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GENETIC DIVERSITY IN BENTGRASS (*AGROSTIS* SPP.)
BY AFLP ANALYSIS AND STUDIES ON DISEASE
RESISTANCE TO *TYPHULA INCARNATA* LASCH

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Georgina V. Vergara

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GENETIC DIVERSITY IN BENTGRASS (*AGROSTIS* SPP.) BY AFLP ANALYSIS AND STUDIES ON DISEASE RESISTANCE TO *TYPHULA INCARNATA* LASCH

by

Georgina V. Vergara

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ABSTRACT

GENETIC DIVERSITY IN BENTGRASS (*AGROSTIS SPP.*) BY AFLP ANALYSIS AND STUDIES ON DISEASE RESISTANCE TO *TYPHULA INCARNATA* LASCH

By

Georgina V. Vergara

Bentgrasses (*Agrostis* spp.) (>220 species) are widely occurring temperate grasses with varied ploidy levels that represent a vast resource for genetic improvement of turfgrass cultivars. Genetic characterization would help in the selection of breeding materials and utilization of germplasm resources. In the first part of this study, 40 plant introductions of 14 *Agrostis* species from 20 countries were studied using fluorescently labeled amplified fragment length polymorphism (AFLP) analyses. Data from 400 AFLP markers and using Unweighted Pair Group Method with Arithmetic Mean (UPGMA) showed genetic similarities between species ranged from 0.62 to 0.98. Principal component analysis (PCA) distinguished seven groups. Dendrogram constructed on the basis of genetic similarities defined groups consistent with the geographic origins and physical and genetic attributes of the species. In the second part of this study, AFLP analyses was performed on old and modern creeping and redtop bentgrasses, selected MSU lines, and plant introductions. Using 355 AFLP markers and clustering analyses, three groups were distinguished. The mean genetic similarity for creeping bentgrasses in the first group was 0.78. Creeping bentgrasses from the US were separated as a subgroup from the European plant introductions. Selected MSU lines were differentiated from modern cultivars. Redtop bentgrasses were found in different groups.

Bentgrasses are susceptible to devastating winter injury caused by gray snow mold (*Typhula incarnata* Lasch). In the third part of this study, 115 random amplified polymorphic DNA (RAPD) markers on 40 isolates of gray snow mold from Michigan, Wisconsin and Minnesota showed mean percentage polymorphism at 48%. Dendrograms constructed showed a wide genetic distance between isolates suggesting high variability and possibly recent colonization. The high variation within populations could be due to outcrossing and recombination. In the last part of the study, controlled screening procedures against *T. incarnata* were developed and used to search for a resistant genotype in creeping bentgrass populations and plant introductions of *Agrostis*. We selected 20 creeping bentgrass genotypes from 890 samples taken from old Northern Michigan golf courses and identified 3 accessions of colonial bentgrasses from 40 plant introductions with potentially useful resistance to *T. incarnata.*

Dedicated to my family

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INTRODUCTION

Classification, distribution and importance of *Agrostis*

Bentgrass is the common name for the closely mowed, fine turfgrass widely seen on putting greens, golf courses and parks in most temperate areas. There are an estimated 220 species of bentgrass distributed throughout the world, on all continents in a variety of habitats. Bentgrass (*Agrostis* spp.) is a perennial or annual outcrossing polyploid (x=7; 2n=14, 28, 42 etc.) belonging to the Poaceae family (Watson and Dallwitz, 1992). Distribution of the species spans shade to open habitats, low to high lands and cool to arctic areas. Some species are helophytic (marsh plants); mesophytic (avoiding extremes of moisture or drought) or xerophytic (susbsisting with relatively little moisture). The importance of the genus is not limited to aesthetic applications as some species are adapted for erosion control and revegetation programs in disturbed areas where grazing, mining and flooding had occurred (Brown and Johnston, 1979; Cornely et al., 1983; Winterhalder, 1990). Some species have important traits such as tolerance to drought and heavy metals. Variation within and between *Agrostis* species represents a potential for genetic improvement of the species for use as turf.

Current significant species in the USA are velvet bentgrass (*A. canina* L.); colonial bentgrass (*A. capillaris* L. or *A. tenuis* Sibth.); dryland or highland bentgrass (*A. castellana* Boiss. & Reut.); creeping bentgrass (*A. palustris* Huds. or *A. stolonifera* L.); red top bentgrass (*A. gigantea* L.); and Idaho bentgrass (*A. idahoensis* Nash). Creeping bentgrass is the premier turfgrass species for closely mowed golf course putting greens (Funk, 1998). Interspecific hybridization, variation in ploidy levels and difficulty in morphological classification has resulted in many synonymous species. Genetic characterization of *Agrostis* species using molecular markers will identify duplicates in the plant introduction collections, define extent of genetic similarity among species and provide directions to future interspecific breeding strategies enabling exploration of germplasm resources.

Introduction and breeding of bentgrasses in the US

Bentgrasses were introduced into the US in the early 1900's and are believed to have come from southern Germany, Holland, England and Belgium. Seeds identified as "south German mixed bentgrass" (Casler, 2002) may have been a mixture of colonial bentgrass, velvet bentgrass and creeping bentgrass, as evident in the mottled appearance of old golf courses.

In the history of bentgrasses in the US, 'Seaside' was the oldest known released cultivar in 1923. 'Seaside' creeping bentgrass, indigenous to coastal regions of Washington and Oregon, was known to be an extremely variable grass that developed into patches of individual strains with different colors, textures and densities. Most seed supplies of cultivar 'Seaside' came from natural stands. In 1955, seeded variety 'Penncross' by Penn State University was released. It took another 30 years before 'Providence' became available in 1987. There are less than 20 known cultivars released in the US between 1923 and 1995. New and improved varieties emerged in the last 15 years increasing the availability of commercial seeded varieties and out-competing vegetative varieties that were susceptible to bacterial wilt and other diseases. Interest in breeding improved creeping bentgrass stems from the demand for better materials with improved agronomic quality, visual appearance and improved resistance to biotic and abiotic factors. Turfgrass breeding involves selection of breeding lines, hybridization and recurrent selection. Most developed cultivars of creeping bentgrass, *A. palustris,* in the US such as 'Penncross','Penn A4' and 'L-93' were obtained from phenotypic recurrent selection of three or more clones descended from selection of promising lines. 'Penn G2' and 'Penn A4', both released in 1995 were part of selections descended from six parental clones. 'L-93' released in 1995 descended from fifteen parental clones. The phylogenetic relationships of creeping bentgrasses in the US are unknown. Newer cultivars may probably be selections coming from the common genetic pool of creeping bentgrasses in the U.S. Genetic variability among creeping bentgrass cultivars was narrow (.007 to .008) as determined using isozyme markers and may be expanded with the use of plant introductions (Warnke et al., 1997). Expanding genetic variability is important because selection becomes more effective when diversity is high.

Information on the extent of genetic variability using more powerful DNA markers among old and new creeping bentgrass cultivars, current breeding lines with disease resistance by natural selection and potential plant introductions as parental materials are important in turfgrass breeding programs.

Turfgrass breeding and use of molecular markers

Molecular markers have become important tools in plant breeding for identification and selection. Isozyme markers were used to distinguish between several creeping bentgrass cultivars (Yamamoto and Duich, 1994; Warnke et al., 1997 and 1998).

Drawbacks to isozyme use are protein instability (Jones 1983) and the limited number of markers (Golembiewski et al., 1997) for biodiversity studies. Research using DNA molecular markers such as restriction fragment length polymorphism (RFLP) markers was applied to differentiate five creeping bentgrass cultivars (Caceres et al., 2000). The potential for sequence characterized amplified regions (SCAR) markers enabled development of species-specific probes for colonial and creeping bentgrasses, but not for dryland bentgrass (Scheef et al., 2003). For biodiversity studies, a more powerful tool in fingerprinting over other PCR-based techniques is by amplified fragment length polymorphism (AFLP) analysis (Vos et al., 1995). The high number of identifiable polymorphism was found useful for distinguishing genotypes, detecting linkages and mapping loci in turfgrasses, such as bermudagrass (Zhang et al., 1999) and zoysiagrass (Ebina et al., 1999). AFLP analysis has not been reported for bentgrass. The technique could be used to differentiate plant introductions of *Agrostis* species and to study diversity among populations of bentgrass breeding materials.

Gray snow mold disease of bentgrass

Bentgrass is susceptible to a wide range of diseases (Vargas, 1994). Snow mold (*Typhula* blight) causes devastating winter injury in turfgrasses. *Typhula* blight is caused collectively by gray snow mold (*Typhula incarnata* Lasch) and speckled snow mold (*T. ishikariensis* Imai) (Hsiang et al., 1999). *Typhula incarnata* is important because the fungus infects other grasses such as *Poa*, *Festuca* and *Lolium* and cereals. The disease economically impacts North America, Russia, Japan and Canada and heavy fungicide applications are currently the only means of control.

Symptoms caused by *T. incarnata* usually appear as circular, water-soaked or straw-colored patches measuring 5 to 15 cm across and may coalesce (Hsiang et al., 1999). Susceptible plants generally show water-soaked lesions and yellowish decaying appearance; plants may be matted and appear slimy with mycelia. Mycelia, basidiospores and or sclerotia produced become inoculum sources leading to new infection. Colonized dead plant tissues decompose and disintegrate, and then the sclerotia fall to the thatch and soil. The sclerotia remain as resting bodies during the summer months. Gray snow mold was more commonly found, than speckled or pink snow mold, on golf areas where snow cover occurred in less than 90 days (Millet, 2000). During periods of longer snowfall, the pathogen infects and kills turfgrass in a significant larger area.

Snow molds are capable of infecting a wide range of plant hosts and bentgrasses have a broad range of susceptibility to the fungus (National Turfgrass Evaluation Program data, Anonymous, 2001). *Typhula incarnata* is ecologically versatile, adapting to a wide geographic range (Matsumoto et al., 1995) and is reported to have several incompatibility alleles based on mating systems (Bruehl and Machtmes, 1978). There is no molecular information to support or describe variability in *T. incarnata*. Genetic characterization using random amplified polymorphic DNA (RAPD) has been used to characterize diversity between *Typhula* species (Hsiang and Wu, 2000) and study variation within pink snow mold, *Microdochium nivale* (Fr.) Samuels (Mahuku et al., 1998). RAPD analysis may be used to differentiate gray snow mold and study variation within and among populations.

Research into snow mold disease resistance has been hampered by the absence of controlled, reliable screening techniques, in addition to difficult identification of pathogenic strains of the fungus. Field screening for snow mold is time consuming and results may be unreliable due to several possible sources of variation such as presence of multiple pathogens, unevenness of moisture or dryness in some areas, etc. Sources of variation in scoring can be minimized using controlled conditions such as constant temperature, concentration and age of inoculum, and the type or strain of inoculum used. A screening procedure to identify resistant plants in replicated trials could increase the reliability of results. Several genotypes may be screened independent of the presence of snow in cold room chambers.

There are no known snow mold resistant cultivars of creeping bentgrass, *A. palustris* or other species of turfgrass. Bentgrasses have a differential range of susceptibilities or resistance to gray snow mold (Hsiang et al., 1999). Old golf courses containing mixed populations of creeping bentgrasses may be possible sources for resistant plants as natural selection pressures have occurred therein allowing the survival of disease resistant genotypes. Another possible source for snow mold resistance is the germplasm or plant introductions of *Agrostis* species. Using controlled screening procedures, different sources can be explored to find bentgrass materials that may be used to improve the creeping bentgrass.

OBJECTIVES

The four chapters in this dissertation aimed to contribute to turfgrass genetics and enhance breeding efforts by:

- Assessing the genetic diversity in 40 plant introductions of 14 *Agrostis* species from 20 countries.
- Comparing the genetic similarities of old and modern creeping bentgrasses, the distinction of selected lines with resistance to gray snow mold, and genetic relationships of tetraploid creeping bentgrass and hexaploid redtop bentgrass genotypes.
- Characterizing diversity in 40 isolates of gray snow mold, *T. incarnata* from populations in Michigan, Minnesota and Wisconsin, USA.
- Developing controlled screening procedures against gray snow mold and searching for a resistant genotype in creeping bentgrass populations and plant introductions of *Agrostis.*

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CHAPTER I

AFLP Analysis of Genetic Diversity in Bentgrass (*Agrostis* spp.)

ABSTRACT

Bentgrasses (*Agrostis* spp.) are widely occurring temperate grasses with more than 220 species that represent a vast resource for genetic improvement of turfgrass cultivars. Bentgrasses are normally outcrossing species and exhibit many ploidy levels. Difficulties in morphological characterization, which are largely subjected to environmental influences, have resulted in many synonymous species and uncertainties in phylogenetic relationships. To study the genetic diversity and relationships between bentgrass species, forty accessions from the USDA's germplasm collection representing fourteen species of *Agrostis* from twenty countries were investigated using fluorescence-labeled AFLP analysis. Four hundred AFLP markers from five chosen primer combinations were used to differentiate between bentgrass accessions using a bulk of 25 genotypes per accession. Genetic similarities between accessions ranged from 0.62 to 0.98 showing no duplication in the collection and a high level of diversity in *Agrostis*. Both principal component analysis and UPGMA dendrogram clearly distinguished seven groups. Genetic relationships between diploids and other polyploids were revealed in the cluster groupings.

Key words: bentgrass, *Agrostis* spp., genetic diversity, AFLP

INTRODUCTION

Bentgrass, *Agrostis* sp. derivation from Greek: grass, forage, is distributed throughout the world and belongs to the Poaceae family (Watson and Dallwitz, 1992). It is a perennial or annual outcrossing polyploid (2n=14, 28, 42 etc.) which is widely used for putting greens, golf courses, parks and forage. Some species were used for erosion control and revegetation programs in disturbed areas where grazing, mining and flooding had occurred (Brown and Johnston, 1979; Cornely et al., 1983; Winterhalder, 1990). Most bentgrass species are widely adapted to temperate climates and occur in a variety of habitats. Variation in *Agrostis* species denotes potential for genetic improvement of the species for use as turf.

Much of the work in classification of *Agrostis* is predominantly based on morphological and cytological features (Bjorkman, 1960). Linnaeaus grouped *Agrostis* based on the presence or absence of awns, panicle shape and color, and orientation of culms and roots. The basic chromosome number of *Agrostis* is x=7 and differences in ploidy level often determine species boundaries. Diploid species (2n=2x=14) are *A. alpina* Leyss., *A. elegans* Walt., *A. canina* L., *A. flaccida* Hack., and *A. nebulosa* Boiss. & Reut. Most bentgrass species such as *A. capillaris* L., *A. castellana* Boiss. & Reut., *A. palutris* Huds. and *A. stolonifera* L. are tetraploids with 2n=4x=28 (Brede and Sellman, 2001). Species such as *A. castellana*, *A. gigantea* R., *A. alba* L., *A. exarata* Trin., *A. clavata* Trin., *A. diegoensis* Vasey and *A. hallii* Vasey are known to exist in tetraploid and hexaploid forms (2n=6x=42). In a wide collection of *A. gigantea,* Jones (1955a) only found hexaploids. Because of the outcrossing nature of bentgrass, ploidy levels need to be identified. Bonos et al. (1999) used laser flow cytometry as a rapid option to validate the number of chromosomes of certain species of *Agrostis.*

Current significant species in the USA are velvet bentgrass (*A. canina* L.); colonial bentgrass (*A. capillaris* L. or *A. tenuis* Sibth.); dryland or highland bentgrass (*A. castellana* Boiss. & Reut.); creeping bentgrass (*A. palustris* Huds. or *A. stolonifera* L.); red top bentgrass (*A. gigantea* L.); and Idaho bentgrass (*A. idahoensis* Nash). Creeping bentgrass is the premier turfgrass species for closely mowed golf course putting greens (Funk, 1998).

Intra- and interspecific hybridization is possible in *Agrostis*, however, very few genetic studies have explained the contribution of diploids to polyploids and their relationship. Hybridization experiments by Davies (1953), using germplasm from the UK, showed that hybrids of *A. stolonifera* x *A. gigantea* were among the easiest to produce and indicated some degree of homology between the two polyploid species. Bivalent formation during meiosis in hybrids between *A. capillaris* L. x *A. vinealis* Schreb., established *A. capillaris* to be a segmental allotetraploid and from hybrids of *A. stolonifera* x *A. canina* that *A. stolonifera* was a strict allotetraploid (Jones, 1955a). The use of interspecific hybrids of *A. tenuis, A. stolonifera* and *A. gigantea* and their offspring helped decode the genome constitution in *Agrostis.* Jones (1955b) suggested that if *A. tenuis* or *A. capillaris* was $A_1A_1A_2A_2$ and *A. stolonifera* was $A_2A_2A_3A_3$, then *A. gigantea* would be $A_1A_1A_2A_2A_3A_3$. The A_2 genomes of the two species were not confirmed to be absolutely identical. Creeping bentgrass (*A. stolonifera*) was also found to hybridize with ticklegrass (*A. scabra* Willd.) and spike bentgrass (*A. exarata* Trin.)(Welsh et al., 1987). *A. scabra* was found to hybridize with *A. trinii* (Probatova and Kharkevich, 1983).

Warnke et al. (1998) used isozyme analysis to study allotetraploid inheritance in creeping bentgrass and found strong genetic evidence for disomic rather than tetrasomic inheritance.

Little is known about bentgrass genetic relationships and there is much confusion regarding assignment to groups. Early efforts to use morphological characters in classification resulted in many synonymous species. *A. stolonifera* L. was listed as being synonymous with *A. alba* var. *palustris* Huds., *A. alba* var. *stolonifera* (L.) Sm., *A. stolonifera* var. *compacta* Hart., *A. stolonifera* var. *palustris* (Huds.) Farw. and *A. maritima* Lam. (Biota of North America Program, BONAP Poaceae Listing; Plant Gene Resources of Canada (GRIN-CA). *A. gigantea* R. is synonymous with three other species (BONAP Poaceae Listing) and may include subspecies on the basis of rhizome and shoot development and the morphology of the spikelet (Dihoru, 1980). Species *A. trinii* Turcz. was found synonymous with *A. vinealis* ssp. *trinii* (Tzvelev, 1971) and *A. flacidda* ssp. *trinii* (Koyama, 1987) while Soreng et al.(2002) taxonomy listed *A. vinealis* Schreb. as being synonymous with *A. canina* ssp. *vinealis* (Turcz.) Hult. Kurchenko (1979) listed *A. trinii* and *A. canina* both from Russia as different species based on morpho-analytical characters and behavior in their natural environments. Differences in phenotypic expressions are oftentimes the result of environmental fluctuations; therefore, DNA fingerprinting is considered a more stable and reliable technique to explore genetic diversity and relationships.

A wide variety of DNA marker technologies have been used to differentiate bentgrass cultivars and species such as isozyme (Yamamoto and Duich, 1994; Warnke et al., 1997), RAPD (Golembiewski et al., 1997, Scheef et al., 2003) and RFLPs (Caceres et al., 2000). These techniques are limited by the low levels of polymorphism at the intra- and interspecific levels. A more powerful tool in fingerprinting for biodiversity studies over other PCR based techniques is by amplified fragment length polymorphism (AFLP) analysis (Zabeau and Vos, 1993; Vos et al. 1995). AFLPs have been used successfully in order to study genetic diversity in crops like rice (*Oryza sativa* L.)(Zhu et al., 1998), cotton (*Gossypium* sp.)(Abdalla et al., 2001) and common bean (*Phaseolus vulgaris*)(Tohme et al., 1996). The high frequency of identifiable polymorphism is useful for distinguishing among genotypes, detecting linkages and for mapping loci in turfgrasses. Zhang et al. (1999) used AFLPs to differentiate bermudagrass (*Cynodon* spp.) genotypes and determine genetic relationships among genotypes. In addition, they showed that the use of fluorescence-based detection of AFLPs has improved both fragment scoring and data handling. Ebina et al. (1999) constructed an AFLP-based genome map of zoysiagrass (*Zoysia* spp.) and developed a linkage map of QTLs associated with some major traits. AFLPs have not been used to study bentgrass. Understanding bentgrass diversity would facilitate the efficient use of germplasm accessions to combine the favorable agronomic and disease resistance traits to produce superior cultivars.

The objectives of this research were to study the genetic diversity between forty Plant Introduction (PI) accessions comprising fourteen species of *Agrostis* from twenty countries and determine the genetic relationships among the species based on AFLP profiles.

MATERIALS AND METHODS

Plant materials and DNA extraction

Bulked leaf samples from 25 plants each of 40 Plant Introduction (PI) accessions (source: USDA, Regional Plant Introduction Station, Pullman, Washington, USA) of *Agrostis* species from different countries were used (Table 1.1, Figure 1.1). Warnke et al. (1997) compared different number of samples per bulk from the same accession in bentgrass and suggested the least variation using 25 plants per bulk for analysis to minimize against sampling errors. Tissues were ground in liquid nitrogen (Extraction buffer: Tris-HCl, SDS, NaCl) and precipitated using chloroform, sodium acetate and ethanol. All samples were treated with RNAse and twice reprecipitated. Extracted DNA was stored in 1% TE buffer. DNA quality was checked by running 5 μl of the undigested samples in 1% agarose gel containing TBE buffer and compared to EcoR1 digested samples. DNA quantification was performed using DyNA Quant 200 Fluorometer.

AFLP analysis

Approximately 150-200 ng of DNA was used to do AFLP analysis for each accession. Digestion was conducted using two restriction enzymes, EcoR1, a six base pair cutter and Mse-1, a four base pair cutter. The AFLP procedure used in this study was as described by Vos et al. (1995) with modifications. Pre-amplification was done on PTC-100 thermal cycler (MJ Research) using 72°C for 2 min, 30 cycles of 94°C for 30 sec, 60°C for 30 sec, 72°C for 1 min, followed by elongation at 72°C 5 min and 4°C hold. PCR products from initial ligation of adapters were checked on 1.5% TBE agarose gel. Combinations of fluorescent(*) dye labeled E and M primers each with 3 selective nucleotides at the 3' ends were used. The following E* primers were used: E*-ACA and E*-AGC. The following M primers were tested: M-CAT, M-CAG, M-CGG, M-CTT, M-CCT. The 15 μl selective amplification mixture consisted of 15 pmol E*-primer, 75 pmol M-primer , 2 mM dNTP, 1.5 mM PCR buffer, 37.5 mM $MgCl_2$, 1.0 unit Taq Polymerase and 1 μl pre-amplified product in deionized distilled water. The PCR cycling sequence used for selective amplification was as recommended by Vos et al. (1995). Products from selective amplification were checked initially on 1.5% agarose and diluted four times with 0.1 TE buffer. For separation on acrylamide gels, samples consisted of 0.5 μl of the amplified product and 0.5 μl loading dye. The samples were denatured at 95 °C for 5 minutes prior to loading and ran on a 6% Long Ranger polyacrylamide gel with 0.7X TBE buffer using a Licor Gel Analyzer at a constant 800 v for 6 hours at 50°C.

Chromosome analysis

Root tips of species with an unknown number of chromosomes were collected and fixed in 3:1 ethanol (95%), acetic acid (Farmer's fixative). The root tip was rinsed and placed in 3N HCl for 10 to 20 minutes to soften. On a glass slide, the meristematic region was cut and squashed with a blunt end of the needle and a drop of acetocarmine dye was added. Slides were viewed under a phase contrast microscope to determine the number of chromosomes from a several mitotic cells. Chromosome numbers were determined for PI195197 and PI299461, *A. lachnantha* Nees; PI230236, *A. munroana* Aitch. & Hemsl.; PI283174, *A. transcaspica* Litv.; PI477045, *A. hygrometrica* Litv. and PI362190, *A. mongolica* Roshev.

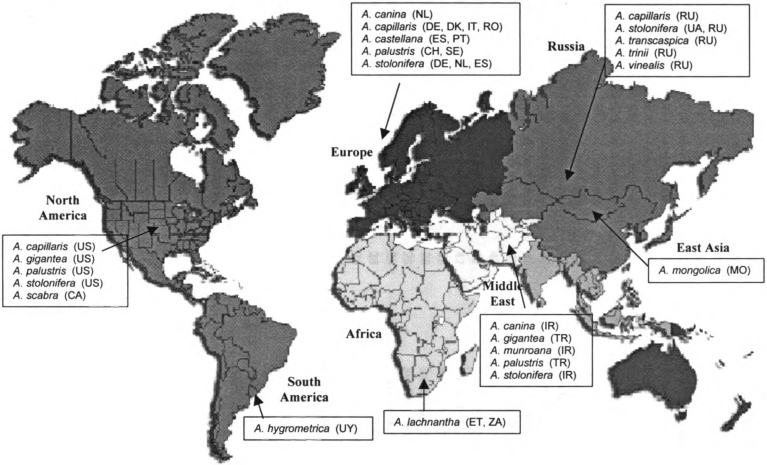

Figure 1.1. World regional sources of 40 accessions of *Agrostis* species.

Legend: CA = Canada; CH = Switzerland; DE = Germany; DK = Denmark; ES = Spain; ET = Ethiopia; IR = Iran; IT = Italy; MO = Mongolia; NL = Netherlands; PT = Portugal; RO = Romania; RU = Russia and former USSR; SE = Sweden; TR = Turkey; UA = Ukraine; US = America; UY = Uruguay; ZA = South Africa

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Table 1.1. List of plant introductions (PI), species, number of chromosomes and geographic origin of bentgrass (*Agrostis* spp.) accessions.

