Ecology and Taxonomy of *Leptosphaerulina spp*. Associated with Turfgrasses in the United States

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Abstract

Leptosphaerulina spp. are common fungi that have been reported to colonize several turfgrass species. Controversy exists regarding the relationship of *Leptosphaerulina spp.* and their turfgrass hosts. The fungus has been classified as a saprophyte, senectophyte, weak pathogen, and pathogen of turfgrasses. There has also been conflicting reports regarding the delineation of species within the genus *Leptosphaerulina*. Because of the uncertainty regarding the ecology and taxonomy of the genus in relation to turfgrasses the present study was undertaken. The ITS and EF-1α gene regions were sequenced and analyzed to compare to the multiple taxonomic schemes reported in the literature. The ITS region offered no resolution of species; however, the phylogeny of the EF-1α gene was consistent with the six-species model of Graham and Luttrell. Inoculation experiments were performed on unstressed and artificially stressed plants to determine whether the fungi are pathogens, senectophytes, or saprophytes of turfgrasses. Perennial ryegrass and creeping bentgrass plants were stressed by placing them in a dew chamber set at 38ºC, 100% R.H., and no light for two and one days respectively. Plants were inoculated with cultures of *Leptosphaerulina* isolated from turfgrasses, and maintained at optimum conditions reported for infection and colonization. There was no visible difference between inoculated and uninoculated plants, and examination of cleared and stained leaves with a light microscope revealed spores that germinated and produced appressoria, but failed to penetrate the epidermal cells. The lack of infection and colonization suggests that *Leptosphaerulina spp.* are saprophytes of turfgrasses.

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Introduction

Leptosphaerulina leaf blight is a common problem of amenity turfgrasses during humid weather in the United States. The disease is characterized by a general leaf blighting of small patches to large stands of turfgrasses. *Leptosphaerulina spp.* are most frequently found colonizing necrotic creeping bentgrass (*Agrostis stolonifera* L.), perennial ryegrass (*Lolium perenne L.*) and Kentucky bluegrass leaves (*Poa pratensis* L.) from the late spring through fall. Inconsistencies exist in the literature as to the species of *Leptosphaerulina* that incite the disease as well as the ecological relationship between the fungus and the turfgrass. Additionally, little is known about the survival, pathogenic potential, and control of the fungus (Smith et al., 1989). It was for these reasons that the present study to investigate the etiology of Leptosphaerulina leaf blight was undertaken.

Chapter 1

Taxonomy of *Leptosphaerulina spp*. in the United States

Introduction

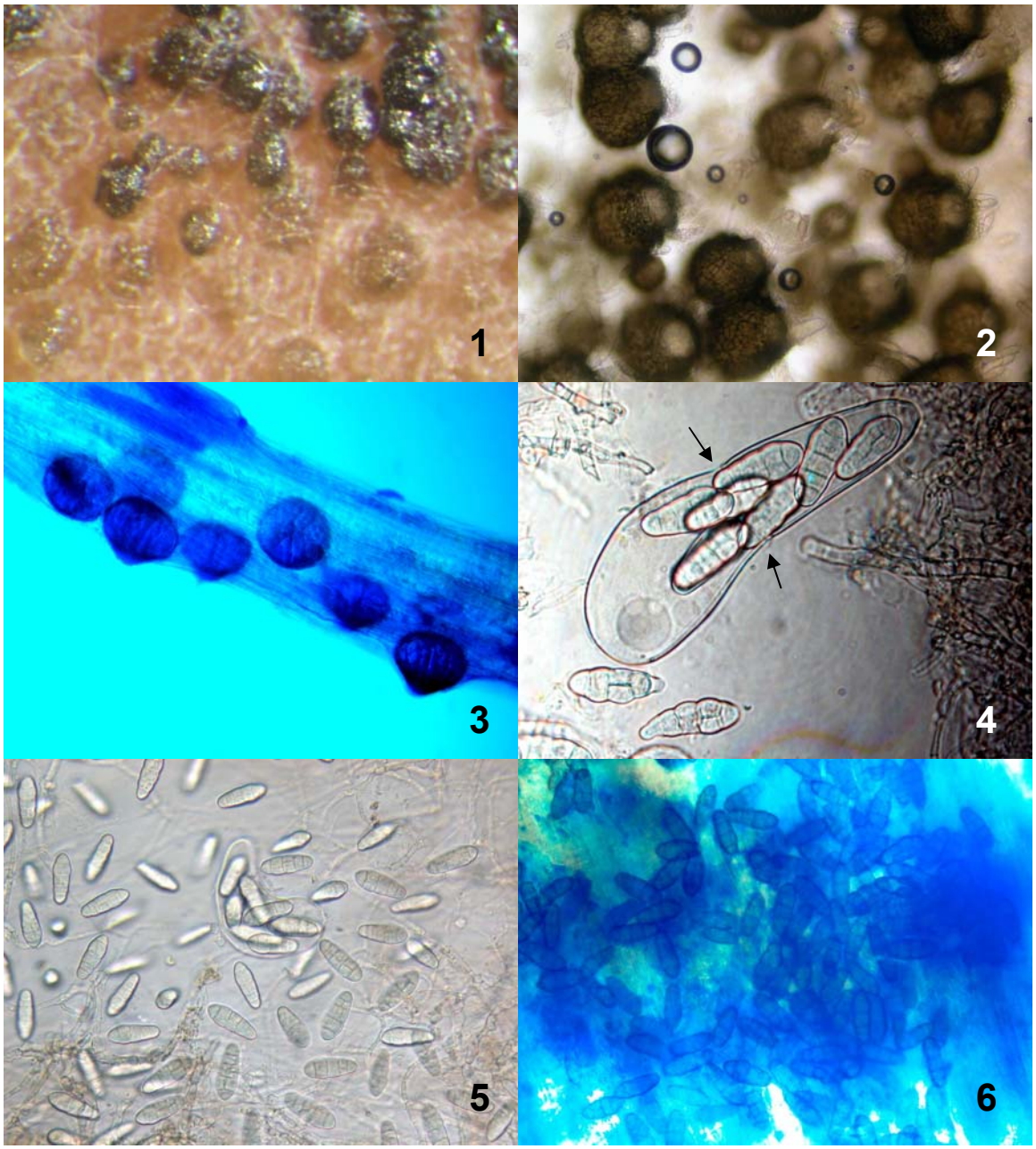
The Genus *Leptosphaerulina* McAlpine

The genus *Leptosphaerulina* comprises roughly twenty-five species that are endemic to North America, South America, Europe, Asia, Africa, and Australia (Inderbitzin et al., 2000; Roux, 1986; Irwin and Davis, 1985; Graham and Luttrell, 1961; McAlpine, 1902). The genus was erected by Daniel McAlpine in 1902, designating *Leptosphaerulina australis* as the type specimen. The fungus was described from apricot (*Prunus armeniaca* L.) leaves as follows:

> Perithecia gregarious, covered or slightly erumpent, globose, with slightly papillate mouth, membranaceous, of parenchymatous texture, pale brown by transmitted light, average 150 μ diam. Asci shortly clavate or saccate, distichous or tristichous, 8-spored, 75-80 x 28-50 μ, average 75 x 37 μ. Sporidia at first hyaline, ultimately brown, elongated oblong, rounded at both ends, 5- septate, not constricted at septa, with longitudinal septa generally in the two median divisions, 30-32 x 11 μ.

Leptosphaerulina australis was also reported by McAlpine to occur on plants in the genera *Dolichos*, *Poa*, and *Lolium* (McAlpine, 1902). The first and only discovery of an anamorphic stage of *Leptosphaerulina* was reported in a study from the Karoo region of South Africa. *Leptosphaerulina chartarum* was described to be the teleomorph of *Pithomyces chartarum* (Berk. and Curt.) Ellis, a pathogen of caltrop (*Tribulus terrestris* L.) (Roux, 1986). Luttrell (1979) stated that species within the genus may suppress anamorphic stages because the teleomorphic stage fulfill the asexual niche by being homothallic, maturing quickly, having repeated cycles, and serving as a dispersal stage.

Species in the genus *Leptosphaerulina* (Pleosporales) are filamentous ascomycetes that produce dark colored pseudothecia (Figures 1-3) (Ericksson, 1999). The development and morphology of the pseudothecial centrum in the genus is consistent with Luttrell's *Dothidea*-type (Wu and Hanlin 1992b; Denison and Carlstrom, 1968; Luttrell, 1951; Wehmeyer, 1955). The Dothidea-type centrum is characterized by dark-colored pseudoparenchyma cells enclosing an aparaphysate locule containing a fascicle of asci (Luttrell, 1951). Asci of *Leptosphaerulina spp*. are shortly clavate to saccate, and have bitunicate wall structure (Figure 4) (Graham and Luttrell, 1961; McAlpine, 1902). Bitunicate asci are characterized by an inner extensible wall (endotunica) that ruptures through an outer inextensible wall (ectotunica) (Ericksson, 1981). Ascospores are forcefully discharged through a pore at the vertex of the endotunica (Luttrell, 1951). Ascospores of *Leptosphaerulina* are hyaline to brown in color and ellipsoid, cylindrical, or oblong in morphology (Figures 5-6). They are phragmosporous or muriform with zero to several longitudinal septa and at least one transverse septum (Graham and Luttrell, 1961; Inderbitzin et al., 2000).



Figures 1-6. 1.) Developing pseudothecia of L114 on 20% V-8 juice agar. 2.) Crush mount of pseudothecia showing emerging asci. The culture was isolated from a necrotic perennial ryegrass leaf and grown on 20% V-8 agar. 3.) Pseudothecia on a necrotic creeping bentgrass leaf. 4.) Bitunicate ascus of L107 with endotunica rupturing through the exotunica (arrows indicate rupture point). 5.) Ascospores of L108 in V-8 juice inoculum broth 6.) Ascospores on a necrotic perennial ryegrass leaf.

Leptosphaerulina spp. in the United States

Leptosphaerulina vignae Tehon and Stout was the first species of *Leptosphaerulina* described in the United States from necrotic leaf spots on cowpea (*Vigna sinensis* Hassk.) in Illinois (Tehon and Stout, 1928). The type of the genus, *L. australis*, was initially described as *Pleosphaerulina zeicola* Stout from Indian corn (*Zea Mays* L.) in Illinois (Stout, 1930). Additional hosts reported for the *L. australis* in the United States include species from the following genera: *Agrostis*, *Festuca*, *Ligustrum*, *Lolium*, *Panicum*, *Poa*, *Rosa*, *Trifolium* (Couch, 1995; Graham and Luttrell, 1961; Wehmeyer, 1955). Zeller (1935) added the species *L. sidalceae* Zeller from *Sidalcea campestris* Greene in Oregon. The author described the asci of this species as cylindrical (Zeller, 1935). This is inconsistent with the type specimen and other members of the genus, which have shortly clavate or saccate asci (McAlpine, 1902). Subsequent studies regarding the genus failed to include *L. sidalceae*, which has spore morphology similar to *L. americana*.

Leptosphaerulina trifolii (Rostr.) Petr. was originally described from white clover (*Trifolium repens* L.) as *Sphaerulina trifolii* (Rostr.) Petr. by Rostrup (1898). The fungus was observed colonizing multiple species of *Trifolium* in the United States by Hopkins (1923) in Missouri. The author noted the presence of the fungus on a diseased white clover (*Trifolium repens* L.) specimen that was collected in 1902 and deposited in the University of Missouri herbarium (Hopkins 1923). *Sphaerulina trifolii* was ultimately moved to the genus *Leptosphaerulina* by Petrak in 1959 (Graham and Luttrell, 1961). This species had been placed in multiple genera between its original description in 1898 and Petrak's reclassification in 1959. A. M. Elliot (1961) documented the many complications and changes in nomenclature that have occurred in previous years. *Leptosphaerulina trifolii* has been observed colonizing several host genera including *Agropyron*, *Arachis*, *Glycine*, *Lespedeza*, *Medicago*, *Oryza*, *Panicum*, *Phaseolus*, *Poa*, *Trifolium*, *Vigna*, and *Zea*. (Farr et al., 1995; Graham and Luttrell, 1961).

Luttrell and Boyle (1960) made the first report of *L. arachidicola* Yen, Chen, and Huang, the causal agent of leaf scorch and pepper spot of peanut in the United States. The fungus was isolated from peanut (*Arachis hypogea* L.) leaves in Georgia (Luttrell and Boyle, 1960).

Graham and Luttrell (1961) published a treatise outlining the three existing species and three new species of *Leptosphaerulina* found on forage plants. They described the morphology, ecology and host specificity of the representative species in great detail. Because of the considerable confusion regarding the similarity of several species of *Leptosphaerulina*, *Sphaerulina*, *Pleospora*, *Catharinia*, *Pleosphaerulina*, *Saccothecium*, *Pseudoplea*, and *Pseudosphaeria*, Graham and Luttrell established three new combinations in the genus *Leptosphaerulina*, which antedates the other synonymous genera (Graham and Luttrell, 1961). New species combinations included *L. briosiana* (Poll.) Graham and Luttrell, *L. americana* (Ell. and Ev.) Graham and Luttrell, and *L. argentinensis* (Speg.) Graham and Luttrell. In addition, *L. vignae* was designated a synonym of *L. australis* making it the first report of the species in the United States.

Leptosphaerulina briosiana was originally described as *Pleosphaerulina briosiana* by Pollacci in 1902, from *Medicago spp.* in Italy. In 1914, the fungus was discovered on alfalfa leaves in both Kansas and Wisconsin and was described by Jones (1916) as *P. briosiana* (Melchers, 1915; Jones, 1916). The host range of *L. briosiana* is limited to *Medicago spp.* and *Trifolium spp.* and it is found in the United States wherever alfalfa is grown (Miles, 1925; Miller, 1925; Graham and Luttrell, 1961).

Leptosphaerulina americana was described as *Pleospora americana* by Ellis and Everhart in 1890. The description was based on collections from common vetch (*Vicia sativa* L.) and pea (*Pisum sativum* L.) from Mississippi. In addition to leguminous hosts in the genera *Pisum*, *Trifolium*, and *Vicia*, *L. americana* has been collected from the dead leaves of timothy (*Phleum pratense* L.) (Graham and Luttrell, 1961).

Leptosphaerulina argentinensis was originally described as *Pleosphaerulina argentinensis* by Spegazzini in 1909. The type specimen was collected from jimson weed (*Datura stramonium* L.) in Argentina. This species was first recognized in the United States by Graham and Luttrell from the leaves of kudzu (*Pueraria lobata* [Willd.] Ohwi.), sweet white clover (*Melilotus alba* Desr.), and Johnson grass (*Sorghum halepense* [L.] Pers.), in Pennsylvania and Georgia (Graham and Luttrell, 1961). Additional collections have been made from creeping bentgrass (*Agrostis palustris* Huds.) in Oregon and Brazilian lucerne (*Stylosanthes guianensis* [Aubl.] Sw.) in Australia (Denison and Carlstrom, 1968; Irwin and Davis, 1985).

Despite the fact that the six species reviewed by Graham and Luttrell were morphologically distinct on their respective host plants, isolates grown on artificial media under identical conditions had similar spore shape, size, and septation. Due to these similarities, Booth and Pirozynski (1967) relegated *L. argentinensis*, *L. arachidicola, L. australis*, and *L. briosiana* synonyms of *L. trifolii*. *L. americana* remained a separate species because of its larger, six-septate ascospores.

Jackson and Bell (1968) changed the name of *L. arachidicola* Yen, Chen, and Huang to *L. crassiasca* (Sechet) Jackson and Bell. The authors felt that this was justified because Sechet described a pathogen of peanut, *Pleospora crassiasca* Sechet, from Madagascar a year before Yen, Chen, and Huang described leaf scorch of peanut incited by *L. arachidicola* in Taiwan. Illustrations and symptom descriptions made by Sechet were found to be consistent with the genus *Leptosphaerulina* and Yen, Chen, and Huang's description of *L. arachidicola*.

Irwin and Davis (1985) restored *L. argentinensis* and *L. crassiasca* to distinct species based on spore morphology, and the number of transverse septa. Their observations were made from isolates originating in Australia, including McAlpine's 1902 specimen of *L. australis* from *Dolichos lignosus* L.. At the present time, valid species of *Leptosphaerulina* that have been collected from grasses and herbaceous plants in the United States include: *L. argentinensis*, *L. americana*, *L. crassiasca*, *L. trifolii*, and *L. sidalceae* (Farr et al., 1989). The spore morphology of these species are listed in Table 1.

	Transverse Septa	**Morphology**
L. crassiasca	3 - 4	Ellipsoid & Cylindrical
L. trifolii	3 - 4	Ellipsoid
L. argentinensis	5	Ellipsoid
L. americana	6	Ellipsoid
L. sidalceae	6 - 7	Ellipsoid

Table 1. Ascospore characteristics of Leptosphaerulina species endemic to the United States (Irwin and Davis, 1985; Zeller, 1935).

Species of *Leptosphaerulina* From Turfgrasses in the United States

Species of *Leptosphaerulina* that have been associated with diseased turfgrasses include *L. australis*, *L. argentinensis*, and *L. trifolii* (Couch, 1995; Watschke et al., 1995; Smith et al.; 1989; Shurtleff et al., 1987; Smiley, 1987; Ormond et al., 1970; Denison and Carlstrom, 1968). *L. australis* is currently regarded as a synonym of *L. trifolii*.

