Genetic Diversity for Pink Snow Mold Resistance in Greens-Type Annual Bluegrass

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ABSTRACT

Unseeded annual bluegrass (*Poa annua* L.) is an important component of golf greens in many regions of Canada and the United States. Although this turfgrass species has desirable playing attributes, it suffers from susceptibility to environmental and biological stresses including subfreezing temperatures and snow molds. In this study, we compared 29 genotypes collected from golf greens located in Québec and Ontario for their resistance to pink snow mold (SM). Plants were inoculated with *Microdochium nivale* [(Fries) Samuels & Hallett], causal agent of SM, and incubated under controlled conditions. High levels of variation in SM resistance were detected within the collection and between genotypes. Analysis of the relationship between climatic parameters at the sites of origin and SM susceptibility revealed that level of resistance was positively correlated to the duration of snow cover. Genetic diversity within the *Poa* collection was estimated using the sequence related amplified polymorphism (SRAP) technique. The UPGMA (unweighted-pair group method arithmetic average) dendrogram yielded two main clusters that differed markedly in their proportion of SM-resistant genotypes. Our results show that SM disease is a major selection pressure for the generation of genetic diversity among annual bluegrass biotypes that evolved on golf greens in northern climates. SRAP polymorphisms between bulked genotypes with contrasting resistance to SM were identified and could be used as markers for SM resistance in annual bluegrass.

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Abbreviations: SM, snow mold; SRAP, sequence related amplified polymorphism; UPGMA, unweighted-pair group method arithmetic average; BMP, best management practices.

The primary turf species used for golf course putting green establishment in Canada and northern United States is creeping bentgrass (*Agrostis palustris* Huds.). However, unseeded annual bluegrass (*Poa annua* L.) thrives under intensive management and gains a competitive edge against creeping bentgrass until it ultimately dominates many golf green putting surfaces (Huff 1996).

Poa annua is an allotetraploid ($2n = 4x = 28$) most likely derived from the hybridization of two diploid species, *P. infirma* Kunth and *P. supina* Schrader (Tutin, 1957). *Poa annua* has evolved as a continuum of life type-cycles from a fully annual biotype (*P. annua* var. *annua)* to the perennial type [*Poa annua* var. *reptans* (Hauskn.) Timm.] found on putting greens (Beard et al., 1978). Selection pressure provided by intensive management on golf greens has resulted in the development of high quality perennial genotypes of annual bluegrass with desirable morphological and aesthetic attributes (Huff, 1999). These have high tiller density and dark color, are well adapted to extremely low height of cuts and are competitive under shady, cool, and humid conditions.

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In spite of these attributes, annual bluegrass has been historically considered as a weed rather than as a usable turf-type grass with phenotypic variability reflecting potential sources of useful genes. However, recent efforts have been undertaken to develop commercial sources of greens-type annual bluegrass suitable for new putting green construction, overseeding, or damage repair (Huff, 1999; Kind, 1997). Such breeding programs need to consider adaptation to environmental conditions in cultivar development to ensure the reliability and long term survival of the seeded material.

One major weakness of greens-type annual bluegrass is its susceptibility to environmental stresses (Beard et al., 1978; Tompkins et al., 2004). Winter damage to putting greens is a major problem for golf courses in cold climates. A combination of impermeable covers with insulating materials provides protection of annual bluegrass golf greens against subfreezing temperatures during winter (Dionne et al., 1999). However, cool and humid conditions along with atmospheric modifications can occur under these long lasting insulating covers (Rochette et al., 2006), which promote dehardening and the development of low temperature diseases that can severely affect winter survival.

Snow molds are the most prevalent and destructive winter diseases affecting cool-season turf in northern locations in the northern United States and Canada (Nelson, 2004). Snow mold diseases often result in extensive damage to golf greens that significantly decrease the quality of putting surfaces for many weeks in the spring and cause major economic losses associated with green repairs and lost revenues. The psychrophilic fungus *Microdochium nivale* (Fries) Samuels & Hallett is the most widespread snow mold pathogen, and it causes pink snow mold (SM) on turf and forage grasses (Tronsmo et al., 2001). While gray snow mold diseases caused by *Typhula* species are especially destructive where snow cover lasts over 3 mo (Hsiang et al., 1999), pink snow mold can cause severe circular patches with just a month of snow cover, or smaller irregular patches in the absence of snow cover (Smiley et al., 2005). *Microdochium nivale* is also favored by high humidity and ambient temperatures between 0 and 7°C (Tani and Beard, 1997) that occur underneath persistent snow covers or impermeable tarps applied onto golf greens as protection against freezing damage.

