unweighted pair-group method using arithmetic averages (UPGMA).

Analysis was performed individually for each RAPD primer with all 17 field isolates. In addition, mean similarity values for each isolate by isolate comparison were calculated by averaging similarity coefficients for all RAPD primers. These mean values were also converted to genetic distances with the above formula and used in the UPGMA clustering procedure. Genetic distance values for single ascospore progeny from the isolate LK30 were generated from tests using two RAPD primers (Table 3.2) and were analyzed using the UPGMA

clustering procedure.

Results

Collection and Isolation of *Leptosphaeria korrae* A total of 17 isolates (Table 3.1) were collected from a Kentucky bluegrass lawn in Guelph, Ontario, which showed numerous patches typical of NRS. The patches were first observed by a lawn care specialist in 1989, and the symptoms persisted and increased into 1993. Repeated observation of the site revealed increases of approximately 140% in the number of patches, from 1991 to 1993. Isolates collected in 1992 (Table 3.1) were obtained from patches first observed that year. During the sampling period in November 1992, a number of pseudothecia were observed on roots and crowns of samples.

Amplification of *Leptosphaeria korrae* DNA Screening of the 100 RAPD primers resulted in the selection of 13 potentially useful primers, for the detection of genetic polymorphism in isolates of *L. korrae* (Table 3.2). These primers generated simple banding patterns which revealed DNA polymorphisms between *L. korrae* isolates. Among these, seven primers were used to test DNA from the 17 Guelph isolates (Table 3.1). These tests resulted in the detection of 35 reproducible amplification products (RAPD markers). Depending on the isolate-primer combination, between 3 and 7 bands were consistently amplified and ranged in size from 0.29 kb to 1.8 kb. All seven primers demonstrated polymorphisms between isolates. Figure 3.1 illustrates the comparative banding profiles of DNA from the 17 field isolates when amplified with primer 105.

Primers 105 and 125 (Table 3.2) were used to test for

polymorphisms in eight single ascospore progeny from isolate LK30, which was fruited on wheat in the greenhouse. A total of 15 bands were amplified which ranged in size from 0.28 kb to 1.3 kb. Significant polymorphism was obtained from DNA of single ascospore isolates tested with primer 125 (Fig 3.2), but none was observed with primer 105.

Genetic Similarity Between *Leptosphaeria korrae* Isolates

The extent of similarity between pairs of isolates and the number of isolates clustering together varied with the primer used. When similarity coefficients for all pairs of isolates were averaged across the seven RAPD primers and converted to relative genetic distance values, the analysis showed four clusters of isolates using an average distance between clusters of 0.62 as a cut-off point (Fig 3.3; Appendix C). A phenogram based on the relative genetic distances for all isolates across all primers was constructed using the Neighbour UPGMA method in the software package Phylip (version 3.52c) (Fig 3.3). Results from the Neighbour UPGMA method was also found to be in close agreement with the SAS® UPGMA cluster analysis (Appendix C).

Figure 3.4 shows the disease distribution, and location where each isolate was collected. Each isolate was assigned a letter, A, B, C or D, to represent one of the four groups in which it was clustered. The distribution pattern of the four cluster groups shows some clumping, but is generally interspersed. Group "A" is found clumped in two separate areas at the north end of the lawn, but a single group "A" isolate is also found at the south end of the lawn. Group "B" isolates are found close to the centre and at the southeast

corner of the lawn and are separated from each other by approximately 5 m. Group "B" is the only group not represented in the cluster of isolates collected from the northwest corner of the lawn. The isolates belonging to group "C" are found at both the north and south ends of the lawn. The group "C" isolates at the north end of the lawn are interspersed with both group "A" and group "D" isolates. The group "C" isolates at the south end are found in pairs, approximately 4 m apart. Group "D" isolates were only found in the northwest corner of the lawn.

In order to determine if the genetic polymorphism observed in *L. korrae* was generated through meiosis, single ascospore cultures derived from a single pseudothecium were examined using RAPD analysis. There were no differences observed in the banding pattern produced for the eight single ascospore siblings and RAPD 105, however, RAPD 125 showed significant polymorphism between these isolates. Using results from RAPD 125 and the eight ascospore siblings, analysis with the SAS® UPGMA cluster procedure produced three clusters of isolates, separated by a genetic distance of 0.63 (Appendix C). A phenogram using the Neighbour UPGMA method in the software package Phylip (version 3.52c) was also constructed using the RAPD 125 and single ascospore data (Fig 3.5).

Table 3.1 Source of *Leptosphaeria korrae* isolates used in PCR-based DNA polymorphism assay.

Isolate[1] ID No.	Date collected	Location collected
LK11	Sept 1991	Independence Rd. Guelph, Ont.
LK15	Sept 1991	Independence Rd. Guelph, Ont.
LK16	Sept 1991	Independence Rd. Guelph, Ont.
LK27	June 1992	Independence Rd. Guelph, Ont.
LK29	June 1992	Independence Rd. Guelph, Ont.
LK35	Sept 1991	Independence Rd. Guelph, Ont.
LK36	June 1992	Independence Rd. Guelph, Ont.
LK37	June 1992	Independence Rd. Guelph, Ont.
LK38	June 1992	Independence Rd. Guelph, Ont.
LK39	June 1992	Independence Rd. Guelph, Ont.
LK40	June 1992	Independence Rd. Guelph, Ont.
LK41	June 1992	Independence Rd. Guelph, Ont.
LK42	June 1992	Independence Rd. Guelph, Ont.
LK43	June 1992	Independence Rd. Guelph, Ont.
LK44	June 1992	Independence Rd. Guelph, Ont.
LK45	Nov. 1991	Independence Rd. Guelph, Ont.
LK46	Nov. 1991	Independence Rd. Guelph, Ont.
LK30	May 1992	Markham, Ontario
LK30a	Feb. 1993	single ascospore progeny of LK30
LK30b	Feb. 1993	single ascospore progeny of LK30
LK30c	Feb. 1993	single ascospore progeny of LK30
LK30d	Feb. 1993	single ascospore progeny of LK30
LK30e	Feb. 1993	single ascospore progeny of LK30
LK30f	Feb. 1993	single ascospore progeny of LK30
LK30i	Feb. 1993	single ascospore progeny of LK30
LK30j	Feb. 1993	single ascospore progeny of LK30

[1] **All isolates from the Guelph study site were collected from separate NRS patches. Single ascospore siblings were derived from a single pseudothecium produced by the isolate LK30**

Table 3.2 RAPD primer sequences obtained from the Biotechnology Laboratory at University of British Columbia (RAPD primer synthesis project - Set #2) which gave reproducible banding patterns with DNA from *Leptosphaeria korrae* isolates.