Molecular-Based Phylogenetics

In addition to morphological, ecological, and histological techniques, the use of molecular biology based tools have become very popular in taxonomic studies. Particularly, the polymerase chain reaction (PCR) cloning and sequencing of specific gene regions have proven valuable for phylogenetic inference. The internal transcribed spacer (ITS) regions of the rRNA gene have been used extensively to study phylogenetic relationship at the species and genus level (Seifert et al., 1995, Hillis and Dixon, 1991, White et al., 1990). The two ITS regions (ITS-1, and ITS-2) flank the 5.8S rRNA gene and separate the 18S, 5.8S, and 28S rRNA genes. The spacers include signals that are involved with the processing of gene transcripts. Several primers have been constructed for this region by utilizing highly conserved sequences of the 18S, 5.8S, and 28S rRNA genes (Hillis and Dixon, 1991, White et al., 1990).

An additional DNA region that has been useful for phylogenetic inference is the translational elongation factor 1α (EF-1α). EF-1α catalyses the energy dependent binding of aminoacyl-tRNAs to ribosomes and accounts for upwards of 2% of the total protein of growing cells (Mita et al., 1997; Wendland and Kothe, 1997). This region has a high level of variability that makes it ideal for resolving interspecific as well as intraspecific relationships (Carbone and Kohn, 1999). O'Donnell et al. (1998) and Jimenez-Gasco et al. (2002) used the variation in the EF-1α region to distinguish between pathogenic lineages of *Fusarium oxysporum* Schlecht ex Fr. after the ITS region and other loci afforded little or no species resolution.

Currently, there have been no molecular studies that have addressed phylogenetic relationships of *Leptosphaerulina spp*. at the species level. Since morphological and physiologic characters have failed to provide a clear and well accepted delineation of

species, this taxonomic study of the genus using current molecular tools is warranted to re-evaluate the classification schemes established in the literature by testing their ability to distinguish monophyletic lineages.

Materials and Methods

Culture Isolation and Maintenance

Isolates of *Leptosphaerulina spp.* were obtained by adhering colonized turfgrass leaves to petri plate lids using a thin layer of immersion oil or petroleum jelly. Ascospores were forcibly discharged onto 2% water agar leaving visible, single-spore colonies in about two days. The leading edges of individual colonies were transferred to 20% V-8 juice agar (Miller, 1955). Cultures were maintained on Bacto® potato dextrose agar (Becton Dickinson and Company, Sparks, MD) slants at 4°C for short-term storage. For long-term storage and to prevent the possibility of isolate attenuation, the single-spore isolates and cultures received from outside sources were lyophilized as follows. Several flat toothpicks (Forster Inc., Wilton, ME) were sterilized by autoclaving in Bacto® potato dextrose broth (Difco laboratories, Detroit, MI). The toothpicks were transferred to a petri plate containing 20% V-8 juice agar using a sterile tweezers. Three 5 mm plugs of the culture were transferred to the petri plate using a sterile dissecting needle. When the fungus had completely grown over the toothpicks and produced pseudothecia, individual toothpicks were transferred to 12 inch sterile glass tubes that were sealed at one end. The air was removed from the tube under 500 mm Hg vacuum and the open end was sealed using a propane torch. The tubes were stored in a laboratory cabinet at room temperature (25 ± 5°C). At the beginning of each new inoculation experiment, a tube was broken and the toothpick transferred to 20% V-8 juice agar using a sterile tweezers. The resulting culture was the source of inoculum for the experiment.

Leptosphaerulina Cultures and Materials

Four cultures of *Leptosphaerulina spp*. were isolated by the author from turfgrasses in Virginia in the spring and summer of 2001. Additional cultures and preserved materials from various hosts were received from cooperating scientists, culture

collections, and herbaria (Table 2). All of the isolates were identified using the Graham and Luttrell (1961) species concepts, which is the broadest interpretation of the genus. Several isolates are those described in primary literature pertaining to species characterization. Specifically, L114, L122, L123, L124, and L125 correspond to Graham and Luttrell's numbers 926, 943, 863, 1326, and 1412, respectively. Culture L117 is from Yen et al. (1956) that was compared to *L. crassiasca* isolates from the United States by Graham and Luttrell (1961) and deposited in the American Type Culture Collection (Wu and Hanlin, 1991; Jong and Gantt, 1987).

Spore Measurements and Septation

Size and septation of ascospores were recorded for sporulating cultures isolated from turfgrasses to compare to existing species criteria. Cultures were maintained at room temperature (25 ± 5°C) on 20% V-8 juice agar. A portion of the agar containing fresh pseudothecia was removed from each culture using a sterile dissecting needle and placed on a clean microscope slide. A drop of sterile distilled water was added and a cover glass was placed on the sample. Using the wooden handle of the dissecting needle, moderate pressure was applied to the cover glass to liberate mature asci and ascospores from the pseudothecia. The length, width, and number of transverse and longitudinal septa of fifty ascospores per culture were recorded at a magnification of 250X.

DNA Extraction

The DNA extraction protocol was a modification of the method described by Gardes and Bruns (1993). To extract the fungal DNA, 500 µl of 2X CTAB (hexadecyltrimethylammonium bromide) and 1 µl of β-mercaptoethanol were added to a 1.5 ml eppendorf tube. The tubes were filled approximately 1/3 full with a fungal sample and the sample was homogenized using a pestle. The tubes were placed in a 65°C water bath and were shaken every 10 minutes. After 45 minutes, 500 µl of chloroform:isoamyl alcohol (24:1) were added to each tube and the tubes were shaken.

Culture	Collector ID[a]	Host	Location	Collector
L101[3]	*L. australis*	creeping bentgrass	California, U.S.A. (1992)	L.J. Stowell
L104a[4]	*L. australis*	creeping bentgrass	Arizona, U.S.A. (1999)	S.L. Rasmussen
L104b[4]	*L. australis*	creeping bentgrass	Arizona, U.S.A. (1999)	S.L. Rasmussen
L105	*L. australis*	creeping bentgrass	Virginia, U.S.A. (2000)	S.W. Abler
L106	*L. australis*	creeping bentgrass	Virginia, U.S.A. (2000)	S.W. Abler
L107	*L. australis*	perennial ryegrass	Virginia, U.S.A. (2000)	S.W. Abler
L108	*L. australis*	creeping bentgrass	Virginia, U.S.A. (2000)	S.W. Abler
L109[1]	*L. crassiasca*	peanut	Georgia, U.S.A.	R.T. Hanlin
L110[1]	*L. australis*	bermudagrass	Georgia, U.S.A. (1965)	E.S. Luttrell
L112[8]	*L. briosiana*	alfalfa	France	G. Raynal
L113[2]	*L. argentinensis*	unknown	Georgia, U.S.A. (1960)	
L114[8]	*L. trifolii*	white clover	Georgia, U.S.A.	E.S. Luttrell
L115[8]	*L. briosiana*	alfalfa	Minnesota, U.S.A.	R. Wilcoxson
L117[8]	*L. crassiasca*	peanut	Taiwan (1955)	K.T. Huang
L118[8]	*L. trifolii*	white clover	Georgia, U.S.A. (1967)	E.S. Luttrell
L119[6]	*L. chartarum*	grass	AK, New Zealand (1965)	J.M. Dingley
L121[6]	*L. chartarum*	perennial ryegrass	WI, New Zealand (1969)	E.H.C. McKenzie
L122[7]	*L. americana**	red clover	Georgia, U.S.A. (1954)	E.S. Luttrell
L123[1]	*L. americana**	timothy	Pennsylvania, U.S.A. (1960)	K. Leath
L124[1]	*L. australis**	annual bluegrass	Georgia, U.S.A. (1958)	E.S. Luttrell
L125[1]	*L. argentinensis**	kudzu	Georgia, U.S.A (1960)	E.S. Luttrell
L126[1]	*L. americana**	white clover	Georgia, U.S.A.	R.T. Hanlin
B1[5]	*Bipolaris maydis*	corn	Virginia, U.S.A. (1959)	C.W. Roane

Table 2. Culture identification and isolation information. Isolates and preserved specimens were received from R.T.Hanlin[1], N. O'Neill[2], L.J. Stowell[3], S.L. Rasmussen[4], H.L. Warren[5], the International Collection of Micro-organisms from plants (ICMP) [6], New York Botanical Garden[7], and the American Type Culture Collection (ATCC) [8]. *Dried specimens (not viable). [a]All cultures Identified using the key of Graham and Luttrell (1961)

Centrifugal force was applied to the tubes for ten minutes at 13,000 rpm using a Biofuge A fixed-angle centrifuge (American Scientific Products). The supernatant (350 µl) was transferred to a new 1.5 ml tube using a micropipette and 245 µl of cold isopropanol was added. The tubes were maintained at -20°C for at least one hour. Centrifugal force was applied for five minutes at 13,000 rpm and the isopropanol was decanted. The DNA was

washed by adding 500 µl cold 80% ethanol and applying centrifugal force for two minutes at 13,000 rpm. The ethanol was decanted and the excess ethanol in the tube was evaporated in a drying oven. The DNA was suspended in the minimum amount of 1X TE (Tris EDTA) required to dissolve the pellet (50-200 µl).

Polymerase Chain Reaction

The internal transcribed spacer (ITS) region of the rRNA gene and the translation elongation factor 1α (EF-1α) were amplified using the polymerase chain reaction (PCR). The PCR cocktail was modified from White et al. (1990) and contained the following components per reaction: 5.5 µl ddH_2O, 2.5 µl thermophilic 10X buffer, 2.0 µl Mg^{++} (25 mM), 2.0 µl dNTPs (10 µM), 1.25 µl forward primer (10 µM), 1.25 µl reverse primer (10 µM), 0.25 µl bovine serum albumin (BSA) (10 mg/ml), 0.25 µl Taq polymerase (5 units/µl). The oligonucleotide primers used to amplify the ITS region were ITS4 and ITS5 which typically amplify a region between 600 and 800 base pairs (bps) (Gardes and Bruns, 1993). Primers EF1-728F and EF1-986R were used to amplify a 350 bp portion of the EF-1α region of which 250 bps are located in introns (Carbone and Kohn, 1999). The amplification programs (Table 3) were carried out on a Biometra® T-gradient thermoblock thermo cycler. Excess primers and dNTPs were removed from PCR products using Millipore Microcon®-PCR filters (Millipore Co., Bedford, MA).

DNA Sequencing

Each sequencing reaction contained 15-20 ng of cleaned PCR products, 2 µl of a forward or reverse oligonucleotide primer (5 µM), and 4 µl BigDye version 3.0 (Applied Biosystems). The sequencing program (Table 3) was carried out in a Biometra® T-gradient thermoblock thermo cycler. The products of the sequencing reaction were sent to the Virginia Bioinformatics Institute Core Laboratory Facility (VBI-CLF) for automated sequencing using an ABI 377 automated DNA sequencer or an ABI 3100 capillary sequencer (VBI-CLF, 2002).

STEP	PROGRAM		
	ITS[a]	EF-1[a]	Sequencing[b]
Initial Denaturation	3m @ 95°C	8m @ 95°C	2m @ 95°C
Denaturation	30s @ 94°C	15s @ 95°C	30s @ 95°C
Annealing	1m @ 50°C	20s @ 55°C	15s @ 50°C
Elongation	1m 30s @ 72°C	1m @ 72°C	4m @ 60°C
Final Elongation	7m @ 72°C	5m @ 72°C	

Table 3. Programs for PCR reactions and Sequencing reactions.
ITS, EF-1α, and Sequencing programs were modified from White et al. (1990), Carbone and Kohn (1999), and VBI-CLF (2002) respectively.
[a] 35 cycles of Denaturing, Annealing and Elongation.
[b] 25 cycles of Denaturing, Annealing and Elongation.

Sequence Analysis

Lasergene (DNASTAR, Inc.) software was used to contig forward and reverse sequences as well as align sequences. Aligned sequence files were converted to the NEXUS format for use in PAUP* using MacClade (Maddison and Maddison, 1992). PAUP* (Phylogenetic Analysis Using Parsimony) version 4.0β10 (Swofford, 2002) was utilized to analyze the sequence matrix and to create strict consensus trees and for statistical analysis of the cladograms.

Results

Spore Dimensions and Septation

Ascospores from the seven cultures of turfgrass origin (Table 4) were similar in size and averaged from 33.50 μm to 40.52 μm in length and 13.32 μm to 14.98 μm in width. The spores from each culture consistently had four transverse septa and one to two longitudinal septa. According to Graham and Luttrell's (1961) key, all of the turfgrass isolates are most consistent with *L. australis*. Current nomenclature considers *L. australis* a synonym of *L. trifolii*.

Culture	Length[a]	Width[a]	Transverse Septa	Longitudinal Septa
L104a	27.41-**(33.50)**-37.41	11.31-**(13.32)**-17.40	3-**(3.94)**-4	0-**(0.80)**-2
L104b	30.02-**(35.32)**-40.46	11.75-**(13.43)**-15.23	4-**(4.02)**-5	0-**(1.06)**-2
L105	30.45-**(38.06)**-43.94	13.05-**(14.98)**-17.40	3-**(4.0)**-5	0-**(1.60)**-3
L106	30.89-**(35.62)**-41.33	12.18-**(14.10)**-16.97	3-**(3.98)**-5	0-**(1.22)**-3
L107	34.80-**(40.52)**-47.85	13.05-**(14.92)**-18.27	4-**(4.02)**-5	0-**(1.46)**-3
L108	31.76-**(37.31)**-42.63	12.62-**(14.38)**-16.53	4-**(4.04)**-5	0-**(1.30)**-3
L110	27.41-**(33.91)**-41.76	11.75-**(13.62)**-16.10	3-**(3.72)**-5	0-**(1.06)**-3

Table 4. Characteristics of 50 ascospores produced by cultures isolated from turfgrasses. Values are formatted as follows: minimum-(mean)-maximum.
[a] Units for length and width are μm.

ITS Sequence Analysis and Phylogeny

Oligonucleotide primers ITS4 and ITS5 generated DNA sequences of the expected size for the entire ITS region. The actual sequence length for individual isolates ranged from 507 to 634 base pairs (bps). Of the 672 total characters in the alignment (Appendix A), 463 were constant, 136 (20%) were parsimony informative, and 73 were parsimony uninformative. The heuristic search of the sequences using the tree bisection-reconnection (TBR) method generated 641 most-parsimonious trees with a minimum of 239 evolutionary steps. The consistency index (CI) and homoplasy index (HI) excluding uninformative characters were 0.958 and 0.042 respectively. The retention index (RI) = 0.958 and the g1 statistic = -4.85. According to the critical values of Hillis and Huelsenbeck (1992) for the g1 value, the data set is statistically more structured ($P < .01$) than random data. This indicates the presence of adequate phylogenetic signal (Huelsenbeck, 1991). The strict consensus tree (Figure 7) was rooted using *Bipolaris maydis* (Nisikado and Miyake) Shoem. (teleomorph = *Cochliobolus heterostrophus* [Drechsler] Drechsler) as an out group. *Bipolaris maydis* (Pleosporaceae) is a loculoascomycete that is a member of the same family as *Leptosphaerulina*.

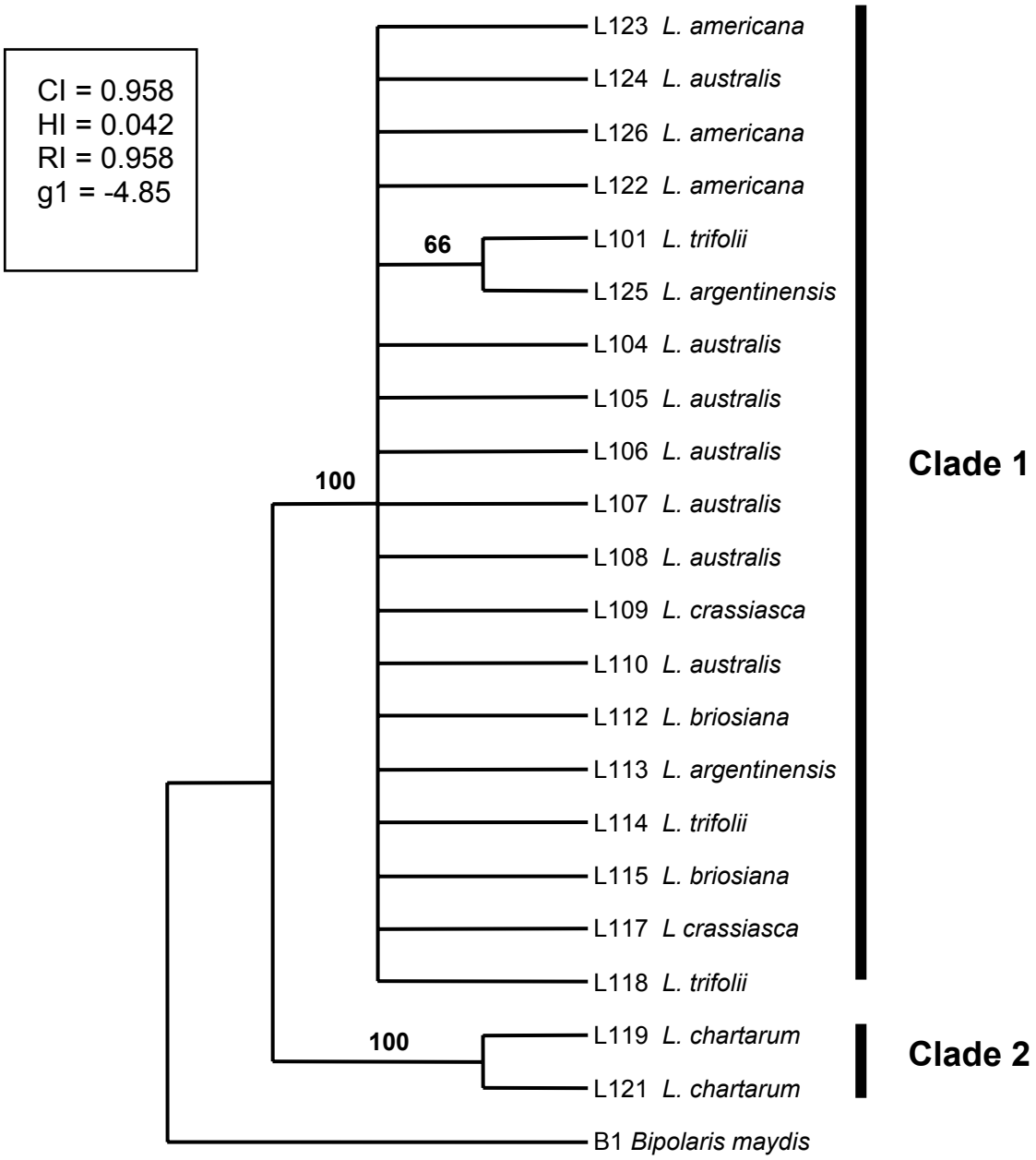


Figure 7. Strict consensus cladogram of ITS sequence data. Numbers on branches indicate bootstrap values (1000 replications). See Table 2 for specific information regarding isolates.

