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 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

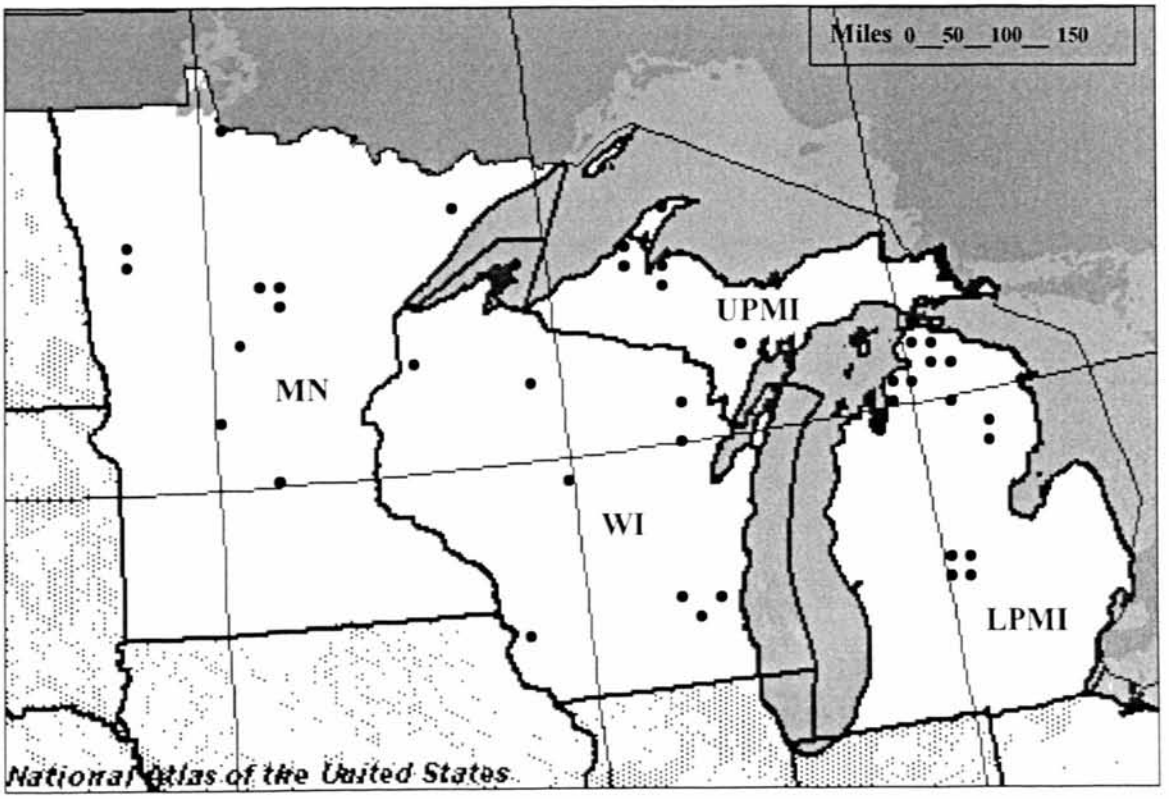

Figure 3.1. Collection sites of gray snow mold clustered in 4 populations based on geographic locations: Minnesota (MN); Wisconsin (WI); Lower Peninsula, Michigan (LPMI) and Upper Peninsula, Michigan (UPMI).

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.