UBC Primer Code	Sequence (5' to 3')
101	GCG GCT GGA G
105[ab]	CTC GGG TGG G
122[a]	GTA GAC GAG C
125[b]	GCG GTT GAG G
145[a]	TGT CGG TTG C
149	AGC AGC GTG G
153[a]	GAG TCA CGA G
171	TGA CCC CTC C
173[a]	CAG GCG GCG T
175[a]	TGG TGC TGA G
178[a]	CCG TCA TTG G
181	ATG ACG ACG G
188	GCT GGA CAT C

[a] RAPD primers used to test Guelph field isolates
[b] RAPD primers used to test single ascospore isolates

Fig 3.1 PCR amplification products from 17 isolates of *Leptosphaeria korrae* and RAPD primer 105. Approximately 10µl from each PCR reaction mixture was loaded per well in a 1.5% agarose gel. Lane 1, a 100 bp DNA ladder; Lane 2; LK11; Lane 3, LK15; Lane 4, LK16; Lane 5, LK27; Lane 6, LK29; Lane 7, LK35; Lane 8, LK36; Lane 9; LK37; Lane 10, LK38; Lane 11, LK39; Lane 12, LK40; Lane 13, LK41; Lane 14, LK42; Lane 15, LK43; Lane 16, LK44; Lane 17, LK45; Lane 18, LK46.

Fig 3.2 PCR amplification products from eight single ascospore isolates of *Leptosphaeria korrae* derived from a single pseudothecia and RAPD primer 125. Approximately 10 µl from each PCR reaction mixture was loaded per well in a 1.5% agarose gel. Lane 1; a 100 bp DNA ladder; Lane 2-9; PCR products from eight single ascospore products.

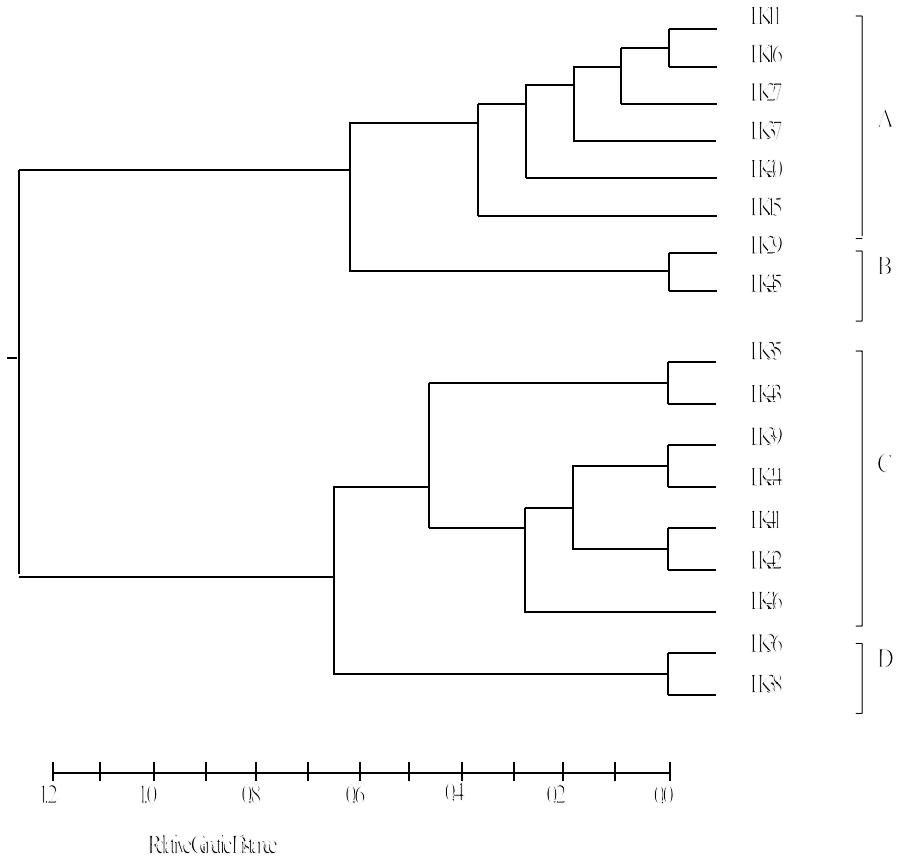

Fig 3.3 Phenogram of the relative genetic distances based on PCR generated RAPD markers, between *Leptosphaeria korrae* isolates collected from a single field site in Guelph, Ontario. Phenogram was constructed using Neighbour UPGMA method in the software package Phylip (version 3.52c).