EF1-1α Sequence Analysis and Phylogeny

Oligonucleotide primers EF1-728F and EF1-986R generated DNA sequences of the expected size for the region. The actual sequence length for individual isolates ranged from 278 to 373 bps. Of the 389 total characters in the alignment (Appendix B), 170 were constant, 157 (40%) were parsimony informative, and 62 were parsimony uninformative. The heuristic search of the sequences using the tree bisection-resection (TBR) method generated 42 most-parsimonious trees with a minimum of 331 evolutionary steps. The CI and HI excluding uninformative characters were 0.818 and 0.182 respectively. The retention index (RI) = 0.881 and the g1 statistic = -1.96. According to the critical values of Hillis and Huelsenbeck (1992) for the g1 value, the data set is statistically more structured ($P < .01$) than random data. This indicates the presence of adequate phylogenetic signal (Huelsenbeck, 1991). The strict consensus tree (Figure 8) was also rooted using *Bipolaris maydis* as the outgroup.

Discussion

Species of *Leptosphaerulina* Associated with Turfgrasses

The ITS phylogenetic tree (Figure 7) consists of two distinct clades with high bootstrap support. Clade 1 includes all of the species that are indigenous to the United States. Two New Zealand isolates of *L. chartarum* comprise clade 2. This species has never been reported in the United States. The ITS tree illustrates a divergence between *L. chartarum* and those individuals found in the United States. All six species defined by Graham and Luttrell (1961) were indistinguishable using the ITS gene. Within clade 1, L101 (*L. trifolii*) and L125 (*L. argentinensis*) grouped together with low (66%) bootstrap support. The ITS data are most consistent with the two species classification scheme of Booth and Pirozynski (1967) except for the fact that the three *L. americana* samples, which were considered a separate species by the authors were indiscernible from the other taxa in clade 1.

Because of the low level of resolution in clade 1, the more phylogenetically informative EF-1α region was chosen to further examine the relationship between the

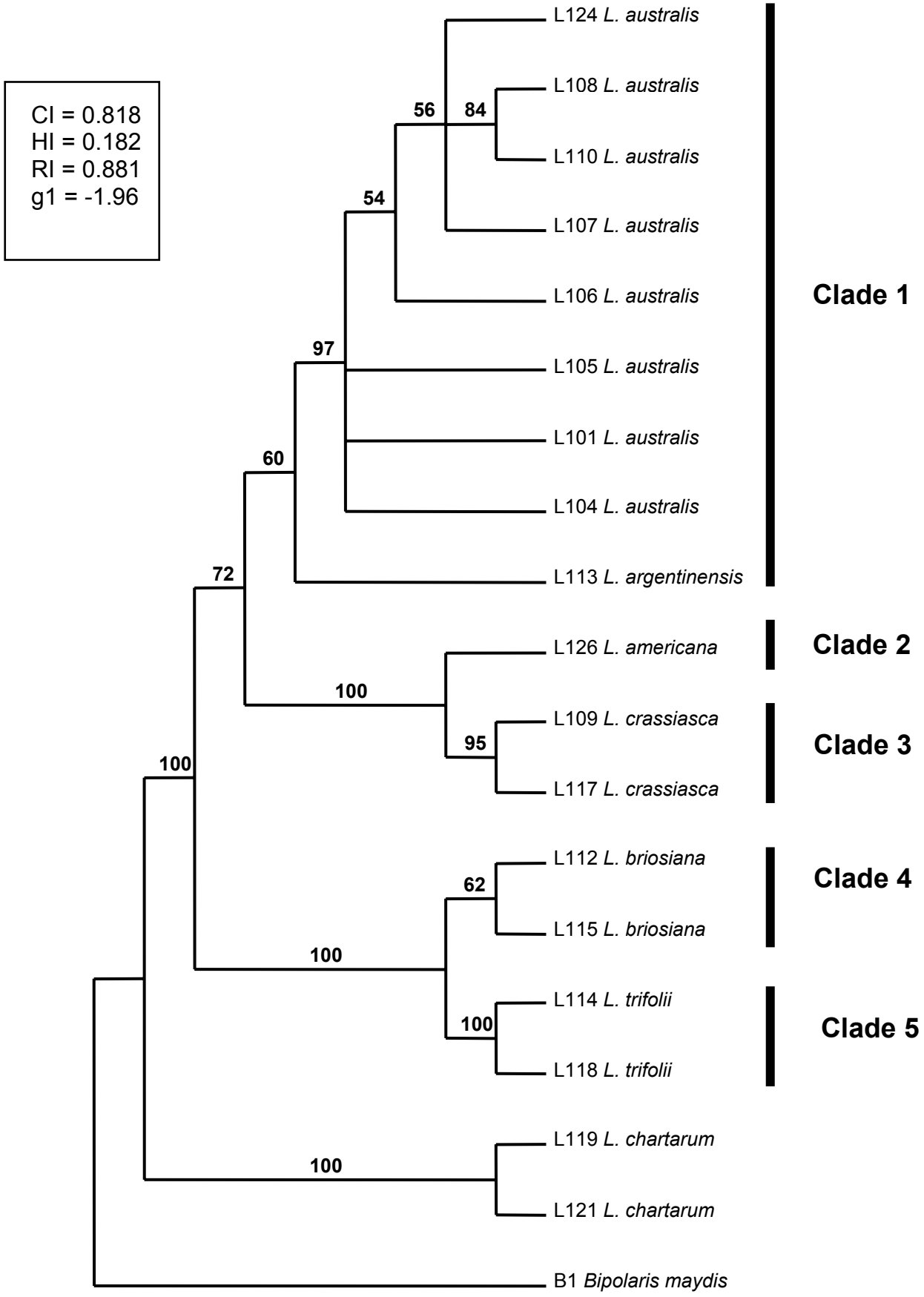


Figure 8. Strict consensus cladogram of EF-1α sequence data. Numbers on branches indicate bootstrap values (1000 replications). See Table 2 for specific information regarding isolates.

reported species of *Leptosphaerulina*. Because of a lack of material, L122 (*L. americana*), L123 (*L. americana*), and L125 (*L. argentinensis*) could not be included in the EF-1α study.

The EF-1α strict consensus tree (Figure 8) confirms the findings of the ITS phylogeny that *L. chartarum*, is distinct from species represented in the United States. The species native to the United States grouped separately from *L. chartarum* with 100% bootstrap support. Unlike the ITS tree, there was adequate resolution and statistical support to separate the isolates from the United States into five monophyletic groups. *Leptosphaerulina trifolii* (clade 5) separated out with 100% bootstrap and is a sister group of *L. briosiana* (clade 4). The lack of strong bootstrap support (62%) for clade 4 could be the result of geographic isolation between L112 and L115 which were isolated from France and the United States respectively. Additionally, the pairwise difference between L112 and L115 is 0.63% (Appendix D) whereas the pairwise difference between the most congruent *L. trifolii* and *L. briosiana* is 2.86%. *Leptosphaerulina crassiasca* comprised clade 3 and was most closely related to *L. americana* (clade 2). Clade 1 consists of the turfgrass isolates of *L. australis* and the single isolate of *L. argentinensis*. *Leptosphaerulina argentinensis* grouped out from *L. australis* with only moderate bootstrap support (60%).

The characterization and sequencing of additional samples of each of the species, especially *L. americana* and *L. argentinensis* must be carried out in order to conclusively define the taxonomy of *Leptosphaerulina*. The analysis of the DNA sequence data in this study is most consistent with the six species classification scheme of Graham and Luttrell (1961). All of the aforementioned species grouped out with at least 60% percent bootstrap support. The EF-1α phylogenetic tree presented and the spore morphology, colony characteristics, growth rate, optimum temperature, host range, pathogenicity, and ecological experiments used by Graham and Luttrell to delimit species in the genus make a compelling argument for the six species classification scheme. With respect to the Graham and Luttrell (1961) key, the species of *Leptosphaerulina* demonstrated to be associated with turfgrasses in the United States are *L. australis* and *L. argentinensis*.

Chapter 2

Ecological Relationship of *Leptosphaerulina spp*. and Turfgrasses

Introduction

Pathogenesis of *Leptosphaerulina* on Legume Hosts

Species of *Leptosphaerulina* incite leaf spot and leaf scorch diseases of several legumes including several agriculturally important hosts such as peanut, alfalfa, white clover, red clover, and soybean (Anahosur and Fazalnoor, 1972; Graham and Luttrell, 1961). Graham and Luttrell (1961) distinguished two ecological groups within the genus. The first group included those species (*L. trifolii*, *L. briosiana*, and *L. arachidicola*) that could cause disease and were only capable of fruiting in necrotic tissues. The second group (*L. australis*, *L. americana*, and *L. argentinensis*) were saprobes that quickly colonize and sporulate on necrotic tissues. Extensive studies of the host-pathogen relationship and disease development of the pathogenic species have been performed for selected legume hosts. Therefore, disease progression of *Leptosphaerulina spp*. is well understood.

Windblown ascospores are the primary means of dispersal for *Leptosphaerulina spp*.. The optimum temperature for the forcible discharge of ascospores from cultures growing on 20% V-8 juice agar is 15-25°C (Wilcoxson and Pandey, 1967; Graham and Lutrell, 1961). Ascospores of *Leptosphaerulina* are enveloped by a mucilaginous sheath that enables the spore to adhere to the leaf surface and adsorb free water (Wu and Hanlin, 1992; Furtado and Olive, 1971; Tehon and Stout; 1928). Under favorable conditions, germination of ascospores occurs very rapidly. Martinez and Hanson (1963) reported one hundred percent germination of *L. briosiana* ascospores after five hours in sterile distilled water at 16, 20 and 24°C. Similarly, Sundheim and Wilcoxson (1965), described eighty percent germination of ascospores on alfalfa leaves maintained at 25°C for twelve hours in a moist chamber. The optimum temperature for germination of *L. briosiana* and *L. trifolii* ascospores is 24°C and 20°C respectively (Martinez and Hanson, 1963; Graham and Luttrell, 1961). Germ tube morphology was described by Wu and Hanlin (1992a).

They found that *L. crassiasca* produced long germ tubes that were septate and terminated in a distinct appressorium as well as short non-septate germ tubes that lacked a distinct appressorium. Additionally, Sundheim and Wilcoxson (1965) observed appressoria that formed between *L. briosiana* ascospores and the cuticle of alfalfa leaves with in the absence of germ tubes.

In most cases, penetration occurs directly through cuticle and epidermal cell wall via a penetration peg (Wu and Hanlin, 1992a; Sundheim and Wilcoxson, 1965; Miles, 1925; Hopkins, 1923). Host penetration through open stomates has been less frequently observed (Wu and Hanlin, 1992a). Sundheim and Wilcoxson (1965) observed that alfalfa chloroplasts surrounding the penetration site appeared granular, swollen, and irregular in shape eight to twelve hours after inoculation with *L. briosiana*. This was followed by disruption of nuclei, an increase in the number of vacuoles, and plasmolysis. After 24 hours, the only remaining cellular remnants were nuclei, cell wall fragments, and plastids. Hyphal colonization by the fungus was observed in the necrotic portion of the lesion, but was absent in the chlorotic tissue. Several researchers noted that the walls of the ascospores remained on the center of the resulting leaf lesions long after penetration of susceptible legume hosts occurred (Miles, 1925; Hopkins, 1923; Jones, 1916).

The infection and colonization of peanut leaves by *L. crassiasca* was similar to the observations of Sundheim and Wilcoxson (1965) with alfalfa and *L. briosiana* (Wu and Hanlin 1992a). *Leptosphaerulina crassiasca* only penetrated the epidermal cells of peanut leaves and intercellular hyphae grew among mesophyll cells without penetrating them. The adjoining cells possessed cytological disturbances similar to alfalfa cells affected by *L. briosiana*.

The fact that cells near, but not penetrated by hyphae were disrupted suggests that the fungus produced an extracellular toxin. Sundheim and Wilcoxin (1965) filtered the hyphae from a liquid culture of *L. briosiana* and discovered that when concentrated, the filtrate produced lesions on alfalfa leaves that were similar to those that occur naturally. Efforts to characterize the toxin were unsuccessful; however, the compound was determined to have acid and amino groups.

Martinez and Hanson (1963) reported the optimum temperature for the development of alfalfa leaf spots to be 20°C. Barbetti (1991) investigated several

temperature and humidity regimes to determine the conditions favorable for disease development in alfalfa. The day/night temperature regimes in the study included: 15/10°C, 18/13°C, and 21/16°C. Symptoms incited by *L. trifolii* were most severe on leaves at the 18/13°C regime whereas symptoms on petioles were most severe at the 21/16°C regime. Leaves incubated in high humidity conditions for 72-96 hours had the highest number of lesions per leaf, whereas plants incubated in high humidity for less than 48 hours had few lesions.

Leptosphaerulina and Turfgrass Hosts

Reports of *Leptosphaerulina*-like fungi associated with diseased turfgrass in the United States were made as early as 1934. A lawn rot incited by a "*Helminthosporium-Pleospora sp.*" associated with a general, widespread decay of bentgrass was described by Clinton (1934) from Connecticut. He described the fungus as having a parasitic *Helminthosporium* stage and a saprophytic *Pleospora* stage. In Texas, Dunlap (1944) found an ascomycete belonging to the genus *Pleospora* sporulating on dead and dying leaves of Kentucky bluegrass and bentgrass respectively. At the time of the aforementioned publications, *Pleospora trifolii*, *P. hyalospora*, and *P. americana* were synonyms of species currently considered to be *L. trifolii* and *L. americana*. Because of the synonomy and the fact that there are no known species of *Pleospora* considered turfgrass pathogens, Clinton (1934) and Dunlap (1944) were most likely describing Leptosphaerulina leaf blight.

Leptosphaerulina leaf blight is presently thought of as a common problem in the humid areas of the United States, occurring mostly on creeping bentgrass (*Agrostis stolonifera* L.), Kentucky bluegrass (*Poa pratensis* L.), and perennial ryegrass (*Lolium perenne* L.) (Shurtleff, et al., 1987; Smiley, 1987). Other turfgrass species described as being susceptible include annual bluegrass (*Poa annua* L.), colonial bentgrass (*Agrostis tenuis* Sibth.), red fescue (*Festuca rubra* L.), tall fescue (*Festuca arundinacea* Schreb.), annual ryegrass (*Lolium multiflorum* Lam.), and bermudagrass (*Cynodon dactylon* (L.) Pers. (Couch, 1995). The initial symptom described for the disease is leaf tip yellowing. The blighted area shifts from yellow to brown and expands toward the leaf sheath. In severe cases individual blades become necrotic and shrivel. Minute brown pseudothecia

develop on the dead tissue (Couch, 1995; Watschke, et al., 1995; Smith et al., 1989; Shurtleff et al., 1987; Smiley, 1987). In some instances, water-soaked spots that quickly turn white may also be present. These bleached spots are similar to lesions caused by dull mowers, frost, or heat stress (Shurtleff et al., 1987; Smiley, 1987). Symptoms of Leptosphaerulina leaf blight often resemble those of Ascochyta leaf blight, dollar spot, Nigrospora leaf blight, Pythium blight, and Septoria leaf spot (Shurtleff et al., 1987).