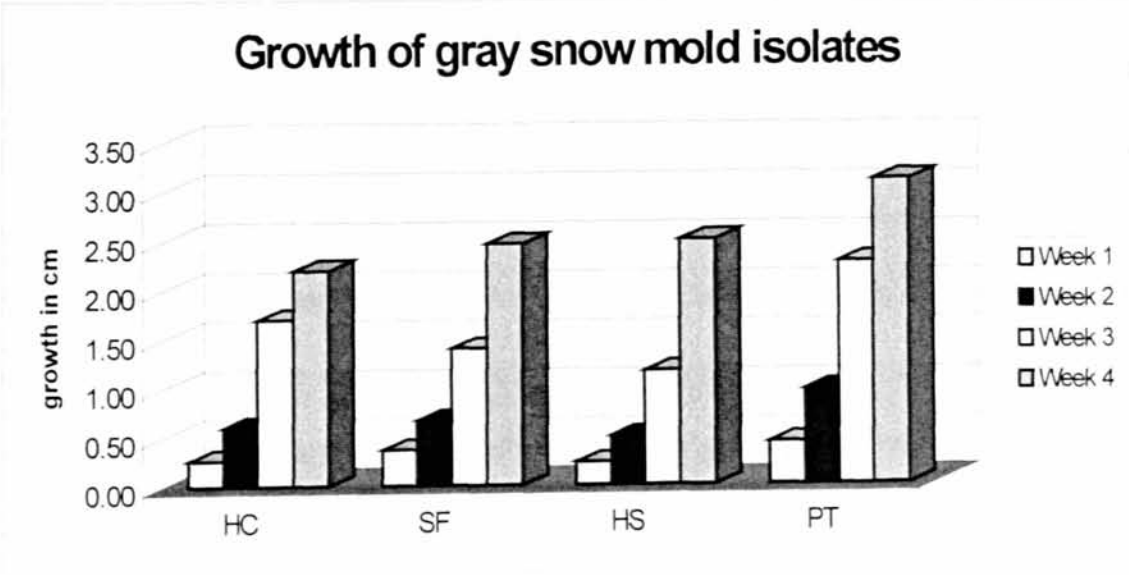

Figure 3.2 Radial growth in four weeks of different gray snow mold isolates *in vitro* from Michigan. ***P*** values < 0.05 as follows: Week 1 = 0.09; Week 2 = 0.02; Week 3 = 0.02 and Week 4 = 0.09.

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
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..																				