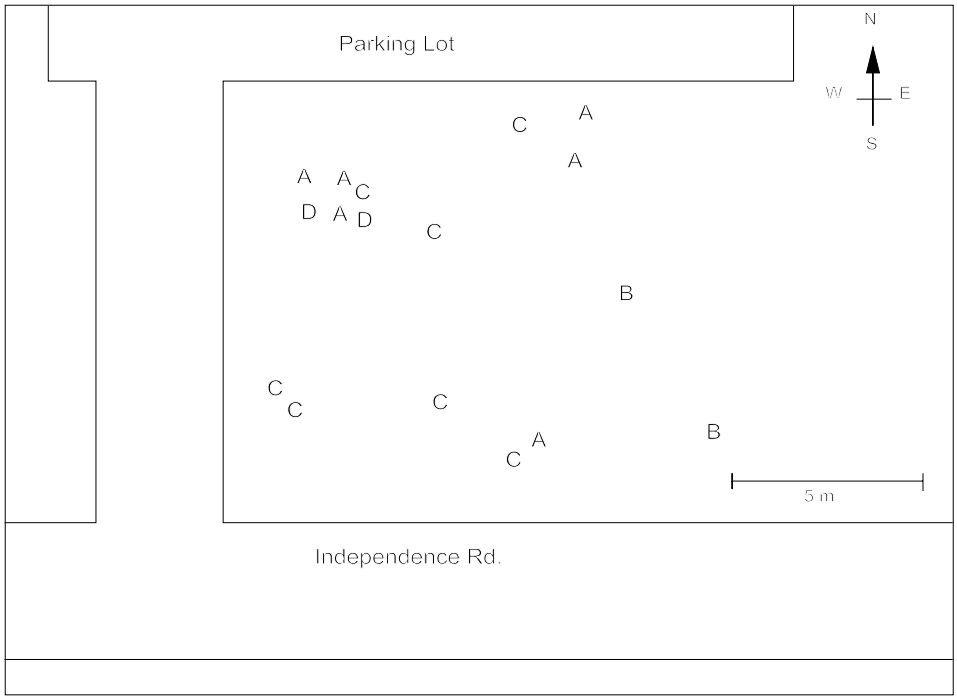

Fig 3.4 Disease distribution map for the 17*Leptosphaeria korrae* isolates collected from the study site in Guelph Ontario. All isolates have been assigned a letter, A, B, C, or D, representing one of the four types of isolates described through the clustering of the data from the polymorphism assay.

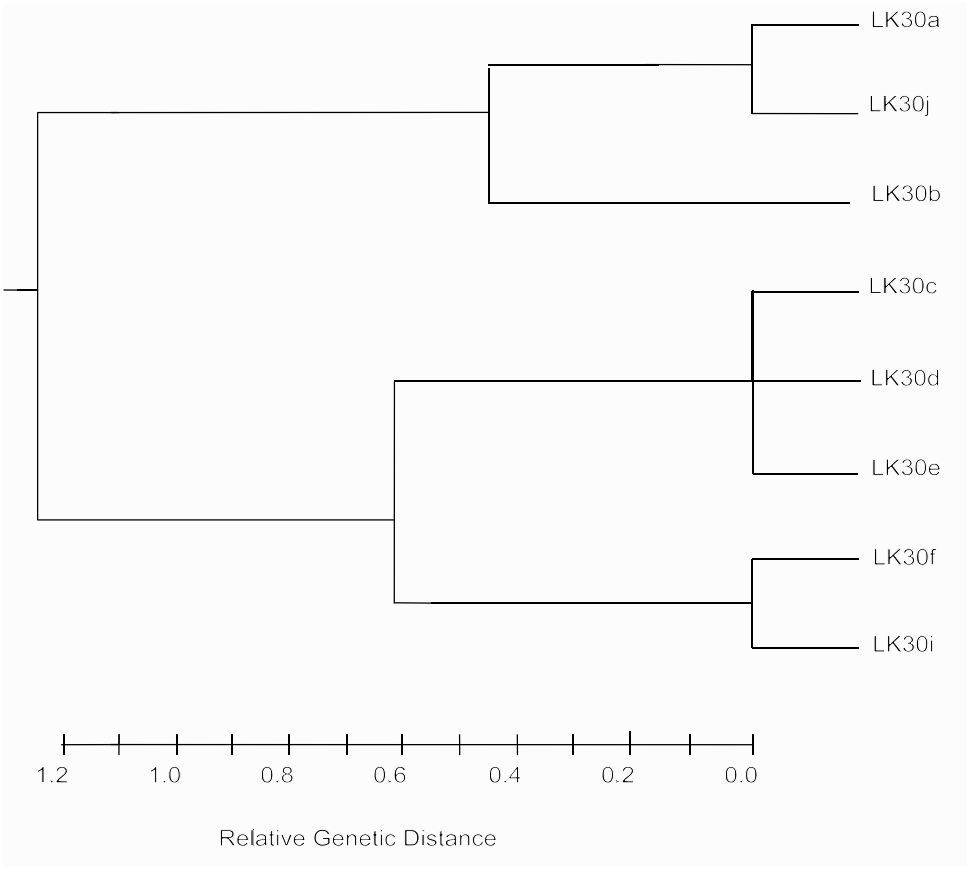

Fig 3.5 Phenogram of the relative genetic distances based on PCR generated RAPD markers, between single ascospore progeny from a single pseudothecium *Leptosphaeria korrae* isolate LK30. Phenogram was constructed using Neighbour UPGMA method in the software package Phylip (version 3.52c).

Discussion

In this study, DNA polymorphisms of *L. korrae* from sibling ascospore cultures and isolates collected from a single field site were detected using RAPD-PCR. Each of seven RAPD primers used to test isolates collected from the lawn in Guelph, generated banding patterns that showed some variation among the three replicate amplifications. These inconsistent bands may have been artifacts resulting from experimental error such as variation in the PCR amplification mixtures. For example, the presence of RNA which was not removed in the extraction procedure, can make it difficult to accurately determine and standardize the DNA concentration between samples (Ellsworth et al., 1993). Furthermore, differences in banding patterns have been noted if a standardized ratio of primer to template DNA concentration is not achieved across all samples (Ellsworth et al., 1993). There were, however, bands which were consistently amplified in all three replicates, and only those bands were included in the analysis.

In this study, it was uncertain whether the variability in banding patterns was due to the inconsistent amplification of legitimate bands or the presence of artifacts, as mentioned above. Omission of the bands which were not consistent ensured the reliability of the assay, but may have altered its ability to detect the true degree of genetic relatedness, excluding additional useful information (Kresovich et al., 1992).

Genetic polymorphisms have been found among single

ascospores derived from a single stroma of the homothallic species *H. truncatum*, and may be due to genetic rearrangement occurring during meiosis or during the parasexual cycle (Yoon and Glawe, 1993). Genetic recombination during the parasexual cycle has been shown to occur in *Penicillium roqueforti* DNA by the loss of functional antibiotic resistance genes (Durand et al., 1992). However, the parasexual cycle may only involve a small percentage of the individual fungal nuclei (Olive, 1963).

The work by Yoon and Glawe (1993) shows the isolates of *H. truncatum* separated into two distinct groups with a high degree of genetic difference. The genetic difference between the groups is described by an average distance of 0.7085. A slightly lower value was used to separate the groups of *L. korrae* isolates in this study. An average genetic distance of 0.62 clustered the *L. korrae* field isolates into four groups. This value indicated that the groups differed from each other by approximately 62% of the 35 RAPD markers generated.