There has been confusion regarding the ecological relationship between turfgrass hosts and the incitant. Watschke et al. (1995) refer to *Leptosphaerulina trifolii* as a pathogen of turfgrasses. In contrast, *Leptosphaerulina spp*. were regarded as saprobes of dead turfgrass leaves by Graham and Luttrell (1961). Most descriptions of the disease refer to *Leptosphaerulina spp*. as "weakly pathogenic" or senectopathic (Couch, 1995; Smith et al., 1989; Shurtleff et al., 1987; Smiley, 1987). Senectophytes are organisms that infect and colonize tissues that have begun the process of senescence (Couch, 1995). According to Couch (1995), in addition to the natural growth cycle, several stresses induce senescence of turfgrass leaves. Senescence-inducing stresses include: parasitic organisms, high and/or low temperature, low light, unbalanced nutrients, anaerobic soil, low mowing, dethatching, and side effects of pesticides. Recognized disorders of turfgrasses caused by senectophytes include Curvularia blight incited by six species of *Curvularia* and anthracnose incited by *Colletotrichum graminicola* (Ces) Wils. *Colletotrichum graminicola* only infected and colonized annual bluegrass (*Poa annua* L.) and red fescue (*Festuca rubra* L.) leaves that had been stressed by high air temperature (Couch, 1995). Likewise, *Curvularia lunata* (Wakker) Boedijn. only colonized Penneagle creeping bentgrass (*Agrostis stolonifera* L.) leaves that had been subjected to high air temperature and high relative humidity (Muchovej, 1984). The author has regularly found *Leptosphaerulina spp*. on diseased turfgrasses in conjunction with other pathogens including *Magnaporthe grisea* (Herbert) Barr (anamorph: *Pyricularia grisea* [Cke.] Sacc.), *Fusarium spp*. and *Sclerotinia homoeocarpa* Bennett as well as plants infested with insect pests. Smiley, (1987) reported Leptosphaerulina leaf blight from turfgrasses grown under stressful soil conditions, stressed by herbicides, and on freshly laid sod.

There are no reports of any researcher fulfilling Koch's Postulates with any *Leptosphaerulina* species and turfgrass. Additionally, pathogenicity experiments are

absent from the literature describing Leptosphaerulina leaf blight of turfgrasses. The fact that *Leptosphaerulina spp*. have been found colonizing declining turfgrasses is not enough evidence to assume a causal relationship. Therefore, pathogenicity tests must be performed to verify the ecological relationship between *Leptosphaerulina spp*. and turfgrasses.

Senescence Research

High temperature stress has been shown to artificially accelerate senescence and has been used by many researchers to induce the onset of senescence (Muchovej and Couch, 1987; Thomas and Stoddart, 1980; Wittenbach, 1977). Chlorophyll degradation is a normal component of the aging process in plants that can be used to track progression of senescence (Smart, 1994). When referring to a single physiological change that occurs in senescent leaves that could be used as a model for induced senescence experiments, Thomas and Stoddart (1980) stated that "Perhaps the ideal component process of senescence to use in such a test would be chlorophyll breakdown, since it is characteristic of senescence, has reasonably sound genetic basis, and behaves consistently in inhibitor experiments." Decreased chlorophyll content of senescing grasses has been documented for wheat (*Triticum aestivum* L.), rye (*Secale cereale* L.), corn (*Zea mays* L.), and creeping bentgrass (*Agrostis stolonifera* L.) (Lu et al., 2001; Lu and Zhang, 1998; Muchovej, 1986; Kar and Feierabend, 1984).

Unlike for legumes, the exact relationship between species of *Leptosphaerulina* and turfgrasses has not been clearly established. This study was undertaken in order to answer the question regarding the pathogenicity of *Leptosphaerulina spp*. to turfgrasses.

Materials and Methods

Temperature Limit Experiment

Isolates L104a, L104b, L105, L106, L107, and L108 were utilized to determine an upper temperature threshold for the growth of *Leptosphaerulina spp*. from turfgrasses. Nine 5 mm plugs of actively growing cultures were removed and placed upside-down in the center of a petri plate containing 20% V-8 juice agar. Three plates of each isolate

were wrapped in foil and were maintained at room temperature (25 ± 5°C), 30°C, and 35°C. After 18 days, plates that did not show growth were transferred to room temperature for 13 days to check for resumption of growth.

Inoculation Experiments

Experimental inoculations were employed to investigate the relative plant health at which *Leptosphaerulina spp*. are able to infect and colonize plant tissues. Growth chamber conditions used in the experiment have been shown to be optimal for disease development in legumes, with the only variable being the leaf vigor of the turfgrass plants. Turfgrass plants were stressed using the high air temperature stress (HATS) regime of Muchovej and Couch (1987). HATS conditions were 38°C air temperature with 100% relative humidity, and complete darkness. Muchovej and Couch (1987) induced senescence of Penneagle creeping bentgrass leaves by maintaining them at HATS conditions for eighteen hours. Figure 9 is a graphical representation of the inoculation experiments. To test the validity of the inoculation procedure, red clover (*Trifolium pratense* L.) plants were inoculated with the turfgrass isolates (L104b, L105, L106, and L107). Slight pepper spot signs and symptoms were evident on most inoculated leaves after 3 days (Figure 15), with L104b inoculated leaves showing the most severe symptoms. The red clover plants in this experiment showed some symptoms (pinpoint chlorotic spots) of insect damage prior to inoculation.

Plant Maintenance

Styrofoam cups (236.5 ml [8.0 fl. oz.], Kroger Co., Cincinnati, OH) were used as pots for the inoculation experiments. To allow for drainage, four holes were melted into the bottom of each cup using a soldering iron. Discs of Acclaim® natural singlefold towels (Fort James Corp., Deerfield, IL) were placed in the bottom of each cup to prevent leakage of the rooting medium through drainage holes. Profile® ceramic soil amendment (Profile Products LLC, Buffalo Grove, IL) was used as the rooting medium. Pots were filled with rooting medium 0.5 cm from the top edge. Certified seed of each variety was weighed and evenly distributed on the rooting medium. Palmer III and Fiesta

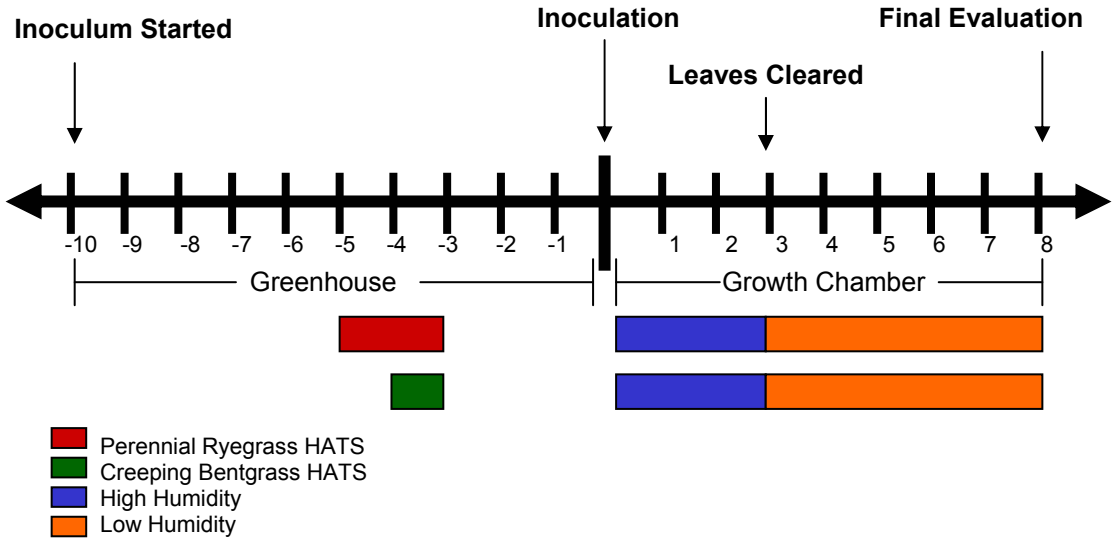


Figure 9. Timeline for inoculation experiments. Each hash on the grid corresponds to one day. The day of inoculation is represented as day zero. Leaves were cleared on day 5 of the second repetition of the inoculations. HATS = **H**igh **A**ir **T**emperature **S**tress.

II perennial ryegrass were seeded at a rate of 0.15g / pot and Crenshaw creeping bentgrass was seeded at rate of 0.02g / pot. The turfgrass cultivars were chosen based on their susceptibility to other pathogens such as *Pyricularia grisea* (Cke.) Sacc. and *Sclerotinia homoeocarpa* Bennett (NTEP, 2002). Enough rooting medium was sprinkled on the pots to partially cover the seed. Seeded pots were placed in flats and transported to the greenhouse where they were sub-irrigated until roots established. After this time, the pots were watered with an overhead sprinkler and fertilized as needed. Perennial ryegrass plants were maintained at a height of 5 cm using a Black and Decker® GS500 Grass Shear, whereas creeping bentgrass plants were maintained at a height of 1 cm.

Plant Nutrition

Ten days prior to inoculation (Day -10) the plants were watered with a modified Hoagland's solution (Hoagland and Snyder, 1933). The solution (Table 5) was prepared

by bringing 5.0 ml of the $Ca(NO_3)_2 \cdot 4H_2O$ stock solution, 5.0 ml of the KNO_3 stock solution, 2.0 ml of the $MgSO_4 \cdot 7H_2O$ stock solution, and 1.0 ml of KH_2PO_4 stock solution up to a total volume of one liter with distilled water. The iron solution (5.0 ml) and the micronutrient solution (1.0 ml) were then added. The plants were watered with the modified Hoagland's solution for the remainder of the inoculation experiment.

Stock Solutions 1 Molar	**Micronutrient Solution grams / liter**	**Iron Solution grams / liter**
$Ca(NO_3)_2 \cdot 4H_2O$	2.5g H_3BO_3	5.0g tartaric acid
KNO_3	1.54g $MnSO_4 \cdot H_2O$	5.0g $FeCl_2$
KH_2PO_4	2.2g $ZnSO_4 \cdot 7H_2O$	
$MgSO_4 \cdot 7H_2O$	0.05g $CuCl_2 \cdot 2H_2O$	
	0.08g MoO_3	

Table 5. Components of modified Hoagland's nutrient solution. Adapted from Hoagland and Snyder (1933).

Plants that received HATS treatment will be referred to as "stressed" whereas plants that did not receive HATS treatment will be referred to as "non-stressed". Perennial ryegrass pots that were to be artificially stressed were clipped and removed from the greenhouse five days before inoculation (Day -5) and placed into a dew chamber (Percival Manufacturing Co., Boone IA) for two days. The dew chamber was set according to the high air temperature stress and darkness regime (HATS) of Muchovej and Couch (1987). The pots were sub-irrigated with the modified Hoagland's solution while in the dew chamber. Creeping bentgrass plants that were artificially stressed were clipped and placed into the dew chamber set at HATS conditions for one day beginning four days before inoculation (Day -4). All stressed plants were moved back to the greenhouse three days before inoculation and were not clipped until prior to inoculation. Plants that were not stressed were clipped at the same time as stressed plants and remained in the greenhouse for the entire ten days.

Inoculum Preparation

Inoculum was started ten days prior to inoculation (Day –10). The procedure for the preparation of inoculum was adapted from Martinez and Hanson (1963). 20 ml of V-8 juice liquid medium (200 ml V-8 juice, 800 ml distilled water, 0.030 g streptomycin sulfate) was dispensed into 100 X 15 mm plastic petri plates (Catalog # 08-757-12, Fisher Scientific, Pittsburg, PA). The petri plates were inoculated using cultures grown on 20% V-8 agar. A sterile cork borer was used to make 5 mm plugs in the agar. Four plugs were placed equidistant from each other in the liquid medium using a sterile dissecting needle. Seven plates per culture were prepared in this manner. The plates were stored on a laboratory bench at room temperature (25 ± 5°C) for ten days. At the end of ten days, ascospore production was evident from the dark brown discoloration on the petri plate lids (Figure 10). This discoloration was the result of the numerous ascospores that were forcibly discharged onto the lid of each petri plate.

Inoculation

For each experiment, four pots of stressed plants and four pots of unstressed plants were sprayed with sterile V-8 juice broth. These plants were the uninoculated controls with which inoculated plants were compared. For each isolate, four stressed and four unstressed replicates were sprayed with the inoculum medium containing that isolate. Each experiment used a different variety of turfgrass. The cultures used to inoculate the Palmer III and the Fiesta II perennial ryegrass were those isolated from turfgrasses (L104b, L105, L106, L107, L108, and L110). Cultures used in the Crenshaw creeping bentgrass experiments were L104b, L105, L106, and L107. Each experiment was repeated once resulting in a total of six experiments for the three turfgrass varieties.

To prepare the inoculum, 0.625 g (0.5% of total volume) of gelatin was added to a Waring Commercial Blendor™ containing 25 ml sterile distilled water, and blended on high speed for five seconds. The contents of five petri plates (100 ml) were added and homogenized for two minutes, alternating high and low speeds. A hemacytometer was utilized to estimate the spore concentration of the inoculum. The number of spores in the four 1 mm^2 corners of the grid were counted and the sum was multiplied by 2500 to

determine the number of spores/ml of inoculum (Hansen, 2002). This was repeated and the two numbers were averaged.

The tips of the elongating leaves of the plants were clipped with a scissors immediately before inoculation to simulate fresh wounds caused by mowing. Inoculum was sprayed on the plants using a carbon dioxide sprayer equipped with a single Teejet 8003VS flat fan nozzle. The inoculum was sprayed from a height of 30 cm with a nozzle pressure of 275.8 kPa (40 p.s.i). The pots were placed in flats in a growth chamber set at 22°C with 12 hours light/dark. Pots were sub-irrigated with the nutrient solution and misted with sterile distilled water. Clear plastic domes were placed over the flats to maintain continuous free moisture on the leaf surfaces. On day three the domes were removed and inoculated plants were compared to control plants. Leaves from each pot were cleared and stained for examination by means of a light microscope. Plants were checked through day eight, and then discarded.

Chlorophyll Extraction

The chlorophyll content of the elongating leaves from four stressed and four non-stressed pots was compared using the protocol of Barnes et al. (1992). The top 5 mm of the elongating leaves of the plants were clipped using a scissors. They were then mixed and a 0.05 g sample was placed in a glass vial containing 10 ml dimethyl sulfoxide (DMSO). The vials were heated in a 60°C water bath for one hour. The optical density at 648 and 665 nm (A^{665}, A^{648}) was recorded for 250 µl samples using a SpectraMax Plus® spectrophotometer (Molecular Devices). Calculations of total chlorophyll were made using the formula described in Barnes et al. (1992) where total chlorophyll (µg/ml extract) is equal to $7.49A^{665} + 20.34A^{648}$. Means of the stressed and unstressed grasses were statistically compared for homogeneity of variance using Bartlett's test, and for analysis of variance. Pesticide Research Manager version 5.0 (Gylling Data Management, Inc.) software was used to calculate statistical significance values.

Leaf Clearing and Staining

Sample leaves were cleared for a minimum of 24 hours in a 1:1 solution of glacial acetic acid and 95% ethanol (Muchovej, 1987; Miles, 1925; Jones, 1916). The leaves were stained with 0.25% aniline blue in lactophenol (10 ml distilled water, 10 g phenol, 10 g glycerin, 10 ml lactic acid) and observed with a light microscope for the presence of fungal structures (Muchovej, 1984). The efficacy of the staining technique was tested using senescent perennial ryegrass leaves colonized by *Curvularia spp*. Spores, germ tubes with appressoria and internal hyphae as well as lesions were visible using the method

Results

Results of the Temperature Experiment

After 18 days at the prescribed temperatures, all cultures maintained at room temperature (25 ± 5°C) and at 30°C had grown to the edge of the pertri plates. The 35°C plates did not exhibit any growth. Two of the plates (L104b and L108) resumed growth after being transferred to room temperature for 13 days. This indicates that the maximum temperature for growth of *Leptosphaerulina spp*. isolated from turfgrasses is between 30°C and 35°C. Additionally, prolonged exposure to air temperature at 35°C was lethal to 16 of the 18 (89%) cultures tested.