**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

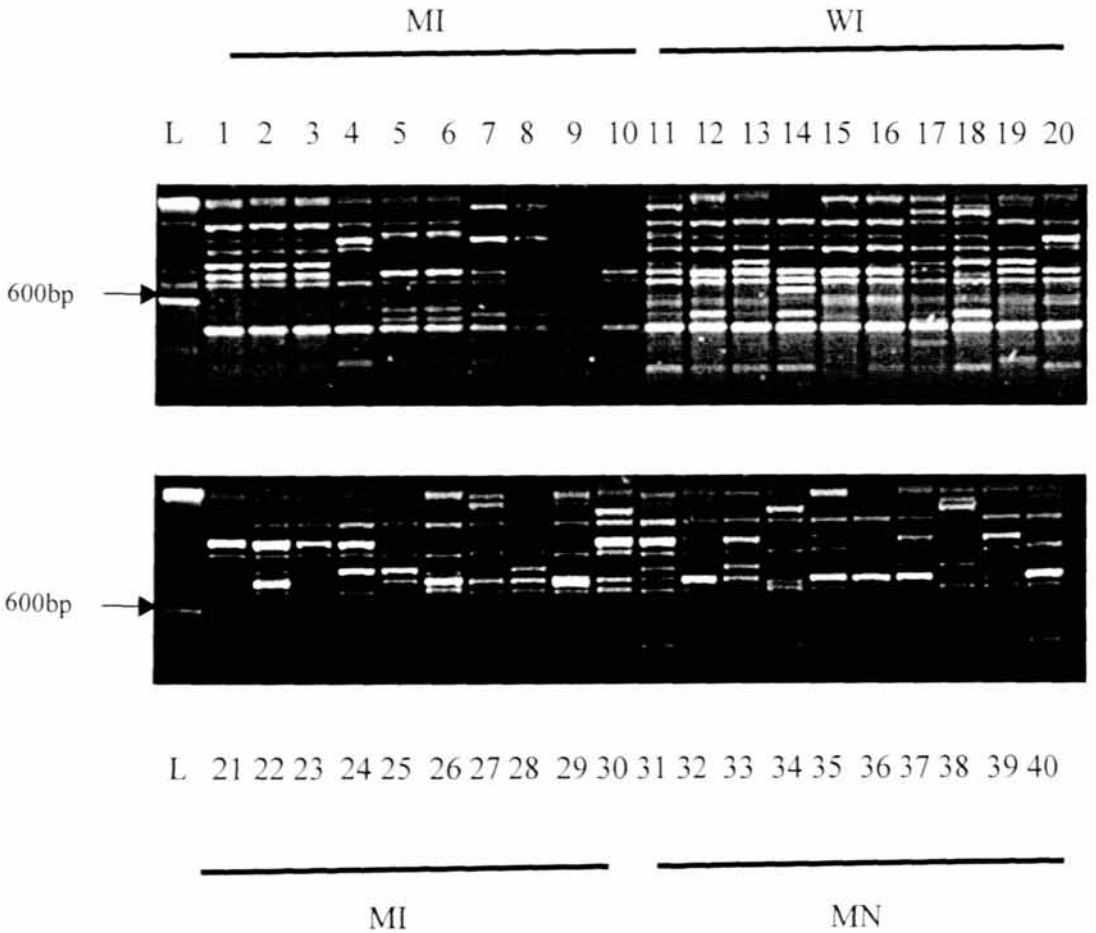

Figure 3.3. RAPD profile of 40 gray snow mold isolates from Michigan (MI), Wisconsin (WI) and Minnesota (MN) using primer OPY-02.(L=100bp DNA ladder). Lane numbers refer to isolated listed in Table 3.1.