The homothallic nature of *L. korrae* was demonstrated by Smiley and Craven Fowler (1984), when single ascospore cultures of *L. korrae* used in a pathogenicity test were found to produce sexual structures. These results have been reconfirmed by single ascospore pairing studies conducted in our lab by Tracey Voorberg (Appendix C, Tables C1 & C2).

The mechanism by which the genetic variability in this homothallic species is generated is unknown. Genetic variation within a species is most commonly generated through sexual reproduction involving two genetically unique but compatible mating types however, this cannot explain

polymorphism arising in homothallic species such as *L. korrae*. One possible explanation that may account for variation between *L. korrae* isolates, is chromosomal length polymorphism generated through meiotic recombination. This phenomena is thought to occur in *L. maculans*, through crossing-over events between parental homologues with chromosomes of unequal sizes (Plummer and Howlett, 1993). Some alternative explanations for the occurrence of genetic variation outside of sexual reproduction are, high degrees of insertions and deletions in fungal DNA (Garber and Yoder, 1985) or, as mentioned earlier, the genetic recombination during the parasexual cycle (McDonald and Martinez, 1990).

In the case of *L. korrae* , if the source of a new infection centre were mycelium from an adjacent NRS patch, both patches would be caused by the same isolate, and the chance of observing any genetic polymorphism would be low. If this was how the pathogen spreads, then it would seem reasonable that new patches would spread radially from a centre point, thus forming large clusters of the same isolate. Should an air-borne ascospore be responsible for a new infection centre, then genetically polymorphic ascospores would establish new patches that would be distinguishable from most of their neighbours. The spread of the disease by ascospores would also give a more random distribution of genetically unique isolates.

The map of disease distribution from the lawn where the *L. korrae* isolates were collected shows that the four groups of isolates were generally interspersed, suggesting ascospores may have played an role in the spread of the disease. For

example, the single group "A" isolate at the south end of the lawn was separated from other group "A" isolates by approximately 10 m, indicating that it may have been initiated by an ascospore. The release of ascospores may also, in part, be responsible for the random distribution throughout the lawn of group "C" isolates. The importance of ascospores in the spread of disease has been cited for the related turf pathogen *G. graminis*. Airborne dissemination of ascospores of *G. graminis*, is thought to account for take-all patch occurring on golf greens seeded with bentgrass following fumigation (Landschoot et al., 1993), as well as the appearance of the take-all in an area with no prior history of the disease (Garrett, 1970).

The distribution pattern of *L. korrae* isolates in the lawn was not completely random and some clumping of the four isolate groups was observed. This was most evident with the two clumps of group "A" isolates at the north end of the lawn. This indicates that lawn care equipment such as aerators and dethatching machines or mycelial spread may have also played a role in the disease spread. Lawn care equipment, however, is generally operated in straight lines parallel to the edge of the lawn. If lawn care equipment were responsible for the disease spread, then the expected distribution pattern would show identical isolate in parallel lines following the same path taken by the machinery. This grid pattern is not obvious in Figure 3.4 and therefore, mycelial growth may be responsible for the clumping of identical isolates.

Conclusions drawn from this study are limited since only one field site was used. However, the genetically diverse

population existing within this small area, strongly suggests that the release of genetically polymorphic ascospores may have been responsible for establishing some of the new infection centres.

GENERAL DISCUSSION

Necrotic ring spot of Kentucky bluegrass is a relatively new disease, and therefore, this study endeavoured to address some fundamental concerns involved with this disease. To achieve the goals set out for this study both morphological and molecular techniques were employed.

This study has shown that NRS is present throughout southern Ontario. The confirmation of *L.korrae* as the causal agent of NRS in Ontario was achieved using established techniques to isolate and then identify the pathogen based on the morphology of its sexual structures. The technique used for the induction of sexual structures of *L. korrae* ultimately provided a means of positive identification of the pathogen. Unfortunately, the total number of isolates identified and the time required for this technique was found to be prohibitive for both diagnostic and research purposes.

In order to fulfil the need for a quick and reliable identification of NRS, *L. korrae*-specific DNA primers were used to test a PCR-based assay for the detection of the pathogen. It was shown that when used with PCR, this species-specific primer pair (LK17S/5.8SC) was able to detect *L. korrae* DNA isolated from pure fungal cultures and infected plant tissue. There were, however, PCR-inhibiting substances present in the DNA samples isolated from infected root samples. These substances lowered the intensity of the *L.*

korrae specific PCR amplification product. Further work using other DNA purification techniques is still required to completely eliminate these inhibitors. Nevertheless, the use of PCR with the LK17S/5.8SC primer pair permits reliable diagnosis of suspected NRS samples within several hours. This is a vast improvement over the eight weeks previously required to identify the pathogen with morphological techniques. The elimination of this lengthy time requirement, should accelerate further studies of the epidemiology of NRS.

Genetic variability in *L. korrae* estimated by RAPD analysis was observed in single ascospore siblings, as well as in a population of Ontario isolates collected from a single field site. The PCR reaction conditions used in this study to generate the observed genetic polymorphism, produced both consistent and inconsistent amplification products. The conservative approach of considering only the consistent bands in the analysis, ensured the reliability of this assay, but may have also reduced the range of detectable polymorphism existing between *L. korrae* isolates.

The mechanism by which this homothallic species generates genetic variability is uncertain. The genetic polymorphism observed suggests that ascospores may play an important role in the genetic variability seen in this population of *L. korrae* field isolates. The distribution pattern produced by the four isolate types identified in this study, suggests that both mycelial growth and the release of ascospores are involved in the spread of NRS.

This study required the completion of each of its objectives in a sequential manner. Initially, proven

morphological techniques were used to confirm the presence of NRS in Ontario, then using PCR technology, the time requirement for pathogen identification was shortened and the accuracy of its detection increased. Finally, the PCR-based assay permitted the identification of many of the isolates used in the study which detected genetic polymorphism in *L. korrae*. Further work is still needed to gain a complete understanding of NRS, but the findings of this study should ultimately invite further research into both the epidemiology of the disease and the biology of its pathogen.

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APPENDIX A

Table A.1 Source of isolates used in the confirmation of *Leptosphaeria korrae* as the causal agent of necrotic ring spot in Ontario. Isolates were tentatively identified on the basis of colony morphology and positive identification was based on the production of sexual structures (pseudothecia).