Results of the Experimental Inoculations

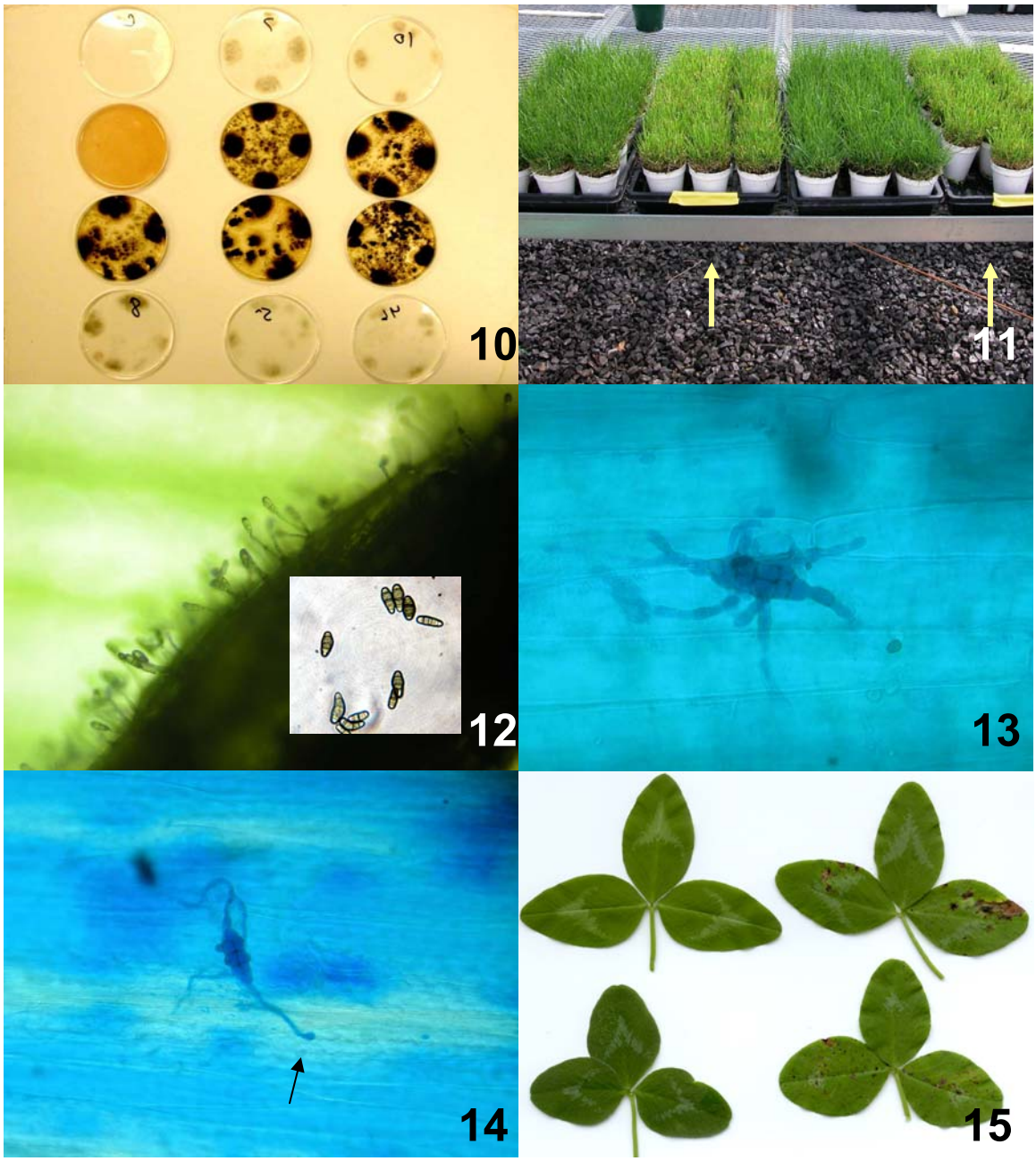
Inoculum spore concentration for all inoculation experiments and isolates ranged from 5,000 spores/ml to 100,000 spores/ml with an average of 24,315 spores/ml. Leaves of stressed plants were visibly chlorotic when compared to leaves of non-stressed plants (Figure 11). In each inoculation experiment, the mean chlorophyll content of stressed plants at the time of inoculation was significantly lower ($P < .001$; $\alpha = 0.01$) when compared to non-stressed plants (Table 6). The decrease in chlorophyll content of the stressed plants indicated a decrease in leaf vigor and the onset of senescence. For the first inoculation repetition of each turfgrass cultivar, a small proportion (10-20%) of the

leaves (including the uninoculated control) exhibited symptoms of leaf dieback. Examination of the leaves with a light microscope revealed that they were colonized by *Curvularia spp* (Figure 12).

			Mean Chlorophyll Content[b]	
Cultivar	Repetition	Plant Age[a]	Non-Stressed	Stressed
Palmer III	1	214	8.24	5.10
Palmer III	2	170	8.58	6.07
Fiesta II	1	234	8.04	4.45
Fiesta II	2	174	8.02	5.55
Crenshaw	1	203	8.29	5.68
Crenshaw	2	249	8.53	5.22

Table 6. Mean chorophyll content of stressed and non-stressed plants on the day of inoculation. Means of all repetitions significantly differ ($P < .001$; $\alpha = 0.01$). [a] Days after seeding. [b] Milligrams chlorophyll per gram of fresh leaf.

Examination of plants from three to eight days post inoculation did not reveal any discernable differences between inoculated and control plants. Cleared and stained leaves showed numerous ascospores, hyphae, and pseudothecia that were deposited on the leaves when inoculated. Many of the ascospores produced germ tubes (Figures 13-14), a number of which had distinct appressoria. No evidence of penetration of the host such as lesion formation or internal hyphae was observed. No pseudothecia or lesions with ascospores near them such as those described by Miles (1925), Hopkins (1923), and Jones (1916) were observed on any of the inoculated leaves.



Figures 10-15. 10.) Inoculum with discharged ascospores on petri plate lids. Clockwise from upper left: Control, L07, L110, L104b, L105, L108. 11.) Palmer III perennial ryegrass plants before inoculation. Arrows indicate stressed plants. 12.) *Curvularia spp*. on stressed Fiesta II perennial ryegrass. Inset: spores of *Curvularia spp*. 13.) Germinating ascospores of L106 on stressed Palmer III perennial ryegrass leaves. 14.) Germinating ascospore of L105 on a creeping bentgrass leaf with a distinct appressorium (arrow). 15.) Uninoculated red clover leaves (left) and leaves inoculated with L104b (right) showing characteristic pepper spot symptoms.

Discussion

Ecological Relationship of *Leptosphaerulina* and Turfgrasses

There was no evidence in this study to support the hypotheses that *Leptosphaerulina spp*. are pathogenic to either non-stressed or senescing turfgrasses. All of the isolates tested for pathogenicity failed to infect and colonize turfgrass plant tissues under conditions proven favorable for disease development on legume hosts. Additionally, no infection or colonization of turfgrass plants artificially stressed using HATS was observed. The senescent state of the stressed test plants was verified in the first repetition of the inoculation experiments by the presence of fungi (*Curvularia spp.*) reported to be senectophytes of turfgrasses. Since the spores deposited on the leaves rapidly germinated and produced appressoria and given the decreased leaf vigor of the test plants, the results presented support the notion that *Leptosphaerulina spp*. are saprophytes of necrotic turfgrass leaves and are unable to infect and colonize living plant tissue.

The fact that *Leptosphaerulina spp*. are often found in stands of turfgrasses colonized by primary pathogens or stressed by other biotic or abiotic factors suggests that these fungi are aggressive secondary colonizers of necrotic tissues. Species of *Leptosphaerulina* rapidly produce conspicuous pseudothecia and large, pigmented ascospores after colonizing necrotic leaves. A cursory examination of affected plants and the presence of these structures may lead to the misdiagnosis of *Leptosphaerulina spp.* as the cause of the declining turfgrasses. It is possible that the primary cause of the decline is masked by the secondary colonization by *Leptosphaerulina spp*.. For instance, Graham and Luttrell (1961) stated that *L. australis*, *L. americana*, and *L. argentinensis*, "Quickly invade and fruit on necrotic tissue and often give the appearance of being responsible for the necrosis." Additionally, Hodges and Madsen (1978) showed that at temperatures above 30ºC the "weak primary-leaf pathogen" *Curvularia geniculata* (Tracy and Earle) Boedijn. aggressively colonized lesions on *Poa pratensis* L. leaves produced by *Bipolaris sorokiniana* (Sacc. In Sorok.) Shoemaker. When leaves were

inoculated with both organisms at 30ºC, *C. geniculata* was more frequently reisolated from resulting lesions.

The results of this research have several practical implications to turfgrass managers in the United States. Since *Leptosphaerulina spp.* are secondary colonizers of necrotic tissues, spraying a fungicide to control these fungi on turfgrasses is expensive, unnecessary, and environmentally irresponsible. To correct the problem, the turfgrass manager must determine the primary cause of the declining turfgrass which could be a multitude of biotic and/or abiotic factors. Determining the underlying cause of the condition may be complicated by the ability of *Leptosphaerulina spp*. to rapidly colonize the moribund turfgrasses.