AFLP No.**	PI No.	Species	Chromosome No.(2n)	Geographic Source (Code)
1	194 697	*A. canina*	14	Netherlands (NL)
2	230 233	*A. canina*	14	Iran (IR)
3	237 717	*A. capillaris*	28	Germany (DE)
4	252 045	*A. capillaris*	28	Italy (IT)
5	469 217	*A. capillaris*	42	USA 'Highland' (USH)
6	311 011	*A. capillaris*	28	Romania (RO)
7	392 338	*A. capillaris*	28	Former Soviet Union (RU)
8	234 685	*A. capillaris*	28	Denmark (DK)
9	578 528	*A. capillaris*	28	USA 'Exeter' (USE)
10	302 830	*A. castellana*	42	Spain (ES1)
11	287 741	*A. castellana*	42	Spain (ES2)
12	240 135	*A. castellana*	42	Portugal (PT1)
13	240 139	*A. castellana*	42	Portugal (PT2)
14	240 133	*A. castellana*	42	Portugal (PT3)
15	240 131	*A. castellana*	42	Portugal (PT4)
16	287 744	*A. castellana*	42	Spain (ES3)
17	240 142	*A. castellana*	42	Portugal (PT5)
18	318 928	*A. castellana*	42	Spain (ES4)
19	240 132	*A. castellana*	28	Portugal (PT6)
20	235 440	*A. palustris*	28	Switzerland (CH)
21	235 541	*A. palustris*	28	Sweden (SE)
22	204 390	*A. palustris*	28	Turkey (TR)
23	578 530	*A. palustris*	28	America (US)
24	269 838	*A. stolonifera*	28	Germany (DE)
25	494 119	*A. stolonifera*	28	Netherlands (NL)
26	494 118	*A. stolonifera*	28	Ukraine (UA)
27	230 235	*A. stolonifera*	28	Iran (IR)
28	318 934	*A. stolonifera*	28	Spain (ES)
29	439 027	*A. stolonifera*	28	Former Soviet Union (RU)
30	195 917	*A. lachnantha*	21*	Ethiopia (ET)
31	299 461	*A. lachnantha*	21*	South Africa (ZA)
32	362 190	*A. mongolica*	28*	Mongolia (MO)
33	477 045	*A. hygrometrica*	28*	Uruguay (UY)
34	230 236	*A. munroana*	21*	Iran (IR)
35	234 681	*A. scabra*	42	Canada (CA)
36	598 462	*A. trinii*	28	Russian Federation (RU)
37	440 110	*A. vinealis*	28	Russian Federation (RU)
38	283 174	*A. transcaspica*	14*	Former Soviet Union (RU)
39	383 584	*A. gigantea*	42	Turkey (TR)
40	443 051	*A. gigantea*	42	America (US)

*Number of chromosomes were determined in this study.

**AFLP No. = designated sample number for accession in the AFLP analysis.

Data Analyses

Gels were visualized using Gene ImagIR 4.0 (Scanalytics, Inc.,VA, USA). Each informative polymorphic band was scored manually as 1 for presence and 0 for absence. Analyses were done using Numerical Taxonomy and Multivariate Analysis System, NTSYS v.2.1 (Rohlf, 2000). Genetic similarities based on Jaccard's coefficients (Jaccard, 1908) were calculated among all possible pairs using the SIMQUAL option and ordered in a similarity matrix. The similarity matrix was run on Sequential, Agglomerative, Hierarchical and Nested clustering, SAHN (Sneath and Sokal, 1973) using Unweighted Pair Group Method with Arithmetic Mean (UPGMA) as an option (Sokal and Michener, 1958). Cophenetic correlation was calculated to measure goodness of fit. Principal component analysis (PCA) was run using SYSTAT to identify the number of groups based on Eigen vectors. The TREE module of NTSYS v.2.1 was used to produce the dendrogram and cluster groupings (Rohlf, 2000).

RESULTS AND DISCUSSION

AFLP fingerprinting and polymorphism level in bentgrass accessions

Selective amplifications were made using different primer combinations from the final products of EcoRI/Mse digestion, adapter ligation and pre-amplification. From the initial eight selective primer combinations, five combinations were chosen for clarity, repetitiveness in duplicated gel runs and high levels of polymorphisms. Four hundred polymorphic markers were scored from the five chosen selective primer combinations. The average number of polymorphic bands across the accessions ranged from 100 to 180 markers per primer combination and 20 to 145 polymorphic bands per individual lane.

Data in Table 1.2 showed the selected primers and number of polymorphic markers scored. The high level of polymorphism revealed the wide genetic diversity between the *Agrostis* accessions. Monte et al (1993) found that if extensive diversity exists among taxa or genus, fewer probes or primer combinations showing high polymorphism may be sufficient to distinguish genotypes. None of the bentgrass accessions shared an identical DNA fingerprint. AFLP profiles between accessions indicated that the collection does not contain duplications. The high level of polymorphism has facilitated analysis of the genetic diversity among bentgrass genotypes.

The most robust primer combination with the highest number of polymorphism was E-ACA/M-CAG, whereas the lowest number was observed with E-AGC/M-CGG. Approximately 12% of the markers were specific for individual *Agrostis* species. Specific AFLP markers were found for *A. lachnantha*, *A. vinealis*, *A. scabra, A. munroana, A. stolonifera/A.palustris, A. transcaspica* and *A. hygrometrica,* whereas no specific markers were detected for *A. canina, A. capillaris, A. castellana, A. mongolica* and *A. gigantea.* Data suggest the possibility of developing probes from specific markers to effectively discriminate between some species in the future.

Diversity between accessions and species

Gaps in phylogenetic information of *Agrostis* have resulted from too few hybridization studies, difficulty in obtaining old botanical records and inconsistencies in morphological characterizations i.e. synonyms, subspecies, and re-classification of some species. New phenotypes with different number of chromosomes and distinct characteristics have been given new species names (Probatova and Karkevich, 1983).

Table 1.2. Number of polymorphic bands obtained from different primer combinations.

Primer Pair	No. Polymorphic Bands
E-ACA/M-CAT	138
E-ACA/M-CAG	142
E-AGC/M-CAT	29
E-AGC/M-CAG	69
E-ACA/M-CGG	21

Adapter and primer sequences were adapted from Vos et al. (1995).

E=5'-GACTGCGTACCAATTC-3', M=5'-GATGAGTCCTGAGTAA-3'.

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Another possible source of uncertainty results from spontaneous natural hybrids where lineage and ploidy levels are not known. In this study, assessment of number of chromosomes of the fourteen species of *Agrostis* from the literature and conducted chromosome analyses confirmed the wide range in ploidy levels (2n=14, 21, 42) (Table 1.1). Physical and cytological examinations of the different species showed that ploidy level may not be indicative of plant size in bentgrass. In the germplasm collection, diploid *A. canina* was much smaller compared to another diploid *A. transcaspica*, which in turn had wider and longer leaves and was larger than a triploid, *A. munroana.* Investigations at the molecular level may provide a clearer understanding of the diversity and relationships in bentgrasses.

In this research, four hundred AFLP markers from five selective primer combinations were used to compare 40 bentgrass accessions. Pairwise similarity coefficients (**sc**) were computed based on shared and unique amplification products using UPGMA (Table 1.3). Based on similarity coefficients, the closest pair would be PI235440 (Switzerland) and PI235541 (Sweden) at 0.98. Both accessions belonged to *A. palustris* (creeping bentgrass), had the same number of chromosomes and were found in Europe. The difference between the two PI lines may be due to minimal within-species allelic variation probably resulting from recombination events. Data in Table 1.3 also showed that the most dissimilar pair would be PI230235, *A. stolonifera* from Iran and PI477045, *A. hygrometrica* from Uruguay. The similarity coefficient was only 0.58 indicating large variability in the genomic constitution. Iran and Uruguay are found geographically in distant hemispheres that separated *A. stolonifera* and *A. hygrometrica*

Table 1.3.1. Genetic similarity coefficients for 40 bentgrass (*Agrostis* spp.) accessions from data of five primer combinations using fluorescence-labeled AFLP

Table 1.3.1. Genetic similarity coefficients for 40 bentgrass (*Agrostis* spp.) accessions from data of five primer combinations using fluorescence-labeled AFLP.

No.**	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20 (..cont. next page)
1	1.00																			
2	0.90	1.00																		
3	0.75	0.72	1.00																	
4	0.79	0.74	0.83	1.00																
5	0.72	0.72	0.84	0.84	1.00															
6	0.74	0.72	0.86	0.86	0.88	1.00														
7	0.73	0.72	0.85	0.83	0.87	0.90	1.00													
8	0.75	0.73	0.87	0.84	0.86	0.89	0.90	1.00												
9	0.75	0.75	0.86	0.86	0.86	0.90	0.88	0.94	1.00											
10	0.74	0.70	0.81	0.84	0.87	0.86	0.87	0.87	0.91	1.00										
11	0.74	0.72	0.80	0.80	0.84	0.84	0.84	0.84	0.88	0.89	1.00									
12	0.77	0.73	0.82	0.84	0.88	0.85	0.85	0.88	0.90	0.92	0.89	1.00								
13	0.76	0.73	0.81	0.84	0.87	0.86	0.85	0.87	0.91	0.92	0.89	0.96	1.00							
14	0.75	0.72	0.82	0.83	0.88	0.85	0.86	0.87	0.89	0.92	0.87	0.95	0.95	1.00						
15	0.73	0.71	0.81	0.81	0.85	0.80	0.82	0.83	0.85	0.87	0.85	0.91	0.90	0.91	1.00					
16	0.72	0.68	0.80	0.80	0.85	0.82	0.83	0.84	0.85	0.89	0.86	0.91	0.90	0.90	0.89	1.00				
17	0.76	0.73	0.83	0.82	0.87	0.86	0.85	0.87	0.90	0.92	0.88	0.96	0.95	0.93	0.90	0.93	1.00			
18	0.76	0.72	0.83	0.84	0.88	0.87	0.86	0.88	0.90	0.90	0.86	0.93	0.94	0.92	0.88	0.89	0.94	1.00		
19	0.76	0.72	0.82	0.82	0.87	0.86	0.86	0.87	0.89	0.89	0.86	0.91	0.92	0.90	0.86	0.88	0.92	0.94	1.00	
20	0.72	0.72	0.68	0.71	0.69	0.72	0.75	0.72	0.72	0.70	0.71	0.72	0.73	0.73	0.69	0.69	0.72	0.72	0.72	1.00
..																				
..																				

**AFLP No. = designated sample number for accession in the AFLP analysis and corresponds to the PI No. listed in Table 1.1

Table 1.3.2. (Continuation). Genetic similarity coefficients for 40 bentgrass (*Agrostis* spp.) accessions from data of five primer combinations using fluorescence-labeled AFLP.

No.**	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20 (..cont next page)
21	0.73	0.72	0.68	0.72	0.69	0.71	0.75	0.71	0.72	0.70	0.71	0.73	0.72	0.73	0.69	0.69	0.72	0.72	0.72	0.98
22	0.71	0.71	0.70	0.71	0.70	0.73	0.76	0.72	0.72	0.72	0.71	0.71	0.71	0.72	0.69	0.70	0.72	0.72	0.73	0.89
23	0.75	0.74	0.69	0.75	0.71	0.71	0.75	0.71	0.73	0.72	0.73	0.73	0.73	0.73	0.71	0.70	0.73	0.73	0.73	0.92
24	0.71	0.70	0.71	0.71	0.70	0.72	0.76	0.72	0.72	0.71	0.71	0.73	0.72	0.73	0.70	0.69	0.72	0.71	0.72	0.91
25	0.70	0.69	0.67	0.69	0.67	0.69	0.72	0.69	0.69	0.69	0.70	0.70	0.69	0.69	0.67	0.67	0.69	0.69	0.69	0.93
26	0.71	0.71	0.70	0.70	0.68	0.69	0.73	0.70	0.70	0.69	0.69	0.71	0.70	0.71	0.68	0.67	0.70	0.70	0.71	0.92
27	0.69	0.68	0.70	0.71	0.70	0.74	0.76	0.71	0.70	0.70	0.69	0.69	0.69	0.69	0.67	0.68	0.70	0.70	0.71	0.81
28	0.72	0.70	0.68	0.71	0.69	0.70	0.73	0.69	0.70	0.71	0.72	0.72	0.71	0.72	0.68	0.70	0.72	0.72	0.72	0.91
29	0.70	0.68	0.75	0.76	0.74	0.74	0.79	0.75	0.75	0.77	0.76	0.76	0.76	0.76	0.76	0.75	0.75	0.74	0.74	0.77
30	0.67	0.64	0.64	0.65	0.62	0.62	0.62	0.62	0.64	0.62	0.65	0.63	0.62	0.63	0.62	0.63	0.63	0.62	0.64	0.65
31	0.66	0.64	0.65	0.66	0.60	0.62	0.63	0.62	0.64	0.63	0.64	0.62	0.62	0.62	0.62	0.63	0.63	0.61	0.63	0.65
32	0.70	0.69	0.74	0.73	0.74	0.74	0.78	0.73	0.73	0.74	0.74	0.74	0.73	0.75	0.71	0.71	0.73	0.72	0.74	0.77
33	0.68	0.60	0.61	0.63	0.60	0.61	0.61	0.61	0.61	0.63	0.64	0.63	0.62	0.61	0.60	0.60	0.61	0.62	0.61	0.62
34	0.73	0.66	0.63	0.66	0.59	0.62	0.62	0.61	0.63	0.63	0.65	0.66	0.65	0.65	0.62	0.64	0.65	0.63	0.65	0.66
35	0.76	0.71	0.69	0.70	0.66	0.66	0.67	0.68	0.67	0.66	0.69	0.70	0.70	0.69	0.69	0.66	0.68	0.68	0.69	0.68
36	0.73	0.70	0.79	0.79	0.77	0.77	0.78	0.81	0.80	0.77	0.75	0.78	0.77	0.76	0.76	0.75	0.77	0.76	0.77	0.65
37	0.75	0.76	0.65	0.68	0.63	0.63	0.63	0.64	0.65	0.63	0.67	0.68	0.66	0.65	0.66	0.63	0.66	0.65	0.65	0.65
38	0.74	0.71	0.73	0.72	0.71	0.73	0.76	0.73	0.73	0.73	0.74	0.73	0.74	0.73	0.69	0.70	0.73	0.72	0.72	0.75
39	0.75	0.71	0.71	0.73	0.70	0.71	0.76	0.71	0.72	0.72	0.75	0.74	0.74	0.73	0.71	0.70	0.73	0.73	0.73	0.79
40	0.76	0.72	0.78	0.82	0.76	0.78	0.80	0.80	0.81	0.78	0.78	0.80	0.79	0.79	0.78	0.76	0.78	0.78	0.80	0.72

**AFLP No. = designated sample number for accession in the AFLP analysis and corresponds to the PI No. listed in Table 1.1

Table 1.3.3 (Continuation). Genetic similarity coefficients for 40 bentgrass (*Agrostis* spp.) accessions from data of five primer combinations using fluorescence-labeled AFLP.

No.**	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40
21	1.00																			
22	0.90	1.00																		
23	0.93	0.88	1.00																	
24	0.93	0.88	0.91	1.00																
25	0.93	0.87	0.92	0.93	1.00															
26	0.93	0.87	0.91	0.93	0.95	1.00														
27	0.82	0.82	0.81	0.82	0.81	0.80	1.00													
28	0.92	0.86	0.91	0.91	0.93	0.93	0.80	1.00												
29	0.78	0.78	0.80	0.80	0.76	0.77	0.76	0.79	1.00											
30	0.67	0.66	0.69	0.67	0.66	0.67	0.63	0.68	0.62	1.00										
31	0.65	0.65	0.69	0.65	0.65	0.66	0.61	0.67	0.62	0.94	1.00									
32	0.78	0.80	0.79	0.79	0.76	0.77	0.77	0.79	0.87	0.62	0.63	1.00								
33	0.63	0.59	0.63	0.61	0.61	0.60	0.58	0.64	0.59	0.62	0.61	0.61	1.00							
34	0.66	0.64	0.68	0.66	0.65	0.65	0.63	0.66	0.64	0.69	0.69	0.66	0.68	1.00						
35	0.69	0.68	0.71	0.69	0.67	0.67	0.65	0.68	0.68	0.68	0.67	0.69	0.66	0.71	1.00					
36	0.67	0.67	0.69	0.68	0.66	0.67	0.67	0.67	0.73	0.62	0.62	0.70	0.59	0.64	0.67	1.00				
37	0.66	0.63	0.68	0.67	0.65	0.67	0.62	0.67	0.65	0.62	0.61	0.65	0.61	0.67	0.69	0.68	1.00			
38	0.75	0.78	0.77	0.76	0.75	0.75	0.71	0.75	0.73	0.63	0.62	0.74	0.62	0.68	0.71	0.73	0.68	1.00		
39	0.80	0.81	0.80	0.79	0.80	0.79	0.73	0.80	0.74	0.63	0.62	0.75	0.64	0.67	0.72	0.74	0.70	0.86	1.00	
40	0.73	0.72	0.77	0.73	0.72	0.73	0.72	0.75	0.77	0.69	0.69	0.76	0.63	0.70	0.71	0.84	0.71	0.77	0.80	1.00

**AFLP No. = designated sample number for accession in the AFLP analysis and corresponds to the PI No. listed in Table 1.1

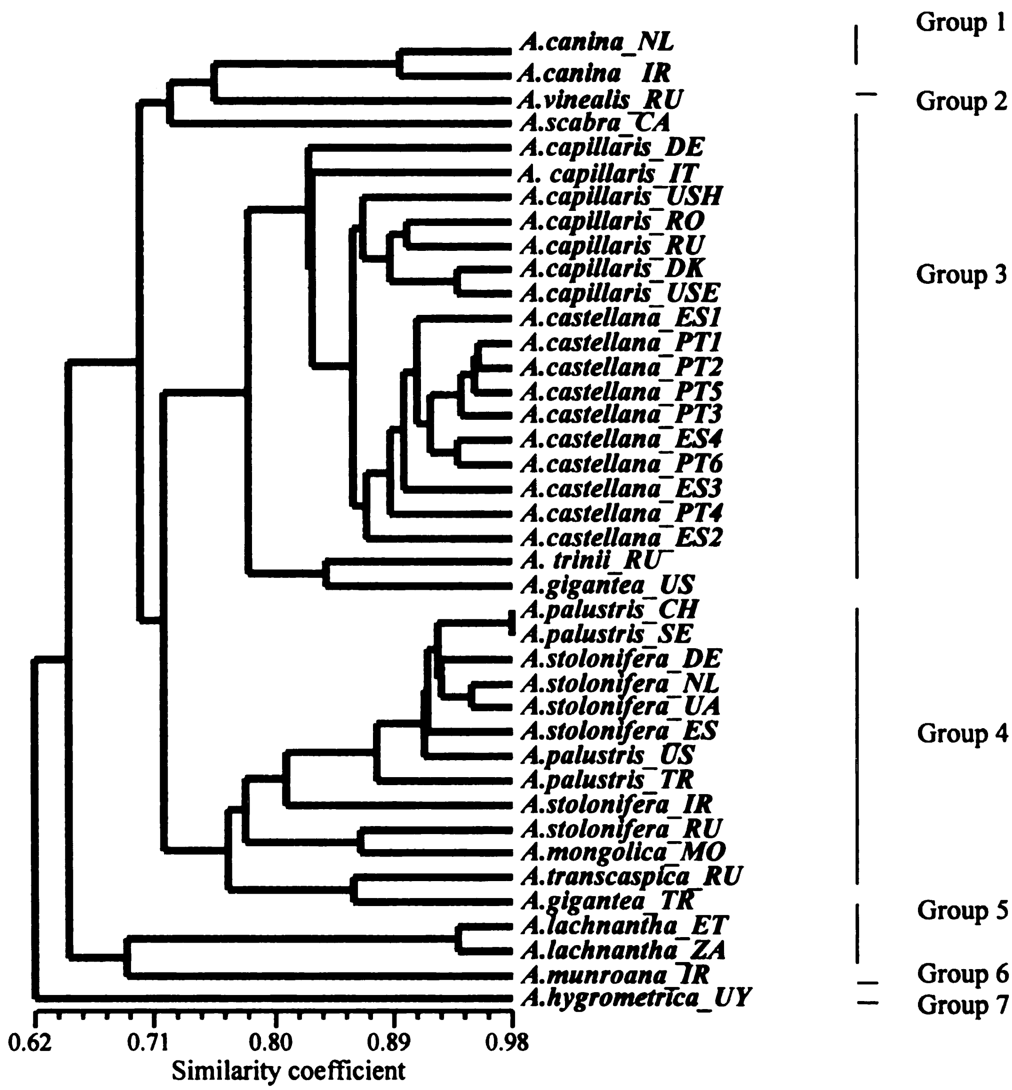

Figure 1.2. UPGMA dendrogram of 40 accessions of 14 *Agrostis* spp. from 20 different countries. PCA analysis distinguishes seven groups based on Eigen values > 1.0.

Legend: CA = Canada; CH = Switzerland; DE = Germany; DK = Denmark; ES = Spain; ET = Ethiopia; IR = Iran; IT = Italy; MO = Mongolia; NL = Netherlands; PT = Portugal; RO = Romania; RU = Russia and former USSR; SE = Sweden; TR = Turkey; UA = Ukraine; US = America; UY = Uruguay; ZA = South Africa

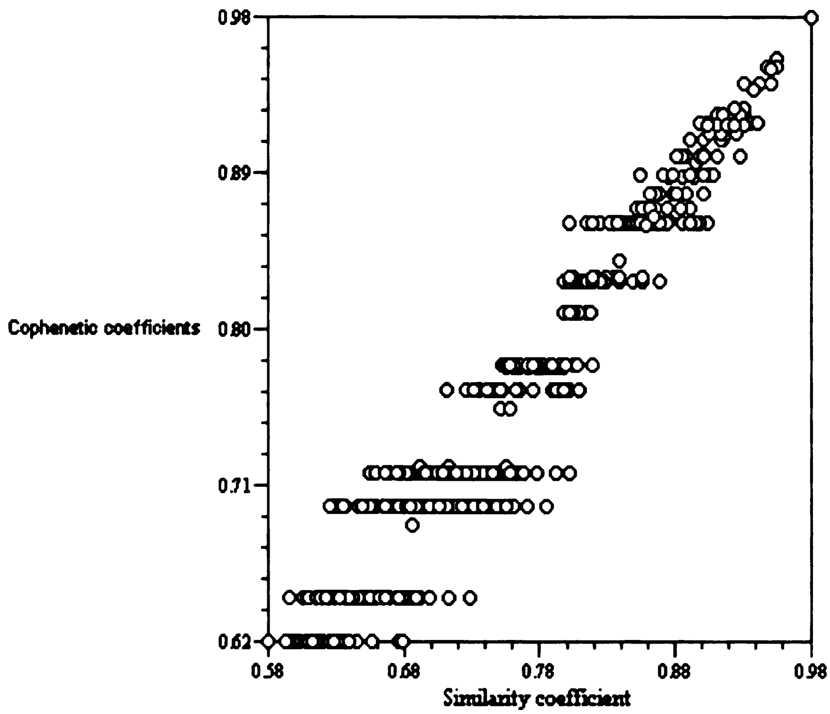

Figure 1.3. Plot analysis of cophenetic correlation and similarity coefficient as a measure of goodness of fit of the similarity indices. r = 0.95951 = normalized Mantel statistic Z; Approximate Mantel t-test: t = 11.9767; P(Z < obs. Z: p = 1.0000).

and AFLP analysis suggested the two species had the least homology, genetic exchange or introduction among the accessions studied.

A single dendogram was generated from the UPGMA cluster analysis with one possible tie found between the closest pair (Figure 1.2). A cophenetic-value (ultrametric) matrix was generated from the coefficients of SAHN's cluster analysis of the distance matrix. The cophenetic correlation was calculated (r = 0.96) as a measure of goodness of fit and the results were plotted in a phenogram (Figure 1.3). Using SYSTAT, a rotated PCA with the bands as observations was used to determine the number of factors or groups based on eigenvalues greater than one. Seven groups were extracted, which explained 72% of the total variance. The dendrogram showed a similarity coefficient of 0.73 for these seven groups.

Group 1 consisted of two accessions of *A. canina* (Netherlands and Iran, **sc** = 0.90) and *A. vinealis* (Russian Federation, **sc** = 0.76 to *A. canina*). Though geographically distant in origin, the two *A. canina* diploids were morphologically similar and AFLP data supported their grouping. In Figure 1, *A. vinealis* grouped closest to *A. canina* than the other twelve species in the study and confirmed their genetic and physical similarity. Species in Group 1 showed very fine, short erect leaves and low growth, but *A. vinealis* was rhizomatous and confirmed earlier descriptions (Hubbard, 1984; Funk, 1998; Brilman, 2001). In catalogues, *A. vinealis* Screb. was listed as being synonymous to eight other species names: *A. canina* ssp. *montana* Hartman, *A. stricta* J.F. Gmel (BONAP Poaceae Listing), *A. syreistschikowii* P.A.S., *A rubra* L., *A. ericetorum* P.B, *A. tenuifolia* M.B., *A. coarctata* E. and *A. pusilla* D. by the Royal Botanic Garden Edinburgh. Jones (1955a) found that *A. canina* ssp. *montana* (*A. vinealis*) also known as brov

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brown velvet bentgrass, was an autotetraploid form of *A. canina* and difficult to distinguish in field specimens. Davies (1953) correlated the morphological difference with ecological preference. Velvet bentgrass occurred in natural wet and damp soils while brown velvet bentgrass was found in heaths and upland ground. Molecular characterization by AFLP confirmed the relationship between *A. canina* and *A. vinealis*. *A. canina* was morphologically distinguished from other species of *Agrostis* physically by having a longer, more pointed ligule and shorter palea than *A. capillaris* or *A. palustris* (Hubbard, 1984). Support that *A. canina* may be grouped separately from the other species was also shown by AFLP data.