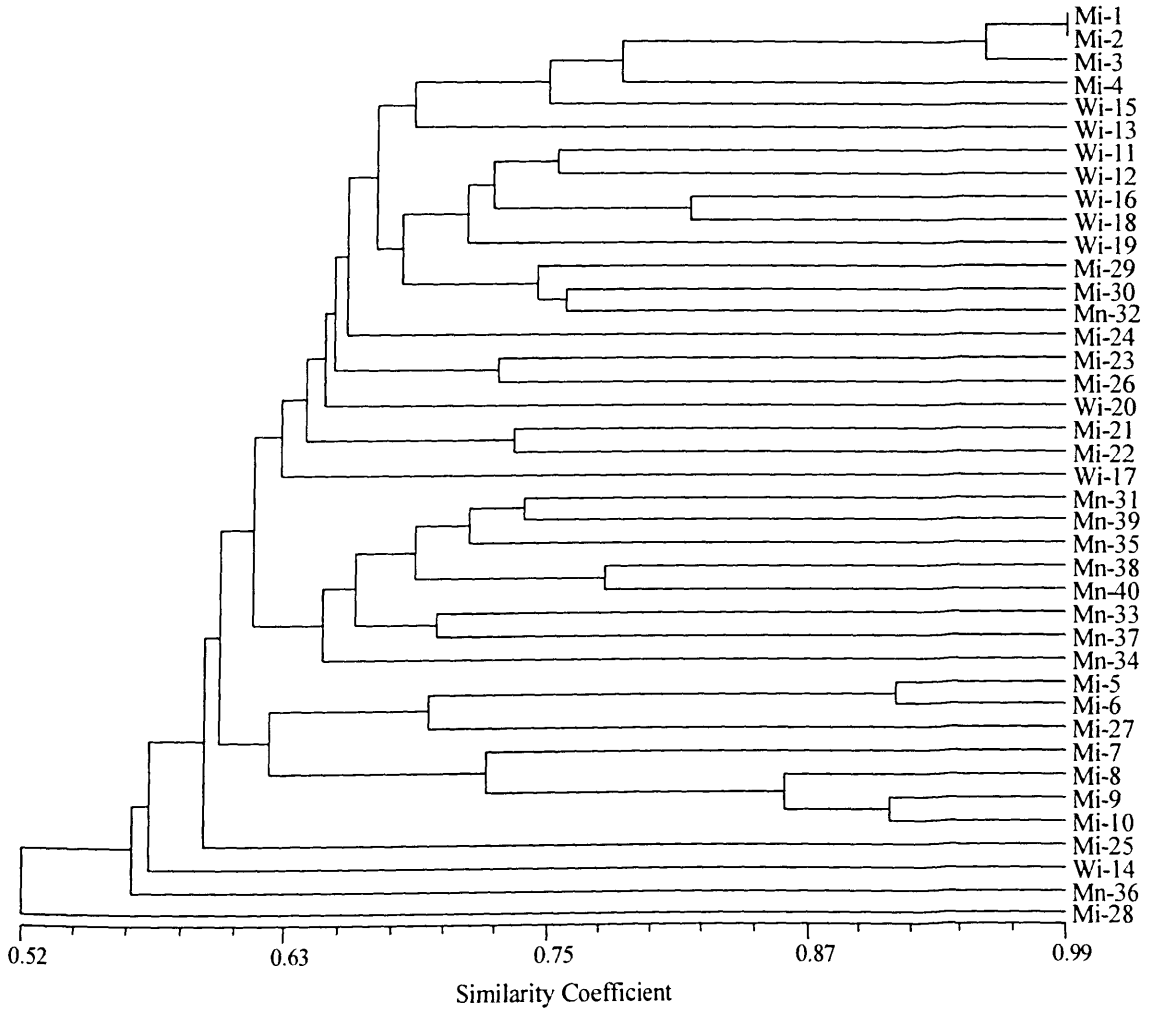

Figure 3.4.1. UPGMA dendrogram of 40 gray snow mold isolates from different locations in Michigan (MI), Wisconsin (WI) and Minnesota (MN), USA based on 115 RAPD markers generated using Dice coefficients, NTSYS program (Rohlf, 2000).