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APPENDIX A

ITS Sequence Alignment

```
[               10        20        30        40        50        60]
[                .         .         .         .         .         .]
L101     ------------------ATGCTTAAGTTCAGCGGGTATCCCTACCTGATCCGAGGTCA   [41]
L104     ------------------ATGCTTAAGTTCAGCGGGTATCCCTAC-TGATCCGAGGTCA   [40]
L105     ------------TA-TGATATGCTTAAGTTCAGCGGGTATCCCTACCTGATCCGAGGTCA   [47]
L106     -TTCCTCCGCTT-ATTGATATGCTTAAGTTCAGCGGGTATCCCTACCTGATCCGAGGTCA   [58]
L107     ----------------------------------------------------CGAGGTCA   [8]
L108     ---------------TGATATGCTTAAGTTCAGCGGGTATCCCTACCTGATCCGAGGTCA   [45]
L109     -TTCCTCCGCTT-ATTGATATGCTTAAGTTCAGCGGGTATCCCTACCTGATCCGAGGTCA   [58]
L110     -TTCCTCCGCTT-ATTGATATGCTTAAGTTCAGCGGGTATCCCTACCTGATCCGAGGTCA   [58]
L112     -----------TTATTGATATGCTTAAGTTCAGCGGGTATCCCTACCTGATCCGAGGTCM   [49]
L113     -TTCCTCCGCTT-ATTGATATGCTTAAGTTCAGCGGGTATCCCTACCTGATCCGAGGTCA   [58]
L114     ------------------------------------------------------------   [0]
L115     -TTCCTCCGCTT-ATTGATATGCTTAAGTTCAGCGGGTATCCCTACCTGATCCGAGGTCA   [58]
L117     -TTCCTCCGCTT-ATTGATATGCTTAAGTTCAGCGGGTATCCCTACCTGATCCGAGGTCA   [58]
L118     -T-----------------------------------------------------------   [1]
L119     ---------GCTTAATTATATGCTTAAGTTCAGCGGGTATCCCTACCTGATCCGAGGTCA   [51]
L121     ---------TTTTATTKATATGCTTAAGTTCAGCGGGTATCCCTACCTGATCCGAGGTCA   [51]
L122     ATTCCTCCGCTT-AATGATATGCTTAAGTTCAGCGGGTATCCCTACCTGATCCGAGGTCA   [59]
L123     -----CGC.TTT-AATGATATGCTTAAGTTCAGCGGGTATCCCTACCTGATCCGAGGTCA   [54]
L124     -TTCCTCCGCTTTAATGATATGCTTAAGTTCAGCGGGTATCCCTACCTGATCCGAGGTCA   [59]
L125     -TTCCTCCGCTTTATTGATATGCTTAAGTTCAGCGGGTATCCCTACCTGATCCGAGGTCA   [59]
L126     -----T-----------ATATGCTTAAGTTCAGCGGGTATCCCTACCTGATCCGAGGTCA   [44]
B1       A------------------AAGTTAAAAATC-GTAAGAGT-----CTTGAT---GGATTA   [33]
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```
[               70        80        90        100       110       120]
[                .         .         .         .         .         .]
L101     AGAGTGTAAAAATGTACTTTTGG-ACGTCGTC-GT-TATGAGT-GCAAA-GCGCGAGATG   [96]
L104     AGAGTGTAAAAATGTACTTTTGG-ACGTCGTC-GT-TATGAGT-GCAAA-GCGCGAGATG   [95]
L105     AGAGTGTAAAAATGTACTTTTGG-ACGTCGTC-GT-TATGAGT-GCAAATGCGCGAGATG   [103]
L106     AGAGTGTAAAAATGTACTTTTGG-ACGTCGTC-GT-TATGAGT-GCAAA-GCGCGAGATG   [113]
L107     AGAGTGTAAAAATGTACTTTTGG-ACGTCGTC-GT-TATGAGT-GCAAA-GCGCGAGATG   [63]
L108     AGAGTGTAAAA-TGTACTTTTGG-ACGTCGTC-GT-TATGAGT-GCAAA-GCGCGAGATG   [99]
L109     AGAGTGTAAAAATGTACTTTTGG-ACGTCGTC-GT-TATGAGT-GCAAA-GCGCGAGATG   [113]
L110     AGAGTGTAAAAATGTACTTTTGG-ACGTCGTC-GT-TATGAGT-GCAAA-GCGCGAGATG   [113]
L112     AGAGTGTAAAA-TGTACTTTTGG-ACGTCGTC-GT-TATGAGT-GCAAA-GCGCGAGATG   [103]
L113     AGAGTGTAAAAATGTACTTTTGG-ACGTCGTC-GT-TATGAGT-GCAAA-GCGCGAGATG   [113]
L114     -----GTAAAAATGTACTTTTKGGACGTCGT.CGTCTATGAGRTGCAAA-GCGCGAGATG   [54]
L115     AGAGTGTAAAAATGTACTTTTGG-ACGTCGTC-GT-TATGAGT-GCAAA-GCGCGAGATG   [113]
L117     -GAGTGTAAAAATGTACTTTTGG-ACGTCGTC-GT-TATGAGT-GCAAA-GCGCGAGATG   [112]
L118     -----GTAAAAATGTACTTTTGG-ACGTCGTC-GT-TATGAGT-GCAAA-GCGCGAGATG   [51]
L119     AAGACG-GTAAATGTGCTTGTTGGACGCGAGCCGTA-GCCCCTCGAGAA---GCGCAATG   [106]
L121     AAGACG-GTAAATGTGCTTGTTGGACGCGAGCCGTA-GCCCCTCGAGAA---GCGCAATG   [106]
L122     AGAGTGTAAAAATGTACTTTTGG-ACGTCGTC-GT-TATGAGT-GCAAA-GCGCGAGATG   [114]
L123     AGAGTGTAAAAATGTACTTTTGG-ACGTCGTC-GT-TATGAGT-GCAAA-GCGCGAGATG   [109]
L124     AGAGTGTAAAAATGTACTTTTGG-ACGTCGTC-GT-TATGAGT-GCAAA-GCGCGAGATG   [114]
L125     AGAGTGTAAAAATGTACTTTTGG-ACGTCGTC-GT-TATGAGT-GCAAA-GCGCGAGATG   [114]
L126     AGAGTGTAAAAATGTACTTTTGG-ACGTCGTC-GT-TATGAGT-GCAAA-GCGCGAGATG   [99]
B1       C-----------CGTCCTTTT-------CTCCTG-ATACAGAGTGCAAA------ATATG   [68]
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```
[              130       140       150       160       170       180]
[                .         .         .         .         .         .]
L101     TACT-GCGCTCCGAAATC-AATACGCCGGCT-GCCAATT-GTTTTAAG-GCGAGT-CTAC   [150]
L104     TACT-GCGCTCCGAAATC-AATACGCCGGCT-GCCAATT-GTTTTAAG-GCGAGT-CTAC   [149]
L105     TACTAGCGCTCCGAAATC-AATACGCCGGCT-GCCAATT-GTTTTAAGAGCGAGT-CTAC   [159]
L106     TACT-GCGCTCCGAAATC-AATACGCCGGCT-GCCAATT-GTTTTAAG-GCGAGT-CTAC   [167]
L107     TACTAGCGCTCCGAAATCCAATACGCCGGCTAGCCAATTAGTTTTAAGAGCGAGTTCTAC   [123]
L108     TACT-GCGCTCCGAAATM-AATACGCCGGCT-GCCAATT-GTTTTAAG-GCGAGT-CTAC   [153]
L109     TACT-GCGCTCCGAAATC-AATACGCCGGCT-GCCAATT-GTTTTAAG-GCGAGT-CTAC   [167]
L110     TACT-GCGCTCCGAAATC-AATACGCCGGCT-GCCAATT-GTTTTAAG-GCGAGT-CTAC   [167]
L112     TACT-GCGCTCCGAAATC-AATACGCCGGCT-GCCAATT-GTTTTAAGAGCGAGT-CTAC   [158]
L113     TACT-GCGCTCCGAAATC-AATACGCCGGCT-GCCAATT-GTTTTAAG-GCGAGT-CTAC   [167]
L114     TACT-GCGCTCCGAAATC-AATACGCCGGCT-GCCAATT-GTTTTAAG-GCGAGT-CTAC   [108]
L115     TACT-GCGCTCCGAAATC-AATACGCCGGCT-GCCAATT-GTTTTAAG-GCGAGT-CTAC   [167]
L117     TACT-GCGCTCCGAAATC-AATACGCCGGCT-GCCAATT-GTTTTAAG-GCGAGT-CTAC   [166]
L118     TACT-GCGCTCCGAAATC-AATACGCCGGCT-GCCAATT-GTTTTAAG-GCGAGT-CTAC   [105]
L119     TGCT-GCGCGAGAGGAGGCAA-GGACCG-CT-GCCAATGAATTT--GGGGCGAGTCC-AC   [159]
L121     TGCT-GCGCGAGAGGAGGCAA-GGACCG-CT-GCCAATGAATTT--GGGGCGAGTCC-AC   [159]
L122     TACT-GCGCTCCGAAATC-AATACGCCGGCT-GCCAATT-GTTTTAAG-GCGAGT-CTAC   [168]
L123     TACT-GCGCTCCGAAATC-AATACGCCGGCT-GCCAATT-GTTTTAAG-GCGAGT-CTAC   [163]
L124     TACT-GCGCTCCGAAATC-AATACGCCGGCT-GCCAATT-GTTTTAAG-GCGAGT-CTAC   [168]
L125     TACT-GCGCTCCGAAATC-AATACGCCGGCT-GCCAATT-GTTTTAAG-GCGAGT-CTAC   [168]
L126     TACT-GCGCTCCGAAATC-AATACGCCGGCT-GCCAATT-GTTTTAAG-GCGAGT-CTAC   [153]
B1       TGCT-GCGCTCCGAAA-CCAGTAGGCCGGCT-GCCAATC-GTTTTAAG-GCGAGTCTCCC   [123]
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[              190       200       210       220       230       240]
[                .         .         .         .         .         .]
L101     ACGCAGAG-GCGAGACAAA--CACCCAACACCAAGCAAAGCTTGAAG-GTACAAATGACG   [206]
L104     ACGCAGAG-GCGAGACAAA--CACCCAACACCAAGCAAAGCTTGAAG-GTACAAATGACG   [205]
L105     ACGCAGAG-GCGAGACAAA--CACCCAACACCAAGCAAAGCTTGAAG-GTACAAATGACG   [215]
L106     ACGCAGAG-GCGAGACAAA--CACCCAACACCAAGCAAAGCTTGAAG-GTACAAATGACG   [223]
L107     ACGCAGAGAGCGAGACAAA--CACCCAACACCAAGCAAAGCTTGAAGAGTACAAATGACG   [181]
L108     ACGCAGAG-GCGAGACAAA--CACCCAACACCAAGCAAAGCTTGAAG-GTACAAATGACG   [209]
L109     ACGCAAAG-GCGAGACAAA--CACCCAACACCAAGCAAAGCTTGAAG-GTACAAATGACG   [223]
L110     ACGCAGAG-GCGAGACAAA--CACCCAACACCAAGCAAAGCTTGAAG-GTACAAATGACG   [223]
L112     ACGCAGAG-GCGAGACAAA--CACCCAACACCAAGCAAAGCTTGAAG-GTACAAATGACG   [214]
L113     ACGCAGAG-GCGAGACAAA--CACCCAACACCAAGCAAAGCTTGAAG-GTACAAATGACG   [223]
L114     ACGCAGAG-GCGAGACAAA--CACCCAACACCAAGCAAAGCTTGAAG-GTACAAATGACG   [164]
L115     ACGCAGAG-GCGAGACAAA--CACCCAACACCAAGCAAAGCTTGAAG-GTACAAATGACG   [223]
L117     ACGCAAAG-GCGAGACAAA--CACCCAACACCAAGCAAAGCTTGAAG-GTACAAATGACG   [222]
L118     ACGCAGAG-GCGAGACAAA--CACCCAACACCAAGCAAAGCTTGAAG-GTACAAATGACG   [161]
L119     GCGCGGAG-GCGGGACAGA--CGCCCAACACCAAGCAGAGCTTGAGG-GTGTAGATGACG   [215]
L121     GCGCGGAG-GCGGGACAGA--CGCCCAACACCAAGCAGAGCTTGAGG-GTGTAGATGACG   [215]
L122     ACGCAGAG-GCGAGACAAA--CACCCAACACCAAGCAAAGCTTGAAG-GTACAAATGACG   [224]
L123     ACGCAAAG-GCGAGACAAA--CACCCAACACCAAGCAAAGCTTGAAG-GTACAAATGACG   [219]
L124     ACGCAGAG-GCGAGACAAA--CACCCAACACCAAGCAAAGCTTGAAG-GTACAMATGACG   [224]
L125     ACGCAGAG-GCGAGACAAA--CACCCAACACCAAGCAAAGCTTGAAG-GTACAAATGACG   [224]
L126     ACGCAAAG-GCGAGACAAA--CACCCAACACCAAGCAAAGCTTGAAG-GTACAAATGACG   [209]
B1       A-GCAAAGAGGGAGACAAAAACGCCCAACACCAAGCAAAGCTTGAGG-GTACAAATGACG   [181]
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[              250       260       270       280       290       300]
[               .         .         .         .         .         .]
L101     CT-CGAACAGGCATGCCCCATGGAATACCAAGGGGCGCAATGTGCGTTC-AAAGATTCGA   [264]
L104     CT-CGAACAGGCATGCCCCATGGAATACCAAGGGGCGCAATGTGCGTTC-AAAGATTCGA   [263]
L105     CTTCGAACAGGCATGCCCCATGGAATACCAAGGGGCGCAATGTGCGTTC-AAAGATTCGA   [274]
L106     CT-CGAACAGGCATGCCCCATGGAATACCAAGGGGCGCAATGTGCGTTC-AAAGATTCGA   [281]
L107     CTTCGAACAGGCATGCCCCATGGAATACCAAGGGGCGCAATGTGCGTTC-AAAGATTCGA   [240]
L108     CT-CGAACAGGCATGCCCCATGGAATACCAAGGGGCGCAATGTGCGTTC-AAAGATTCGA   [267]
L109     CT-CGAACAGGCATGCCCCATGGAATACCAAGGGGCGCAATGTGCGTTC-AAAGATTCGA   [281]
L110     CT-CGAACAGGCATGCCCCATGGAATACCAAGGGGCGCAATGTGCGTTC-AAAGATTCGA   [281]
L112     CT-CGAACAGGCATGCCCCATGGAATACCAAGGGGCGCAATGTGCGTTCCAAAGATTCGA   [273]
L113     CT-CGAACAGGCATGCCCCATGGAATACCAAGGGGCGCAATGTGCGTTC-AAAGATTCGA   [281]
L114     CT-CGAACAGGCATGCCCCATGGAATACCAAGGGGCGCAATGTGCGTTC-AAAGATTCGA   [222]
L115     CT-CGAACAGGCATGCCCCATGGAATACCAAGGGGCGCAATGTGCGTTC-AAAGATTCGA   [281]
L117     CT-CGAACAGGCATGCCCCATGGAATACCAAGGGGCGCAATGTGCGTTC-AAAGATTCGA   [280]
L118     CT-CGAACAGGCATGCCCCATGGAATACCAAGGGGCGCAATGTGCGTTC-AAAGATTCGA   [219]
L119     CT-CGAACAGGCATGCCCCACGGAATACCGAGGGGCGCAATGTGCGTTC-AAAGATTCGA   [273]
L121     CT-CGAACAGGCATGCCCCACGGAATACCGAGGGGCGCAATGTGCGTTC-AAAGATTCGA   [273]
L122     CT-CGAACAGGCATGCCCCATGGAATACCAAGGGGCGCAATGTGCGTTC-AAAGATTCGA   [282]
L123     CT-CGAACAGGCATGCCCCATGGAATACCAAGGGGCGCAATGTGCGTTC-AAAGATTCGA   [277]
L124     CT-CGAACAGGCATGCCCCATGGAATACCAAGGGGCGCAATGTGCGTTC-AAAGATTCGA   [282]
L125     CT-CGAACAGGCATGCCCCATGGAATACCAAGGGGCGCAATGTGCGTTC-AAAGATTCGA   [282]
L126     CT-CGAACAGGCATGCCCCATGGAATACCAAGGGGCGCAATGTGCGTTC-AAAGATTCGA   [267]
B1       CT-CGAACAGGCATGCCCTTTGGAATACCAAAGGGCGCAATGTGCGTTC-AAAGATTCGA   [239]
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[              310       320       330       340       350       360]
[               .         .         .         .         .         .]
L101     -TGATTCACTGAATTCTGCAATTCACACTACTTAT-CGCATTTCGCTGCGTTCTTCATCG   [322]
L104     -TGATTCACTGAATTCTGCAATTCACACTACTTAT-CGCATTTCGCTGCGTTCTTCATCG   [321]
L105     -TGATTCACTGAATTCTGCAATTCACACTACTTATTCGCATTTCGCTGCGTTCTTCATCG   [333]
L106     -TGATTCACTGAATTCTGCAATTCACACTACTTAT-CGCATTTCGCTGCGTTCTTCATCG   [339]
L107     ATGATTCACTGAATTCTGCAATTCACACTACTTATTCGCATTTCGCTGCGTTCTTCATCG   [300]
L108     -TGATTCACTGAATTCTGCAATTCACACTACTTAT-CGCATTTCGCTGCGTTCTTCATCG   [325]
L109     -TGATTCACTGAATTCTGCAATTCACACTACTTAT-CGCATTTCGCTGCGTTCTTCATCG   [339]
L110     -TGATTCACTGAATTCTGCAATTCACACTACTTAT-CGCATTTCGCTGCGTTCTTCATCG   [339]
L112     -TGATTCACTGAATTCTGCAATTCACACTACTTAT-CGCATTTCGCTGCGTTCTTCATCG   [331]
L113     -TGATTCACTGAATTCTGCAATTCACACTACTTAT-CGCATTTCGCTGCGTTCTTCATCG   [339]
L114     -TGATTCACTGAATTCTGCAATTCACACTACTTAT-CGCATTTCGCTGCGTTCTTCATCG   [280]
L115     -TGATTCACTGAATTCTGCAATTCACACTACTTAT-CGCATTTCGCTGCGTTCTTCATCG   [339]
L117     -TGATTCACTGAATTCTGCAATTCACACTACTTAT-CGCATTTCGCTGCGTTCTTCATCG   [338]
L118     -TGATTCACTGAATTCTGCAATTCACACTACTTAT-CGCATTTCGCTGCGTTCTTCATCG   [277]