Group 2 consisted only of PI234681, *A. scabra* (Canada) and AFLP analysis showed that the similarity with *A. canina* was only 0.74. Morphologically, *A. scabra* was also a non-rhizomatous bunch type grass like *A. canina* but the two species differed in ploidy levels. Dissimilarity may come from the hexaploid nature and more diverse genetic composition of the species in Group 2. Known as ticklegrass or hairgrass, *A. scabra* Willd., a delicate species was listed as being synonymous with winter bentgrass (*A. hyemalis* Tuck.) but differed in flowering time (Lawrence, 1986).

Group 3 consisted of three subgroups. Subgroup A consisted of the seven accessions of *A. capillaris* (colonial bentgrass) from seven countries. This indicated that the seven allotetraploid accessions were similar and/or may have originated from common diploid ancestors. Visual classification between colonial and highland bentgrass was difficult. Hubbard (1984) separated them by flower color, ligule size and growth habit. Two accessions of *A. capillaris* (US) cv. 'Highland' and cv. 'Exeter' were reclassified as *A. castellana* (Hubbard, 1984; Shildrick, 1976; Brilman, 2001). Steiner

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and Lupold (1978) supported the suggestion that *A. capillaris* cv. 'Highland' should belong to *A. castellana* based on the percentage distribution of the form of palea apex, the presence and angle of basal hairs and awn types. In this study, AFLP analysis of PI469217 'Highland' and PI578528 'Exeter' showed that though they contained a small number of specific bands present only in *A. castellana*, they clustered more with the *A. capillaris* group. There is no information whether the plant introductions were seeded in open or isolated places during seed increase. Cross contamination at any time may add to the taxonomic variation. Yamamoto and Duich (1994) could not distinguish 'Highland' and 'Exeter' from the other ten colonial bentgrasses using phosphoglucoisomerase, glutamate oxalotransaminase, peroxidase and topoisomerase isozymes. Difference at the Pox-2 locus was observed (Yamamoto and Duich, 1994) but peroxidase isozymes are oftentimes unstable and dependent on stress conditions. Subgroup B consisted of all PI accessions of *A. castellana* from Spain and Portugal with no distinction as to groupings based on origin. The two subgroups, A and B, may share common diploid species progenitors and may be related with *A. capillaris* from Germany and Italy.

Subgroup C consisted of PI598462 *A. trinii* (Russian Federation) and PI443051 *A. gigantea* (USA). Classification of *A. trinii* Turcz. has been unclear and listed as being synonymous to *A. canina* ssp. *trinii* (Turcz.) Hulten (Soreng et al., 2003). Cluster analysis however showed *A. trinii* to be dissimilar to *A. canina* (**sc** = 0.70 to 0.73). Skolovskaya (1938) described *A. trinii* in Siberia and Orient as having both diploid and tetraploid forms. The diploid form of *A. trinii* may have been recognized as subspecies of *A. canina* (Brilman, 2002). PI598462 *A. trinii* was known to be a tetraploid and appeared different phenotypically from *A. vinealis*, an autotetraploid form of *A. canina*.

Agrostis trinii (Russian Federation) could be an allopolyploid with one chromosome set different from *A. canina* and *A. vinealis*, thus forming separate groups. The other member of this subgroup, *A. gigantea* (US) surprisingly was not grouped with the *A. gigantea* (Turkey). Hexaploid *A. gigantea* genomic constitution of $A_1A_1A_2A_2A_3A_3$ may have consisted of A_1A_1 from *A. canina*, with the $A_2A_2A_3A_3$ probably from *A. stolonifera* or $A_1A_1A_2A_2$ from *A. capillaris* (Jones, 1955b). AFLP data supported both possibilities. The two PI lines of *A. gigantea* may possibly have different chromosome sets but share the common A_1A_1 genome. Based on the clustering, PI443051 (USA) may have the $A_1A_1A_2A_2$ from *A. capillaris* while PI383584 (Turkey) may have the $A_2A_2A_3A_3$ from *A. stolonifera*. *A. gigantea* (Turkey) may have a second genomic constitution originating from another species. In this study, *A. transcaspica* (Former USSR) was found to be diploid and clustered closest with the *A. gigantea* (Turkey). This indicated the possibility that *A. transcaspica* was closely related to this species and may be the source for the A_3A_3 genome in *A. gigantea* (Turkey). This relationship would be interesting to examine in future interspecific hybridization and cytogenetic studies.

Group 4 consisted mainly of the creeping bentgrass (*A. palustris* and *A. stolonifera)*, *A. gigantea* (Turkey), *A. mongolica* and *A. transcaspica*. There has been considerable taxonomic confusion regarding creeping bentgrass and whether they should also be *A. stolonifera* with subspecies *palustris*, ssp. *stolonifera* or ssp. *gigantea*. The genetic similarity coefficient between PI accessions in this group ranged from 0.82 to 0.95. Genetic dissimilarity computed from 1- **sc** x 100% (Zhang et al., 1999) ranged from 5 to 18%. Turf breeders believe *A. palustris* (USA) have originated from and frequently outcrossed with materials from Europe. AFLP data supported this idea and results sho

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showed that USA creeping bentgrasses may share some genetic similarity with those from Switzerland and Sweden. The most divergent in *A. stolonifera* would be from Turkey, Iran and the former USSR as opposed to those originating from other parts of Europe. No separation of groups for *A. palustris* and *A. stolonifera* was observed based on AFLP, but they differed slightly from *A. gigantea*. The difference may be due to the third chromosome set of hexaploid *A. gigantea*. Caceres et al.(2000) used RFLP markers to distinguish four creeping bentgrass *A. stolonifera* cultivars from *A. capillaris* 'Highland'. AFLP data supported that PI accessions of *A. stolonifera* from different parts of the globe were in one group and differed from *A. capillaris* (Group 3, Subgroup A). Species *A. stolonifera* also shared slight similarities with *A. mongolica* (**sc** = 0.76 to 0.87*)* and *A. transcaspica* (**sc** = 0.73 to 0.80*)*. PI362190 *A. mongolica* plant type was also stoloniferous and chromosome analysis showed that the bentgrass from Eastern Europe was also a tetraploid. UPGMA analysis grouped *A. mongolica* (Mongolia) closest to *A. stolonifera* (Russia). Because Mongolia and Russia are geographically adjacent, these countries may have similar environmental conditions favorable to both species. The fourth species in Group 4, which comprised mostly of stoloniferous bentgrasses, was *A. transcaspica*, PI283174 (Former USSR). Physical examination showed *A. transcaspica* also to be creeping but differed in leaf characteristics. The species has wider (6 to18 mm) dark green, thick leaves with pointed tips. Soreng et al. (2003) has listed *A. transcaspica* Litv. as being synonymous to *A. stolonifera* ssp. *transcaspica* (Litv.) Tzvelev. AFLP clustering and similarity coefficients indicated that *A. transcaspica*, a diploid may have contributed to the tetraploid or hexaploid creeping bentgrass genome.

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Group 5 consisted of *A. lachnantha* N. (Ethiopia and South Africa). The African bentgrasses were morphologically distinct from the other bentgrass species. PI195917 (Ethiopia) bentgrasses were shorter (4 to 10 inches) than PI299461 (South Africa, >10 inches). The latter also has fewer leaves, harder stalks, and produced very tall flowering panicles (>2 feet) in the greenhouse but were highly sterile. The chromosome number reported here differed from the listing of the Index to Plant Chromosome number (IPCN) with gametophytic count (n=21) and sporophytic 2n=28. Chromosome analysis of 2n=21 may suggest intra- or intercrossing variation during seed increase. The two PI accessions of *A. lachnantha* has **sc**=0.94 based on AFLP analysis. Eleven specific AFLP markers were found that could distinguish *A. lachnantha* species from the other thirteen species.

Group 6 consisted only of PI230236 *A. munroana* (Iran). Background information about *A. munroana* Aitch. & Hemsl. was minimal and was earlier referred to as *Calamagrostis munroana* (Aitch. & Hemsl.) Boiss. in 1884 (Soreng et al., 2003). Chromosome analysis of plants of PI230236 showed 2n=21 which confirmed earlier reports (Gohil and Koul, 1986; Mouinuddin et al., 1994). Physical analysis of the mature triploid plant showed a short plant stature (2 to 6 inches) with fine (2 to 3 mm width), flat, soft, normal green leaves. Species *A. munroana* was observed to be a bunch type bentgrass and early flowering with the florets openly branched. *A. munroana* plants were morphologically distinct from triploids of *A. lachnantha.* Their triploid genome constitutions may be largely unlike and AFLP results showed **sc** = 0.61, thus forming separate groups.

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Group 7 was the most genetically distant from the thirteen other species studied and included *A. hygrometrica* (Uruguay). Seven specific AFLP markers were found for this species. *A. hygrometrica* Nees. has nine synonyms in genus *Agrostis* or *Bromidium* (Soreng et al., 2003). Plant materials from PI477045 were low growing, bunch-type grasses with hard, lengthy flowering panicles. AFLP analysis showed that bentgrass germplasm from Uruguay (South America) formed a separate group from other bentgrasses of Europe, Asia or North America. Distinct ecological conditions among continents and unique germplasm pools from which the *A. hygrometrica* may intercross would differentiate the accessions.

Assessment of genetic diversity in germplasm collections from several geographic locations using AFLP markers has been conducted for *Morus* germplasm (Sharma et al., 2000) and the Triticeae tribe (Monte et al., 1993). Positive correlations were found for cluster groupings and geographic distances. Results in this study indicated that geographically adjacent countries like Spain and Portugal have bentgrass accessions which also clustered together as in Group 3, Subgroup B and bentgrass accessions from distant locations (Iran vs. Uruguay) were in separate groups with low similarity coefficients. Possibilities of genetic introductions may have occurred with migration, selection and breeding among the colonial, highland and creeping bentgrasses from Europe and USA. Local environmental adaptation may play a significant role in *Agrostis* diversity.

AFLP analysis revealed its usefulness for assessing germplasm collection for possible duplications. It may also indicate where incorrect species determinations would be in the GRIN system or germplasm collection. The four hundred polymorphic markers from five chosen primer combinations showed the high level of diversity between the *Agrostis* germplasm and distinguished the seven groups. Ploidy level differences did not give ambiguous results in scoring as highly polymorphic, repetitive and specific bands were found. The high cophenetic correlation showed the goodness of fit of the similarity indices. AFLP analyses supported the hybridization paths between species (Figure 1.4). The dendogram showed the relationships between *A. canina* with *A. vinealis* but not with *A. trinii.* Cluster analysis also showed that two hexaploid *A. gigantea* from different geographic sources (USA and Turkey) were not grouped together, possibly due to different chromosome sets. A possible diploid progenitor would be *A. transcaspica.* The percentage genetic dissimilarity among creeping bentgrasses indicated considerable potential for the improvement of turf. Turfgrass breeders may develop superior cultivars either by crosses with germplasm accessions from the same species or among varying species. Important traits from other *Agrostis* species can be introduced to cultivated bentgrasses and AFLP analysis would be a useful tool to monitor introgression and molecular tagging. Using specific amplified products, sequence characterized amplified primers may be developed to genetically distinguish the different bentgrass species in the future. AFLP analysis may be used in identifying bentgrass genotypes and clusters, constructing core collections and screening for duplicate or misclassified accessions in germplasm collections.

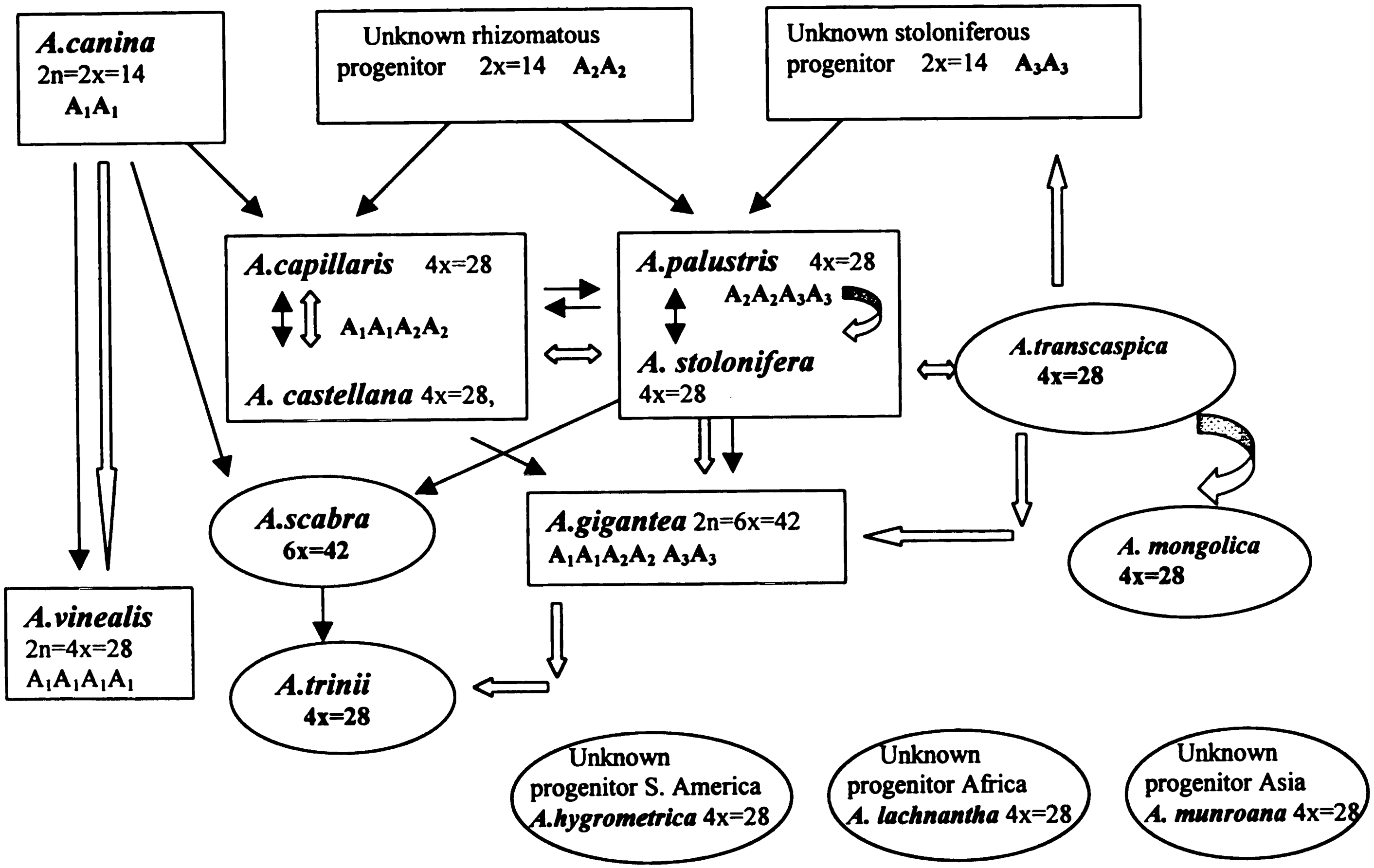

Figure 1.4. Diagram of hybridization pathways of *Agrostis* species (indicated by solid arrows) and relationships supported by AFLP analyses (indicated by unfilled arrows).

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CHAPTER II

Genetic Differentiation of Tetraploid Creeping Bentgrass and Hexaploid Redtop Bentgrass Genotypes by AFLP and Their Use in Turfgrass Breeding

ABSTRACT

The turf industry in the last decade has seen doubling in number of new creeping bentgrass (*Agrostis stolonifera* var. *palustris* Huds. and *A. stolonifera var. stolonifera* Huds.) cultivars, many with unknown variability and lineage. Understanding the genetic diversity of putative parental and wild stocks would be useful in breeding programs. AFLP analysis was conducted to investigate genetic variability among old and new cultivars of creeping bentgrasses, redtop bentgrasses (*Agrostis gigantea* Roth.), plant introductions and selected genotypes with resistance to gray snow mold (*Typhula incarnata* Lasch). Seven chosen primer combinations resulting in 355 polymorphic markers were used to differentiate the bentgrasses. Three groups were extracted using principal component analysis. Using UPGMA analysis, mean similarity coefficients of creeping bentgrass genotypes found in the first group was 0.78. Creeping bentgrasses in the USA were clustered as a subgroup and separated from European plant introductions, indicating that most selection and genetic exchanges in the last fifty years have evolved locally. Redtop bentgrasses were the most diverse and were found in different groups. Selected lines from northern Michigan, MI 20104, MI 20215 and MI 203164 were differentiated from the other cultivars and would be advantageous to use as sources of disease resistant traits and for development of populations for future gene mapping.

Predictive estimates of genetic variation and molecular identification of new cultivars and interrelationships would be important for advanced breeding of bentgrass species.

Key words: AFLP analysis, turfgrass breeding, *Agrostis stolonifera* var. *palustris* Huds., *Agrostis stolonifera* var. *stolonifera* Huds., *Agrostis gigantea* Roth.

INTRODUCTION

Creeping bentgrasses (2n=4x=28) are the premier and most widely used cool-season turfgrasses for golf course putting greens, tees and fairways in the USA (Funk, 1998). Redtop bentgrasses (2n=6x=42) are widely used in seed mixtures for rapid vegetation cover. These two species of bentgrasses are differentiated from other species by their profuse creeping stolons forming dense mats, vigorous shallow roots and their attractive range of bluish-green to grayish appearance. From 1927 to 1994, less than twenty cultivars have been released by different institutions and private seed companies in the USA but within the past eight years, the number of cultivars that has been bred have doubled. Creeping bentgrasses are synthetic products created from crossing of three or more clones and recurrent selection. Improved turf quality and appearance, tolerance to close mowing, regenerative and seed yield potential, and tolerance to diseases are some important parameters for selection. Some of these new cultivars may be closely related but their interrelationships are unknown. Diversity within creeping bentgrasses appears limited as their genetic variability may be narrow (.007 to .08%) as revealed by isozyme polymorphism (Yamamoto and Duich 1994; Warnke et al., 1997). Plant introductions from germplasm collections could be used to widen the genetic base.

Morphologically, creeping bentgrasses are difficult to distinguish and taxonomic confusion has resulted in many synonymous names for the tetraploid species. *Agrostis stolonifera* L. was listed as being synonymous with *A. alba* var. *palustris* Huds.; *A. alba* var. *stolonifera* (L.) Sm; *A. stolonifera* var. *compacta* Hart.; A. *stolonifera* var. *palustris* (Huds.) Farw. and *A. maritima* Lam. (Texas A&M, BONAP Poaceae Listing 2003; Plant Gene Resources of Canada, GRIN-CA Taxonomy, 2003). Hexaploid bentgrasses were referred to as *A. stolonifera* sp. *gigantea* (Huds.), *A. gigantea* L. or *A. alba* sp. *gigantea* Huds. Adding to the taxonomic complexity are other factors; i.e. outcrossing nature of bentgrasses, ability to form interspecific hybrids and environmental fluctuations that may result in differences in phenotypic expressions and inconsistencies in classification. A more stable tool like DNA molecular marker technology may help resolve identification and offer insights to the degree of genetic variability and relationships among cultivars and to help define future breeding strategies.

Molecular marker studies using amplified fragment length polymorphism (AFLP) analyses have been used to differentiate bermudagrass (*Cynodon* spp.) genotypes (Zhang et al., 1999) and to construct a genomic map of zoysiagrass (*Zoysia* spp.) (Ebina et al., 1999). Some bentgrass cultivars and species have been differentiated using random amplified polymorphic DNA (RAPD) (Golembiewski et al., 1997, Scheef et al., 2001) and restriction fragment length polymorphism (RFLP) (Caceres et al., 2000). Our recent work in the study of genetic diversity in the *Agrostis* species using AFLPs has shown the advantage of this technique for biodiversity studies, distinguishing fourteen species of bentgrasses into seven major groups (Vergara and Bughrara, 2003). Stoloniferous bentgrasses from several countries formed a single cluster comprised of *A. stolonifera* L.

or *A. palustris* Huds., *A. gigantea* L., *A. mongolica* Roshev., and *A. transcaspica* Litv. The percentage of genetic dissimilarity (5 to 18%) within plant introductions of creeping bentgrasses indicated considerable potential for the improvement of turf.

Turfgrass breeders are currently interested in developing superior cultivars of *A. stolonifera* var. *palustris* with resistance to major diseases such as dollar spot (caused by *Rutstroemia floccosum*, teleomorph of *Sclerotinia homeocarpa* F.T. Bennett) (Powell, 1998), brown patch (*Rhizoctonia solani* Kuhn) and snow molds (*Typhula* spp. and *Microdochium nivale* Fr.) (Vargas, 1994; Hsiang et al., 1999). In 2000 and 2001, the Michigan State University (MSU) turf breeding program isolated and selected experimental lines, MI 20104, MI 20215 and MI 203164, with resistance to gray snow mold from replicated controlled screening trials. Our objective was to use AFLPs to investigate genetic variability among old and new cultivars of creeping bentgrasses, redtop bentgrasses, plant introductions and differentiate MSU experimental lines of creeping bentgrasses. Predictive estimates of genetic variation will be useful in planning turfgrass breeding programs and developing heterotic populations. Marker data and identified AFLP primer combinations will be useful in future mapping studies.

MATERIALS AND METHODS

Plant materials and DNA extraction

Bulk leaf samples from 25 plants each of cultivars 'Seaside', 'Penncross', 'Providence', 'L-93', 'Penn G2', 'Penn A4', 'S. Redtop', 'Emerald' and redtop bentgrass cultivar 'S. Redtop' were used. Widely used cultivars developed during different periods of time were chosen. Leaf samples from identical selected clones of MI 20104, MI 20215

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and MI 203164 selected from Northern Michigan golf courses in 2000 were included for comparison. In addition, Plant Introductions (PI) of eight creeping bentgrass accessions and two redtop bentgrass accessions (source: USDA Plant Introduction Station, Pullman, WA) from different countries were included (Table 2.1). Fresh leaf tissues were ground in liquid nitrogen and an equal volume of extraction buffer was added. The extraction buffer consisted of Tris-EDTA-HCl, SDS, NaCl with 0.38 g/100 ml buffer of sodium bisulfite. The mixture was incubated at 65 °C for 20 min followed by the addition of 10 ml of 5M potassium acetate. After 20 min incubation with ice in a gyratory shaker, the suspension was centrifuged at 3000 *g* RCF (Sorvall RT7 model) for 20 min at 5 °C. The supernatant was collected to which 2/3 volume of cold isopropanol was added to precipitate the DNA. All DNA samples were treated with RNAse dissolved in TE buffer and twice reprecipitated by using 1/10 volume of 3M sodium acetate followed by two volumes of chilled absolute ethanol. Extracted DNA was stored in 1% TE buffer. DNA quality was checked by running 5 μl of the undigested samples in 1% agarose gel containing TBE buffer and compared to *EcoR*I digested samples. DNA quantification was done using a DyNA Quant 200 Fluorometer (Pharmacia Biotech, CA).

AFLP analysis

Approximately 150 to 200 ng of DNA was used for AFLP analysis of each genotype. Digestion was conducted using two restriction enzymes, *Eco*RI, a six base pair cutter and *Mse*-I, a four base pair cutter. The AFLP procedure used in this study was as described by Vos et al. (1995) with modifications. Pre-amplification was done on a PTC-100 thermal cycler (MJ Research, Inc., MA) using 72°C for 2 min, 30 cycles of 94°C for 30 sec, 60°C for 30 sec, 72°C for 1 min, followed by elongation at 72°C for 5 min and 4°C

Table 2.1. Cultivars, MSU experimental lines and plant introductions (PI) lines of creeping and redtop bentgrasses (*Agrostis* spp.) examined, year released or collected and their sources.

Bentgrass	Scientific name	Year[a]	Source/Origin
Cultivars			
Seaside	*A. stolonifera var. palustris*	1928	Tee-2 Green Corp., US
Penncross	*A. stolonifera var. palustris*	1955	Pure Seed Testing, Inc., US
Providence	*A. stolonifera var. palustris*	1987	Seed Research of OR, Inc., US
L-93	*A. stolonifera var. palustris*	1995	Agri-Biotech Inc., US
Penn G2	*A. stolonifera var. palustris*	1995	Tee-2 Green Corp., US
Penn A4	*A. stolonifera var. palustris*	1995	Tee-2 Green Corp., US
Emerald	*A. stolonifera var. palustris*	1973	Cebeco Intl. Seeds, Inc.,US
S. Redtop	*A. stolonifera var. palustris*	1989	Pickseed West, Inc., US
Experimental Lines			
MI 20104	*A. stolonifera var. palustris*	2000	Michigan State University, US
MI 20215	*A. stolonifera var. palustris*	2000	Michigan State University, US
MI 203164	*A. stolonifera var. palustris*	2000	Michigan State University, US
Plant Introductions			
PI 251 945	*A. stolonifera var. palustris*	1958	USDA PI from Austria (AT)
PI 235 440	*A. stolonifera var. palustris*	1956	USDA PI from Switzerland (CH)
PI 235 541	*A. stolonifera var. palustris*	1956	USDA PI from Sweden (SE)
PI 204 390	*A. stolonifera var. palustris*	1953	USDA PI from Turkey (TR)
PI 269 838	*A. stolonifera var.stolonifera*	1960	USDA PI from Germany (DE)
PI 494 119	*A. stolonifera var.stolonifera*	1984	USDA PI from Netherlands (NL)
PI 230 235	*A. stolonifera var.stolonifera*	1955	USDA PI from Iran (IR)
PI 439 027	*A. stolonifera var.stolonifera*	1978	USDA PI from Russia (RU)
PI 383 584	*A. gigantea*	1972	USDA PI from Turkey (TR)
PI 443 051	*A. gigante*	1980	USDA PI from US

[a] Year of release for cultivars and year collected for experimental lines and PIs.