Alternatives to current preventive applications of fungicides in the fall to control SM are required to implement low pesticide maintenance programs. The development of seed sources more resistant to freezing temperatures and SM is one of the most effective and sustainable approaches to improve winter survival and quality of spring regrowth of turfgrass in a low input system.

Inoculation with *Microdochium nivale* has revealed significant genetic variation in SM resistance in both forage grasses and winter rye (Tronsmo, 1992). Recent breeding advances have demonstrated that genetic variation exists within creeping bentgrass for a range of pest resistances and stress tolerances. Twenty clones with superior multiple pest resistances, including SM diseases, were identified for use in breeding new bentgrass varieties (Casler et al., 2006). In two grass species, orchardgrass (*Dactylis glomerata* L.) and winter rye (*Secale cereale* L.), the broad sense heritability of resistance to *M. nivale* was reported to be 0.79 and 0.49 respectively (Miedaner et al., 1992; Tronsmo et al., 2001), which indicates that improvement of SM resistance through selection is a valuable approach that could be extended to annual bluegrass.

Perennial genotypes of annual bluegrass are uniquely adapted to the management and environmental conditions to which they are exposed, and constitute a unique source of genes for turf quality improvement. Thus, analysis of genetic diversity in annual bluegrass has important implications in the understanding of the evolution of its genome as well as for the management and improvement of this important component of golf greens. For instance, studies have indicated that biotic and abiotic stresses are among the major determinants of evolutionary changes in a plant genome (Nevo, 2001).

Genetic relatedness between populations and genotypes can be estimated by using molecular markers to detect polymorphisms in genomic DNA. Several PCR-based methodologies that can generate large numbers of markers have been developed in recent years (Weising et al., 2005). Sequence related amplified polymorphism (SRAP) is a PCR-based marker system that preferentially targets coding sequences randomly distributed throughout the genome (Li and Quiros, 2001). Forward and reverse primers that respectively allow preferential amplification of exonic and intronic regions uncover polymorphic sequences resulting from variations in the length of introns, promoters, and spacers among genotypes or populations. SRAP, which has been shown to be effective in gene tagging in several species, is highly reproducible and comparatively less expensive to develop than other types of markers (Cravero et al., 2007). SRAPs have been successfully applied to study genetic relationships in a wide range of turfgrass species (Budak et al., 2004a) and to analyze the diversity among naturally occurring genotypes of buffalograss [*Buchloe dactyloides* (Nutt.) Englem.] (Budak et al., 2004b).

Snow mold is a major cause of winter mortality in annual bluegrass. Determination of the extent of genetic variability for SM resistance within that species and the identification of resistant genotypes are necessary steps toward the development of improved cultivars. The objectives of this project were to: (i) develop a screening procedure for SM resistance in annual bluegrass; (ii) identify genotypes of greens-type annual bluegrass with contrasting levels of resistance to SM, caused by *Microdochium*