L119     -TGATTCACTGAATTCTGCAATTCACACTACTTAT-CGCATTTCGCTGCGTTCTTCATCG   [331]
L121     -TGATTCACTGAATTCTGCAATTCACACTACTTAT-CGCATTTCGCTGCGTTCTTCATCG   [331]
L122     -TGATTCACTGAATTCTGCAATTCACACTACTTAT-CGCATTTCGCTGCGTTCTTCATCG   [340]
L123     -TGATTCACTGAATTCTGCAATTCACACTACTTAT-CGCATTTCGCTGCGTTCTTCATCG   [335]
L124     -TGATTCACTGAATTCTGCAATTCACACTACTTAT-CGCATTTCGCTGCGTTCTTCATCG   [340]
L125     -TGATTCACTGAATTCTGCAATTCACACTACTTAT-CGCATTTCGCTGCGTTCTTCATCG   [340]
L126     -TGATTCACTGAATTCTGCAATTCACACTACTTAT-CGCATTTCGCTGCGTTCTTCATCG   [325]
B1       -TGATTCACTGAATTCTGCAATTCACACTACGTAT-CGCATTTCGCTGCGTTCTTCATCG   [297]
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[              370       380       390       400       410       420]
[                .         .         .         .         .         .]
L101     ATGCCAGAACCAAGAGATCCGTTGTTGAAAGTTGTAACTATTAAAGTTTTTCAGAC---G   [379]
L104     ATGCCAGAACCAAGAGATCCGTTGTTGAAAGTTGTAACTATTAAAGTTTTTCAGAC---G   [378]
L105     ATGCCAGAACCAAGARATCCGTTGTTGAAAGTTGTAACTATTAAAGTTTTTCAGAC---G   [390]
L106     ATGCCAGAACCAAGAGATCCGTTGTTGAAAGTTGTAACTATTAAAGTTTTTCAGAC---G   [396]
L107     ATGCCA-AACCAAGARW-CCGTTGTTGAAAGTTGTAACTATTAAAGTTTTTCARAC---G   [355]
L108     ATGCCAGAACCAAGAGATCCGTTGTTGAAAGTTGTAACTATTAAAGTTTTTCAGAC---G   [382]
L109     ATGCCAGAACCAAGAGATCCGTTGTTGAAAGTTGTAACTATTAAAGTTTTTCAGAC---G   [396]
L110     ATGCCAGAACCAAGAGATCCGTTGTTGAAAGTTGTAACTATTAAAGTTTTTCAGAC---G   [396]
L112     ATGCCAGAACCAAGAGATCCGTTGTTGAAAGTTGTAACTATTAAAGTTTTTCAGAC---G   [388]
L113     ATGCCAGAACCAAGAGATCCGTTGTTGAAAGTTGTAACTATTAAAGTTTTTCAGAC---G   [396]
L114     ATGCCAGAACCAAGAGATCCGTTGTTGAAAGTTGTAACTATTAAAGTTTTTCAGAC---G   [337]
L115     ATGCCAGAACCAAGAGATCCGTTGTTGAAAGTTGTAACTATTAAAGTTTTTCAGAC---G   [396]
L117     ATGCCAGAACCAAGAGATCCGTTGTTGAAAGTTGTAACTATTAAAGTTTTTCAGAC---G   [395]
L118     ATGCCAGAACCAAGAGATCCGTTGTTGAAAGTTGTAACTATTAAAGTTTTTCAGAC---G   [334]
L119     ATGCCAGAGCCAAGAGATCCATTGTTGAAAGTTGTAACGATTGTTTGTTTCGGAACA--G   [389]
L121     ATGCCAGAGCCAAGAGATCCATTGTTGAAAGTTGTAACGATTGTTTGTTTCGGAACA--G   [389]
L122     ATGCCAGAACCAAGAGATCCGTTGTTGAAAGTTGTAACTATTAAAGTTTTTCAGAC---G   [397]
L123     ATGCCAGAACCAAGAGATCCGTTGTTGAAAGTTGTAACTATTAAAGTTTTTCAGAC---G   [392]
L124     ATGCCAGAACCAAGAGATCCGTTGTTGAAAGTTGTAACTATTAAAGTTTTTCAGAC---G   [397]
L125     ATGCCAGAACCAAGAGATCCGTTGTTGAAAGTTGTAACTATTAAAGTTTTTCAGAC---G   [397]
L126     ATGCCAGAACCAAGAGATCCGTTGTTGAAAGTTGTAACTATTAAAGTTTTTCAGAC---G   [382]
B1       ATGCCAGAACCAAGAGATCCGTTGTTGAAAGTTGTAATAATTACATTGTTTATACTGACG   [357]
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[              430       440       450       460       470       480]
[                .         .         .         .         .         .]
L101     CTGATTGCAACTGCA------AATGGT-T-TAAATT-GTCCAATCGGCGGGCGGACCCGC   [430]
L104     CTGATTGCAACTGCA------AATGGT-T-TAAATT-GTCCAATCGGCGGGCGGACCCGC   [429]
L105     CTGATTGCAACTGCA------AATGGT-T-TAAATT-GTCCAATCGGCGGGCGGACCCGC   [441]
L106     CTGATTGCAACTGCA------AATGGT-T-TAAATT-GTCCAATCGGCGGGCGGACCCGC   [447]
L107     CTGATTGCAACTGCA------AATGGT-T-TAAATTGTCCAAWTCGGCGGGCGGACCCGC   [407]
L108     CTGATTGCAACTGCA------AATGGT-T-TAAATT-GTCCAATCGGCGGGCGGACCCGC   [433]
L109     CTGATTGCAACTGCA------AATGGT-T-TAAATT-GTCCAATCGGCGGGCGGACCCGC   [447]
L110     CTGATTGCAACTGCA------AATGGT-T-TAAATT-GTCCAATCGGCGGGCGGACCCGC   [447]
L112     CTGATTGCAACTGCA------AATGGT-T-TAAATTTGTCCAATCGGCGGGCGGACCCGC   [440]
L113     CTGATTGCAACTGCA------AATGGT-T-TAAATT-GTCCAATCGGCGGGCGGACCCGC   [447]
L114     CTGATTGCAACTGCA------AATGGT-T-TAAATT-GTCCAATCGGCGGGCGGACCCGC   [388]
L115     CTGATTGCAACTGCA------AATGGT-T-TAAATTTGTCCAATCGGCGGGCGGACCCGC   [448]
L117     CTGATTGCAACTGCA------AATGGT-T-TAAATT-GTCCAATCGGCGGGCGGACCCGC   [446]
L118     CTGATTGCAACTGCA------AATGGT-T-TAAATT-GTCCAATCGGCGGGCGGACCCGC   [385]
L119     GTAATGCTAGATGCA------AAAAGAGT-TTATTCGGTTCCAACGGCAGGTTGCCCCGC   [442]
L121     GTAATGCTAGATGCA------AAAAGAGT-TTATTCGGTTCCAACGGCAGGTTGCCCCGC   [442]
L122     CTGATTGCAACTGCA------AATGGT-T-TAAATT-GTYCAATCGGCGGGCGGACCCGC   [448]
L123     CTGATTGCAACTGCA------AATGGT-T-TAAATT-GTCCAATCGGCGGGCGGACCCGC   [443]
L124     CTGATTGCAACTGCA------AATGGT-T-TAAATT-GTCCAATCGGCGGGCGGACCCGC   [448]
L125     CTGATTGCAACTGCA------AATGGT-T-TAAATT-GTCCAATCGGCGGGCGGACCCGC   [448]
L126     CTGATTGCAACTGCA------AATGGT-T-TAAATT-GTCCAATCGGCGGGCGGACCCGC   [433]
B1       CTGATTGCAACTGCATAAAAAAAAGGTTTATGGTTTGGTCCTGGTGGCGGGCGAACCCGC   [417]
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```
[              490       500       510       520       530       540]
[                .         .         .         .         .         .]
L101     CGAGGAA--ACGTAAG-TACTCAAAAGACATGGGTAAGAGATAG-CAGGCA--AAG----   [480]
L104     CGAGGAA--ACGTAAG-TACTCAAAAGACATGGGTAAGAGATAG-CAGGCA--AAG----   [479]
L105     CGAGGAA--ACGTAAG-TACTCAAAAGACA-GGGTAA-AGATAG-CAGGCA--AAG----   [489]
L106     CGAGGAA--ACGTAAG-TACTCAAAAGACATGGGTAAGAGATAG-CAGGCA--AAG----   [497]
L107     CGAGGAA--ACGTAAG-TACTCAAAAGACATGGGTAAGAGATAG-CAGGCA--AAG----   [457]
L108     CGAGGAA--ACGTAAG-TACTCAAAAGACATGGGTAAGAGATAG-CAGGCA--AAG----   [483]
L109     CGAGGAA--ACGTAAG-TACTCAAAAGACATGGGTAAGAGATAG-CAGGCA--AAG----   [497]
L110     CGAGGAA--ACGTAAG-TACTCAAAAGACATGGGTAAGAGATAG-CAGGCA--AAG----   [497]
L112     CGAGGAA--ACGTAAG-TACTCAAAAGACATGGGTAAGAGATAG-CAGGCA--AAG----   [490]
L113     CGAGGAA--ACGTAAG-TACTCAAAAGACATGGGTAAGAGATAG-CAGGCA--AAG----   [497]
L114     CGAGGAA--ACGTAAG-TACTCAAAAGACATGGGTAAGAGATAG-CAGGCA--AAG----   [438]
L115     CGAGGAA--ACGTAAG-TACTCAAAAGACATGGGTAAGAGATAG-CAGGCA--AAG----   [498]
L117     CGAGGAA--ACGTAAG-TACTCAAAAGACATGGGTAAGAGATA.GCAGGCA--AAG----   [497]
L118     CGAGGAA--ACGTAAG-TACTCAAAAGACATGGGTAAGAGATAG-CAGGCA--AAG----   [435]
L119     CGAGGGAGAACAGAAGGTGCTCGTAAAGTAAGGATGGCAGTCGTGCGTGCGTTAAGGGGT   [502]
L121     CGAGGGAGAACAGAAGGTGCTCGTAAAGTAAGGATGGCAGTCGTGCGTGCGTTAAGGGGT   [502]
L122     CGAGGAA--ACGTAAG-TACTCAAAAGACATGGGTAAGAGATAG-CAGGCA--AAG----   [498]
L123     CGAGGAA--ACGTAAG-TACTCAAAAGACATGGGTAAGAGATAG-CAGGCA--AAG----   [493]
L124     CGAGGAA--ACGTAAG-TACTCAAAAGACATGGGTAAGAGATAG-CAGGCA--AAG----   [498]
L125     CGAGGAA--ACGTAAG-TACTCAAAAGACATGGGTAAGAGATAG-CAGGCA--AAG----   [498]
L126     CGAGGAA--ACGTAAG-TACTCAAAAGACATGGGTAAGAGATAG-CAGGCA--AAG----   [483]
B1       CCAGGAA--ACAACAAGTGCGCAAAAGACATGGGTGAAAAAAATAT-----TTCAGCCGG   [470]
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```
[              550       560       570       580       590       600]
[                .         .         .         .         .         .]
L101     CCTACAACTCTAGG-------TAATGAT-CCTTCCGC-------AGGTT------CACC-   [518]
L104     CCTACAACTCTAGG-------TAATGAT-CCTTCCGC-------AGGTT------CACC-   [517]
L105     CCTACAACTCTAGG-------TAATGAT-CCTTCCGC-------AGGTT------CACC-   [527]
L106     CCTACAACTCTAGG-------TAATGAT-CCTTCCGC-------AGGTT------CACC-   [535]
L107     CCTACAACTCTAGG-------TAATGAT-CCTTCCGC-------AGGTT------CACC-   [495]
L108     CCTACAACTCTAGG-------TAATGATTCCTTCCGC-------AGGTT------CACC-   [522]
L109     CCTACAACTCTAGG-------TAATGAT-CCTTCCGC-------AGGTT------CACC-   [535]
L110     CCTACAACTCTAGG-------TAATGAT-CCTTCCGC-------AGGTT------CACC-   [535]
L112     CCTACAACTCTAGG-------TAATGAT-CCTTCCGC-------AGGTT------CACC-   [528]
L113     CCTACAACTCTAGG-------TAATGAT-CCTTCCGC-------AGGTT------CACC-   [535]
L114     CCTACAACTCTAGG-------TAATGAT-CCTTCCGC-------AGGTT------CACC-   [476]
L115     CCTACAACTCTAGG-------TAATGAT-CCTTCCGC-------AGGTT------CACC-   [536]
L117     CCTACAACTCTAGG-------TAATGAT-CCTTCCGC-------AGGTT------CACC-   [535]
L118     CCTACAACTCTAGG-------TAATGAT-CCTTCCGC-------AGGTT------CACC-   [473]
L119     CCGAAGACTCCGCAAACCTCCCAAGGATTGTTTCCGCTCCTCGGGGGCCGCGACGCACCC   [562]
L121     CCGAAGACTCCGCAAACCTCCCAAGGATTGTTTCCGCTCCTCGGGGGCCGCGACGCACCC   [562]
L122     CCTACAACTCTAGG-------TAATGAT-CCTTCCGC-------AGGTT------CACC-   [536]
L123     CCTACAACTCTAGG-------TAATGAT-CCTTCCGC-------AGGTT------CACC-   [531]
L124     CCTACAACTCTAGG-------TAATGAT-CCTTCCGC-------AGGTT------CACC-   [536]
L125     CCTACAACTCTAGG-------TAATGAT-CCTTCCGC-------AGGTT------CACC-   [536]
L126     CCTACAACTCTAGG-------TAATGAT-CCTTCCGC-------AGGTT------CACC-   [521]
B1       CC-GCAAAGCCAGG--CCTTC-----ATATTTT-------------GTTGTG--------   [501]
```

```
[              610       620       630       640       650       660]
[                .         .         .         .         .         .]
L101     ---TACSGAAACCT--TG----TTACG-AAC-------------------------   [539]
L104     ---TACGGAAACCT--TG----TTACG--------C-------------------------   [536]
L105     ---TACGGAAACCT--TG----TT-----------------------------------   [542]
L106     ---TACGGAAACCT--TG----TTACG-ACTTTTACTT-------CCTTTAAATGACC--   [576]
L107     ---TACGGAAACCT--TG----TTAC-----------------------------------   [512]
L108     ---TACGGAAACCT--TG----TTACG-----------------------------------   [540]
L109     ---TACGGAAACCT--TG----TTACG-ACTTTTACTT-------CCTT------------   [567]
L110     ---TACGGAAACCT--TG----TTACG-ACTTTTACTT-------CC--------------   [565]
L112     ---TACGGAAACCT--TG----TT-----------------------------------   [543]
L113     ---TACGGAAACCT--TG----TTACG-ACTTTTACTT-------CC--------------   [565]
L114     ---TACGGAAACCT--TG----TTACG-ACTTTTACTT-------CC--------------   [506]
L115     ---TACGGAAACCT--TG----TTACG-ACTTTTACTT-------CC--------------   [566]
L117     ---TACGGAAACCT--TG----TTACA-AC.TTT--TT-------ACTT-------CC--   [567]
L118     ---TACGGAAACCT--TG----TTCRA-CTTTTT--TT-------ACTT-------CC--   [505]
L119     TGATTTGAAAGGTTAATGATCCTTCCGCAGGTTCACCTACGGAAACCTTGTTACGA.CTT   [622]
L121     TGATTTGAAAGGTTAATGATCCTTCCGCAGGTTCACCTACGGAAACCTTGTT-CGAC.TT   [621]
L122     ---TACGGAAACCT--TG----TTACG-ACTTTTACTT-------CC--------ACC--   [569]
L123     ---TACGGAAACCT--TG----TTAC-----------------------------------   [548]
L124     ---TACGGAAACCT--TG----TTC------------------------------------   [552]
L125     ---TACGGAAACCT--TG----TTACG-AACTTT--TT-------ACTT-------CC--   [568]
L126     ---TACGGAAACCT--TG----TTAC-----------------------------------   [538]
B1       -------------TAATGATCCCTCCGCAGGTTCACCTACGGAGACCTTGTTACGA.CTT   [548]
```

```
[              670  ]
[                .  ]
L101     ------------   [539]
L104     ------------   [536]
L105     ------------   [542]
L106     --------AAGA   [580]
L107     ------------   [512]
L108     ------------   [540]
L109     ------------   [567]
L110     ----------AA   [567]
L112     -----------A   [544]
L113     ----------AA   [567]
L114     ----------AA   [508]
L115     ----------AA   [568]
L117     ----------AA   [569]
L118     ----------AA   [507]
L119     TTTTACTTCCAA   [634]
L121     TTT---------   [624]
L122     --------C---   [570]
L123     -----------A   [549]
L124     ------------   [552]
L125     ----------AA   [570]
L126     ------------   [538]
B1       TTT-ACTTCCAA   [559]
```