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hold. PCR products from initial ligation of adapters were checked on 1.5% TBE agarose gel. Combinations of fluorescent (*) dye labeled E and M primers each with 3 selective nucleotides at the 3' ends were used. The following E* primers were used: E*-ACA and E*-AGC. The following M primers were tested: M-CAT, M-CAG, M-CGG, M-CGA, M-CTT, M-CCT. The 15 µl selective amplification mixture consisted of 15 pmol E*-primer, 75 pmol M-primer , 2 mM dNTP, 1X PCR buffer, 37.5 mM $MgCl_2$, 1.0 unit Taq polymerase in deionized distilled water. The PCR cycling sequence used for selective amplification was as recommended by Vos et al. (1995). Products from selective amplification were checked initially on 1.5% agarose and diluted four times with 0.1 TE buffer. For separation on acrylamide gels, samples consisted of 0.5 µl of the amplified product and 0.5 µl loading dye. The samples were denatured at 95°C for 5 min prior to loading and run on a 6% Long Ranger polyacrylamide gel with 0.7X TBE buffer using a LI-COR DNA Analyzer 4200 (Lincoln, NE) at a constant 800 v for 6 hours at 50°C.

Data analyses

Gels were visualized using the software Gene ImagIR 4.0 (Scanalytics, Inc.,VA). Each informative polymorphic band was scored manually as 1 for presence and 0 for absence. Analyses were done using Numerical Taxonomy and the Multivariate Analysis System, NTSYS v.2.1 (Rohlf, 2000). Genetic similarities based on Jaccard's coefficients (Jaccard, 1908) were calculated among all possible pairs using the SIMQUAL option and ordered in a similarity matrix. The similarity matrix was run on Sequential, Agglomerative, Hierarchical and Nested clustering, SAHN (Sneath and Sokal, 1973) using the Unweighted Pair Group Method with the Arithmetic Mean (UPGMA) as an option (Sokal and Michener, 1958). Cophenetic correlation was calculated to measure goodness of fit. Principal component analysis (PCA) was run using CPCA option (NTSYS) to identify the number of groups based on eigenvectors and verified using SYSTAT. The three dimensional PCA plot was generated from the NTSYS program using AFLP markers as observations and bentgrass genotypes as the operational taxonomic units (OTUs). The TREE module of NTSYS v.2.1 was used to produce the dendrogram (Rohlf, 2000).

RESULTS and DISCUSSION

From the initial twelve primer combinations, seven combinations were chosen for clarity and repetitiveness in duplicated gel runs (Table 2.2). A total of 380 polymorphic markers were initially scored for the 21 genotypes from the seven combinations. Cultivar Seaside had data from only four of the seven primer combinations. Two UPGMA analyses were performed, first using 248 markers for 21 genotypes which included Seaside and secondly, 355 markers for 20 genotypes, excluding 'Seaside'. The number of bands varied with each primer combination ranging from 100 to 150 bands and the number of polymorphic markers ranged from 25 to 100 per individual lane. Primer combination E-ACA/M-CAT gave the most robust polymorphism. Pairwise similarity coefficients (**sc**) were computed based on shared and unique amplification products using UPGMA (Table 2.3). The genetic similarity coefficients ranged from 0.56 to 0.93. The most similar bentgrass genotypes were the plant introductions (PI) from Switzerland and Sweden (**sc**=0.93) and the most dissimilar would be 'Penncross' and PI 443051 (**sc**=0.56).

Table 2.2. Number of polymorphic bands obtained from different primer combinations.

Primer Pair	No. of Polymorphic Bands	
	20 genotypes	21 genotypes
E-ACA/M-CAT	98	94
E-ACA/M-CAG	25	22
E-ACA/M-CGG	61	60
E-ACA/M-CGA		31
E-AGC/M-CAT	64	62
E-AGC/M-CGG		60
E-AGC/M-CGA		26
Total markers	248	355

Adapter and primer sequences were adapted from Vos et al. (1995).
E=5'-GACTGCGTACCAATTCA-3', M=5'-GATGAGTCCTGAGTAAC-3'.

Table 2.3. Genetic similarity coefficients* for 21 genotypes of creeping and redtop bentgrasses using fluorescence-labeled AFLP technique.

Bentgrass Genotypes	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1 Penncross	1.00																			
2 Providence	0.72	1.00																		
3 L-93	0.71	0.83	1.00																	
4 Penn G2	0.76	0.81	0.79	1.00																
5 Penn A4	0.74	0.76	0.78	0.79	1.00															
6 Emerald	0.73	0.76	0.79	0.81	0.81	1.00														
7 MI 20104	0.72	0.78	0.79	0.77	0.78	0.88	1.00													
8 MI 20215	0.75	0.79	0.77	0.80	0.76	0.78	0.80	1.00												
9 MI 203164	0.78	0.83	0.81	0.80	0.77	0.78	0.79	0.86	1.00											
10 S. Redtop	0.60	0.67	0.63	0.63	0.63	0.65	0.63	0.65	0.66	1.00										
11 PI 251 945_AT	0.69	0.73	0.74	0.74	0.75	0.75	0.74	0.77	0.76	0.65	1.00									
12 PI 235 440_CH	0.72	0.78	0.78	0.79	0.79	0.81	0.79	0.77	0.82	0.66	0.82	1.00								
13 PI 235 541_SE	0.74	0.82	0.79	0.80	0.78	0.80	0.79	0.79	0.81	0.68	0.81	0.93	1.00							
14 PI 204 390_TR	0.68	0.73	0.73	0.74	0.73	0.74	0.73	0.74	0.73	0.68	0.74	0.80	0.80	1.00						
15 PI 269 838_DE	0.72	0.76	0.74	0.78	0.75	0.78	0.77	0.80	0.77	0.67	0.80	0.84	0.84	0.78	1.00					
16 PI 494 119_NL	0.73	0.79	0.77	0.81	0.79	0.80	0.80	0.79	0.79	0.67	0.80	0.83	0.84	0.78	0.87	1.00				
17 PI 230 235_IR	0.60	0.59	0.60	0.60	0.63	0.61	0.62	0.63	0.61	0.64	0.62	0.63	0.64	0.68	0.62	0.65	1.00			
18 PI 439 027_RU	0.58	0.61	0.64	0.60	0.63	0.61	0.61	0.63	0.63	0.62	0.64	0.63	0.63	0.62	0.61	0.64	0.72	1.00		
19 PI 383 584_TR	0.66	0.65	0.67	0.68	0.70	0.66	0.67	0.69	0.68	0.64	0.72	0.71	0.72	0.77	0.70	0.73	0.65	0.60	1.00	
20 PI 443 051_US	0.55	0.55	0.56	0.55	0.57	0.56	0.55	0.56	0.55	0.59	0.54	0.59	0.59	0.56	0.58	0.59	0.67	0.59	0.63	1.00
21 Seaside	0.81	0.66	0.60	0.64	0.62	0.62	0.58	0.64	0.66	0.54	0.60	0.61	0.64	0.57	0.62	0.63	0.54	0.50	0.58	0.46

*Data from bentgrass genotypes 1 to 20 came from 355 AFLP markers and data from 'Seaside' came from 248 AFLP markers.

Using the subroutine programs of NTSYS, a rotated PCA with the markers as observations was used to determine the number of groups based on Eigen values greater than one. Three groups were extracted which explained 58% of the total variance. The first component accounted for 44% of the total variance with cultivar 'S.Redtop' bearing the lowest component loading (Figure 2.1). Seventeen genotypes were found distinguished by the first component. The second component, which accounted for 8% of the variation, separated Groups 1 and 2. The third component accounted for 5% of the variation and distinguished PI 443051.

From the two UPGMA analyses, dendrograms were generated. The two dendrograms did not differ in terms of genotypes within a cluster but only in the estimates of similarity coefficients. These slight differences (0.01 to 0.02 **sc**) could be due to the deletion or addition of allelic markers considered for an additional genotype. The higher number of markers used would predict a more accurate dendrogram presented in Figure 2.2. The dendrogram also indicated where 'Seaside' would cluster. The cophenetic correlation was calculated (r = 0.95) as a measure of goodness of fit of the similarity indices. The cophenetic values were plotted against the distances derived from SAHN's cluster analysis and the phenogram was plotted in Figure 2.3. Mean similarity coefficient for all creeping bentgrasses in the first group was 0.78. Similarity coefficient was lower between the redtop bentgrasses (**sc**=0.64). Group 2 consisted of *A. palustris* (Iran and Russia) and Group 3 consisted only of PI 443051, *A. gigantea* (US). The dendrogram showed a similarity coefficient of 0.64 for the three groups.

'Seaside' was found to be most similar to 'Penncross' with **sc**= 0.81 based on UPGMA analysis using data from 248 AFLP markers (Table 2.3). The dendrogram

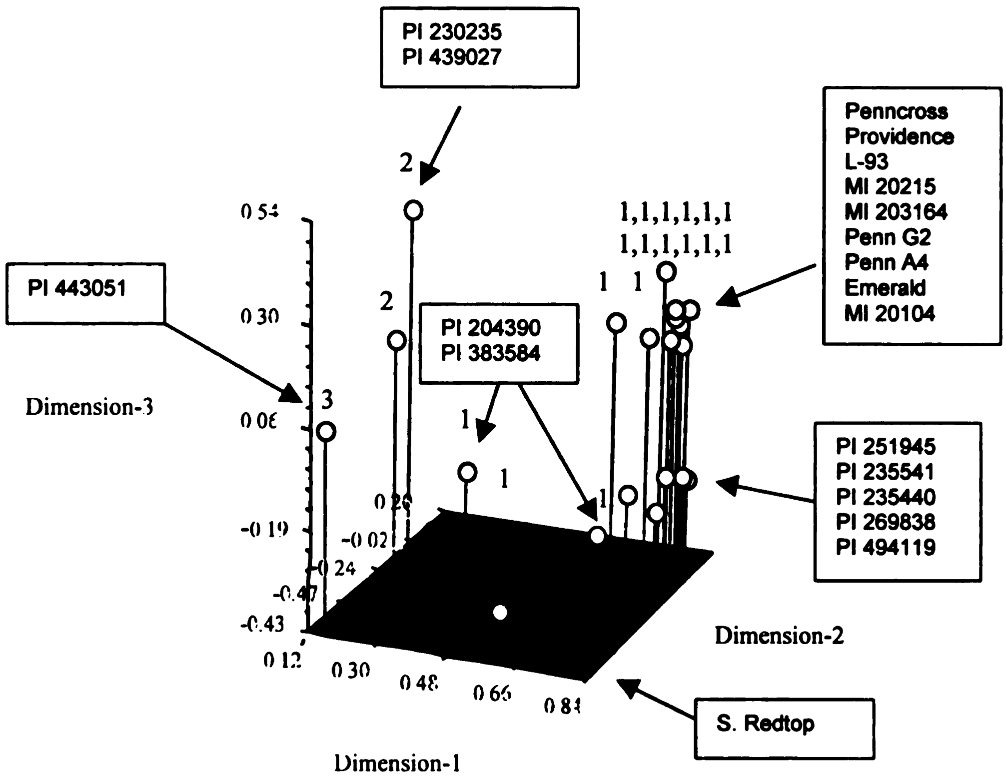

Figure 2.1. Three dimensional plot of principal component analysis (PCA) using 355 AFLP markers (observations) and bentgrass genotypes defining three groups marked as 1, 2 and 3 from the plot options of NTSYS v.2.1 (Rohlf, 2000).

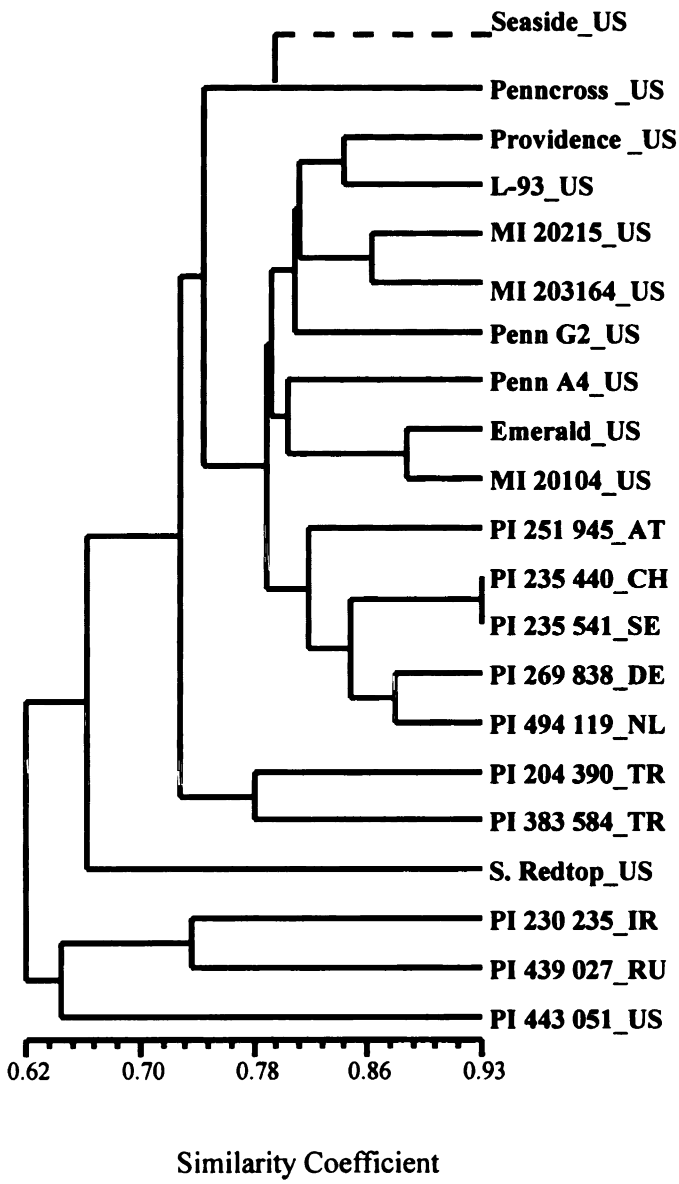

Figure 2.2. UPGMA dendrogram of creeping and redtop bentgrasses using data from 355 AFLP markers (solid lines) and genetic similarity of 'Seaside' using data from 248 AFLP markers (dashed lines).

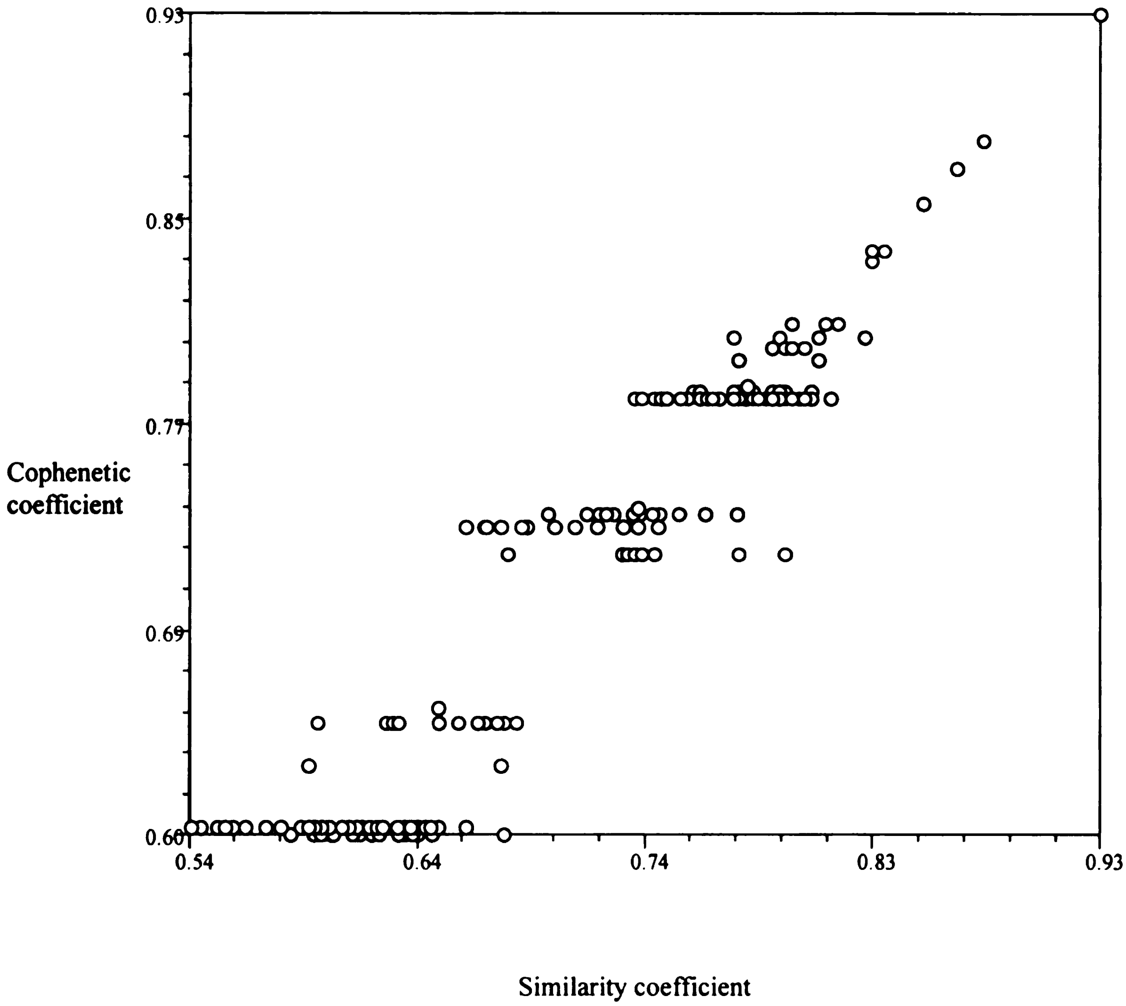

Figure 2.3. Plot analysis of cophenetic and similarity coefficients as a measure of goodness of fit of the similarity indices. r = 0.94808 = normalized Mantel statistics Z; Approximate Mantel t-test: t= 5.1548; P(Z<obs. Z: p = 1.0000).

using data from 355 markers showed that 14 of the 17 genotypes in Group 1, namely 'Penncross', 'Providence', 'L-93', 'Penn-G2', 'Penn A4', 'Emerald', 'MI 20104', 'MI 20215', 'MI 203164', PI 251945 (Austria), PI 235440 (Switzerland), PI 235541 (Sweden), PI 269838 (Denmark) and PI 494119 (Netherlands), were clustered close together at **sc**=0.78. Cultivar 'Penncross' may share genetic similarities with modern cultivars by a mean **sc**=0.75. The fourteen genotypes were slightly differentiated from PI 204390 and PI 383584 (Turkey) and 'S. Redtop'. Cultivar 'S. Redtop' was the most genetically distant in the first group. 'MI 20104' was most similar with 'Emerald' with **sc**=0.88 while 'MI 20215' and 'MI 203164' were more similar to 'Providence' with **sc**=0.79 and 0.83 respectively. 'Providence' and 'L-93' had a high **sc**=0.83 and shared close identity with 'MI 20215' and 'MI 203164' as well. Dendrogram results showed the three genotypes of *A. gigantea* did not group together. PI 443051, *A. gigantea* (US) separated from cultivar 'S. Redtop' (US) and from PI 383584 (Turkey).

Differentiating old and new cultivars of creeping bentgrasses

Most developed cultivars of creeping bentgrass, *A. stolonifera* var. *palustris*, in the USA such as 'Penncross', 'Penn A4', 'L-93' and others came from phenotypic recurrent selection of three or more clones descended from selection of adapted lines which were thought to be from earlier European origin. Warnke et al. (1997) used isozyme polymorphisms to distinguish between several creeping bentgrasses. Eighteen bentgrasses were divided into two groups based on cluster analyses. The first group included 10 cultivars ('Penncross', 'Emerald', 'Cobra', 'Crenshaw', 'Seaside', 'Penneagle', 'Putter', 'Trueline', 'Viper' and '18th Green'). With the exception of 'Crenshaw', they were all strongly aligned with creeping cultivars. 'Seaside' is thought to be the oldest within the group and may have provided some of the germplasm used in the development of some bentgrasses in the grouping. Their genetic distance calculated from Nei's distance formula ranged from 0.007 to .08. The second group contained the cultivars 'Pennlinks', 'Southshore', 'ProCup', 'Lopez', 'Providence', 'SR1020', 'National' and 'Cato'. Both groups were differentiated from PI 251945, which is of European origin by genetic distance of 0.27. They found genetic differences to be small within cultivars and suggested that the PI line could be a source to broaden genetic diversity of bentgrasses in the USA.

In this study, UPGMA similarity coefficients from AFLP showed that 'Seaside' (1928), the oldest seeded cultivar used, and 'Penncross' (1955) were clustered closely but evidently showed genetic dissimilarity at 19%. Genetic dissimilarity was computed from 1-**sc** x 100 (Zhang et al., 1999). 'Seaside' creeping bentgrass, indigenous to coastal regions of Washington and Oregon, was known to be an extremely variable grass that develops into patches of individual strains with different colors, textures and densities. Most seed supplies came from natural stands. Hence to date after more than seventy-five years, 'Seaside' remains to be a highly heterogenous population.

In Figure 2.2, more recent cultivars, 'Providence' (1987) and 'L-93' (1995) were also clustered together with **sc**=0.84. The genetic dissimilarity between 'Providence' and 'L-93' is 16% and both are differentiated from 'Seaside' as much as 34%. 'Providence' was a consistently top rated (best five) bentgrass in the National Turfgrass Evaluation Program (NTEP 98-8) Putting Green and Fairway Test (Morris, 1997), and in trials throughout the world. The progenitors of 'Providence' were five clones that Dr. Richard Skogley of the University of Rhode Island selected as being unique and superior to

'Penncross' since 1965 (Seed Research Reports of Oregon, 2003). The newer cultivars, 'Penn G2' and 'Penn A4' (1995) were clustered together at **sc**=0.80 and differentiated from its early predecessor 'Penncross'. Pennsylvania State University developed cultivars with the epithet 'Penn'. 'Providence', 'Penn G2' and 'Penn A4' were reported as having better resistance to dollar spot in NTEP trials (Moris, 2001a) and would be useful as genetic stocks. Cultivars 'Seaside', 'Penncross' and 'Emerald' were in the same cluster using AFLP and supported similar findings by Warnke et al.(1997). Another similar observation to their findings from our results was that PI251945 (Austria) has a range of **sc**=0.65 to 0.77 to USA cultivars. PI251945 and other PIs from Switzerland, Sweden, Denmark and Netherlands would be useful to expand genetic variability in creeping bentgrass. In contrast to the results of Warnke et al. (1997), AFLP analysis showed that the genetic differences among USA cultivars were higher than the previous estimate using isozyme. Variability is important because selection becomes more effective when diversity is high.

Creeping bentgrass from Turkey (Central Asia) was the most distant in the first group and would be highly useful and informative for future mapping studies. Redtop bentgrass cultivar 'S. Redtop' in group 1 cluster was shown to share some genetic similarity with the tetraploid creeping bentgrasses. In contrast to creeping bentgrasses, redtop bentgrass is not used as fine turf but would be an important source of other important traits like drought and heavy metal tolerance (Winterhalder, 1990). Creeping bentgrasses (USA) clustered separately as a subgroup from the PIs from Europe at **sc** = 0.78, an indication that most development, selection and genetic exchanges in the last fifty years occurred locally. Support for European lineage for USA creeping bentgrasses was also shown by AFLP results in Group 1 in this study. Previous findings comparing genetic similarities of plant introductions of *Agrostis* species using AFLPs showed that bentgrasses from the USA were closer to European PIs than accessions coming from other parts of the world (Vergara and Bughrara, 2003).

Differentiating MSU experimental lines with other creeping bentgrasses

MSU experimental lines were collected from old (>50 years) Northern Michigan golf courses which have not been overseeded for the last 10 years. Materials from these golf courses have been through natural selection pressures for abiotic and biotic stresses making them excellent genetic stocks for turf breeding programs. Data from AFLPs showed that the selected experimental lines were differentiated from other creeping bentgrasses and showed the closest genetic similarity to 'Providence', 'L-93' and 'Emerald'. The two cultivars, 'L-93' and 'Emerald' are currently internationally known for their dark, dense and aggressive growth habit. 'L-93' was rated premier out of 25 cultivars in the National Bentgrass Test – 1998 (NTEP 02-3, Morris, 2001b) on fairways and tees and also performed better in heat stress than 'Penncross' (Huang and Xu, 2001). Cultivar 'Emerald', is a descendant of a single synthetic clone originating from Sweden and is widely used in blends of two or more creeping bentgrasses in the USA. 'Emerald' is important as it also has moderate tolerance to heat like 'Providence', as compared to most cool-season bentgrasses. The high similarity coefficients (0.80 to 0.88) to top rated modern cultivars provided predictive estimates by which MI 20104, MI 20215 and MI 203164 with resistance to snow mold disease may be used to improve performance of newer cultivars without radically altering their genetic components. Correspondingly, cultivars to which the **sc** values were less gave indication of the high genetic diversity favorable for studies on combining abilities. Populations derived from crosses with large differences in polymorphic markers may be used to map the snow mold disease resistance trait.

Results from dendrogram analysis showed that germplasm materials or plant introductions from Europe may also be used to widen the genetic base of modern USA creeping bentgrasses. The estimated mean genetic dissimilarity among all creeping bentgrass genotypes was 0.78 and suggested considerable diversity from which selection for improved cultivars may be generated. Creeping bentgrass from other subgroups (Central Asia) which are more differentiated by AFLPs would correspondingly further increase genetic variability. Diverse parental combinations would create segregating populations of various heterotic groups from which superior clones may be selected.