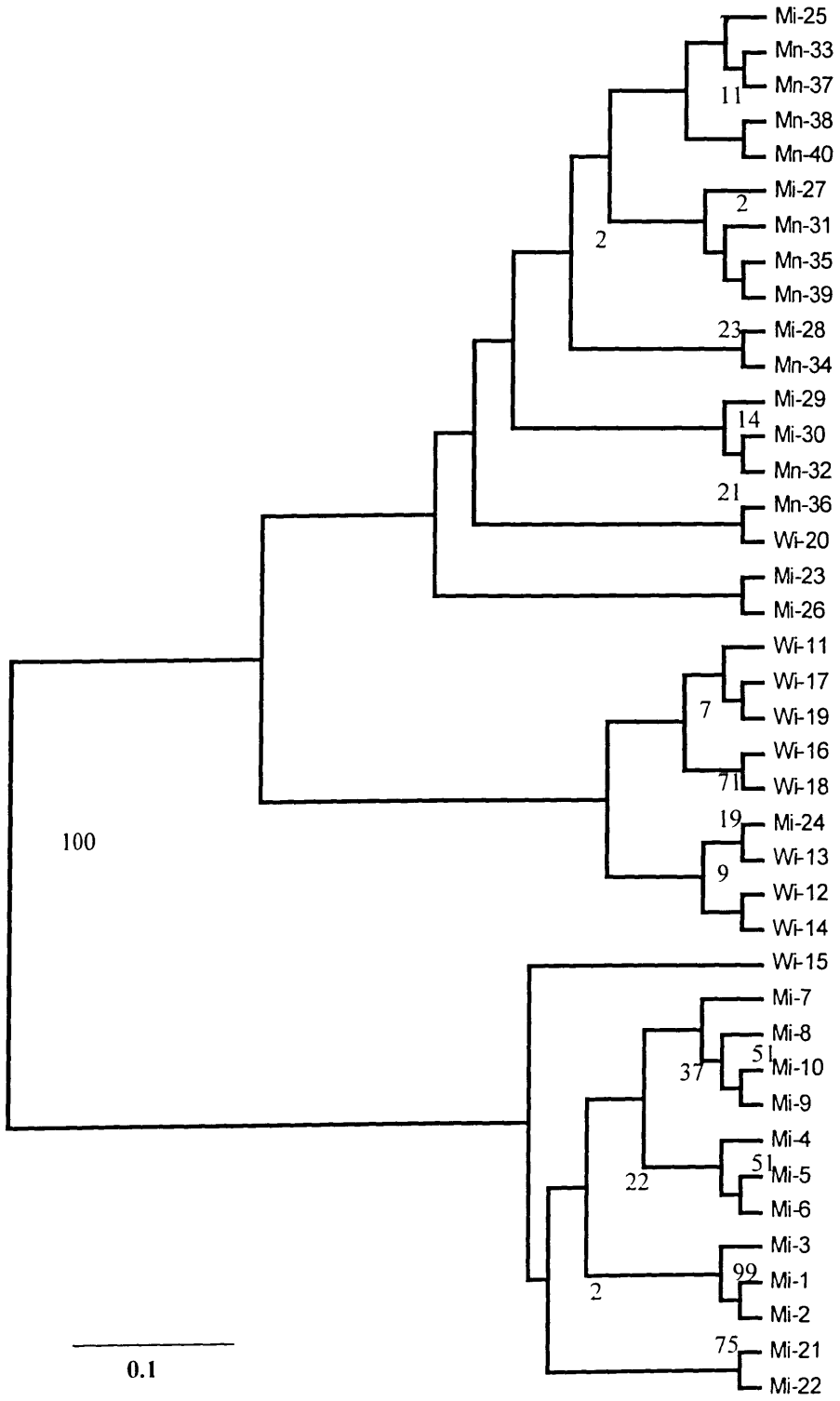

Figure 3.4.2. Dendrogram of 40 gray snow mold isolates based on 115 RAPD markers using Nei-Li genetic distance, neighbor joining (NJ) method and resampling options of FreeTree (Pavlicek et al., 1999) and TreeView (Page, 2001) programs. Numbers beside node indicate relative bootstrap frequencies.

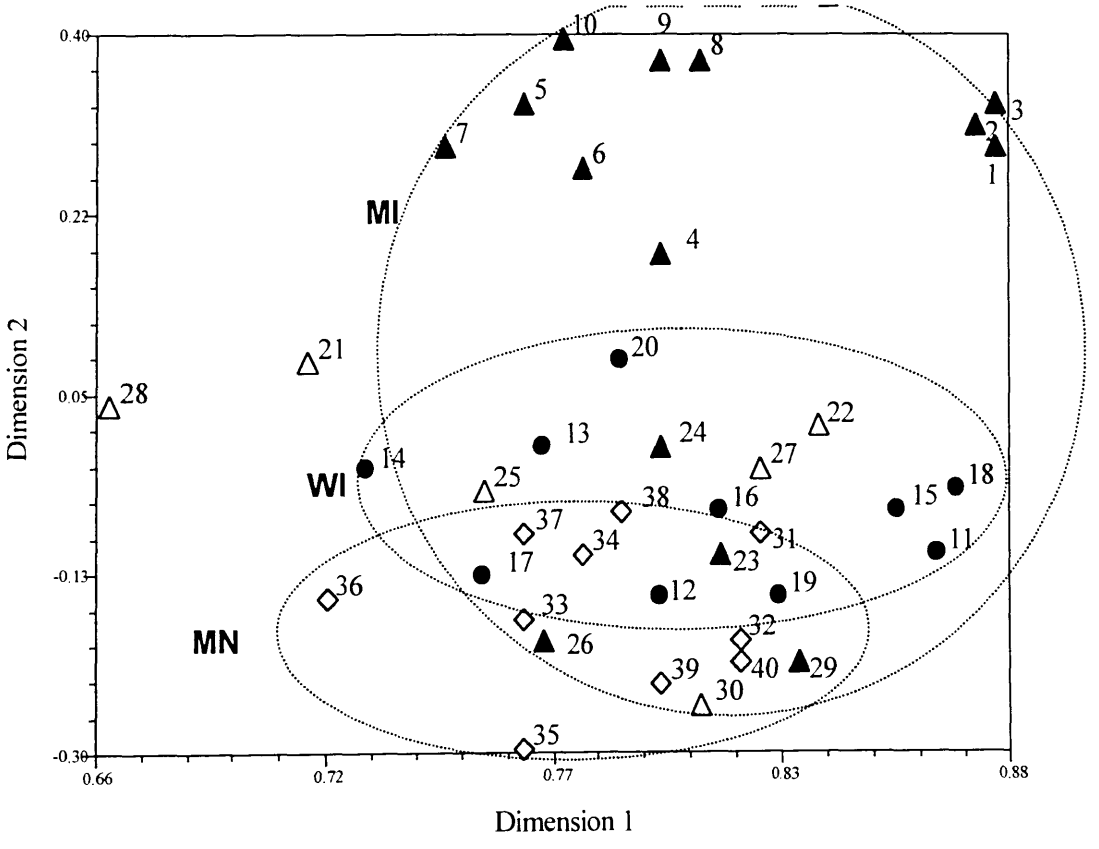

Figure 3.5. Scatterplot of the first two dimensional scales for 40 gray snow mold isolates based on principle component analysis of RAPD profile. Number correspond to isolate as listed in Table 3.1.

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 ± 0.11
MN	0.17	0.00			0.26 ± 0.14
UMI	0.03	0.06	0.00		0.34 ± 0.20
WI	0.13	0.19	0.10	0.00	0.30 ± 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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