ID NUMBER (ALTERNATE ID No.)	DATE OF ISOLATION/ SUBCULTURE	HOST	SOURCE	TENTA-TIVE ID	POSI-TIVE ID
90-447-1 (LK-06)	91/4/3 slant stock	KB	Leslie McDonald, BC Min. Agriculture	YES	YES[A]
90-447-2 (LK-07)	91/4/3 slant stock	KB	Leslie McDonald, BC Min. Agriculture	YES	YES[A]
89-570 (LK-05)	91/4/3 slant stock	KB	Leslie McDonald, BC Min. Agriculture	YES	YES[A]
90-794 (LK-08)	91/4/3 slant stock	KB	Leslie McDonald, BC Min. Agriculture	YES	YES[A]
90-797 (LK-10)	91/4/3	KB	Leslie McDonald, BC Min. Agriculture	YES	YES[A]
90-796 (LK-09)	91/4/3	KB	Leslie McDonald, BC Min. Agriculture	YES	YES[A]
K1	91/05/02	KB	Kennedy Lawn Supply	YES	NO[A]
K2	91/05/02	KB	Kennedy Lawn Supply	YES	NO[A]
OLC-1	91/05/24	KB	Lockie Wye. Dufferin Land Serv. 5 Elm Ave. Orangeville, Ont. L9W 3G3	YES	NO[A]
OLC-2 (LK-04)	91/05/24	KB	Lockie Wye. Dufferin Land Serv. 5 Elm Ave. Orangeville, Ont. L9W 3G3	YES	YES[A]
PPO91-1	91/05/31	CB	Lambton Golf Course	NO	NT
PPO91-2	91/06/01	CB	Bruce Dodson, Hawkridge Golf Course, RR#1, Orillia, Ont. L3V 6H2	NO	NT
PPO91-3	91/06/01	CB	Bruce Dodson, Hawkridge Golf Course, RR#1, Orillia, Ont. L3V 6H2	NO	NT

PPO91-4	91/06/02	KB	Rudy Brown, CAT, Ridgetown, Ont. N0P 2C0	NO	NT
PPO91-5	91/06/02	KB	Rudy Brown, CAT, Ridgetown, Ont. N0P 2C0	NO	NT
PPO91-6	91/06/02	KB	Rudy Brown, CAT, Ridgetown, Ont. N0P 2C0	NO	NT
PPO91-7	91/06/11	KB	G. Harpenuk R.R #1 Port Colbourne Ont. Pest Clinic #91-127	YES	NO^
PPO91-8	91/07/04	CB	D. McNeil, Greenhills Golf Club, RR#3 Lambeth, N0L 1S0, 519-652-5033	NO	NT
PPO91-9	91/07/04	CB	D. McNeil, Greenhills Golf Club, RR#3 Lambeth, N0L 1S0	NO	NT
PPO91-10	91/07/04	CB	D. McNeil, Greenhills Golf Club, RR#3 Lambeth, N0L 1S0	YES	NO^
PPO91-11	91/07/05	KB	Metro Weed Control, 205 Champaigne Dr.#6, Downsview. Pest Clinic #91-420	NO	NT
PPO91-12 (LK-01)	91/07/05	KB	Imperial Rd., Guelph Pest Clinic #91-513	YES	YES^
PPO91-13	91/07/08	CB	London Highlands, 205 Commissioners London. Pest Clinic.	NO	NT
PPO91-14	91/07/08	CB	Greenhills Country Club, Lambeth, Ont. Pest Clinic.	NO	NT
PPO91-15	91/07/08	CB	Greenhills Country Club, Lambeth, Ont. Pest Clinic.	NO	NT
PPO91-16	91/07/10	CB	Emerald Hills Pest Clinic.	NO	NT
PPO91-17	91/07/10	CB	Emerald Hills Pest Clinic.	NO	NT
PPO91-18	91/07/23	KB	25 Woodland Glen Guelph. c/o J.Cruickshank, Outdoor Services.	YES	NO^
PPO91-19	91/07/23	KB	Annette Anderson. 3 Crowe, Guelph, Ont.	YES	NO^
PPO91-20	91/07/25	AB	Bluewater Golf Course, Bayfield. Pest Clinic.	NO	NT
PPO91-21	91/07/26	KB	Chemlawn-Woodbridge 901 Rowntree Dairy Woodbridge	YES	NO^
PPO91-22 (LK-21)	91/07/26	CB	Chemlawn-Woodbridge 61 Binscarth Cres Thornhill	YES	YES^

PPO91-23	91/07/26	CB	Chemlawn-Woodbridge 54 Helena Gdns Thornhill	NO	NT
PPO91-24 (LK-02)	91/08/07	KB	Paul Grobe/Grobe Nurseries-McDonald's at Sportsworld, King St. Kitchener.	YES	YES^A
PPO91-25	91/08/07	KB	Paul Grobe/Grobe Nurseries- McD. Sp. Wrld, corner	YES	NO^A
PPO91-26 (LK-03)	91/08/07	KB	Paul Grobe/Grobe Nurseries-Kieswetter Motors, near sign, 4202 King St. E, Kitchener.	YES	YES^A
PPO91-27 (LK-20)	91/08/07	KB	Paul Grobe/Grobe Nurseries-Kieswetter Motors	YES	YES^A
PPO91-28	91/08/09	KB	Paul Grobe/Grobe Nurseries-Ardelt, near sign, 115 Ardelt St. Kitchener	NO	NT
PPO91-29 (LK-19)	1/08/09	KB	Paul Grobe/Grobe Nurseries-Ardelt, new patch	YES	YES^A
PPO91-30	91/08/09	KB	Paul Grobe/Grobe Nurseries-Hurlbut Corp. near hedge, #20 Hansen at Ardelt	NO	NT
PPO91-31	91/08/09	KB	Paul Grobe/Grobe Nurseries-Hurlbut Corp.	NO	NT
PPO91-32 (LK-18)	91/08/13	KB	Jay Terryberry, 3396 Avondale, Windsor. N9E 1X6	YES	YES^A
PPO91-33	91/08/13	N/A	Pest Clinic #91-576	YES	NT
PPO91-34 (LK-12)	91/08/13	N/A	Pest Clinic #91-619	YES	YES^A
PPO91-35	91/08/13	N/A	Pest Clinic #91-739	NO	NT
PPO91-36	91/08/14	N/A	Stevensville Lawn Service, 3639 Main St. E Stevensville Ont. L0S 1S0	NO	NT
PPO91-37	91/08/14	AB	Art Ostwald, whitevale Golf Club, P.O. Box 30, whitevale, Ont., L0H 1M0	NO	NT
PPO91-38	91/08/14	AB	Art Ostwald, whitevale Golf Club, P.O. Box 30, whitevale, Ont., L0H 1M0	YES	NT
PPO91-39	91/08/20	KB	Kelly's Lawn Care RR#3 Peterborough	YES	NO^A
PPO91-40	91/08/20	KB	Pest Clinic Sample #91-806	YES	NO^A