Genetic dissimilarity of tetraploid and hexaploid creeping bentgrasses

AFLP analysis indicated that most tetraploid creeping bentgrasses were grouped together and separated from hexaploid stoloniferous bentgrasses (Figure 2.2). PI 204390 (*A. palustris*, Turkey) was the most genetically distant among the tetraploid creeping bentgrasses in this study and may share closer genomic constitution with PI 383584 (*A. gigantea*, Turkey). Having similar geographic origins, possible genetic introductions between the progenitors of the two species may have occurred and allowed them to evolve sympatrically. Interspecific hybridizations between tetraploid and hexaploid bentgrasses like *A. stolonifera* x *A. gigantea* were known to occur naturally and are easy to produce (Davies, 1953; Jones,1955). The ability of cross-speciation enhances opportunities to transfer other important traits, e.g. tolerance to heavy metals and poor soils, drought tolerance, and vigorous growth habit found in *A. gigantea* to creeping bentgrass.

Hexaploid bentgrasses, *A. gigantea* have been found to be genetically diverse within species and differentiated from the tetraploid *A. stolonifera var. palustris* using AFLPs (Table 2.3). The three *A. gigantea* genotypes were found to group separately from each other. S. Redtop (US) has only a **sc** = 0.59 with PI 443051, *A. gigantea* (US). This indicated that their genomic constitutions ($A_1A_1A_2A_2A_3A_3$) may be dissimilar and may have descended from different diploid and tetraploid progenitors. Much of the extensive variation in *A. gigantea* could have been generated by multiple or repeated cycles of hybridization of multiple origins. Our findings in the AFLP analysis of a number of *Agrostis* species were similar and indicated that only the A_1A_1 genome may be shared by the different accessions of *A. gigantea* (Vergara and Bughrara, 2003). Future cytogenetic and hybridization studies may be done to explain their differences.

Applications to Turfgrass Breeding

Turf breeders may gain advantage in selection when breeding materials are highly heterogenous and populations could be efficiently differentiated. Difficulty in differentiating outcrossing allopolyploid turfgrass species morphologically may be overcome with AFLP analyses. This study has shown considerable genetic dissimilarities between old and new cultivars, germplasm and MSU experimental lines. Dendrogram analysis revealed that USA creeping bentgrass cultivars have locally evolved and differentiated from European germplasm. Genetic similarity coefficients may predict which cultivars are more similar or distant and help define strategies for breeding. The AFLP data suggested that *A. stolonifera* var. *palustris* cultivars in the USA are highly heterogenou

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heterogenous but may be further diversified with materials from Europe and Turkey. Experimental materials from MSU with disease resistance to snow mold could be used to improve cultivars. Other important traits found in plant introductions of *A. gigantea,* may be used to improve 'S. Redtop' or transferred to creeping bentgrass cultivars by interspecific hybridization. In the future, the identified primer combinations and polymorphic marker data identified herein may be used to monitor introgression, map important traits, and used for protection of indigenous materials and developed cultivars.

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CHAPTER III

Genetic Variability of the Gray Snow Mold (*Typhula incarnata* Lasch)

ABSTRACT

Randomly amplified polymorphic DNA (RAPD) markers were used to assess the genetic diversity of isolates of gray snow mold, *Typhula incarnata*, taken from infected turfgrasses from 40 various locations in Northern USA. Data from 115 markers using 37 RAPD primers showed 48% polymorhism. The genetic similarity coefficients 0.57 to 0.99 between isolates indicate the wide genetic diversity of the fungi. Dendrograms from Unweighted Pair Group Method with Arithmetic Mean (UPGMA) clustering and neighbor joining (NJ) bootstrap analyses, showed similar clades and suggest possible recent colonization from common founder populations. Partitioning of the genetic variance using analysis of molecular variance (AMOVA) of 4 populations based on geographic locations: Lower Peninsula, Michigan; Upper Peninsula, Michigan, Wisconsin and Minnesota showed that genetic variation attributable among populations and within populations was 12.67% and 87.33% respectively. No correlation was found between geographic distance and pairwise genetic distance of the groups. High outcrossing and sexual recombination of *T. incarnata* may likely be key factors explaining their genetic variability as shown with the low Fixation index (FST) and high average of genetic diversity per loci within populations.

Key words: Gray snow mold, RAPD, genetic diversity, *Typhula incarnata*

INTRODUCTION

Typhula incarnata is an economically important pathogen affecting winter cereals and perennial grasses. The fungi are known to occur in cool temporal to boreal regions of the northern hemisphere, in countries such as Russia, Canada, North America, Europe, Japan and other Nordic countries (Smith, 1989). Snow mold blight, collectively caused by gray snow mold (*T. incarnata*) and speckled snow mold (*T. ishikariensis*) is ranked as the second most damaging turfgrass disease after dollar spot of the Great Lakes Region by the golf course superintendents (Anonymous, 1996).

The wide host specificity of the gray snow mold among turfgrasses is shown in the pathogen's ability to cause diseases in *Agrostis*, *Poa*, *Festuca* and *Lolium*. On golf courses, the pathogen is ubiquitously found on the putting greens comprised of bentgrasses (*Agrostis* sp.), and fairways comprised of bluegrasses (*Poa* sp.) and other bentgrass mixtures. Lateral transit of the soil-borne pathogen across genera boundaries contributes to its presence across a wide geographic range. *T. incarnata* has been described as more versatile than *T. ishikariensis* due to its ability to adapt to less favorable environments such as shallower and shorter duration of snow cover (Jacobs and Bruehl, 1986; Matsumoto and Tajimi, 1985). The fungus is more common on golf courses with less than 90 days of snow cover (Millet, 2000). The disease symptoms usually appear as circular, water-soaked or straw-colored patches measuring 5 to 15 cm across and may coalesce. Plants may be matted, appear slimy with mycelium and may be covered with dust giving it a gray-white appearance, hence the name "gray snow mold" (Jackson and Fenstermacher, 1969). On diseased turfgrass, *T. incarnata* produces numerous round, orange to brown colored bodies called sclerotia which remain as resting bodies during the summer months and become an inoculum source when temperatures fall. Sclerotia of *Typhula* snow molds are used to distinguish the different species during field collections. Hsiang et al. (1999) provides a comprehensive review of the biology of snow molds in turfgrasses.

The complexity of *T. incarnata* was revealed by the study of incompatibility alleles in mating classes. *T. incarnata* is a tetrapolar species, producing viable basidiospores and vigorous monokaryons. Bruehl and Machtmes (1978) found four mating classes based on clamp connections suggesting that there may be 39 alleles of both incompatibility loci A and loci B in a field sample of 32 field dikaryons. In fungi, locus A is responsible for the initial formation of clamps, nuclear pairing, conjugate nuclear division and septation of clamps while locus B is responsible for nuclear migration and fusion of clamp tip. Both loci are multi-allelic. The high fecundity of *T. incarnata* and large number of incompatibility alleles suggest that this species utilizes its sexual stage frequently. It is possible that genetic variants of the pathogen allow it to infect a wide range of hosts. In Japan, the fungus has a wide geographic range and this is ascribed to its ecological versatility (Matsumoto et al., 1995). Studies have not found variation in mycelial growth rates among isolates from Japan but sclerotia from longer and heavier snowfall regions germinated faster than those from areas of less persistent snow cover. Despite all the information, there are no known molecular studies to support or further describe the genetic diversity in *T. incarnata.*

Many approaches have been developed to look at the genetic variability of various organisms. Randomly amplified polymorphic DNA (RAPD, Welsh and McClelland, 1990; Williams et al., 1990) has been a popular choice due to its simplicity, speed, low cost and availability of primer kits. For minute living organisms like slow-growing fungi where DNA isolation is difficult and sparse in concentration, simple polymerase chain reaction (PCR) through RAPD would be an ideal choice. The technique has been used previously to examine genetic diversity in several fungi (Grajal-Martin et al. 1993; Huff et al, 1994). RAPD has been used to characterize populations of pink snow mold, *Microdochium nivale* (Mahuku et al., 1998) and speckled snow mold (Hsiang and Wu, 2000). The genetic diversity of pink snow mold isolates from turfgrass using RAPD analyses revealed the low level of genetic differentiation among populations. For speckled snow mold, RAPD analyses distinguished among the three main *Typhula* species, the distinction of *T. ishikariensis* var. *idahoensis* from var. *canadensis* and uncovered variation within isolates of the same species (Hsiang and Wu, 2000).

The objectives of this study were to examine the genetic variability of 40 *T. incarnata* isolates growing on and presumed pathogenic to turfgrasses, from Michigan, Wisconsin and Minnesota, U.S. Also, genetic variation within and among populations in 4 different geographic locations: the Lower and Upper Peninsula of Michigan, Wisconsin and Minnesota, U.S. using RAPD analysis were of interest. Understanding the population structure and genetic variability of gray snow mold will help in disease resistance studies for turfgrasses.

MATERIALS AND METHODS

Pathogen

T. incarnata samples were collected from Hancock Turfgrass Research Center in East Lansing, Michigan on April, 2001 and from golf course areas in Northern Michigan in April

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in April 2002. Isolates from a sod farm in Lansing, Michigan were obtained courtesy of the Department of Plant Pathology at Michigan State University (MSU). Samples from Minnesota and Wisconsin were collected by researchers from Department of Plant Pathology, University of Wisconsin-Madison through collaboration in 2002 and DNA samples were sent to the Turfgrass Genetics Laboratory of Crops & Soil Sciences at MSU, courtesy of Dr. G. Jung (Table 3.1). Sclerotia from collected sites were grown, purified and maintained in potato dextrose agar (PDA) at 5 °C. PDA broth was prepared using 37 grams dehydrated DIFCO potato dextrose agar in 1 L. distilled water followed by sterilization for 30 min. Mycelial growth was compared for samples obtained from Michigan golf courses.

DNA preparation

Fungal mycelia were grown for two months in PDA prior to genomic DNA extraction. DNA from mycelia was extracted using a modified extraction buffer to eliminate proteins from the agar. The standard buffer consisted of Tris-EDTA-HCl at pH 8.0, SDS, NaCl with 0.38 g of sodium bisulfite per hundred ml of the buffer. Proteinase K (14 mg/ml in 10mM Tris-HCl) was added at a volume of 400 ul per 400 ul extraction buffer. The mixture was incubated at 65 °C for 20 min. followed by the addition of an equal volume of 25:1 chloroform:isoamyl alcohol. Centrifugation was performed at 2800 *g* RCF (Sorvall RT7 model) for 20 min at 5 °C. The supernatant was collected to which 2/3 volume of cold isopropanol was added to precipitate the DNA. All DNA samples were treated with RNAse dissolved in TE buffer and twice reprecipitated by using 1/10 volume of 3M sodium acetate followed by two volumes of chilled absolute ethanol.

Table 3.1. List of sampling areas, cities and USA states for *Typhula incarnata* isolates used.

No.	CODE	SOURCE	CITY[1]	STATE[2]
1	MI-LN 1	Michigan State University	East Lansing	MI
2	MI-LN 2	Michigan State University	East Lansing	MI
3	MI-LN 3	Michigan State University	East Lansing	MI
4	MI-LN-8	Sod Farm	Lansing	MI
5	MI-HS 4	Birchwood Golf Course (GC)	Harbor Springs	MI
6	MI-HS 4-1	Birchwood GC	Harbor Springs	MI
7	MI-HS 5	Birchwood GC	Harbor Springs	MI
8	MI-PT 4-7	Walloon	Petoskey	MI
9	MI-PT 4-8	Walloon	Petoskey	MI
10	MI-PT 5	Walloon	Petoskey	MI
11	WI-NE 75.16.1	Perry's Landing	Marion	WI
12	WI-NE 68.17.1	Nicolet	Laona	WI
13	WI-NW 54.7.3	Park Falls	Park Falls	WI
14	WI-NW 70.14.3	Spooner	Spooner	WI
15	WI-SE 62.8.1	Meadow Springs	Jefferson	WI
16	WI-SE 65.3.5	Moor Downs	Waukesha	WI
17	WI-SE 96.15.2	The Squires	Port Washington	WI
18	WI-SW 61.3.3	Prairie du Chien	Prairie du Chien	WI
19	WI-SW 76.18.5	Towne	Edgerton	WI
20	WI-SW 84.18.1	The Valley	Muskego	WI
21	MI-LSE 2-5	L'Anse GC	L'Anse, UP	MI
22	MI-LSE 9-5	L'Anse GC	L'Anse, UP	MI
23	MI-Long 1-2	Lakewood Blackshire	Oscoda	MI
24	MI-Long 15-2	Lakewood Blackshire	Oscoda	MI
25	MI-PL-6-3	Portage Lake	Houghton, UP	MI
26	MI-Tree 5-1	Treetops	Gaylord	MI
27	MI-KML 2-2	Keeweenaw MTM Lodge	Copper Harbor, UP	MI
28	MI-Glad 4-5	Gladstone GC	Gladstone, UP	MI
29	MI-IR 7-2	Indian River GC	Indian River	MI
30	MI-PL 2-4	Portage Lake	Houghton, UP	MI
31	MN-CW 11-1	Crosswoods GC	Cross Lake	MN
32	MN-CW 18-4	Crosswoods GC	Cross Lake	MN
33	MN-OH 3-1	Oak Harbor GC	Baudette	MN
34	MN-CW 9-2	Crosswoods GC	Cross Lake	MN
35	MN-LP 18-1	Long Prairie GC	Long Prairie	MN
36	MN-IM 9-3	Ironman GC	Detroit Lakes	MN
37	MN-IM 4-1	Ironman GC	Detroit Lakes	MN
38	MN-NCL 3-3R	Northland GC	North Mankato	MN
39	MN SN 2	Superior National	Lutsen	MN
40	MN WF 2-1	Whitefish	Pequot Lakes	MN

[1]UP = Upper Pensinsula
[2] MI = Michigan; WI = Wisconsin ; MN = Minnesota , USA.

Extracted DNA was collected by microcentrifugation at 8,000 *g* RCF (Marathon 16 model, Fisher Scientific) for 30 sec. and the supernatant was discarded. DNA was re-dissolved at room temperature and stored in 1% TE buffer. DNA quality was checked by running 5 μl of the undigested samples in 1% agarose gel containing TBE buffer. All DNA sample concentrations were quantified using DyNA Quant 200 Fluorometer (Pharmacia Biotech, CA) and concentrations were adjusted to equal volumes of approximately 8 ng/ul prior to RAPD analysis.

RAPD Analyses

Decamer random primers from Operon kits (Operon Technologies, Inc. Alameda, CA, USA) and primer sequences used by Hsiang & Wu (2000) for RAPD analysis of other snow molds were tested for PCR amplification. Synthesized primers were made by MWG Biotech, NC. Thirty-seven primers were chosen for strong signals and reproducibility. The primers, sequences and annealing temperature are listed in Table 3.2. The DNA amplification mixture consisted of 2.5 ul of DNA (~20 ng), 2 ul of 1mM dNTP mix, 2 ul of 25 mM $MgCl_2$, 2 ul of 10 ng/ul Primer, 1 unit Taq Polymerase in 0.2 ul volume and distilled deionized H_20 for a complete volume of 20 ul. PCR amplification was performed in a thermal cycler, PTC-100 (MJ Research, Inc., MA) with one cycle of 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 sec, 40 °C for 30 sec and 72 °C for 1 min and a final cycle of 72 °C for 15 min. Only 3 RAPD primers were amplified at 36 °C temperature and the other 34 RAPD primers gave good amplification products at 40 °C. Fragments were separated in 1.5% TBE agarose gel and stained with ethidium bromide. PCR reactions and gel analyses were conducted at least two times for each primer for replication and verification of results.

Analyses of genetic similarity

Bands were scored for presence as 1 or absence as 0. Missing or ambiguous bands (approximately < 2.0%) were designated as 999. Analyses were done using Numerical Taxonomy and the Multivariate Analysis System, NTSYS v.2.1 (Rohlf, 2000). Genetic similarities or similarity coefficients (**sc**) based on Dice's estimate (Dice, 1945) were calculated among all possible pairs using the SIMQUAL option and ordered in a similarity matrix. Landry and Lapointe (1996) compared several coefficients for use with RAPD markers and suggested the use of Jaccard and Dice coefficients for 12 markers and more. The similarity matrix was run on Sequential, Agglomerative, Hierarchical and Nested clustering (SAHN) (Sneath and Sokal, 1973) using the Unweighted Pair Group Method with the Arithmetic Mean (UPGMA) as an option (Sokal and Michener, 1958). Principal component analysis (PCA) was run after doing correlation between similarity coefficients using *T. incarnata* isolates as operational taxonomic units (OTUs). The two dimensional plot was generated from Eigen vectors and Eigen values calculated from EIGEN option. The TREE module of NTSYS v.2.1 was used to produce the dendrogram (Rohlf, 2000).

Data from RAPD markers were subjected to bootstrap analysis using another software, FreeTree (Pavlicek et al., 1999). The distance similarity matrix was computed using the Nei-Li option, followed by neighbor joining (NJ) clustering method with resampling analysis using 100 repetitions. On the reference tree, the bootstrapping values were copied into the bracketed form of the tree. The form of the tree was copied and pasted into the TreeView program (Page, 2001) to draw the dendrogram. The NJ dendrogram contained the bootstrap information.

Analyses of Molecular Variance (AMOVA)

The isolates were assigned to 4 populations based on proximal geographic locations as Lower Peninsula, Michigan (LPMI), Upper Peninsula, Michigan (UPMI), Wisconsin (WI) and Minnesota (MN) (Figure 3.1, Table 3.2). To compare the populations' genetic diversity, AMOVA (Arlequin 2.0) program enabled partitioning of the RAPD variation between and within groups using variance and covariance components. Fixation index (FST), pairwise difference between populations and average diversity per loci were determined using the software package.

RESULTS AND DISCUSSION

Gray snow mold mycelial growth *in vitro*

Sclerotia of the fungi from East Lansing (HC), Lansing (SF), Harbor Spring (HS), Petoskey (PT) were grown in PDA and were measured weekly for mycelial growth. HC and SF represented Mid-Michigan while HS and PT were from Northern Michigan. Mean mycelial growth was calculated by taking the growth differences between weekly measurements for all isolates and taking the grand average. The mean growth rate was established at 0.32 cm. per week. Gray snow mold has been described as a weak saprophyte, slow growing with varying cultural habits *in vitro* (Hsiang et al., 1999). Analysis of variance on the radial growth of the different isolates using 3 replicates per

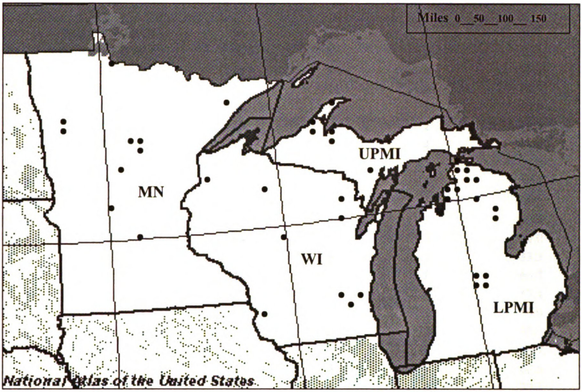

Figure 3.1. Collection sites of gray snow mold clustered in 4 populations based on geographic locations: Minnesota (MN); Wisconsin (WI); Lower Peninsula, Michigan (LPMI) and Upper Peninsula, Michigan (UPMI).

Table 3.2. RAPD primers and sequence, annealing temperature and percentage polymorphism found in gray snow mold. (OP = Operon primer kits; P = Biotechnology Laboratory, British Columbia primer).

RAPD	Sequence 5' to 3'	Anneal (°C) Temperature	No. of Bands Visible	No. of Bands Polymorphic	Percentage Polymorphism
P143	TCG CAG AAC G	36	2	1	50.0
P162	CTA GAT GTG C	36	5	2	40.0
P177	TCA GGC AGT C	36	7	4	57.1
P715	CCA CCA CCC A	40	11	6	54.5
P731	CCC ACA CCA C	40	6	3	50.0
P732	CAC CCA CCA C	40	5	2	40.0
P701	CCC ACA ACC C	40	9	4	44.4
OPA-08	GTG ACG TAG G	40	7	5	71.4
OPA-20	GTT GCG ATC C	40	9	5	55.6
OPC-04	CCG CAT CTA C	40	6	3	50.0
OPC-05	GAT GAC CGC C	40	7	3	42.9
OPC-06	GAA CGG ACT C	40	6	3	50.0
OPC-07	GTC CCG ACG A	40	6	2	33.3
OPC-08	TGG ACC GGT G	40	12	8	66.7
OPC-10	TGT CTG GGT G	40	7	4	57.1
OPC-13	AAG CCT CGT C	40	2	1	50.0
OPC-15	GAC GGA TCA G	40	6	2	33.3
OPC-16	CAC ACT CCA G	40	3	2	66.7
OPC-19	GTT GCC AGC C	40	8	5	62.5
OPC-20	ACT TCG CCA C	40	6	2	33.3
OPY-02	CAT CGC CGC A	40	14	7	50.0
OPY-03	ACA GCC TGC T	40	2	1	50.0
OPY-05	GGC TGC GAC A	40	10	4	40.0
OPY-06	AAG GCT CAC C	40	6	4	66.7
OPY-07	AGA GCC GTC A	40	5	3	60.0
OPY-10	CAA ACG TGG G	40	5	2	40.0
OPY-13	GGG TCT CGG T	40	7	4	57.1
OPY-14	GGT CGA TCT G	40	6	5	83.3
OPY-15	AGT CGC CCT T	40	6	3	50.0
OPY-16	GGG CCA ATG T	40	7	3	42.9
OPY-17	GAC GTG GTG A	40	5	3	60.0
OPY-18	GTG GAG TCA G	40	5	1	20.0
OPY-19	TGA GGG TCC C	40	5	2	40.0
OPY-20	AGC CGT GGA A	40	9	2	22.2
OPX-01	CTG GGC ACG A	40	8	3	37.5
OPX-06	ACG CCA GAG G	40	7	3	42.9
OPX-13	ACG GGA GCA A	40	10	3	30.0
Total			247	120	
Mean			6.7	3.2	48.7

clone for each week are summarized in Table 3.3 and growth progress was graphed in Figure 3.2. Significant differences in mycelial growth between isolates were observed in the 2nd and 3rd week. No significant variation in radial growth was found in 1st and the 4th week although the isolates from Petoskey (Northern Michigan) appeared to have germinated and elongated faster in the 1st week and contributed to a significant difference in the F-tests for the 2nd and 3rd week. Isolates from Mid-Michigan were found to achieve the growth size of those from Northern Michigan in four weeks. Northern Michigan has longer and more severe snow patterns than Mid-Michigan and may have provided selection pressures leading to variation in germination and growth patterns of these psychrophilic organisms. However, no significant differences in sclerotial and mycelial growth were detected at the end of this four week study. The results support previous findings comparing growth of isolates in Japan (Matsumoto et al., 1995). Information on mycelial growth rate will be useful for the selection of vigorous inoculum, timing for DNA extraction and for planning inoculum preparation in future disease resistance screening experiments. A growth period of two months was later established as good for a full plate of mycelial growth for DNA extraction and for transfer to cornmeal agar for future inoculation work.

RAPD analyses and genetic similarities

Thirty-seven RAPD primers or 60% of the tested primers yielded at least one scorable fragment. The 37 primers used, their sequences, annealing temperature and number of amplified bands are presented in Table 3.2. The number of bands ranged from 2 to 14 with a mean of 6.7 bands/primer. The percentage mean polymorphism was

Table 3.3. Analysis of variance on the radial mycelial growth of gray snow mold isolates *in vitro* from different locations in Michigan.

Source	No. of Samples	Mean mycelial growth (cm.) in weeks 1	2	3	4
East Lansing (HC)	3	0.27 ± 0.2	0.60 + 0.1	1.70 ± 0.2	2.20 ± 0.2
Lansing (SF)	3	0.37 ± 0.3	0.67 ± 0.3	1.40 ± 0.2	2.47 ± 0.2
Harbor Spring (HS)	3	0.23 ± 0.2	0.50 ± 0.2	1.17 ± 0.2	2.50 ± 0.3
Petoskey (PT)	3	0.43 ± 0.2	0.97 ± 0.4	2.27 ± 0.3	3.10 ± 0.5
F-Values		3.03	5.09*	6.51*	3.13

* Significantly different P<0.05.

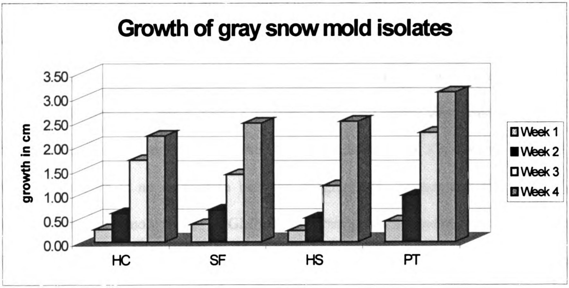

Figure 3.2 Radial growth in four weeks of different gray snow mold isolates *in vitro* from Michigan. ***P*** values < 0.05 as follows: Week 1 = 0.09; Week 2 = 0.02; Week 3 = 0.02 and Week 4 = 0.09.

Legend: Hancock Research Center, East Lansing (HC); Sod Farm,Lansing (SF); Harbor Spring (HS); and Petoskey (PT)

48.7%. The high level of discriminatory bands confirms RAPD as an ideal choice for genetic variability testing. A sample RAPD profile using OPY-02, the primer yielding the highest number of observed bands is shown in Figure 3.3.