APPENDIX B

EF-1α Sequence Alignment

```
[               10        20        30        40        50        60]
[                .         .         .         .         .         .]
L101     T--TTACTTGAAGGAAACCCTTACCGAGTTCGGCGGCTTCCTATGAGG-TTGTTAGTATC   [57]
L104     ------CTTGAAGGAA-CCCTTACCGAGTTCGGCGGCTTCCTATGAGG-TTGTTAGTATC   [52]
L105     T--T-ACTTGAAGGAA-CCCTTACCGAGTTCGGCGGCTTCCTATGAGG-TTGTTAGTATC   [55]
L106     T--T-ACTTGAAGGAA-CCCTTACCGAGTTCGGCGGCTTCCTATGAGG-TTGTTAGTATC   [55]
L107     ------CTTGAAGGAA-CCCTTACCGAGTTCGGCGGCTTCCTATGAGG-TTGTTAGTATC   [52]
L108     ------CTTGAAGGAA-CCCTTACCGAGTTCGGCGGCTTCCTATGAGG-TTGTTAGTATC   [52]
L109     TGATTACTTGAAGGAA-CCCTTRCCGAGTTSGGCGGCTTCCTATAAGACTTGTTAGCTTC   [59]
L110     ------CTTGAAGGAA-CCCTTACCGAGTTCGGCGGCTTCCTATGAGG-TTGTTAGTATC   [52]
L112     ------CTTGAAGGAA-CCCTTACCGAGTTCGGCGGCTTCCTGTGAGATTTGTTAGCGTT   [53]
L113     ------CTTGAAGGGA-CCCTTWCCGAGTTCGGCGGCTTCCTGTTAAACATATTAGCATC   [53]
L114     T---TACTTGAAGGAA-CCCTTACCGAGTTCGGCAGCTTCCTATAAGATTTGTTAGCGTT   [56]
L115     T---TACTTGAAGGAA-CCCTTACCGAGTTCGGCGGCTTCCTGTGAGATTTGTTAGCGTT   [56]
L117     T---TACTTGAAGGAA-CCCTTACCGAGTTCGGCGGCTTCCTATAAGACTTGTTAGCTTC   [56]
L118     T---TACTTGAAGGAA-CCCTTACCGAGTTCGGCAGCTTCCTATAAGATTTGTTAGCGTT   [56]
L119     T---TACTTGAAGGAA-CCCTTACCAAGTTCGGCAGCTTCCTGTAAGGAAAGTCAGTGGT   [56]
L121     T---TACTTGAAGGAA-CCCTTACCAAGTTCGGCAGCTTCCTGTGAGGAAAGTCAGTGAT   [56]
L124     ------CTTGAAGGAA-CCCTT.CCGAGTTCGGCGGCTTCCTATGAGG-TTGTTAGTATC   [52]
L126     T---TACTTGAAGGAA-CCCTTACCGAGTTCGGCGGCTTCCTGTAATACCTGTTAGCTTC   [56]
B1       T---TACTTGAAGGAA-CCCTTMCCGAGTTCGGCGGCTTCCTGTGAGGCTTGTCAGCATT   [56]
```

```
[               70        80        90       100       110       120]
[                .         .         .         .         .         .]
L101     ATGAAGA--GT--ATAACGAAGTAGTATAGGGCG-----------GTGCAAC-----CC   [96]
L104     ATGAAGA--GT--ATAACRAAGTAGTATAGGGCG-----------GTGCAAC-----CC   [91]
L105     ATGAAGA--GT--ATAACGAAGTAGTATAGGGCG-----------GTGCAAC-----CC   [94]
L106     ATGAAGA--GT--ATAACGAAGTAGTATAGGGCG-----------GTGCAAC-----CC   [94]
L107     ATGAAGA--GT--ATAACGAAGTAGTATAGGGCG-----------GTGCAAC-----CC   [91]
L108     ATGAAGA--GT--ATAACAAAGTAGTATAGGGCG-----------GTGCAAC-----CC   [91]
L109     ATGAAGGTTGT--ATGAGGAAGTAGTATAGGGCA-----------GTGCAAC-----CC   [100]
L110     ATGAAGA--GT--ATAACAAAGTAGTATAGGGCG-----------GTGCAAC-----CC   [91]
L112     ATGAAGAC--T--CTAAAGAAATTGTATAGGGCG-----------GTGCAAC-----CC   [92]
L113     ATGAAG--TAT--GTAACGTAGTAACATAGGGCA-----------GTGCAAC-----CC   [92]
L114     ATGAAGAC--T----AAAAAAATTGTACAGGGCG-----------GTGCAAC-----CC   [93]
L115     ATGAAGAC--T--CTAAAGAAATTGTATAGGGCG-----------GTGCAAC-----CC   [95]
L117     ATGAAGGTTGT--ATGAGGAAGTAGTATAGGGCA-----------GTGCAAC-----CC   [97]
L118     ATGAAGAC--T----AAAAAAATTGTACAGGGCG-----------GTGCAAC-----CC   [93]
L119     CTGAATCTTGAAAA-ATGGTGACAACGAATCATGAAGTGACCCAGGGT---AGGTTGGGC   [112]
L121     CTGGATCTTGAAAA-ATAATGACAACAAATCATGAAGTGACTCAGGGT---AGGTTGGGC   [112]
L124     ATGAAGA--GT--ATAACGAAGTAGTATAGGGCG-----------GTGCAAC-----CC   [91]
L126     ATGAAGATCGT--ATGAGGAAGTAGTATAGGGCA-----------GTGCAAC-----CC   [97]
B1       GCGCAGTCAATTAGTAGGTAGAGACTATCAAGCGCAAAGACTAAATGTCCAAGGATGACC   [116]
```

```
[              130       140       150       160       170       180]
[                .         .         .         .         .         .]
L101     TTGATCGAAATCAAAAAAAA-----CTCGCGATGCAAAATG-CAATGTGGGGC---ATG-   [146]
L104     TTGATCGAAATCAAAAAAAAAAAA-CTCGCGATGCAAAATG-CAATGTGGGGC---ATG-   [145]
L105     TTGATCGAAATCAAAAAAAA-----CTCGCGATGCAAAATG-CAATGTGGGGC---ATG-   [144]
L106     TTGGTCGAAATCAAAAAAAAA---ACTCGCGATGCAAAATG-CAATGTGGGGC---ATG-   [146]
L107     TTGATCGAAATCAAAAAAAAAAA--CTCGCGATGCAAAATG-CAATGTGGGGC---ATG-   [144]
L108     TTGATCGAAATCAAAAAAAAAAAA-CTCGCGATGCAAAATG-CAATGTGGGGC---ATG-   [145]
L109     TTGATCAAAGTCAAAAAAA------CTCGTGATGCAAAATG-CAATGTGGGGC---ATG-   [149]
L110     TTGATCGAAATCAAAAAAAAAAAA-CTCGCGATGCAAAATG-CAATGTGGGGC---ATG-   [145]
L112     TTGATTAAAGTCAAGAA--------CTCGCGATGCAAAATG-CAATGTGGAAC---ATG-   [139]
L113     TTCATSGAAG----AAAAAAAAAATCTCGCGATGCAAAATG-CAATGTGGGGC--CATG-   [144]
L114     TTGATTAAAGTCAAGAA--------CTCGCGATGCAAAATG-CAATGTGGAAC---ATG-   [140]
L115     TTGATTAAAGTCAAGAA--------CTCGCGATGCAAAATG-CAATGTGGAAC---ATG-   [142]
L117     TTGATCAAAGTCAAAAAAA------CTCGTGATGCAAAATG-CAATGTGGGGC---ATG-   [146]
L118     TTGATTAAAGTCAAGAA--------CTCGCGATGCAAAATG-CAATGTGGAAC---ATG-   [140]
L119     CGACAAGATATCCAACAGCAAAAGATT---GCCGCGAAGTAACTTGAAGGTACAAACCAT   [169]
L121     CGACAAGATATCCAACAGCAAAAGATT---GCCACGAAGTAACTTGAAGGTACAAACCAT   [169]
L124     TTGATCGAAATCAAAAAAAAAAAAACTCGCGATGCAAAATG-CAATGTGGGGC---ATG-   [146]
L126     TTGATCAAAGTCAAAAAA-------CTCGTGATGCAAAATG-CAATGTGGGGC---ATG-   [145]
B1       TTGAT-GTCGTTGAAGTTGGTGGTGGTGGTGCGTGGTGGTGGTGGGAAATTGCATGT   [175]
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[              190       200       210       220       230       240]
[                .         .         .         .         .         .]
L101     GTGTTGAAGT-TGGCGAGCTCTC-AAAGCGG-------ACTAGCGCTAGTGC-GCAAAAA   [196]
L104     GTGTTGAAGT-TGGCGAGCTCTC-AAAGCGG-------ACTAGYGCTAGTGC-GCAAAAA   [195]
L105     GTGTTGAAGT-TGGCGAGCTCTC-AAAGCGG-------ACTAGCGCTAGTGC-GCAAAAA   [194]
L106     GTGTTGAAGT-TGGCGAGCTCTC-AAAGCGG-------ACTAGCGCTAGTGC-GCAAAAA   [196]
L107     GTGTTGAAGT-TGGCGAGCTCTC-AAAGCGG-------ACTAGTGCTAGTGC-GCAAAAA   [194]
L108     GTGTTGAAGT-TGGCGAGCTCTC-AAAGCGG-------ACTAGTGCTAGTGC-GCAAAAA   [195]
L109     GTGTTGAAGT-TGGCGAGCTCCC-GAAACGG-------ACTAGCGCTAGTGC-GCAAAAA   [199]
L110     GTGTTGAAGT-TGGCGAGCTCTC-AAAGCGG-------ACTAGTGCTAGTGC-GCAAAAA   [195]
L112     GCGTTGAAGT-TGGCGAGCTCCC-AAAGCGG-------ACTAGTGCTAGTGC-GCAAAAA   [189]
L113     GTGTTGAAGT-TGGCGAGC-CCCCAAGATGG-------ACTAGCGCTAGTGCTGCAAAAA   [195]
L114     GCGTTGAAGT-TGGCGAGCTCCC-AAAGCG-------GACTAGTGCTAGTGC-GCAAAAA   [190]
L115     GCGTTGAAGT-TGGCGAGCTCCCCAAAGCBGC-----GACTAGTGCTAGTGC-GCAAAAA   [195]
L117     GTGTTGAAGT-TGGCGAGCTCCC-GAAACGG-------ACTAGCGCTAGTGC-GCAAAAA   [196]
L118     GCGTTGAAGT-TGGCGAGCTCCC-AAAGCG-------GACTAGTGCTAGTGC-GCAAAAA   [190]
L119     CCACTGT-GCGTCATCGGATTTTGGTGTCGCTGAGTAACTTAGAGGGGCTAGCGCGAAAA   [228]
L121     CCGCTGT-GCGTCATCGGATTTTGGTGTCGCTGAGTAACTTAGAGGGGCTAGCGCGAAAA   [228]
L124     GTGTTGAAGT-TGGCGAGCTCTC-AAAGCGG-------ACTAGTGCTAGTGC-GCAAAAA   [196]
L126     GTGTTGAAGT-TGGCGAGCTTCC-GAAACGG-------ACTAGCGCTAGTGC-GCAAAAA   [195]
B1       GCGTCATAGTGTTGCTGGTTCGCGAAGGCCGCATCCAGACTAGCGCTAGCAGCGTAAAA-   [234]
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[              250       260       270       280       290       300]
[               .         .         .         .         .         .]
L101     GCTCGCACAACCCCACCA--------AAAATGCCCCTCAGCGTGCGATAAGATCCGAATC   [248]
L104     GCTCGCACAACCCCACCA--------AAAATGCCCCTCAGCGTGCGATAAGATCCGAATC   [247]
L105     GCTCGCACAACCCCACCA--------AAAATGCCCCTCAGCGTGCGATAAGATCCGAATC   [246]
L106     GCTCGCACAACCCCACCA--------AAAATGCCCCTCAGCGTGCGATAAGATCCGAATC   [248]
L107     GCTCGCACAACCCCACCA--------AAAATGCCCCTCAGCGTGCGATAAGATCCGAATC   [246]
L108     GCTCGCACAACCCCACCA--------AAAATGCCCCTCAGCGTGCGATAAGATCCAAATC   [247]
L109     GCTCGCACAACCCCACCA--------AAAATGCCCCTCAATGTGCGATAAGATCCGAGGA   [251]
L110     GCTCGCACAACCCCACCA--------AAAATGCCCCTCAGCGTGCGATAAGATCCAAATC   [247]
L112     GCTCGCACAACCCCACCAC-------AAAATGCCCCTCAGCGTGCGATAAGATCAAAGGC   [242]
L113     GCTCGCACAACCCCACCA--------AAAATGCCCCTCAGCATGCGATAAGATCCGAGAC   [247]
L114     GCTCGCACAACCCCACCA--------AAAATGCCCCTCAGCGTGCGATAAGATCAAAGGC   [242]
L115     GCTCGCACAACCCCACCCA-------AAAATGCCCCTCAGCGTGCGATAAGATCARAGGC   [248]
L117     GCTCGCACAACCCCACCA--------AAAATGCCCCTCAATGTGCGATAAGATCCGAGGA   [248]
L118     GCTCGCACAACCCCACCA--------AAAATGCCCCTCAGCGTGCGATAAGATCAAAGGC   [242]
L119     TTTCGCACAACCCCACCTAAGCACCAAAATTGCCCCTCA--GTGCGATAAG-CCAGAAAA   [285]
L121     TTTCGCACAACCCCACCTAAGCACCAAAATTGCCCCTCA--GTGCGATAAG-CCAGAAAA   [285]
L124     GCTCGCACAACCCCACCA--------AAAATGCCCCTCAGCGTGCGATAARATCCGAATC   [248]
L126     GCTCGCACAACCCCGCCA--------AAAATGCCCCTCAATATGCGATAAGATCCGAGGA   [247]
B1       GTTCGCACAACCCCACCAC----CCAAAAATGCCCCTCAT--TGCGATAAGCCCRAAAAT   [288]
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[              310       320       330       340       350       360]
[               .         .         .         .         .         .]
L101     ---CGTTCGTGAA----CTGCAGCTGAATGCGATATTGCCGCT-CAAA--GAAAAGTT-T   [297]
L104     ---CGTTCGTGAA----CTGCAGCTGAATGCGATATTGCCGCT-CAAA--GAAAAGTT-T   [296]
L105     ---CGTTCGTGAA----CTGCAGCTGAATGCGATATTGCCGCT-CAAA--GAAAAGTT-T   [295]
L106     ---CGTTCGTGAA----CTGCAGCTGATTGCGATATTGCCGCT-CAAA--GAAAAGTT-T   [297]
L107     ---CGTTCGTGAA----CTGCAGCTGATTGCGATATTGCCGCT-CAAA--GAAAAGTT-T   [295]
L108     ---CGTTCGTGAA----CTGCAGCTGATTGCGATATTG----------------------   [278]
L109     ---CGTTCGTCAAATGGTTGCAGCTGAAGGCAATATTGCCACC-CTAA--GAAATGCT-T   [304]
L110     ---CGTTCGTGAA----CTGCAGCTGATTGCGATATTGCCGCT-CAAA--GAAAAGTT-T   [296]
L112     ---TATTTGCCAAA-GACTGCAGSCGAATGCGATATTGCCACC-TGAA--GAAATATT-T   [294]
L113     ---CGTTCGCGAA----CTGTAGCTGATTGCGGTATTGCCACT-TAAA-AGAAAAGTT-T   [297]
L114     ---AATTTGCCAAAAGATTGCAGCCGAACGCGGTATTGCCACC-TGAA--GAAATATT-T   [295]
L115     ---TATTTGCCAAA-GACTGSAGCCGAATGCGATATTGCCACC-TGAA--GAAATATK-T   [300]
L117     ---CGTTCGTCAAATGGTTGCAGCTGAAGGCAATATTGCCACC-CTAA--GAAATGCT-T   [301]
L118     ---AATTTGCCAAAAGATTGCAGCCGAACGCGGTATTGCCACC-TGAA--GAAATATT-T   [295]
L119     TGGCGGGGGCAACGCAGTGAGAGCGCAATCAAATGCCAGCGCCGCAAACGGGAAAATTGT   [345]
L121     TGGCGGGGGCAACGCAGTGAGAGCGCAATCAAATGCCAGCGCCGCAAACGGGAAAATTGT   [345]
L124     ---CGTTCGTGAA----CTGCAGCTGATTGCGATATTGCCGCT-AAAA--RAAAAGTT-T   [297]
L126     ---CGTTCGTCAAATGGTTGCAGCTGAAGGCAATGTTGCCACC-CAAA--AAAA-GCT-T   [299]
B1       GGGCGCGCGCTCAGAAGTTGTAATGGACAGTGGTGGGGGTTTGGWGAA-AGAAAAGTC-A   [346]
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[              370       380         ]
[                .         .         ]
L101     AT-CGTACCTTCTCGAACTTCTCGATGA-   [324]
L104     AT-CGTACCTTCTCGAACTTCTCGATG--   [322]
L105     AT-CGTACCTTCTCGAACTTCTCGATGA-   [322]
L106     AT-CGTACCTTCTCGAACTTCTC------   [319]
L107     AT-CGTACCTTCTCGAACTTCTCGATGAG   [323]
L108     ----------------------------   [278]
L109     AT-CGTACCTTCTCGAACTTCTCGATGA-   [331]
L110     AT-CGTACCTTCTC--------------   [309]
L112     AT-CGTACCTTCTCGAACTTCTCGAT---   [319]
L113     AT-CGTACCTTCTCGAACTTCTCGATG--   [323]
L114     AT-CGTACCTTCTCGAACTTCTCGATGA-   [322]
L115     AT-CGTACCTTCTCGAACTTCTCGATGA-   [327]
L117     AT-CGTACCTTCTCGAACTTCTCGATGA-   [328]
L118     AT-CGTACCTTCTCGAACTTCTCGATGA-   [322]
L119     GTGCATACCTTCTCGAACTTCTCGATGA-   [373]
L121     GTGCATACCTTCTCGAACTTCTCGATGA-   [373]
L124     AT-CGTACCTTCTCGAACTTCTC------   [319]
L126     AT-CGTACCTTCTCGAACTTCTCGAT---   [324]
B1       AGGCATACCTTCTCGAACTTCTCGATGAG   [375]
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APPENDIX C

Pairwise Differences of ITS Sequences

	L123	L124	L126	L122	B1	L101
L124	0.00921346					
L126	0.00183930	0.00372912				
L122	0.00741271	0.00180604	0.00179616			
B1	0.16942893	0.17019030	0.16977254	0.17420726		
L101	0.00187166	0.00190145	0.00187138	0.00384296	0.17442544	
L104	0.00187835	0.00190046	0.00187820	0.00000000	0.16931005	0.00000000
L105	0.00183890	0.00000000	0.00185439	0.00000000	0.16937593	0.00000000
L106	0.00920399	0.00367904	0.00179256	0.00355449	0.18440737	0.00381590
L107	0.00800771	0.00821888	0.00800654	0.00614481	0.16318214	0.00606442
L108	0.00187824	0.00190046	0.00188591	0.00000000	0.17346331	0.00000000
L109	0.00748493	0.00544189	0.00000000	0.00354293	0.17262402	0.00564436
L110	0.00922524	0.00365532	0.00180566	0.00176970	0.17473075	0.00381985
L112	0.00369635	0.00182375	0.00186255	0.00183193	0.17180751	0.00000000
L113	0.00922524	0.00365532	0.00180566	0.00176970	0.17473075	0.00381985
L114	0.00373085	0.00381939	0.00376801	0.00173611	0.15596871	0.00591260
L115	0.00923567	0.00366201	0.00180248	0.00177908	0.17628099	0.00382699
L117	0.00745909	0.00550117	0.00000000	0.00714190	0.17300366	0.00552330
L118	0.00570444	0.00000000	0.00575045	0.01167697	0.15947600	0.01199592
L119	0.23501097	0.23116630	0.23258142	0.23263893	0.26544949	0.23298267
L121	0.23264728	0.23016270	0.23329361	0.23201483	0.27031085	0.23370142
L125	0.00922985	0.00366594	0.00179616	0.00713393	0.17640807	0.00000000

	L104	L105	L106	L107	L108	L109
L105	0.00000000					
L106	0.00000000	0.00000000				
L107	0.00608065	0.00606534	0.00611154			
L108	0.00000000	0.00000000	0.00000000	0.00610250		
L109	0.00180804	0.00177792	0.00175087	0.00802938	0.00180485	
L110	0.00000000	0.00000000	0.00173740	0.00612913	0.00000000	0.00175747
L112	0.00000000	0.00000000	0.00000000	0.00815924	0.00000000	0.00179251
L113	0.00000000	0.00000000	0.00173740	0.00612913	0.00000000	0.00175747
L114	0.00180832	0.00176991	0.00364800	0.00808102	0.00178891	0.00365696
L115	0.00000000	0.00000000	0.00173441	0.00822523	0.00000000	0.00175439
L117	0.00366903	0.00181878	0.00697749	0.00805104	0.00366881	0.00356632
L118	0.00584932	0.00000000	0.01347450	0.01014664	0.00585462	0.01358897
L119	0.23085144	0.22849311	0.24426350	0.25477973	0.23118992	0.23825069
L121	0.23153192	0.22731751	0.23661250	0.25556919	0.22992091	0.23373397
L125	0.00000000	0.00000000	0.00694766	0.00612742	0.00000000	0.00709948

	L110	L112	L113	L114	L115	L117
L112	0.00000000					
L113	0.00000000	0.00000000				
L114	0.00175131	0.00176991	0.00175131			
L115	0.00000000	0.00000000	0.00000000	0.00174825		
L117	0.00528522	0.00182532	0.00528522	0.00747227	0.00527599	
L118	0.01165041	0.00000000	0.01165041	0.01394045	0.01163004	0.00947105
L119	0.23651761	0.23348823	0.23651761	0.24803081	0.23766421	0.23839763
L121	0.23286299	0.23088992	0.23286299	0.24979535	0.23403303	0.23481385
L125	0.00529753	0.00000000	0.00529753	0.00763263	0.00530075	0.00529198

	L118	L119	L121
L119	0.25078237		
L121	0.25262585	0.00481516	
L125	0.01142995	0.23749377	0.23388608

APPENDIX D

Pairwise Differences of EF-1α Sequences

	L124	L126	B1	L105	L101	L104
L126	0.11312689					
B1	0.33067814	0.34779775				
L105	0.00947676	0.10422342	0.31101492			
L101	0.00945255	0.10391156	0.31010720	0.00000000		
L104	0.00628972	0.10527220	0.32153547	0.00000000	0.00000000	
L106	0.00959886	0.11134513	0.32317263	0.00630559	0.00633287	0.00647568
L108	0.00673728	0.11957071	0.33055469	0.01428955	0.01424006	0.00699234
L109	0.09982876	0.03416691	0.34120718	0.08749419	0.08723654	0.08901020
L110	0.00965693	0.12181313	0.33424637	0.01308181	0.01303591	0.00646756
L112	0.11085303	0.14191569	0.31203794	0.10858743	0.10852697	0.10600409
L113	0.09659814	0.12600335	0.33198738	0.09302935	0.09303167	0.09196299
L114	0.12797822	0.15108065	0.31327778	0.12406864	0.12360392	0.11926678
L115	0.11153819	0.14127736	0.31144986	0.10776377	0.10742835	0.10626856
L117	0.09977355	0.03400590	0.34030068	0.08746514	0.08719628	0.08871225
L118	0.12797822	0.15108065	0.31327778	0.12406864	0.12360392	0.11926678
L119	0.41102615	0.41026551	0.44430315	0.39486530	0.39370063	0.39420259
L121	0.41086268	0.42045495	0.43868381	0.39474878	0.39356998	0.39059132
L107	0.00314239	0.11106710	0.32038555	0.00616760	0.00616243	0.00311857

	L106	L108	L109	L110	L112	L113
L108	0.01392666					
L109	0.09528840	0.09770302				
L110	0.01291505	0.00000000	0.10556638			
L112	0.11599854	0.10358333	0.12611531	0.11299305		
L113	0.09327284	0.10543551	0.12254983	0.10177043	0.13963282	
L114	0.13239041	0.11529249	0.12815233	0.12358736	0.02862560	0.15401179
L115	0.11592376	0.10799324	0.12477319	0.11696172	0.00626463	0.13943537
L117	0.09524904	0.09718277	0.00000000	0.10516828	0.12570591	0.12206092
L118	0.13239041	0.11529249	0.12815233	0.12358736	0.02862560	0.15401179
L119	0.40574223	0.44630796	0.41329470	0.42117652	0.42195120	0.42170233
L121	0.40552020	0.43865293	0.42335716	0.41404679	0.41856486	0.42507496
L107	0.00627594	0.00693133	0.09449274	0.00656339	0.10871735	0.09193674

	L114	L115	L117	L118	L119	L121
L115	0.03136414					
L117	0.12764382	0.12439815				
L118	0.00000000	0.03136414	0.12764382			
L119	0.41622740	0.41330975	0.41163349	0.41622740		
L121	0.41293037	0.40993205	0.42162454	0.41293037	0.02412869	
L107	0.12477865	0.10856499	0.09422641	0.12477865	0.39696455	0.39677566

APPENDIX E

Vita

Steven W. Abler

Steven William Abler was born on November 5, 1975 to Roger and Mary Abler in Fond du Lac, Wisconsin. He graduated from Lowell P. Goodrich High School in Fond du Lac in 1994. Steve received a Bachelor of Science degree from the University of Wisconsin-Oshkosh as a Biology major in 1999. While at Oshkosh, Steve enrolled in an introductory Mycology class taught by Dr. Stephen P. Bentivenga. The class sparked his interest in Mycology and Plant Pathology. In the spring of 1998, Steve and Dr. Bentivenga received a Faculty-Undergraduate Student Collaborative Research Grant from the UW-Oshkosh to investigate the potential role of arbuscular mycorrhizal fungi in the restoration of an abandoned surface mine to a native tallgrass prairie ecosystem. Working with grasses appealed to Steve, and he looked for a way to increase his knowledge of fungi and plants.

Steve joined the turfgrass pathology laboratory of Dr. Houston B. Couch as a Master's candidate in the fall of 1999. The field of turfgrass pathology was ideal for Steve, because it incorporated many of his interests. In addition to his thesis work, Steve diagnosed diseased turfgrass samples from throughout the United States and participated in Dr. Couch's fungicide efficacy trials. Steve is a member of the American Phytopathological Society, The Golf Course Superintendents Association of America, the Beta, Beta, Beta, Biological Honor Society and an honorary member of the Northern Great Lakes Golf Course Superintendents Association. On August 18, 2001 Steve married Rebecca Belling.