PPO91-41	91/08/22	KB	Mrs. Vye Pace, 187 Cole Rd. Guelph, N1G 4A2	NO	NT
PPO91-42 (LK-17)	91/08/22	KB	Robert Bag, 189 Cole Rd. Guelph, N1G 4A2	YES	YES^
PPO91-43 (LK-16)	91/09/20	KB	POLYCON Ltd., Independence Rd. Guelph, Ont.	YES	YES^
PPO91-44 (LK-15)	91/09/20	KB	POLYCON Ltd., Independence Rd. Guelph, Ont.	YES	YES^
PPO91-45 (LK-35)	91/09/20	KB	POLYCON Ltd., Independence Rd. Guelph, Ont.	YES	YES^
PPO91-46 (LK-11)	91/09/20	KB	POLYCON Ltd., Independence Rd. Guelph, Ont.	YES	YES^
PPO91-47 (LK-23)	91/09/27	KB	Kennedy Lawn Spray Barrie Ont.	YES	YES^
PPO91-48	91/09/27	KB	Kennedy Lawn Spray Barrie Ont.	NO	NT
PPO91-49	91/09/27	KB	Kennedy Lawn Spray Barrie Ont.	NO	NT
PPO91-50 (LK-13)	91/09/27	KB	Lawn Service Plus Ottawa Ont.	YES	YES^
PPO91-51	91/09/29	KB	Pest Clinic #91-928	NO	NT
PPO91-52 (LK-22)	91/09/29	KB	Pest Clinic #91-919a	YES	YES^
PPO91-53 (LK-14)	91/09/29	KB	Pest Clinic #91-919b	YES	YES^
PPO91-54	91/09/29	CB	Bay of Quinte Country Club Belleville, Ont.	NO	NT
PPO91-55	91/11/20	KB	Pest Clinic: Kelly's Lawn Care	YES	NT
PPO91-56	91/11/25	KB	Pest Clinic #91-10667	NO	NT
PPO91-57	91/11/26	KB	Pest Clinic #91-1099	YES	NO^
PPO91-58 (LK-30)	92/5/11	KB	Weedman, Markham, Ont.	YES	YES^
PPO91-59	92/5/11	KB	Weedman, Markham, Ont.	YES	NO^
PPO91-60 (LK-34)	92/5/11	KB	Weedman, Stouffville, Ont.	YES	YES^
PPO91-61 (LK-25)	92/5/11	KB	Weedman, Markham, Ont.	YES	YES^
PPO91-62	92/5/11	KB	Weedman, Markham, Ont.	YES	NO^
PPO91-63	92/5/11	KB	Weedman, Markham, Ont.	YES	NO^
PPO92-64	92/5/11	KB	Weedman, Markham, Ont.	YES	NO^
PPO92-65	92/5/12	KB	Weedman, Markham, Ont.	NO	NT
PPO92-66 (LK-26)	92/5/12	KB	Weedman, Markham, Ont.	YES	YES^
PPO92-67	92/5/12	KB	Sandra Cook, Erin	YES	NT
PPO92-68 (LK-31)	92/5/12	KB	Nutri-Lawn, #1 Ancaster, Ont.	YES	YES^

PPO92-69	92/5/12	KB	Nutri-Lawn, #2 Ancaster, Ont.	YES	NT
PPO92-70 (LK-32)	92/5/12	KB	Nutri-Lawn, #3 Ancaster, Ont.	YES	YES[A]
PPO92-71	92/5/12	KB	Weedman Port Elgin, Ont.	NO	NT
PPO92-72	92/5/20	KB	Dol Sod Farm, Ont.	NO	NT
PPO92-73	92/5/29	CB	Compact Sod Farm, Ont.	NO	NT
Gg-02	92/6/10	CB	Nutri-Lawn, Victoria, B.C. (Suspected TAP)	TAP	NT
Gg-01	92/6/10	CB	Nutri-Lawn, Victoria, B.C. (Suspected TAP)	TAP	NT
PPO92-74	92/6/29	KB	(POLY CON) Guelph, Ont. GENOTYPE TESTING	YES	NT
PPO92-75 (LK-36)	92/6/29	KB	(POLY CON) Guelph, Ont. GENOTYPE TESTING	YES	YES[B]
PPO92-76 (LK-37)	92/6/29	KB	(POLY CON) Guelph, Ont. GENOTYPE TESTING	YES	YES[B]
PPO92-77 (LK-38)	92/6/29	KB	(POLY CON) Guelph, Ont. GENOTYPE TESTING	YES	YES[B]
PPO92-78 (LK-39)	92/6/29	KB	(POLY CON) Guelph, Ont. GENOTYPE TESTING	YES	YES[B]
PPO92-79 (LK-40)	92/6/29	KB	(POLY CON) Guelph, Ont. GENOTYPE TESTING	YES	YES[B]
PPO92-80 (LK-27)	92/6/29	KB	(POLY CON) Guelph, Ont. GENOTYPE TESTING	YES	YES[A]
PPO92-81 (LK-41)	92/6/29	KB	(POLY CON) Guelph, Ont. GENOTYPE TESTING	YES	YES[B]
PPO92-82	92/6/29	KB	(POLY CON) Guelph, Ont. GENOTYPE TESTING	YES	NT
PPO92-83 (LK-42)	92/6/29	KB	(POLY CON) Guelph, Ont. GENOTYPE TESTING	YES	YES[B]
PPO92-84 (LK-43)	92/6/29	KB	(POLY CON) Guelph, Ont. GENOTYPE TESTING	YES	YES[B]
PPO92-85 LK-44)	92/6/29	KB	(POLY CON) Guelph, Ont. GENOTYPE TESTING	YES	YES[B]
PPO92-86 (LK-28)	92/6/29	KB	(POLY CON) Guelph, Ont. GENOTYPE TESTING	YES	YES[A]
PPO92-87	92/6/29	KB	(POLY CON) Guelph, Ont. GENOTYPE TESTING	YES	NT
PPO92-88 (LK-29)	92/6/29	KB	(POLY CON) Guelph, Ont. GENOTYPE TESTING	YES	YES[A]
PPO92-89	92/6/29	KB	(POLY CON) Guelph, Ont. GENOTYPE TESTING	YES	NT
PPO92-90	92/7/2	KB	Pest Clinic #95-510	YES	NT