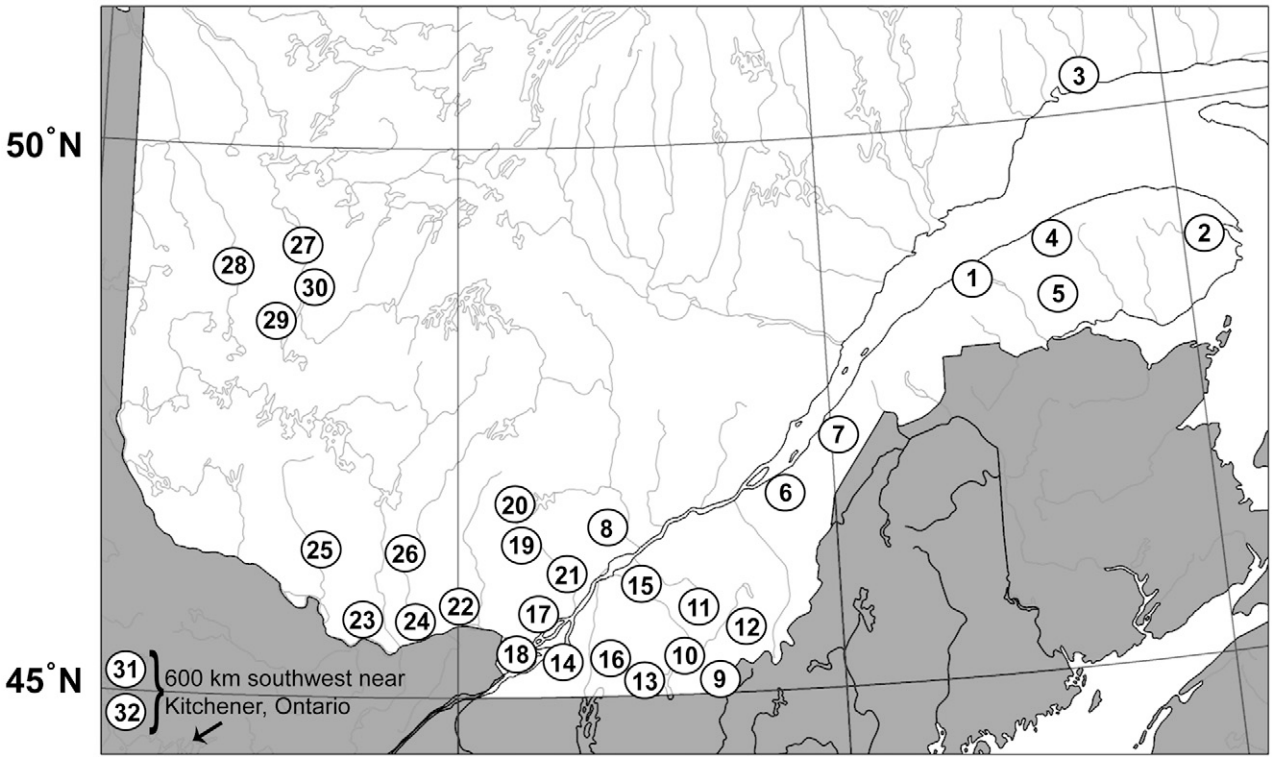

Figure 1. Location of golf courses in the province of Québec where greens-type genotypes of annual bluegrass were collected, plus two samples from Ontario.

nivale; (iii) identify geoclimatic factors linked with the level of resistance of the genotypes and; (iv) analyze the genetic diversity in resistance to SM among greens-type annual bluegrass genotypes collected across eastern Canada. This information will be used to assist the development of seed sources more broadly adapted to winter stresses. This project will also contribute to the development of best management practices (BMP) to optimize winter survival of turfgrass while reducing pesticide use.

MATERIALS AND METHODS

Plant Material

Thirty-two genotypes of greens-type annual bluegrass (*Poa annua* var. *reptans* [Hauskn.] Timm.) from different geoclimatic environments were collected on 30 greens located on different golf courses across Québec (45°N to 50°N, 65°W to 80°W) and two located on the same golf course in southern Ontario (Fig. 1, Table 1). On each green, a single 5-cm diameter core was collected in a zone covered with annual bluegrass. Genotypes were maintained and vegetatively propagated at the Agriculture and Agri-Food Canada Research Centre, Québec. Tillers of each of the 32 genotypes of greens-type annual bluegrass were transplanted individually in a 164 mL-volume Cone-tainer (SC10 super cell low density, Stuewe and Sons, Inc., Corvallis, OR, U.S.) filled with sand amended with 20% peat (Topdress with peat for greens #02–01, Sols Champlain, Mascouche, QC, Canada) and placed in IPL Rigipots trays (IP110, Stuewe and Sons, Inc., Corvallis, OR, U.S.). Plants were grown for 4 wk under the following environmentally-controlled conditions: photoperiod, 16 h; day-time temperature, 22°C; and night-time temperature, 17°C. Artificial lighting was provided by a mixture of high pressure sodium and metal halide 400 W lamps (PL Light Systems, Beamsville, ON, Canada) with a photosynthetic photon flux density of 600 to 800 μmol photons m^{-2} s^{-1}. Plants were watered daily and fertilized once a week with 20–20–20 + micronutriments (1 g L^{-1}).