Of the 120 polymorphic bands observed, 115 were chosen based on reliability. Genetic similarities were obtained using Dice estimates. Similarity coefficients ranged from 0.58 to 0.95 between different isolates from different locations (Table 3.4). Isolates no. 1 and 2 (MI), (numbered according to Table 3.1) are clones proving Koch's postulate, since Mi-2 was the pathogenic fungus recovered from plants infected with Mi-1, continuously re-cultured every three months within a year. Genetic similarity was 100%, suggesting no recombination within the infected plants. Mi-3, an isolate from the same sampling site has 95% genetic similarity to Mi-1 and Mi-2, indicating variability arising probably from allelic diversity and recombination. The next closest pair was Mi-5 and Mi-6 (**sc**=0.93) and Mi-8, 9, 10 (**sc** > 84%). Several isolates have **sc**=0.58 from pairwise comparisons, as in isolates from Detroit Lakes, MN versus those taken from East Lansing, MI (MN-36 vs. MI-1, Table 3.4); from Laona, WI versus Gladstone, MI (WI-12 vs. MI-28); from Spooner, WI versus North Mankato, MN (WI-14 vs. MN-36). Apparently, there is no correlation between geographical distance and the similarity coefficient for gray snow mold. UPGMA dendrogram produced and cluster analysis also showed no specific grouping, indicating that the isolates were highly heterogenous (Figure 3.4 and 3.5). Principal component analysis on the correlation of similarity coefficients between isolates indicate 2 groups based on eigen values greater than 1. The first component accounted for 71% of the variance and the second component accounted for only 2%, which did not really define specific groups. Scatter plot of the 40 gray snow

Table 3.4.1. Genetic similarity coefficients using Dice estimates for 40 gray snow mold (*T. incarnata*) isolates from data of 115 RAPD markers using 37 primers.

No.**	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1	1.00																			
2	0.99	1.00																		
3	0.95	0.96	1.00																	
4	0.82	0.81	0.83	1.00																
5	0.74	0.75	0.78	0.82	1.00															
6	0.75	0.76	0.76	0.79	0.93	1.00														
7	0.75	0.76	0.81	0.84	0.80	0.85	1.00													
8	0.79	0.81	0.85	0.76	0.78	0.73	0.91	1.00												
9	0.85	0.86	0.89	0.74	0.77	0.77	0.84	0.93	1.00											
10	0.79	0.81	0.83	0.72	0.77	0.77	0.87	0.93	0.96	1.00										
11	0.73	0.72	0.75	0.71	0.68	0.66	0.72	0.77	0.74	0.74	1.00									
12	0.63	0.62	0.67	0.68	0.67	0.63	0.65	0.63	0.64	0.67	0.74	1.00								
13	0.73	0.72	0.72	0.75	0.64	0.68	0.71	0.68	0.69	0.70	0.71	0.68	1.00							
14	0.65	0.64	0.63	0.67	0.68	0.70	0.64	0.61	0.68	0.72	0.69	0.69	0.68	1.00						
15	0.79	0.78	0.79	0.74	0.66	0.69	0.73	0.76	0.78	0.74	0.71	0.72	0.70	0.69	1.00					
16	0.73	0.72	0.70	0.66	0.64	0.64	0.69	0.71	0.76	0.72	0.73	0.68	0.65	0.63	0.71	1.00				
17	0.70	0.69	0.69	0.70	0.64	0.67	0.73	0.70	0.68	0.66	0.71	0.63	0.67	0.61	0.70	0.71	1.00			
18	0.72	0.71	0.73	0.74	0.73	0.74	0.74	0.73	0.71	0.67	0.76	0.70	0.63	0.65	0.74	0.82	0.75	1.00		
19	0.67	0.66	0.69	0.68	0.63	0.66	0.70	0.74	0.69	0.68	0.75	0.69	0.71	0.60	0.74	0.66	0.73	0.76	1.00	
20	0.73	0.73	0.71	0.72	0.68	0.70	0.80	0.80	0.75	0.75	0.69	0.59	0.69	0.63	0.73	0.69	0.61	0.73	0.73	1.00
..																				
..																				

**No. = designated sample number for isolate listed in Table 3.1

Table 3.4.2. (Continuation). Genetic similarity coefficients using Dice estimates for 40 gray snow mold (*T. incarnata*) isolates from data of 115 RAPD markers using 37 primers.

No.**	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
21	0.70	0.69	0.71	0.64	0.67	0.67	0.64	0.72	0.74	0.74	0.64	0.59	0.57	0.64	0.64	0.63	0.59	0.68	0.64	0.67
22	0.77	0.77	0.82	0.73	0.70	0.68	0.73	0.80	0.81	0.78	0.73	0.67	0.64	0.63	0.76	0.70	0.72	0.77	0.74	0.75
23	0.74	0.74	0.73	0.68	0.63	0.67	0.73	0.72	0.74	0.72	0.73	0.62	0.66	0.62	0.70	0.70	0.72	0.70	0.70	0.70
24	0.68	0.68	0.68	0.67	0.69	0.74	0.74	0.72	0.76	0.78	0.70	0.68	0.70	0.68	0.68	0.69	0.67	0.71	0.64	0.67
25	0.70	0.70	0.74	0.68	0.69	0.69	0.78	0.80	0.81	0.79	0.67	0.57	0.65	0.59	0.71	0.63	0.67	0.68	0.68	0.67
26	0.65	0.64	0.67	0.69	0.69	0.71	0.75	0.72	0.72	0.67	0.69	0.70	0.68	0.67	0.74	0.68	0.68	0.72	0.70	0.71
27	0.70	0.70	0.74	0.73	0.77	0.77	0.78	0.76	0.78	0.76	0.72	0.68	0.62	0.68	0.66	0.67	0.65	0.75	0.68	0.63
28	0.68	0.70	0.70	0.63	0.66	0.63	0.75	0.76	0.79	0.76	0.61	0.58	0.58	0.59	0.69	0.63	0.64	0.61	0.62	0.68
29	0.70	0.68	0.71	0.69	0.63	0.63	0.75	0.74	0.75	0.74	0.76	0.64	0.67	0.61	0.71	0.70	0.70	0.68	0.70	0.70
30	0.66	0.65	0.65	0.69	0.63	0.61	0.73	0.72	0.75	0.70	0.72	0.70	0.62	0.63	0.75	0.70	0.64	0.70	0.63	0.65
31	0.70	0.71	0.74	0.68	0.70	0.72	0.73	0.74	0.81	0.79	0.75	0.70	0.64	0.63	0.70	0.66	0.65	0.70	0.74	0.66
32	0.70	0.69	0.72	0.79	0.74	0.72	0.76	0.69	0.72	0.67	0.72	0.67	0.68	0.65	0.73	0.66	0.70	0.78	0.72	0.74
33	0.64	0.66	0.67	0.64	0.69	0.69	0.73	0.73	0.76	0.76	0.67	0.61	0.60	0.61	0.71	0.63	0.66	0.66	0.68	0.63
34	0.66	0.66	0.66	0.66	0.70	0.69	0.80	0.76	0.71	0.76	0.73	0.63	0.62	0.66	0.66	0.65	0.68	0.66	0.68	0.67
35	0.69	0.68	0.70	0.68	0.64	0.65	0.78	0.73	0.76	0.76	0.71	0.65	0.64	0.58	0.69	0.66	0.68	0.63	0.70	0.64
36	0.61	0.60	0.63	0.67	0.65	0.65	0.82	0.74	0.75	0.76	0.64	0.65	0.66	0.65	0.66	0.65	0.65	0.65	0.65	0.74
37	0.72	0.71	0.71	0.74	0.71	0.71	0.79	0.76	0.81	0.78	0.66	0.62	0.70	0.62	0.74	0.66	0.66	0.69	0.69	0.71
38	0.73	0.74	0.74	0.64	0.65	0.67	0.76	0.78	0.79	0.75	0.68	0.59	0.69	0.57	0.73	0.67	0.65	0.66	0.70	0.67
39	0.71	0.70	0.68	0.67	0.67	0.70	0.78	0.76	0.78	0.79	0.70	0.64	0.67	0.66	0.75	0.70	0.73	0.66	0.71	0.70
40	0.72	0.71	0.70	0.68	0.66	0.66	0.73	0.73	0.74	0.72	0.71	0.60	0.66	0.58	0.74	0.70	0.65	0.72	0.74	0.71

**No. = designated sample number for isolate listed in Table 3.1

Table 3.4.3 (Continuation). Genetic similarity coefficients using Dice estimates for 40 gray snow mold (*T. incarnata*) isolates from data of 115 RAPD markers using 37 primers.

No.**	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40
21	1.00																			
22	0.78	1.00																		
23	0.64	0.74	1.00																	
24	0.65	0.70	0.71	1.00																
25	0.63	0.77	0.70	0.62	1.00															
26	0.63	0.72	0.77	0.68	0.75	1.00														
27	0.62	0.70	0.70	0.67	0.67	0.73	1.00													
28	0.58	0.67	0.70	0.63	0.68	0.68	0.67	1.00												
29	0.67	0.77	0.75	0.69	0.76	0.70	0.69	0.70	1.00											
30	0.58	0.66	0.64	0.70	0.69	0.68	0.67	0.62	0.77	1.00										
31	0.64	0.72	0.67	0.64	0.71	0.67	0.73	0.64	0.71	0.71	1.00									
32	0.65	0.75	0.68	0.66	0.71	0.73	0.74	0.63	0.77	0.79	0.75	1.00								
33	0.62	0.68	0.66	0.65	0.74	0.68	0.74	0.67	0.70	0.74	0.71	0.74	1.00							
34	0.69	0.68	0.68	0.69	0.67	0.68	0.74	0.70	0.70	0.69	0.70	0.67	0.76	1.00						
35	0.63	0.69	0.69	0.66	0.70	0.69	0.75	0.64	0.77	0.71	0.77	0.73	0.75	0.73	1.00					
36	0.58	0.70	0.66	0.65	0.73	0.68	0.69	0.62	0.68	0.70	0.65	0.71	0.65	0.66	0.73	1.00				
37	0.61	0.70	0.67	0.66	0.77	0.72	0.73	0.66	0.70	0.70	0.74	0.75	0.80	0.73	0.77	0.75	1.00			
38	0.62	0.71	0.70	0.67	0.74	0.66	0.69	0.68	0.70	0.70	0.75	0.69	0.70	0.70	0.73	0.67	0.77	1.00		
39	0.65	0.71	0.77	0.70	0.72	0.73	0.76	0.68	0.72	0.74	0.80	0.73	0.76	0.77	0.80	0.78	0.80	0.77	1.00	
40	0.59	0.67	0.67	0.66	0.73	0.69	0.73	0.66	0.71	0.73	0.70	0.73	0.75	0.73	0.76	0.72	0.81	0.82	0.82	1.00

**No. = designated sample number for isolate listed in Table 3.1

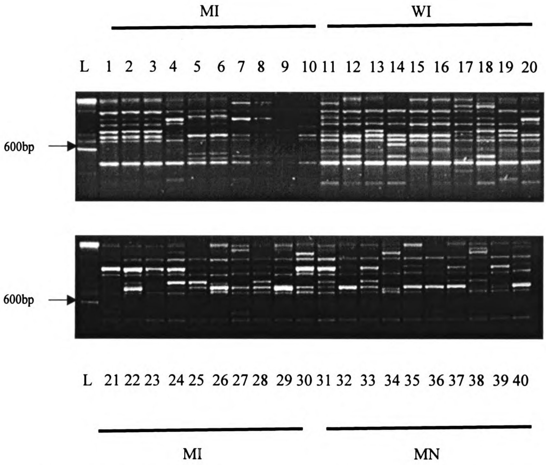

Figure 3.3. RAPD profile of 40 gray snow mold isolates from Michigan (MI), Wisconsin (WI) and Minnesota (MN) using primer OPY-02.(L=100bp DNA ladder). Lane numbers refer to isolated listed in Table 3.1.

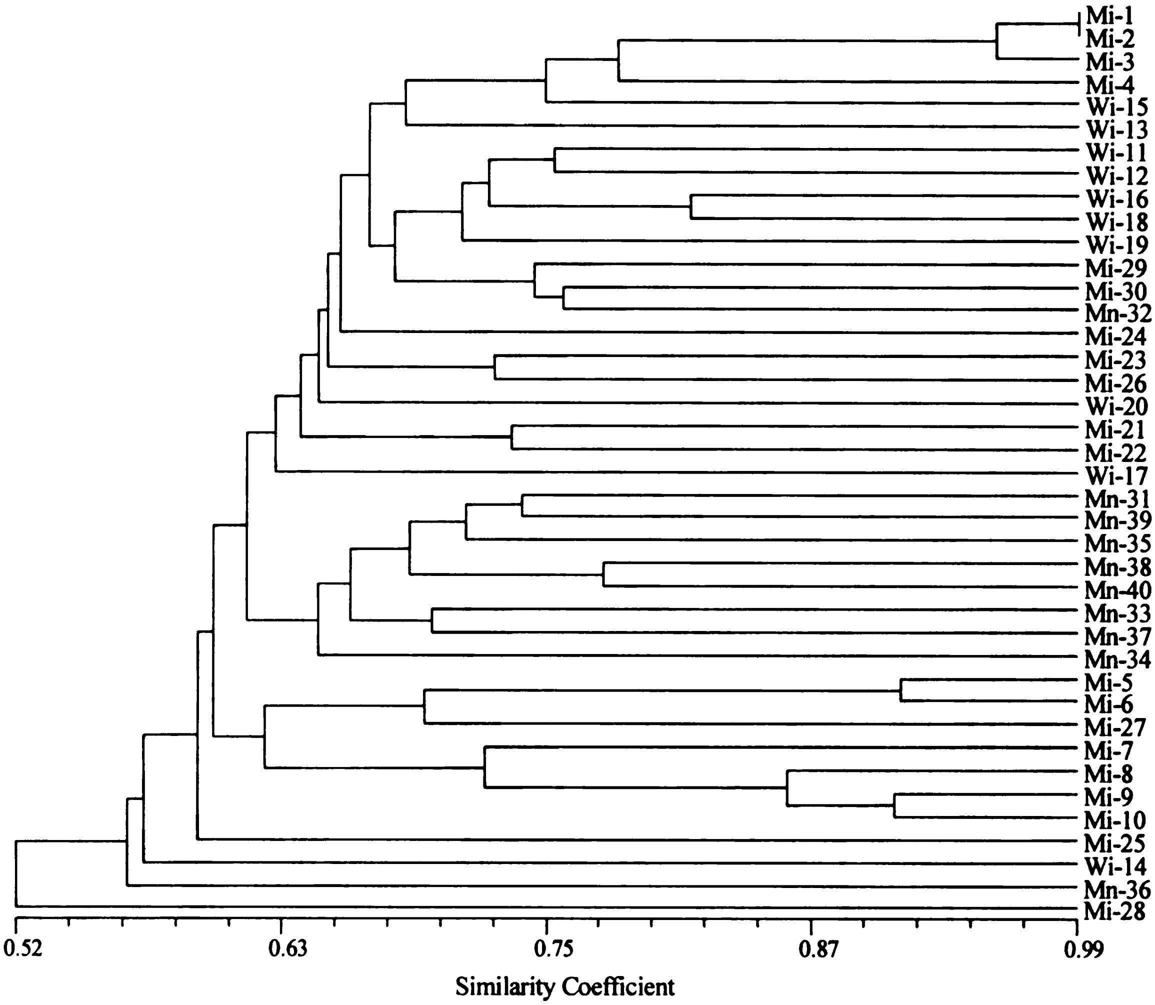

Figure 3.4.1. UPGMA dendrogram of 40 gray snow mold isolates from different locations in Michigan (MI), Wisconsin (WI) and Minnesota (MN), USA based on 115 RAPD markers generated using Dice coefficients, NTSYS program (Rohlf, 2000).

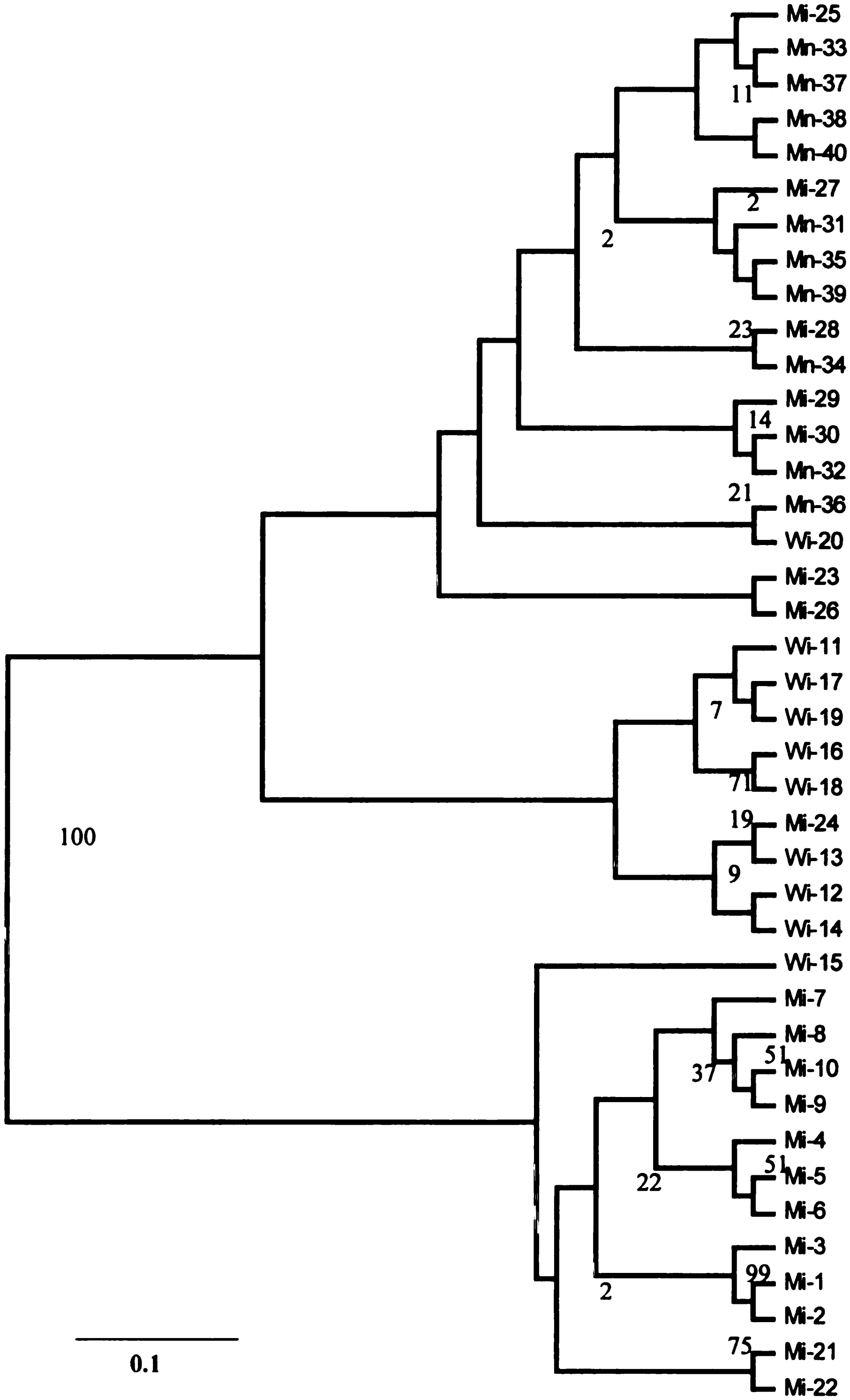

Figure 3.4.2. Dendrogram of 40 gray snow mold isolates based on 115 RAPD markers using Nei-Li genetic distance, neighbor joining (NJ) method and resampling options of FreeTree (Pavlicek et al., 1999) and TreeView (Page, 2001) programs. Numbers beside node indicate relative bootstrap frequencies.

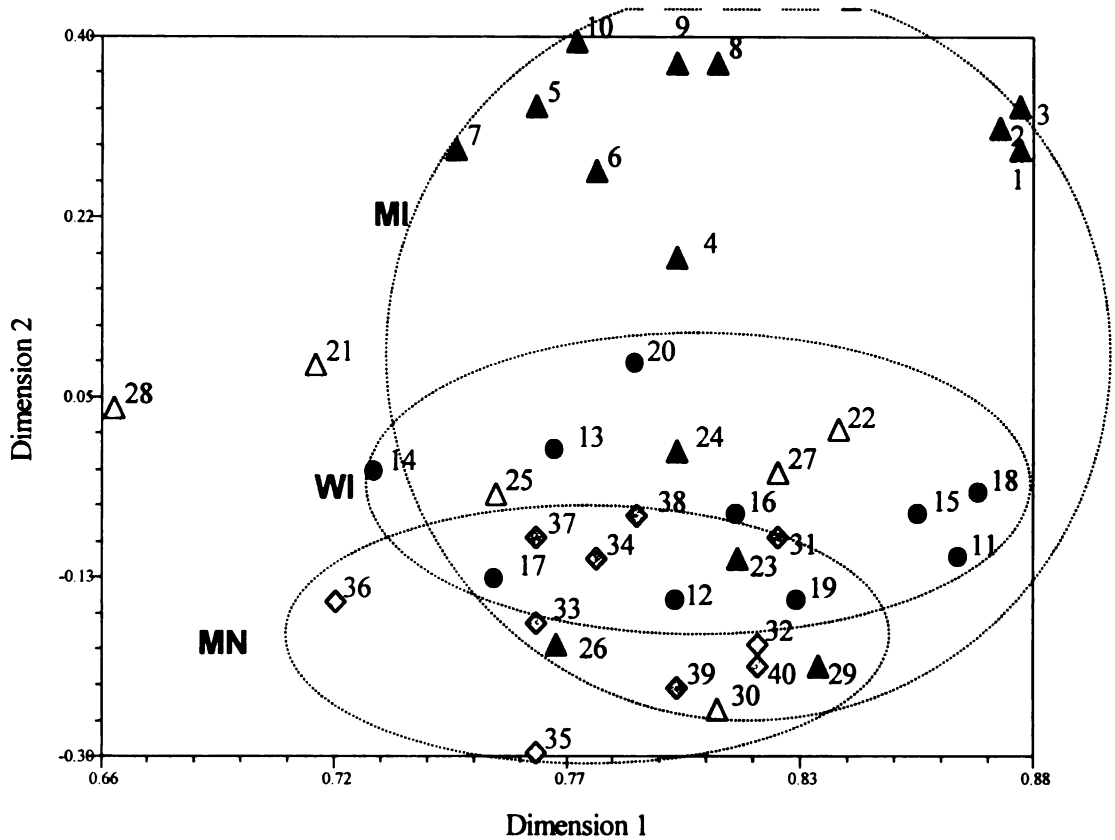

Figure 3.5. Scatterplot of the first two dimensional scales for 40 gray snow mold isolates based on principle component analysis of RAPD profile. Number correspond to isolate as listed in Table 3.1.

Legend: Michigan ▲, Upper Michigan △
Wisconsin ●
Minnesota ◇

mold isolates using the first 2 dimensional scales showed that distribution between isolates coming from different geographical sources overlapped (Figure 3.5). The intertwined cluster corresponded to the first major group defined by PCA, with Mi-28 and Mi-21, representing a minor group, presumably outliers defined by the second component.

The distance data was used to generate a single NJ tree after carrying out 100 replicates on the bootstrap test. The UPGMA tree for the 115 RAPD markers was shown Figure 3.4.1 and the NJ tree was given in Figure 3.4.2. While the two dendrograms differed topographically or in general appearance, the small clades were broadly similar for some of the isolates. As an example, in the UPGMA tree, Mi-1, Mi-2, Mi-3 belonged to one clade and would similarly appear as a subclade for the NJ tree. Also in the UPGMA tree, Mi-7, Mi-8, Mi-9 and Mi-10 would be a subclade; Mi-5, Mi-6 and Mi-7 would be another subclade and the two subclades would belong to the same single clade for the NJ tree. Isolate pairs of Mi-33 and Mi-37; Mn-38 and Mn-40 or Wi-16 and Wi-18 would appear as pairs also in both trees. Isolates with low bootstrap frequencies of 1 and 2 (Mi-28 or Mn-36, respectively) from the NJ tree were found in different clades in the UPGMA tree. The bootstrapping method revealed that there could possibly be three major clades and was found to have a good resolution to distinguish the isolates than by the UPGMA method. The first major clade in the NJ tree composed of 18 fungal isolates from the three states, Michigan, Minnesota and Wisconsin. The second major clade included 8 isolates from Wisconsin and Mi-24 (Michigan) while the third major clade consisted of 12 isolates from Michigan and Wi-15 (Wisconsin).

From the two dendrograms, the long branches in the UPGMA tree indicated the wide genetic diversity of the *T. incarnata* isolates and the relatively long branches in the NJ tree also gave indication of the wide genetic distance between clades. The low bootstrap values for the shorter nodes in the NJ tree suggest relatively recent clonal differentiation probably arising from the same founder group. Similar results were found for trichomonad parasites using the RAPD method and using repeated bootstrap analysis (Hampl et al., 2001).

The results generally showed that gray snow molds were highly variable. Small groups or clades could be discerned using a resampling approach. Heterogeneity can arise from several factors by way of sexual recombination as *T. incarnata* is highly outbred (Bruehl and Machtmes, 1978), selection pressures from the environment or from the fungi's complex tetrapolar sexual mating systems. With different mating classes, the fungus may have different structures for male and female hyphae or heterothallic in nature which may increase out crossing. Future studies should be made to define genetic variation in association with mating groups.

AMOVA analyses

An analysis of molecular variance of the genetic distances between populations defined by geographic clustering indicate that 12.67% of the genetic variation was attributable among populations and 87.33% within populations (Table 3.5). The presence of high variation within populations in contrast to lower levels of genetic differentiation among geographical locations confirms the hypothesis that migration results in low levels

Table 3.5 Summary of AMOVA of populations of gray snow mold based on 4 geographic locations, Michigan (MI), Upper Michigan (UMI), Wisconsin (WI) and Minnesota (MI).

Source of Variation	Degrees of Freedom	Sum of squares	Variance components	Percentage of variation
Among populations	3	64.60	1.29	12.67
Within populations	36	321.3	8.93	87.33
Total	39	386.00	10.22	
Fixation Index	FST :	0.1267		

Table 3.6. Population pairwise difference (distance method) and average genetic diversity/loci in 4 different populations of gray snow mold according to geographic locations.