PPO92-91	92/7/10	FSC	Pest Clinic #92-648	NO	NT
PPO92-92	92/7/10	KB	Pest Clinic #92-687	NO	NT
PPO92-93	92/7/10	KB	Pest Clinic #92-693	NO	NT
PPO92-94	92/7/10	CB	Pest Clinic #92-707	NO	NT
PPO92-95	92/7/10	KB	Pam Charboneau (GTI)	YES	NT
PPO92-96	92/7/11	KB	Pest Clinic #92-578	NO	NT
PPO92-97	92/7/11	KB	Pest Clinic #92-683	NO	NT
PPO92-98	92/7/11	CB	Osprey Valley Golf	NO	NT
PPO92-99	92/8/4	CB	Markham Golf, Markham, Ont. (Suspect TAP)	NO	NT
PPO92-100	92/8/10	CB	Victoria Park Golf, (Suspect TAP)	NO	NT
PPO92-101	92/8/31	KB	Bracebridge, Ont.	NO	NT
PPO92-102	92/10/8	KB	Manderly Sod, Pinecourt Que.	YES	NT
PPO92-103	92/10/8	KB	Innerkip Golf Course Innerkip, Ont.	YES	NT
PPO92-104	92/10/8	KB	Ontario Hydro	YES	NT
PPO92-105	92/10/8	CB	King Valley Golf, (TAP Sample)	NO	NT
PPO92-106	92/10/8	KB	Bruceridge, Ont.	YES	NT
PPO92-107	92/10/8		Pest Clinic #92-1022	YES	NT
PPO92-108	92/10/8	KB	Pest Clinic #92-1085	YES	NT
PPO92-109	92/10/8	KB	Pest Clinic #92-1062	NO	NT
PPO92-110	2/10/8	KB	Pest Clinic #92-922	YES	NT
PPO92-111 (LK-24)	92/10/8	KB	Lewis Weed Exterminator (PSEUDOTHECIA ON ROOTS)	YES	YES
PPO92-112	92/10/8	KB	Weedman, Scarborourgh, Ont.	YES	NT
PPO92-113	92/10/22	PR	Weedman, Niagara Falls, Ont.	NO	NT
PPO92-114	92/10/30	AB	City of Hamilton,	NO	NT
PPO92-115	92/10/30	CB	Carrie Foss WSU (TAP)	TAP	NT
PPO92-116	92/10/30	CB	Carrie Foss WSU (TAP)	TAP	NT
PO92-117 (LK-45)	92/11/15	KB	POLYCON (3 Samples/ring, A, B, C) (PSEUDOTHECIA ON ROOTS)	YES	YES
PPO92-118 (LK-46)	92/11/15	KB	POLYCON (3 Samples/ring, A, B, C) (PSEUDOTHECIA ON ROOTS)	YES	YES
PPO92-119	92/11/	CB	L. MacDonald B.C. Min. Ag. # 92-746 (TAP)	TAP	NT

PPO92-120	92/11/	CB	L. MacDonald B.C. Min. Ag. # 92-778 (TAP)	TAP	NT
PPO92-121	93/1/4	CB	L. MacDonald B.C. Min. Ag. # 92-727 (TAP)	TAP	NT
PPO92-122	93/1/4	KB	Nature Plus Inc.	NO	NT
PPO93-123	93/7/20	KB	POLYCON Ltd., Independence Rd. Guelph, Ont.	YES	NT
PPO93-124	93/7/20	KB	POLYCON Ltd., Independence Rd. Guelph, Ont.	YES	NT
PPO93-125	93/7/20	KB	POLYCON Ltd., Independence Rd. Guelph, Ont.	NO	NT
PPO92-126	93/7/20	KB	POLYCON Ltd., Independence Rd. Guelph, Ont.	YES	NT
PPO92-127	93/7/20	KB	POLYCON Ltd., Independence Rd. Guelph, Ont.	YES	NT
PPO92-128	93/7/10	KB	POLYCON Ltd., Independence Rd. Guelph, Ont.	YES	NT
PPO93-129	93/7/10	KB	POLYCON Ltd., Independence Rd. Guelph, Ont.	YES	NT

NA = Information not available.
NT = Isolate not tested.
YES[A]= Isolate tested, and positive identification based on production of sexual structures.
NO[A] = Isolate tested, but did not produce sexual structures.
YES[B]= Positive identification based on PCR-based probe.
TAP = Take-all patch.

APPENDIX B

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90-796   -TCCCCGTTGGTGAACCAGCGGAAGG-ATCATTACAGAATA------------GTAACAGG--CCCAAAGTG-CA 58
LK-4     -TCCCCGTTGGTGAACCAGCGGAAGG-ATCATTACACAGTATA----------GTAACAGG--CCCAAAGTG-CA 60
UNITY    -TCCCCGTTGGTGAACCAGCGGAAGG-ATCATTACCC-TTCTATCAGAGGATTGGTGTCAGGATTTCGGCCTTTGG 73
LEROY    -TCCCCGTTGGTGAACCAGCGGAAGGGATCATTACCCATTT-TCAAAGCACTGCG------GGCCTCGATCAGTGG 68
NC       -TCCCCGTTGGTGAACCAGCGGAAGGGATCATTACAGAGTTG----------CAAAACTCCCACAAACCATCGCG 64