Screening for Pink Snow Mold Resistance

Snow mold resistance was assessed for 29 genotypes that were amenable to propagation and that maintained vigorous growth under environmentally-controlled conditions. The steps of the screening method for SM resistance are summarized in Table 2 and detailed as follows. Step 1: Annual bluegrass plants were incubated for 2 wk in a growth chamber set to 2°C and 250 μmol photons m^{-2} s^{-1} photosynthetic photon flux density under 8 h photoperiod for acclimation to low temperatures; Step 2: To achieve the second stage of cold acclimation of greens-type annual bluegrass (Dionne et al., 2001), plants were transferred to a programmable freezer set at –2°C for an additional period of 2 wk in the dark; Step 3: Half of the plants were inoculated at 0.01 g cm^{-2} with a mixture of four isolates of *Microdochium nivale* grown for 2 wk on wheat bran, dried, ground, and kept at –20°C. The four isolates of *M. nivale* var. *nivale* were of diverse host origin (one from British Colmbia *P. annua*, two from Ontario *P. pratensis*, and one from Ontario *A. stolonifera*). The inoculum had been grown on wheat bran for 3 wk, consisted of hyphal-infested dried and ground wheat bran. Autoclaved inoculum was similarly applied to control plants. Control and inoculated plants were incubated 7 wk at 2°C and 98% relative humidity. Percentage of humidity in boxes was monitored with humidity sensors (Traceable Humidity Temperature Pen, Control Company, Friendswood, TX, U.S.). To maintain a high

Table 1. Geographical origin of each genotype and of corresponding nearest weather station.

Genotype	Genotype origin		Nearest weather station	
	Latitude	Longitude	Latitude	Longitude
3	50°11′ N	66°38′ W	50°13′ N	66° 16′ W
4	49°8′ N	66°30′ W	49° 7′ N	66° 28′ W
5	48°28′ N	67°26′ W	48° 31′ N	66° 27′ W
6	46°48′ N	71°11′ W	46° 49′ N	71° 6′ W
7	46°58′ N	69°47′ W	46° 58′ N	69° 46′ W
8	46°15′ N	72°57′ W	46° 16′ N	73° 1′ W
9	45°17′ N	71°58′ W	45° 22′ N	71° 49′ W
10	45°24′ N	71°54′ W	45° 25′ N	71° 40′ W
11	45°24′ N	71°54′ W	45° 25′ N	71° 40′ W
12	45°16′ N	71°54′ W	45° 22′ N	71° 49′ W
13	45°1′ N	72°6′ W	45° 1′ N	72° 6′ W
14	45°19′ N	73°16′ W	45° 19′ N	73° 15′ W
15	45°53′ N	72°29′ W	45° 52′ N	72° 28′ W
16	45°34′ N	73°12′ W	45° 37′ N	73° 7′ W
17	45°31′ N	73°39′ W	45° 25′ N	73° 55′ W
18	45°28′ N	74°9′ W	45° 30′ N	74° 4′ W
19	46°7′ N	74°29′ W	46° 7′ N	74° 28′ W
20	46°7′ N	74°36′ W	46° 7′ N	74° 28′ W
21	45°54′ N	74°08′ W	45° 58′ N	74° 0′ W
22	45°39′ N	74°56′ W	45° 42′ N	74° 55′ W
23	45°29′ N	75°39′ W	45° 22′ N	75° 43′ W
24	45°29′ N	75°39′ W	45° 22′ N	75° 43′ W
25	45°30′ N	75°47′ W	45° 31′ N	75° 46′ W
26	45°35′ N	75°25′ W	45° 31′ N	75° 46′ W
27	48°34′ N	78°7′ W	48° 34′ N	78° 08′ W
28	48°14′ N	79°1′ W	48° 3′ N	77° 47′ W
30	48°6′ N	77°47′ W	48° 3′ N	77° 47′ W
31	43°25′ N	80°28′ W	43° 27′ N	80° 22′ W
32	43°25′ N	80°28′ W	43° 27′ N	80° 22′ W

level of humidity, the trays containing annual bluegrass were covered with two layers of wet paper towels (Kimtuff Wiper, Kimberly-Clark, Dallas, TX, U.S.), sprayed as needed with distilled water and placed in covered plastic boxes. The end parts of the paper towels were kept soaking in water at the bottom of the boxes to maintain towels wet consistently by capillary transfer; Step 4: After 7 wk, the trays containing the plants were removed from the boxes and placed in a growth chamber for 1 wk of regrowth under step 1 conditions, and plant injury was evaluated. The extent of fungal growth was evaluated at the end of cold incubation and was not correlated with plant injury after a 1-wk regrowth, suggesting that some genotypes might have high genetic tolerance for foliar injury yet show strong recovery and regrowth from the crown.