Population	MI	MN	UMI	WI	Average genetic diversity/loci
MI	0.00				0.19 $\pm$ 0.11
MN	0.17	0.00			0.26 $\pm$ 0.14
UMI	0.03	0.06	0.00		0.34 $\pm$ 0.20
WI	0.13	0.19	0.10	0.00	0.30 $\pm$ 0.16

of differentiation between geographical locations and that sexual recombination and dissemination result in higher levels of genetic diversity (Mahuku et al., 1998).

Fixation index (FST) measures the reduction in heterozygosity in an individual due to non-random mating within its subpopulation due to genetic drift (Wright's F-statistics). The index value of 0 indicates panmixis (random mating) and the value of 1 indicates extreme isolation. The FST index for *T. incarnata* population, based on geographic groups based on permutation in the AMOVA was estimated at 0.13. To compare the FST index, most large vertebrates with higher number of nuclear genes and capacity for recombination and even capable of migration would have a mean value of FST <0.2. The relatively low FST index for *T. incarnata* suggests that heterozygosity may be highly maintained more through random mating.

Population pairwise difference or genetic distance between the 4 populations were measured and presented in Table 3.6. The results indicated close values for genetic distance between populations, MN and WI (0.19) compared to MN vs. LPMI (0.17). Geographic distance hence does not significantly differentiate the populations of snow mold between the three states. The population genetic distance between LPMI and UPMI was only 0.03, suggesting no significant difference in genetic diversity between the two populations. The average genetic diversity per loci was estimated for each population (Table 3.6) and isolates from Upper Peninsula, Michigan (UPMI) had the highest diversity (0.34) followed by WI (0.30), MN (0.26) and LPMI (0.19).

In summary, RAPD markers were used to assess genetic diversity in *T. incarnata* and demonstrated the vast differences in isolates genetically. In other fungi like speckled snow mold, RAPD was able to isolate clonal groups of *T. ishikariensis* var. *idahoensis* and var. *canadensis*. Our results from the dendrograms, 2 dimensional scale plot, AMOVA analyses however all indicate that isolates of *T. incarnata* may not be sufficiently diverged and isolated to identify clonal groups. The high amount of genetic variability found within geographic groups probably was attributable to high rates of sexual recombination and random mating. Geographic distance was unlikely a major contributing factor population differentiation due to human interference by way of transport, contaminated equipment, similar selection pressures from fungicide applications, same source of infected sod (Smith et al., 1989) and similarity of hosts or cultivars of turfgrasses at different locations. The results obtained from gray snow mold are similar to those for pink snow mold, where genetic diversity among individuals within populations was high (Mahuku et al., 1998). High variability have also been found for other sexually producing fungi in local populations (Huff et al., 1994; Morjane et al. 1994). Immigration of propagules from outside areas and presence of a sexual state would increase genetic diversity (Morjane et al., 1994). RAPD markers would be highly useful as a simple tool in future investigations to characterize variation in *T. incarnata* according to mating groups, host specificity, virulence and study how mitigating forces of selection, recombination and migration may affect the population genetics of this species.

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CHAPTER IV

Disease Resistance Screening of Bentgrass to *Typhula incarnata* Lasch

ABSTRACT

Bentgrass (*Agrostis* spp.) is susceptible to a wide range of diseases. One economically devastating disease is gray snow mold blight, caused by *Typhula incarnata* Lasch. There are no known resistant cultivars of creeping bentgrass (*A. palustris*) or any other turfgrass species to *T. incarnata*. Research into snow mold disease resistance is hampered by the absence of rapid screening techniques, in addition to poor identification of pathogenic strains of the fungus. Using a controlled screening procedure described herein, we have selected 20 creeping bentgrass genotypes from 890 samples from old Northern Michigan golf courses and identified 3 accessions of colonial bentgrasses (*A. capillaris*) with strong resistance to the gray snow mold. Six commercial creeping bentgrass cultivars 'L-93', 'Penn A4', 'Penn G2', 'Penncross', 'Providence' and 'Emerald' were all found susceptible. The resistant genotypes identified will be useful to the development of creeping bentgrass cultivars.

Key words: Snow mold resistance, controlled screening, creeping bentgrass, colonial bentgrass

INTRODUCTION

Snow mold diseases are important causes of winter injury in grasses and cereals in North America, Canada, Russia, Japan, and the Nordic countries. *Typhula* snow molds are known by different names such as gray snow mold (*T. incarnata),* snow scald, speckled snow mold (*T. ishikariensis)* and *Typhula* blight (collectively caused by *T. incarnata* and *T. ishikariensis*) (Vargas, 1994). Gray snow mold (*T. incarnata*) causes serious damage in turfgrass and is common on golf courses in climates with less than 90 days of snow cover (Millet, 2000). The pathogen is very slow growing and symptoms caused by the different species are difficult to distinguish (Hsiang et al, 1999). Symptoms usually appear as circular, water-soaked or straw-colored patches measuring 5 to 15 cm across. Plants may be matted and appear slimy with mycelium. Usually only the leaves appear diseased and dead and the crown may survive to produce new leaves in the spring. Mycelium, basidiospores or sclerotia that are produced may be sources of inoculum that lead to new infection. Colonized dead plant tissues decompose and disintegrate, and then the sclerotia fall to the thatch and soil where they oversummer, and remain as resting bodies during the summer months. Susceptible plants generally show the water-soaked lesions and a yellowish decaying appearance. The thin crust of mycelium is white and may be covered with dust giving it a gray-white appearance (hence the name "gray snow mold") (Jackson and Fenstermacher, 1969).

Gray snow mold is a cold-loving or 'psychrophilic' organism. Snow molds have the ability to modify their intracellular conditions favorable to survival (Snider et al., 2000). The lack of high ice nucleation activity combined with the presence of antifreeze activity in all fungal fractions indicates that snow molds can moderate their environment to inhibit or modify intra- and extracellular ice formation, which helps explain their ability to grow at subzero temperatures under snow cover. Pathogenicity of the fungi is not dependent on ice nucleation activity to cause freeze wounding of host plants. Another factor contributing to snow mold survival may be the pathogen's capacity to utilize a broad range of substrates, from live tissues to dead organisms. Jacobs and Bruehl (1986) theorized that *T. incarnata* is unable to establish itself sufficiently in its host to cause disease problems, while Matsumoto and Tajimi (1985) described the fungus more as an 'opportunistic parasite' attacking senescent or moribund plant tissues beneath the snow, where the low temperature reduces the activity of other antagonistic microflora.

There is little information regarding the pathogenicity and variability of the gray snow mold. Snow mold resistance evaluation among bentgrass cultivars is currently done in the field with the National Turfgrass Evaluation Procedure (NTEP) trials using naturally infected plots. In the 1995 NTEP trial, twenty seven *A. palustris* varieties from twenty four sites were observed to show a wide range of resistance to the *Typhula* species, but no variety was found to be strongly resistant. Results from NTEP scored in 2000 at the Michigan State University Hancock Research Center showed snow mold disease infection ranged from 20% to 90% of the plot area. Although NTEP provides information on the performance of bentgrass cultivars, compounding biotic or abiotic factors, e.g. the presence of multiple pathogens, competition, and the moisture or dryness in some areas, limit the applicability of the findings and do not give an accurate description of a cultivar's resistance. Field studies are also dependent on the duration of snow cover and melt.

Under controlled conditions, Millet (2000) inoculated the creeping bentgrass (*A. stolonifera sp. palustris*) cultivar 'Penncross' with isolates of the three snow molds (*T. incarnata, T. ishikariensis* and *T. phacorrhiza*) from Wisconsin area using the Fast Turf screening system. Creeping bentgrass was grown in 35-mm film cans and incubated in cold chambers for three weeks at temperatures of 41 °F and 50 °F. The disease was rated at 21, 28 and 35 days. The results indicated that gray and speckled snow molds were not significantly different from each other in their ability to cause disease. *T. phacorrhiza* was not as strongly aggressive and the author postulated its role as a decomposer, termed 'senectophatic disorder', which colonized dying grass as the other snow molds were becoming less active. The study by Millet (2000) is conceptually important because it highlighted developing a controlled screening procedure against snow mold. Resistance in the host plants however was not precisely determined because all three fungal species were used as inoculants concurrently, disregarding the probability of competition between *Typhula* species. Under field conditions, a thin line separates the circular damaged spots created by different species and these disease patches never overlapped.

Studies measuring resistance in turfgrass and other crops to gray snow mold or snow molds are generally few in number. In grasses, *Lolium perenne* is considered the most susceptible to gray snow mold, followed by *Festuca arundinaceae, A. palustris, Poa annua, P. pratensis* and *F. rubra.* (Hsiang, 1999). Within each species, there can be a broad range of susceptibility among cultivars. Wu and Hsiang (1998) found a strong positive correlation between susceptibility of 12 turfgrass species to *T. incarnata* and their susceptibility to *T. ishikariensis*. There are no known resistant cultivars or species of bentgrass and the severity of damage warrants research to find a resistant genotype under controlled conditions. Despite the low resistance found in creeping bentgrasses, selection of varieties for durable resistance can be done because heritability estimates for snow mold resistance in grasses are high (Gaudet et al., 1999).

Bentgrass, *Agrostis* sp. a derivation from Greek: grass, forage, has about 220 species distributed throughout the world (Watson and Dallwitz, 1992). It is a perennial or annual outcrossing polyploid (x=7, 2n=14, 21, 28, 42, etc.). The wide genetic variability implies potential for finding a resistant plant among the bentgrass species. Other possible sources for snow mold resistance may be in naturally selected clones of creeping bentgrass. To identify such sources, creeping bentgrass (*A. stolonifera sp. palustris*) samples were collected from old Northern Michigan golf courses that have not been sprayed with fungicide or overseeded for the last 10 years. Natural selection and recombination events could have played a significant role in creating resistant creeping bentgrass. Identifying resistant clones through controlled screening experiments would contribute to turfgrass improvement programs. Controlled screening would also enable rapid testing of several materials independent of the presence of snow and with high repeatability.

The objectives of this study were to develop a controlled physiological screening system for resistance to *T. incarnata* and to search for plants resistant to the pathogen from several creeping bentgrass cultivars, naturally selected clones obtained from Michigan and plant introduction lines of *Agrostis* spp.

MATERIAL AND METHODS

Plant materials

The materials were divided into three groups:

a. Creeping bentgrass collected in April 2000 from golf courses in Northern Michigan - Plugs were collected from areas bordering snow mold circular patches. A total of 890 genotypes were used as follows: 220 samples (Population A) for developing the procedure and another 670 samples (Population B) for additional screening.

b. Commercial creeping bentgrasses, 8 to 10 plants each of cultivars, 'L-93', 'Penn A4', 'Penn G2', 'Penncross', 'Providence' and 'Emerald' were used.

c. Forty accessions of *Agrostis* sp. plant introductions, representing 14 species obtained from USDA Washington Pullman Station were used. Seeds of 25 plants per accession were germinated on filter paper and transferred into pots (1 seedling /pot) (Table 4.1).

The plants were grown in No. 2 pots (4x4x4 inches) containing peat soil mixture and supplemented with Peter's fertilizer. Plant height was maintained by clipping at 1.0 inch. Several 'Penncross' plants were also grown in pots and used as a susceptible plant control.

Pathogen

Sclerotia of gray snow mold (*T. incarnata*) were collected from Hancock Turfgrass Research Center in April 2001 and grown in potato dextrose agar (PDA, 39g/L with streptomycin and penicillin antibiotics) at 5 °C for 2 months. Each isolate was then transferred to sterilized cornmeal mixture (1 part cornmeal to 2 parts silica sand with 5% PDA broth, autoclaved for 40 minutes) for multiplication. Growth and incubation in cornmeal was made at 5 °C for another two months. A phase contrast microscope was used to verify the presence of *T. incarnata* in the cornmeal mixture by checking for the

Table 4.1. List of *Agrostis* spp. screened for snow mold resistance and their geographic origins.

Species	No. of accessions	Geographic origins
A. canina	2	Netherlands, Iran
A. capillaris	7	Europe
A. castellana	10	USA, Spain, Portugal
A. palustris	5	USA, Turkey, Europe
A. stolonifera	6	USA, Europe, Russia
A. mongolica	1	Mongolia
A. lachnantha	2	Africa
A. munroana	1	Iran
A. hygrometrica	1	Uruguay
A. scabra	1	Canada
A. trinii	1	Russia
A. vinealis	1	Russia
A. transcaspica	1	Russia
A. gigantea	2	USA, Turkey

presence of mycelia and visually checking for the presence of orange to brown sclerotia. The fastest growing and virulent isolate identified from pre-screening was chosen and used for the controlled screening procedures.

Controlled screening and rating procedure

Initial screening was done in controlled plant growth chambers using day temperature of 7 °C and night temperature of 4 °C for 4 weeks. However the disease did not progress well. After the optimum temperature of 5 ^{o}C was determined, all succeeding tests were performed in the cold room available at the Crop & Soil Sciences farm at Michigan State University. In the first screening, 220 creeping bentgrasses (Population A) from the N. Michigan collection were randomly put in trays at 10 plants per tray. The plants were brought into the cold room (5 ^{o}C) for 3 days for acclimation prior to disease inoculation. Equal amounts of inoculum (1 g) were put into the center of each pot and covered with moistened cheesecloth. To compare treatments of inoculated versus uninoculated plants, only 9 plants/tray were inoculated and 1 pot was left untreated. The trays were filled with water and put inside plastic bags to maintain high humidity and optimize disease severity. Visual inspection and scoring for resistant and susceptible plants was done at 4 and 6 weeks using Horsfall-Barratt system with a scale of 0 = no disease to 10 = completely dead plant. Susceptible plants were classified by the growth of the infection area as characterized by increased area of soft, watery, yellow or brown lesions and widespread development of mycelia. Two people rated and the means of two ratings were used for analysis. Plants were returned to the greenhouse (GH) after 6 weeks and scores were taken again after 3 days. The previously yellowing leaves would appear brown and dried at this time. Recovery was scored after another 10 days (1, 3, 5, 7, 9, 10 scale, with 10 as 100% recovered). Statistical analysis using Proc GLM option of SAS was done to test significance between uninfected and infected treatments. The plants were ranked based on the disease scores and recovery.

Candidate resistant plants were divided into three clones and screened for a second time using 'Penncross' as the susceptible check, and all plants in the tray were inoculated. Screening for the resistant lines from Population B (670 creeping bentgrass), the 6 commercial cultivars and 40 plant introductions followed the procedure described above using completely randomized design (CRD). Statistical Analysis Software (SAS version 8.0) was used for data analyses. Arcsin transformation on percentage means for each score was calculated and F-Test was generated using the PROC GLM with LSD options of SAS.

RESULTS AND DISCUSSION

Screening creeping bentgrass populations

The growth conditions in the cold room of 5 °C temperature and high humidity were found favorable for *T. incarnata*. At 4 weeks, fungal mycelia were visible on creeping bentgrass and leaves appeared water-soaked and slightly yellowed (Figure 4.1.A). At 6 weeks, lesions were turning brown on whole leaves of some of the inoculated plants with a wider spread of infected area (Figure 4.1.B). Some plants appeared to be entirely damaged and this was more apparent when plants were brought to the greenhouse and scored after 3 days (Figure 4.1.C). The results of the controlled screening system using two populations of creeping bentgrass from old Michigan golf courses: population A (220 plants) and population B (670 plants), are shown in Table 4.2. In both populations, significant differences between the two treatments, inoculated and uninoculated (as control) were observed at the 4th week, 6th week and after 3 days in the greenhouse. Control plants were mostly rated as 0 (no disease) but a few plants were rated as 1 or 2 (for slightly yellowed). This is largely in contrast to the inoculated plants where the mean disease scores in six weeks ranged from 5.51 (50-60% infected) to 7.53

Figure 4.1. **A.** Creeping bentgrass at the 4th week of snow mold infection, left bottom pot in the tray is uninoculated. **B**. Disease after the 6th week, left top pot in the tray is uninoculated. **C**. Trays on benches at 3 days in the greenhouse **D**. PI lines showing susceptible plants and accessions with good recovery. *"Images in this dissertation are presented in color."*

Table 4.2. Analysis of variance of snow mold disease inoculation in creeping bentgrass using inoculated and uninoculated (control) as treatments in two populations from N. Michigan.

Population	Treatments and F-values[3]	N	Snow mold Rating[1] 4th week	6th week	GH 3d	Recovery Means[2]
A. 220 plants	Control	23	0.95 ± 1.5	1.24 ± 2.0	1.87 ± 2.7	8.2 ± 2.8
	Inoculated	197	5.51 ± 1.2	6.46 ± 1.2	7.53 ± 1.1	3.2 ± 2.1
	F-value		228.15**	241.97**	266.07**	190.04**
B. 670 plants	Control	63	0.84 ± 1.0	1.40 ± 1.6	2.40 ± 1.9	8.07 ± 2.2
	Inoculated	607	5.67 ± 1.1	6.83 ± 1.1	8.02 ± 1.0	2.73 ± 2.2
	F-value		898.78**	993.72**	787.1**	480.28**

[1] Disease severity was rated on as Horsfall-Baratt scale of 0 (no infection) to 10 (completely dead). Scores are the means of N (number of samples).

2 Recovery rated on a scale of 0 (no recovery) to 10 (complete recovery).

[3] Ratings were converted into percentages as in 1=10% and 10=100%. Arcsin transformation on done on the percentage values and run using Proc GLM option of the SAS system.

* *Treatment means were found significant different at P<0.05.

(70-80%, highly infected). There were significant differences between the two treatments, uninoculated and inoculated, suggesting the inoculation treatment was effective to cause disease. The frequency pattern of snow mold disease scores in population B was graphed and generally follows a bell distribution curve (Figure 4.2). The continuous distribution pattern is suggestive of a horizontal type of resistance controlled by a few minor genes. In contrast, if resistance trait was a vertical resistance type controlled by a major gene, frequency data distribution will be in 2 discrete columns.

Recovery was measured after another 10 days in the greenhouse. Uninoculated creeping bentgrasses recovered significantly better than the inoculated plants. Recovery for infected plants was generally low at 27% to 32%.

From population A, 28 plants were chosen as candidate resistant plants after ranking. Another 59 plants were chosen as candidate resistant plants from population B. The candidate resistant plants were divided into three clones and subjected to a second screening. A second replicated screening was necessary to determine accuracy of the first rating for the resistant genotypes as disease escapes could be factor for erroneous rating. Replication and testing against a highly susceptible check, 'Penncross' ensured better selection for the resistant genotype.

The results of replicated screening of selected genotypes from population A and B are shown in Table 4.3 and Table 4.4. Analysis of variance (F-test) and t-tests (LSD) showed significant differences in the 6th week, GH 3 days rating and the means of the three ratings (Table 4.3.1). Ratings were not found significant across the 28 creeping bentgrass genotypes at the 4th week ($P<0.10$) and but were highly significant at the 6th week and GH at $P<.05$. Nine genotypes (Nos. 15, 4, 21, 6, 8, 26, 22, 24 and 28)

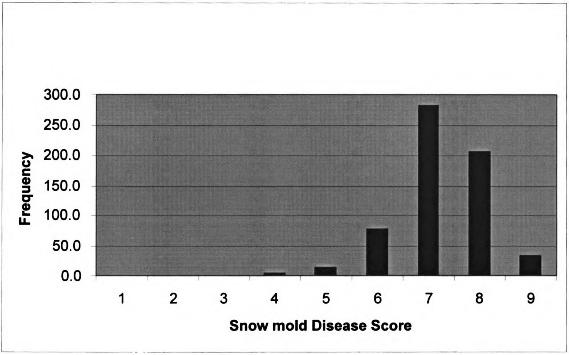

Figure 4.2. Distribution of disease rating scores in 670 creeping bentgrass genotypes.

Table 4.3.1. Disease ratings and analysis of variance of candidate resistant lines of creeping bentgrass from Population A to snow mold (*Typhula incarnata*) using a completely randomized design with 3 replicates.

Candidate Resistant line [3]	Snow mold Rating[1,2] 4th Week	6th Week	GH 3 days	Means
15	3.5	4.5	5.5	4.8 †
4	4.5	5.3	7.8	5.9 †
21	6.0	5.5	7.2	6.2 †
6	5.7	6.3	7.0	6.3 †
8	4.5	7.7	6.7	6.3 †
26	4.7	6.3	8.0	6.3 †
22	5.0	6.8	7.7	6.5 †
24	5.7	6.0	7.8	6.5 †
28	5.2	7.0	7.3	6.5 †
2	5.8	6.3	7.7	6.6
9	5.8	6.7	7.3	6.6
20	6.0	5.8	8.0	6.6
27	5.8	6.8	7.2	6.6
17	6.2	6.7	7.3	6.7
19	5.8	7.2	7.3	6.8
25	5.7	5.8	7.3	6.8
3	6.0	7.5	7.3	6.9
11	6.3	7.1	7.3	6.9
12	5.8	6.8	8.2	6.9
23	6.5	6.7	7.5	6.9
10	5.3	7.5	8.2	7.0
7	6.2	7.5	7.5	7.1
13	6.8	7.2	7.5	7.1
16	6.5	7.3	7.3	7.1
1	6.7	7.5	7.5	7.2
5	6.5	7.2	8.0	7.2
18	6.5	7.8	7.3	7.2
14	7.3	7.8	7.5	7.5
Penncross	7.0	7.8	8.3	7.7
F-test (P<.0001)[4]	1.62	1.69**	2.07**	1.84**
LSD (P=0.05)	1.8	1.8	1.0	1.1

[1] Disease severity rated on a Horsfall-Barratt scale of 0 (no infection) to 10 (completely dead).

[2] Each score was taken independently by 2 raters and the mean was calculated for 3 replicates. '4th' and '6th' week ratings were taken in the cold room at 5°C. 'GH 3 days' ratings were taken 3 days in the greenhouse after pots were taken out of the cold room.

[3] A total of 28 candidate resistant lines were selected from 220 individual creeping bentgrass genotypes and divided into three replicates.' Penncross' was used as the susceptible plant control.

[4] Ratings were converted into percentages as in 1=10% and 10=100%. Arcsin transformation was done on the percentage values and run using Proc GLM option of the SAS system for calculation of F values.

* Scores were found significantly different at P<0.10.
** Scores were found significantly different at P<0.05.

† Disease rating means were found to be significantly different from 'Penncross'.

Table 4.3.2. Recovery ratings of 28 selected creeping bentgrass lines (Population A) from snow mold infection using CRD with 3 replicates (ranked from the best genotype).

	Recovery Rating[1, 2]
Candidate Resistant Line	Means
2	8.7 †
8	8.0 †
15	8.0 †
22	7.7 †
24	7.7 †
9	7.0 †
17	7.0 †
25	7.0 †
26	7.0 †
7	6.7 †
23	6.7 †
6	6.3 †
18	6.3 †
21	6.3 †
3	6.0 †
5	6.0 †
27	6.0 †
28	6.0 †
14	5.3 †
19	5.3 †
20	5.3 †
10	5.0
13	4.7
11	4.0
16	4.0
Penncross	4.0
1	3.7
4	3.3
12	2.0
F test (P<0.0001)[3]	2.33**
LSD(P=0.05)	2.90

[1] Recovery rated on a scale of 0 (no recovery) to 10 (complete recovery).

[2] Each score was taken independently by 2 raters and the mean for 3 replicates.

[3] Ratings were converted into percentages as in 1=10% and 10=100%. Arcsin transformation on done on the percentage values and run using the Proc GLM option of the SAS system.

** Scores were found significantly different at P<0.05.

† Recovery ratings were found to be significantly better than 'Penncross'.

Table 4.4.1. Disease ratings and analysis of variance of candidate resistant lines of creeping bentgrass from Population B to snow mold using a completely randomized design with 3 replicates.

Candidate Resistant line [3]	Snow mold Rating[1,2]			
	4th Week	6th Week	GH 3 days	Means
336	3.0	4.3	5.0	4.1 †
226	3.0	3.5	6.7	4.4 †
223	3.2	5.0	5.2	4.4 †
560	3.2	4.8	5.3	4.4 †
367	3.2	3.5	6.7	4.4 †
191	3.3	3.8	6.3	4.5 †
585	3.3	3.3	7.0	4.6 †
79	3.5	4.8	5.8	4.7 †
249	3.5	4.3	6.3	4.7 †
645	3.2	4.0	7.0	4.7 †
247a	4.0	4.5	6.0	4.8 †
247b	3.7	5.2	6.0	4.9 †
484	4.3	4.5	6.0	4.9 †
261	3.3	4.7	7.2	5.1 †
587	4.0	4.2	7.2	5.1 †
595	3.5	4.7	7.2	5.1 †
148	4.5	4.8	6.2	5.2
152	3.8	5.2	6.7	5.2
242	4.5	5.2	6.0	5.2
110	4.7	5.2	6.0	5.3
194	4.3	5.3	6.2	5.3
121	4.3	5.2	6.5	5.3
122	3.7	4.7	7.7	5.3
404	4.5	5.3	6.3	5.4
156	4.5	5.2	6.7	5.4
368	4.5	4.2	7.7	5.4
640	4.5	4.2	7.7	5.4
117	4.0	5.0	7.7	5.6
175	4.7	5.0	7.0	5.6
239	4.3	5.8	6.5	5.6
648	4.2	4.7	7.8	5.6
544	4.3	5.3	7.2	5.6
639	4.7	4.3	8.2	5.7
313	4.2	4.8	8.2	5.7
656	4.3	4.7	8.2	5.7
406	5.0	4.8	7.5	5.8
392	5.0	5.5	7.0	5.8
227	5.2	5.7	6.8	5.9
647	4.8	5.8	7.0	5.9
193	4.7	5.8	7.5	6.0
370	4.5	5.8	7.7	6.0
435	4.5	5.3	8.2	6.0
527	4.5	5.7	7.8	6.0
39	4.5	6.0	7.7	6.1
636	5.0	6.2	7.0	6.1
49	5.2	5.5	7.5	6.1
289	5.3	5.8	7.0	6.1
635	5.2	5.3	7.7	6.1
6	5.2	5.3	7.8	6.1
528	5.5	4.7	8.3	6.2

Penncross	3.5	6.8	8.8	6.2
146	4.8	6.3	7.7	6.3
604	5.8	5.5	7.7	6.3
644	5.7	5.5	8.2	6.4
145	5.5	5.7	8.3	6.5
570	5.7	6.0	8.2	6.6
291	5.2	6.7	8.2	6.7
649	5.5	6.7	8.0	6.7
522	5.8	6.7	7.8	6.8
F-test (P<.0001)[4]	2.28**	2.37**	2.92**	3.20**
LSD (P=0.05)	1.4	1.4	1.4	1.0

[1] Disease severity rated on a Horsfall-Barratt scale of 0 (no infection) to 10 (completely dead).