90-796   G---CACAAACT-GCATGGGCGGGTTATGTCTATTACCCTTGTTTAT--TGAGTAACCTA----------TGTTTC 118
LK-4     G---CACAAACT-GCATGGGCGGGTTATCTCTATTACCCTTGTTTATATTGAGTA-CCTA---------TTGTTTC 122
UNITY    CTTACTTTCTGGCCCTTTCCTTTCTGATTC----TACCCATGTTTTTT--GCGTA--CTA--------TTTGTTTC 134
LEROY    C---------GGCAGTCTACTT--TGATTC----TGCCCATGTTTTTT-GCGGTA--CTA-------TTTGGTTTC 119
NC       AATCTTACCCGTACGGTTGCCT-CGGCG-CTGGCGGTCC-GGAA-----AG-GC---CTT-CGGGCCTCCCGGATC 127

90-796   CTT-GGTGGGCTTGCCTGCCAAAA-GGA----CACCCCATTGAACCT-ATTTA---TTTTT-AATCAG-CGTC--T 181
LK-4     CTT-GGTGGGCTTGCCTGCCAAAA-GGA----CACCCCATTGAACCT-ATTTA---TTTTT-AATCAG-CGTTC-T 185
UNITY    CTTNGGTAGGCTTGCCTGCCAAAA-GGA--GGTACC-TTTTCCTACC-ACTT-GCAATTGC-AGTCAG-CGTCAGT 202
LEROY    CTTGGGTGGGCTTGCCCGCCAAAAAATT--GGATCCCC-TAAA-ACCAACTT-GCAATTGC-AGTCAG-CGTCAGT 187
NC       CTCGGGTCTCCC-GCTCGCGGCTGCCCGCCGGAGTGCCG-AAACT-AAACTCTTGATTTTT-ATGTCTCTCTGAGT 199

90-796   TGAA-T-AACAATAAT-AATTAC-AACTTTCAACAACGGNNCTCTTGGTTCTGGCATCGATGAAGAACGCAGC-   250
LK-4     TGAA-T-AACAATAATTAATAATGTAA-TTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGC-   255
UNITY    TACACTGTA------TAAATT-ACTTCTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGC-   268
LEROY    AACACTGTAA-----TAAATT-ACTTCTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGC-   254
NC       AA-ACTTTTAA---AT-AAGTCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGC-   267
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Fig B.1 Alignment of the ITS-1 sequences with flanking 17S and 5.8S rDNA from *Leptosphaeria korrae* LK-4 and 90-796, *Leptosphaeria maculans* Unity and Leroy , and *Neurospora crassa* NC. Position of the primers are underlined. LK17S primer position starts at base number 63 for LK-4 and base number 61 for 90-796. The 5.8SC primer position for both *L. korrae* isolates is shown at the 3' end of the sequence. The 17S and 5.8S rRNA sequences of *N. crassa* are described by Chambers et al. (1986) and are represented within the shaded area and aligned with *L. korrae* and *L. maculans*. Sequencing and primer development was performed by Dr. B. Xue and Dr. P Goodwin at the University of Guelph.

APPENDIX C

Table C.1 ***Leptosphaeria korrae*** **isolate LK01 was paired with itself and five of its single spore progeny were paired in all possible combinations to study its sexual behaviour. Pseudothecia were produced, indicated by "*", in 14 of the pairings. All isolates except LK01.4 produced pseudothecia when paired with themselves. These results indicate that** ***L. korrae*** **isolate LK01 is homothallic.**

	LK01	LK01.1	LK01.2	LK01.3	LK01.4	LK01.5
LK01	*					
LK01.1		*	*	*	*	*
LK01.2			*	*	*	*
LK01.3				*	*	-
LK01.4					-	*
LK01.5						*

Table C.2 ***Leptosphaeria korrae*** **isolate LK05 was paired with itself and five of its single spore progeny were paired in all possible combinations to study its sexual behaviour. Pseudothecia were produced, indicated by "*", in 15 of the pairings. All isolates produced pseudothecia when paired with themselves. These results indicate that** ***L. korrae*** **isolate LK05 is homothallic.**

	LK05	LK05.6	LK05.7	LK05.8	LK05.9	LK05.10
LK05	*					
LK05.6		*	*	*	*	*
LK05.7			*	*	*	*
LK05.8				*	*	-
LK05.9					*	*
LK05.10						*

Table C.3 SAS® UPGMA cluster analysis data generated from genetic distances for 17 field isolates of *Leptosphaeria korrae* averaged across seven RAPD primers.

(Average linkage cluster analysis)

Number of Clusters	Clusters Joined		Pseudo F	Pseudo t**2	Normalized RMS Distance	
16	LK11	LK16	149.49	.	0.08	
15	CL16	LK27	102.76	2.11	0.11	
14	CL15	LK37	77.85	2.26	0.14	
13	LK41	LK42	74.16	.	0.16	12
	LK39	LK44	72.18	.	0.17	11
	LK35	LK43	72.75	.	0.18	10
	LK29	LK45	71.81	.	0.20	9
	CL14	LK40	66.61	4.14	0.22	8
	CL12	CL13	64.97	2.80	0.22	7
	LK36	LK38	60.49	.	0.34	6
	CL8	LK46	54.89	4.44	0.36	5
	CL9	LK15	51.44	9.43	0.41	4
	CL11	CL6	44.69	7.72	0.47	3
	CL5	CL10	38.90	14.72	0.62[1]	
2	CL4	CL7	56.03	6.59	0.63	
1	CL3	CL2	.	56.03	1.30	

[1] The genetic distance value of 0.62 was the lowest value showing over 50% differences between clusters and therefore was chosen arbitrarily to separate unique clusters.

Table C.4 SAS® UPGMA cluster analysis data generated from genetic distances for eight single spore isolates of *Leptosphaeria korrae* with RAPD primer 105.

(Average Linkage Cluster Analysis)

Number of Clusters	Clusters Joined		Pseudo F	Pseudo t**2	Normalized RMS Distance
7	C	D	.	.	0.00
6	CL7	E	.	.	0.00
5	F	I	.	.	0.00
4	A	J	.	.	0.00
3	CL4	B	61.71	.	0.45
2	CL6	CL5	28.09	.	0.63[1]
1	CL3	CL2	.	28.09	1.30

[1] The genetic distance value of 0.63 was the lowest value showing over 50% differences between clusters and therefore was chosen arbitrarily to separate unique clusters.