[2] Each score was taken independently by 2 raters and the mean for 3 replicates. '4th' and '6th' week ratings were taken in the cold room at 5°C. 'GH 3 days' was ratings taken 3 days in the greenhouse after pots were taken out of the cold room.

[3] A total of 59 breeding lines were selected from 670 individual creeping bentgrass genotypes and divided into three replicates. 'Penncross' was used as the susceptible plant control.

[4] Ratings were converted into percentages as in 1=10% and 10=100%. Arcsin transformation was done on the percentage values and run using Proc GLM option of the SAS system.

** Scores were found significantly different at P<0.05.

† Disease rating means were found to be significantly less than 'Penncross'.

Table 4.4.2. Recovery ratings of selected creeping bentgrass lines to snow mold (*Typhula incarnata*) using CRD with 3 replicates.

	Recovery Rating[1, 2]
Breeding Line	Means
194	7.9 †
336	7.4 †
79	7.3 †
152	7.2 †
6	7.1 †
242	7.1 †
554	6.9 †
175	6.7 †
404	6.7 †
247b	6.6 †
640	6.5 †
484	6.3 †
570	6.3 †
223	6.2 †
560	6.2 †
585	6.2 †
122	6.1 †
522	6.1 †
247a	6.0 †
587	6.0 †
645	6.0 †
110	5.9 †
249	5.8
227	5.8
239	5.8
39	5.7
370	5.7
649	5.7
636	5.5
639	5.5
604	5.4
49	5.3
648	5.3
289	5.3
121	5.2
145	5.2
226	5.2
656	5.2
117	5.0
291	5.0
644	5.0
595	4.9
191	4.8
313	4.8
146	4.8
261	4.7
367	4.4
647	4.3
368	4.2
406	4.2

193	4.1
148	4.0
528	3.8
635	3.7
435	3.5
527	3.0
Penncross	2.9
F test (P<0.0001)[3]	2.77**
LSD(P=0.05)	2.08

[1] Recovery rated on a scale of 0 (no recovery) to 10 (complete recovery).

[2] Each score was taken independently by 2 raters and the mean for 3 replicates.

[3] Ratings were converted into percentages as in 1=10% and 10=100%. Arcsin transformation on done on the percentage values and run using the Proc GLM option of the SAS system.

† Recovery means were found to be significantly better than 'Penncross'.

** Scores were found to be significantly different at P<0.05.

performed significantly better than susceptible check 'Penncross' from the replicated trials and candidate resistant line No. 15 was found to be the most resistant genotype. Recovery from disease was also found to be significantly different among genotypes (Table 4.3.2). Genotype No. 15 was ranked third in recovery. Twenty-one lines recovered better than 'Penncross'. Genotype No. 4 which had the second best resistant score or low disease rating had poor recovery indicating that resistance and recovery are independent traits. Eight genotypes could be considered as potential breeding materials with good snow mold resistance and significant recovery from cold and disease treatments.

Significant differences among disease rating means of 59 candidate resistant plants were found from Population B at the 4^{th} week, 6^{th} week and GH 3 days rating stages (Table 4.4.1). Sixteen genotypes were found with significantly better disease resistance than 'Penncross'. Genotype No. 336 was found to be the most resistant and with the second best recovery (Table 4.4.2). From these 16 best resistant genotypes, only 12 plants were selected as breeding lines because 4 genotypes did not recover well. The line with the second least disease rating, No. 226, did not perform better than 'Penncross' supporting previous findings that resistance and recovery are independent traits. A total of 20 resistant genotypes were found from 890 clones taken from Northern Michigan old golf courses.

Screening commercial creeping bentgrass cultivars

Six of the most popular commercial creeping bentgrass cultivars 'L-93', 'Penn A4', 'Penn G2', 'Penncross', 'Providence' and 'Emerald', were subjected to the developed snow mold screening procedure using complete randomized design with 8 to 10 replicates per cultivar. Significant differences were found among them for disease rating means and percentage recovery (Table 4.5). 'L-93', 'Penn A4', 'Penn G2', 'Penncross' and 'Providence' were grouped together by t-tests (LSD) and were not significantly different in mean disease ratings. Cultivar 'Emerald' was grouped separately and hence appeared as more susceptible than the first group. No commercial cultivar was found resistant.

The six commercial cultivars significantly varied in recovery performance with 'L-93' having the best rating. The results support the ratings from NTEP that 'L-93' is the superior creeping bentgrass cultivar. 'Penncross' showed 39% mean recovery and this is close to the previous recovery ratings of 4.0 (40% recovery) in the trials using Populations A and B in the first two tests. 'Penn G2', 'Penn A4' and 'Providence' had low recovery ratings and did not differ as a group based on t-tests.

Screening of PI Lines

Forty plant introductions were subjected to the snow mold screening procedure, with 24 to 25 representative genotypes per accession. Disease rating means and analysis of variance showed that ten accessions significantly performed better than 'Penncross' (Table 4.6.1). The ten accessions were species of *A. canina*, *A. capillaris*, *A. vinealis* and *A. mongolica*. The best performing colonial bentgrasses (*A. capillaris*) came from Europe. *T. incarnata* is widely distributed in Europe, Russia and parts of Northern Hemisphere and bentgrasses originating from these areas may have potential resistance to the pathogen. None of the creeping bentgrass species (*A. stolonifera* or *A. palustris*) was

Table 4.5 . Performance and analysis of variance of commercial creeping bentgrass cultivars using CRD in controlled snow mold screening experiments, their disease rating means[1] and percentage recovery [2].

Genotype	No. of Samples	Disease Rating Means[3]	Percentage Recovery[3]
L-93	10	5.9 ± 0.54[a]	61.00 % ± 10.22[a]
Penn A4	8	5.6 ± 0.48[a]	19.37 % ± 13.74[c]
Penn G2	10	5.4 ± 0.41[a]	27.00 % ± 13.78[c]
Penncross	10	5.8 ± 0.76[a]	39.00 % ± 7.74[b]
Providence	10	5.5 ± 0.20[a]	16.50 % ± 7.09[c]
Emerald	10	6.5 ± 0.71[b]	29.50 % ± 3.29[bc]
F-test		5.28**	31.54**
LSD		0.9	16.08

[1]Disease severity rated on Horsfall-Baratt scale of 0 (no infection) to 10 (completely dead). Disease rating means is the average of mean ratings at the 4th week, 6th week and at 3 days in the greenhouse taken independently by 2 raters and mean for given number of samples.

[2] Recovery was taken at 17 days in the greenhouse and means of the percentage recovery of the number of samples.

[3] Means followed by the same letter are not significantly different.

** Scores were found significantly different at P<0.05

Table 4.6.1. Disease ratings and analysis of variance of resistance to gray snow mold of different bentgrass species with 'Penncross' (as susceptible control) using CRD with 24 to 25 genotypes per accession.

		Snow mold Rating[1, 2]			
Species (MSU No.)	N	4th Week	6th Week	GH 3 days	Means
A. capillaris MSU-4	24	4.1	6.4	6.6	5.7 †
A. capillaris MSU-6	25	4.5	6.3	6.4	5.7 †
A. canina MSU-2	25	4.4	6.3	6.9	5.9 †
A. canina MSU-1	25	4.0	5.7	8.8	6.2 †
A. capillaris MSU-3	25	4.2	6.6	7.7	6.2 †
A. capillaris MSU-8	25	5.9	7.2	5.4	6.2 †
A. vinealis MSU-37	25	4.5	6.4	7.8	6.2 †
A. capillaris MSU-9	25	5.8	7.3	5.7	6.3 †
A. mongolica MSU-32	25	4.3	6.7	7.9	6.3 †
A. capillaris MSU-5	25	5.9	7.2	6.0	6.4 †
A. gigantea MSU-40	25	4.9	6.6	8.1	6.5
A. capillaris MSU-7	25	5.5	7.1	7.1	6.6
A. lachnantha MSU-30	25	4.6	6.5	8.6	6.6
A. hygrometrica MSU-33	25	4.3	6.7	8.7	6.6
A. scabra MSU-35	25	4.5	6.0	9.2	6.6
A. trinii MSU-36	25	5.5	6.9	7.5	6.6
A. stolonifera MSU-29	25	4.7	6.9	8.4	6.7
A. castellana MSU-16	25	4.9	7.0	8.2	6.7
A. castellana MSU-18	25	5.0	6.7	8.5	6.7
A. castellana MSU-13	25	5.2	6.9	8.2	6.8
A. castellana MSU-15	24	5.5	7.2	7.9	6.9
A. castellana MSU-17	25	5.5	7.4	7.9	6.9
A. palustris 'Penncross'	25	5.5	7.4	7.9	6.9
A. castellana MSU-14	25	5.3	7.3	8.2	6.9
A. castellana MSU-12	25	5.6	7.2	8.1	7.0
A. castellana MSU-11	25	5.0	7.1	8.8	7.0
A. stolonifera MSU-27	25	5.2	6.9	8.9	7.0
A. transcaspica MSU-38	25	5.6	7.3	8.2	7.0
A. palustris MSU-20	25	5.3	7.2	8.6	7.0
A. gigantea MSU-39	25	5.5	7.2	8.4	7.0
A. stolonifera MSU-25	25	5.0	7.5	8.8	7.1
A. stolonifera MSU-26	25	5.9	7.4	8.0	7.1
A. palustris MSU-21	25	5.8	7.3	8.4	7.2
A. palustris MSU-23	25	5.6	7.3	8.6	7.2
A. munroana MSU-34	25	5.4	6.9	9.3	7.2
A. castellana MSU-10	25	5.6	7.1	9.0	7.2
A. stolonifera MSU-24	25	5.8	7.7	8.3	7.3
A. castellana MSU-19	25	5.6	7.2	9.0	7.3
A. palustris MSU-22	25	5.9	7.7	8.3	7.3
A. stolonifera MSU-28	25	5.8	7.3	9.2	7.4
A. lachnantha MSU-31	25	5.4	7.7	9.3	7.5
F test ($P<0.0001$)[3]		6.5**	5.8**	11.2**	8.38**
LSD($P=0.05$)		0.6	0.5	0.7	0.4

[1] Disease severity rated on a Horsfall-Barratt scale of 0 (no infection) to 10 (completely dead).

2 Each score was taken independently by 2 raters and the mean for 25 replicates. '4th' and '6th' week

[3] Ratings were taken in the cold room at 40°F. 'GH 3 days' were ratings taken 3 days in the greenhouse after pots were taken out of the cold room.

[4] Ratings were converted into percentages as in 1=10% and 10=100%. Arcsin transformation on done on the percentage values and run using the Proc GLM option of the SAS system.

† Disease rating means found to be significantly different from 'Penncross'.

** Scores were found significantly different at $P<0.05$.

Table 4.6.2. Recovery ratings of accesssions of *Agrostis* species to gray snow mold (*Typhula incarnata*) using CRD with 24 to 25 genotypes per accession.

		Recovery Rating[1, 2]
MSU No.	Species	Means
MSU-8	*A. capillaris*	6.1 †
MSU-9	*A. capillaris*	6.0 †
MSU-5	*A. capillaris*	5.3 †
MSU-7	*A. capillaris*	4.1
MSU-26	*A. stolonifera*	3.9
MSU-6	*A. capillaris*	3.7
Penncross	*A. palustris*	3.5
MSU-2	*A. canina*	3.4
MSU-4	*A. capillaris*	2.9
MSU-14	*A. castellana*	2.9
MSU-36	*A. trinii*	2.9
MSU-12	*A. castellana*	2.8
MSU-13	*A. castellana*	2.8
MSU-17	*A. castellana*	2.8
MSU-32	*A. mongolica*	2.8
MSU-15	*A. castellana*	2.6
MSU-16	*A. castellana*	2.6
MSU-22	*A. palustris*	2.5
MSU-20	*A. palustris*	2.4
MSU-3	*A. capillaris*	2.1
MSU-21	*A. palustris*	2.1
MSU-30	*A. lachnantha*	2.1
MSU-18	*A. castellana*	2.0
MSU-37	*A. vinealis*	2.0
MSU-40	*A. gigantea*	1.9
MSU-23	*A. palustris*	1.7
MSU-38	*A. transcaspica*	1.7
MSU-24	*A. stolonifera*	1.6
MSU-39	*A. gigantea*	1.6
MSU-19	*A. castellana*	1.5
MSU-25	*A. stolonifera*	1.5
MSU-29	*A. stolonifera*	1.5
MSU-1	*A. canina*	1.4
MSU-11	*A. castellana*	1.2
MSU-27	*A. stolonifera*	1.1
MSU-33	*A. hygrometrica*	0.9
MSU-10	*A. castellana*	0.8
MSU-28	*A. stolonifera*	0.3
MSU-34	*A. munroana*	0.3
MSU-35	*A. scabra*	0.3
MSU-31	*A. lachnantha*	0.2
F test ($P<0.0001$)[3]		14.96**
LSD($P=0.05$)		1.0

1 Recovery rated on a scale of 0 (no recovery) to 10 (complete recovery).

2 Each score was taken independently by 2 raters and the mean for 25 replicates.

3 Ratings were converted into percentages as in 1=10% and 10=100%. Arcsin transformation on done on the percentage values and run using the Proc GLM option of the SAS system.

† Recovery means were found to be significantly different from 'Pencross'.

** Scores were found significantly different at α =0.05.

found to be significantly different from the susceptible check 'Penncross' (*A. palustris).* Colonial bentgrasses and *A. mongolica* have the same ploidy level (2n=4x, tetraploid) as creeping bentgrasses and may potentially be donors of snow mold resistance. However *A. mongolica* had very poor recovery. Only three accessions showed significant recovery after snow mold infection (Table 4.6.2). These three accessions were all colonial bentgrasses.

In summary, a suitable physiological screening technique was developed against gray snow mold (*T. incarnata*) that would enable rapid, reproducible and controlled determination of resistance. We selected 20 resistant creeping bentgrass genotypes from 890 samples from old Northern Michigan golfcourses. Original populations of bentgrasses in temperate North America were derived from highly heterogenous populations of South German bentgrass mixtures (Casler et al., 2003). Decades of natural selection must have eliminated many unadapted plants in favor of plants with pest resistance and stress tolerances needed to survive management and edaphic factors that define their local environment (Casler et al., 1996 and 2003). Natural variation existing in old golf courses has been useful as a foundation for several creeping bentgrass programs (Engelke et al., 1995; Hurley et al., 1994).

Current top commercial creeping bentgrasses, 'L-93', 'Penn A4', 'Penn G2', 'Penncross', 'Providence' and 'Emerald' were all found susceptible to gray snow mold. Among the PI accessions or 14 *Agrostis* species, some accessions of colonial bentgrasses (*A. capillaris*) appears to have the best resistance to snow mold. The 2001 National Bentgrass test sponsored by the USDA and National Turfgrass Federation findings showed that from the snow mold complex ratings of 26 bentgrass cultivars grown on a fairway or tee, only one cultivar of colonial bentgrass, SR 7100 was found to be resistant to snow mold. Future experiments need to compare the colonial bentgrass selections and re-check SR7100 resistance to gray snow mold, and or screen for resistance against other isolates of gray, speckled or pink snow molds.

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APPENDIX A

Potential for Detached-Leaf Assay for Gray Snow Mold Screening

Detached-leaf assay is a rapid, economical way to do pathogenicity tests and the procedure has been used to study many host-pathogen interaction systems in dicots (Dufresne, 2000); large leaf blade monocots such as rice and corn (Pitkin et al., 1999), but not on small leaf blade grasses. Unavailability of disease screening methods for gray snow mold, temperature sensitivity and slow-growing characteristics of the fungus (Table 3.2, Chapter III) led to initial skepticism whether detached-leaf assay could be use as an alternative to whole plant physiological screening against *T. incarnata*. To assess the feasibility of the procedure, six leaves each of susceptible bentgrass cultivars, 'Penncross' and 'L-93' and resistant bentgrass, 'MSU 20215' and 'MSU-8' were utilized. Three treatments were performed: a) no inoculation, b) inoculated at the leaf center (pathogen invasion through stomates) and c) inoculated at the base (pathogen invasion through wounded site). The leaves were swabbed with 10% sodium hypochlorite, rinsed 3x in sterilized dionized distilled water and placed on moistened sterile filter paper in petri dishes. On the petri dish cover, a single layer of sterilized cheesecloth was attached to hold moisture and for diffused lighting. Agar plugs (2 cm.diam.) with gray snow mold mycelia were applied at the center or base of the leaves. The plates were sealed with parafilm. The experiment was conducted in the cold room at 5 ^{o}C.

After 4 weeks, visual inspection showed that leaves of the resistant lines MSU 20215 and MSU-8 did not show any signs of yellow discoloration or infection despite low lighting, susceptible plants, 'L-93' and 'Penncross' inoculated at the center or at the site of wounding showed 40% to 90% symptoms of yellow discoloration in patches or whole leaf, and the leaves from resistant plants showed 'localized necrosis' at the site where the inoculum was applied (Table A.1). Since none of the control plants showed the symptoms of yellow patches, the experiments indicated that discoloration was associated with disease infection and not due to wounding or cold treatment. This finding is important as an additional support to the validity for a rating system to be used in the developed system for controlled screening against gray snow mold in bentgrasses. The advantage of the detached leaf assay would be an ability to initially screen large populations of genotypes using smaller resources for inoculum, less space for cold room and easier handling, sufficing the problem or need for large greenhouse spaces to maintain populations. Selected candidate resistant plants may then be further subjected to screening with replicates on whole plant basis to measure plant recovery as well.

The leaves from resistant plants manifested 'localized necrosis' or 'browning' indicative of oxidation of phenols, hypersensitive response (HR) or cell death (Goodman and Novacky, 1994; Hammerschmidt and Nicholson, 1999) at the site of inoculation that may probably restrict further invasion of the pathogen. More investigation through cytological analysis is needed to show that mycelial growth was inhibited in resistant plants, as HR may only be a single part of the defense strategy. Efficiency of the detached leaf assay should be compared with the controlled whole plant screening method on a wider scale. Future experiments may also contrast the differences if any, in basic leaf resistance and whole plant resistance.

Table A. 1. Disease reactions to gray snow mold using detached leaf assay 30 days after inoculation depicted by presence (+), absence (-) and mean percentage disease symptoms per leaf .

Treatments & Plant Material	Disease Reactions		
	Yellowing (Mean % /leaf)	Lesion(s) expansion	Localized necrosis
A. Uninoculated	-	-	-
B. Center inoculated			
Susceptible 'Penncross'	+ (70%)	+	-
Susceptible 'L-93'	+ (60%)	+	-
Resistant 'MSU20015'	+ (5%)	-	+
Resistant 'PI-8'	-	-	+
C. Wound inoculated			
Susceptible 'Penncross'	+ (80%)	+	-
Susceptible 'L-93'	+ (70%)	+	-
Resistant 'MSU20015'	-	-	+
Resistant 'PI-8'	-	-	+

APPENDIX B

Changes in Carbohydrate Levels in Bentgrass During Cold and Disease Treatments

The nature of gray snow mold resistance is still unknown. In general, genetics of resistance to gray snow mold in winter wheat appears to be probably polygenic and non-specific (Gaudet and Larouche, 1999). In winter barley, resistance may possibly be controlled by a single major gene (Cavelier, 1989). Previous studies implying the polygenic nature of disease resistance to snow mold in winter wheat (*Triticum aestivum* L) report the involvement of A) pathogenesis related (PR) proteins such as chitinase, B-1,3-glucanase, peroxidase, gamma-thionin, and a lipid transfer protein (Gaudet et al., 2000); B) changes in osmotic potential during cold hardening (Gaudet et al., 2001) and C) changes in the form and quantity of carbohydrates that may directly affect fungal development within the plant (Gaudet et al., 1999). Resistant winter wheat cultivars possessed higher levels and more highly polymerized fructans than susceptible cultivars. Nakajima and Abe (1994) suggested that depletion of carbohydrate reserves, as affected by duration of snow cover is a major determining factor in snow mold resistance. Yoshida et al (1998) similarly found that snow mold resistant wheat cultivars tended to metabolize carbohydrates more slowly, suggesting that the enzymatic metabolism of carbohydrates to cryoprotective sugars differed between the two contrasting types of resistance during winter stress.

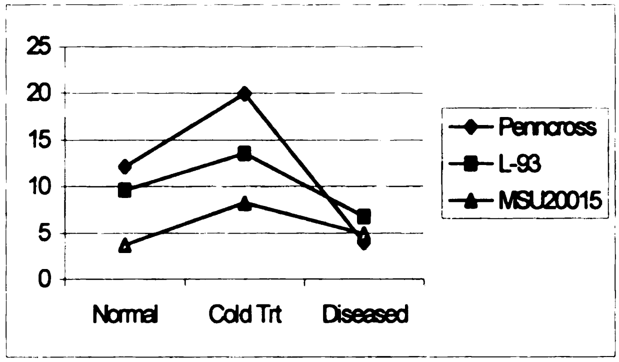
Disease resistance screening for gray snow mold in bentgrasses from my preceding work showed a wide range of resistance, that was probably under polygenic control, and the mechanism for leaf and crown resistance may also be different and independent as suggested by the differences in recovery reactions.

As a preliminary study to evaluate the changes in total non-structural carbohydrate (TNC) levels in snow mold resistant and susceptible creeping bentgrasses and to see whether TNC differences existed between the leaf and crown, susceptible cultivars ‘Penncross’ and ‘L-93’ and resistant line ‘MSU 20215’ were used for the study. Leaf samples for carbohydrate analysis were collected 5 cm. above the base of the plant and crown samples were cut 2 cm. above the soil base. The leaf and crown tissues were rinsed in deionized distilled water 3x and stored in –80°C before freeze-drying. Three different treatments were conducted as follows: A) no treatment or control B) 3 days cold treatment and C) gray snow mold infection for 4 weeks. Plant tissues were prepared for analysis following the procedures of Smith (1981). Freeze-drying was done at –80°C for 90 minutes for the crowns and 60 minutes for the leaves. Samples were ground by hand until powder-like and placed in 50 ml flasks and re-dried at 70°C for another 30 minutes before sealing bottles. Samples were sent to University of Missouri for TNC analysis and analyzed following Smith (1981) procedure developed at the University of Wisconsin-Madison.

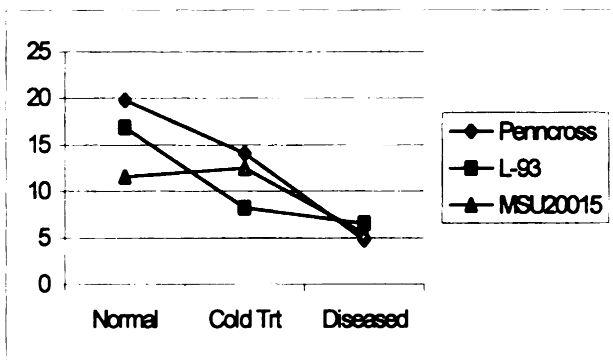
The results generally indicated an increase in TNC levels after 3 days of cold treatment in the leaves for both resistant and susceptible plants (Table A.2, Figure A.1). The percentage increase in TNC in leaves after cold treatment ranged from 41.12% (L-93), 64.7% (Penncross), and 121% in the resistant plant (MSU20215). In the crowns, the

Table A.2. Changes in total non-structural carbohydrate (TNC) levels in bentgrass following 3 days of cold treatment or four weeks of disease inoculation.

Tissue & Plant Material	TNC levels				
	Control	Cold	% Difference	Disease	% Difference
Leaves:					
Penncross	12.1	19.93	64.7 %	3.94	-80.2 %
L-93	9.59	13.54	41.1 %	6.72	-50.3 %
MSU20015 (R)	3.71	8.21	121.3 %	4.87	-40.7 %
Crown:					
Penncross	19.76	14.06	- 28.8 %	4.74	-66.3 %
L-93	16.81	8.18	-51.3 %	6.5	-20.5 %
MSU20015	11.57	12.48	7.8 %	5.39	-56.8 %



LEAVES



CROWN

Figure A. 1. Changes in the total non-structural carbohydrate levels in leaves and crown.

TNC levels decreased by 28.8% (Penncross) and 51.13% (L-93) in the two susceptible lines, while TNC increased by 7.8% in the resistant plant (MSU20215). The data are preliminary but indicate that there may be an association of increased TNC with cold acclimation and higher TNC in resistant creeping bentgrass. After 4 weeks of gray snow mold infection, TNC levels in the leaves dropped for both resistant and susceptible plants. The percentage decrease in TNC were 80.2% (Penncross), 50.3% (L-93) and lowest at 40.7% (MSU20215). The percentage decreases of TNC levels in the crowns were 66.3% (Penncross), 20.5% (L-93) and 56.8% (MSU20215). More studies should be conducted to explain the TNC differences in the crown at this point and whether TNC levels at the crown could be associated with snow mold resistance.

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