Percentage of plant injury was assessed visually after 1 wk of regrowth using the Horsfall and Barratt visual scale (Couture, 1980). The 0 to 11 scale was expanded to accommodate one additional injury category at both ends of the scale, whereby 0.5 was used for injury percentage limits 0 to 1.5% and 10.5 for injury-free percentage limits of 0 to 1.5%. For data analysis, injury scores were transformed to their corresponding mid-percentage values. Mid-percentage values for the 0.5 and 10.5 scores were 0.96 and 99.04%, respectively.

To validate the method, two screenings were made sequentially. Twenty genotypes were assessed for their resistance to SM in the two screening tests with an additional nine genotypes screened only during the second one. The experiments were conducted using a completely randomized block design with 10 replicates, and the same mixture of isolates of *M. nivale* was used in both tests. The first screening included 400 Cone-tainers [20 genotypes × 2 treatments (inoculated and control) × 10 blocks], while the second screening, with 29 genotypes, included 580 Cone-tainers. Analysis of variance with nested factors using the MIXED procedure of SAS was applied to the percentage of injury of each genotype (SAS Institute, 1999). LS means comparisons were used to statistically compare the susceptibility of the genotypes to SM.

Geoclimatic Characterization of the Genotypes

To assess the relationship between winter conditions at the site of collection and genetic potential for SM resistance, we used records from the meteorological station nearest to each site of collection (Table 1). Several climatic parameters (30 yr record; 1971–2000) for the months of December, January, February, and March including days with snow cover, monthly average of daily maximum and minimum temperatures, total rain, and snow precipitation were selected. These climatic variables were analyzed with SM percentage of injury recorded in the second screening to estimate Pearson's correlation coefficients (SAS Institute, 1999).

DNA Extraction

Genomic DNA was extracted from 5 g of each genotype tested by the cetyltrimethylammonium bromide (CTAB) method of Rogers and Bendich (1988). DNA fragments were separated by electrophoresis at 5 V cm^{-1}, in a 0.8% agarose gel with 1x Tris Borate EDTA running buffer (pH 8.0). Ethidium bromide was incorporated into the gel at a concentration

Table 2. Screening method for pink snow mold resistance of annual bluegrass.

Step	Duration	Activity	Temperature	Relative humidity (day/night)	Photon flux density	Photoperiod
	wk		°C	%	μmol m^{-2} s^{-1}	h
1	2	First stage of cold acclimation	2	55/70	150	8
2	2	Second stage of cold acclimation	–2	65/65	0	0
3	7	Inoculation and incubation	2	98/98	0	0
4	1	Transfer to regrowth conditions	20	65/80	150	16
		Visual injury assessment				

of 1.0 μg mL^{-1} and DNA was quantified by visual assessment by comparison with the molecular weight marker II (Roche Diagnostics, Laval, QC, Canada). Gel images were captured on gel BioDoc-IT System (UVP, Upland, CA).

Primer Selection and DNA Amplification

A total of 24 SRAP primer pairs were selected in this assay, using seven forward primers and six reverse primers (Table 3). These primers were previously described by Li and Quiros (2001) and Vandemark et al. (2006). The SRAP primer pair combinations used were: Me4-R15, F9-R8, F11-R7, F13-R15, Me2-R14, Me4-em2, F9-em2, F11-em2, F10-R7, F8-R15, F8-R9, Me2-R9, F8-R7, F11-R15, F10-R14, F13-R14, F13-R9, F8-R14, F9-R7, F10-R15, Me4-R8, F13-em2, Me2-em2, F10-R9 (Table 3). DNA polymorphism analysis was conducted with the whole collection with the exception of Genotype 18 that was unavailable at the time DNA was extracted.

Polymerase chain reactions (PCR) were performed in 25 μL volumes containing 50 ng DNA, 0.2 μM of each primer, 200 μM each dNTP, 2.5 m*M* $MgCl_2$, 2.5 U Hotmaster Taq DNA polymerase (Eppendorf, Mississauga, ON, Canada), 2.5 μL 10X buffer and sterile distilled water. The thermal cycling profile for all reactions was 95°C for 3 min, followed by 5 cycles of 94°C for 1 min, 35°C for 1 min, 72°C for 1 min, 35 cycles of 94°C for 1 min, 50°C for 1 min, 72°C for 1 min, and a final extension at 72°C for 7 min. All PCR reactions were performed in an Eppendorf Master Cycler Gradient. Twenty microliters of the amplification products were separated by electrophoresis in a 2% (w/v) agarose gel with 1× TBE buffer, at 75 V for 3 h.

Genetic Similarity Analysis

Normalizations and analyses of SRAP amplification profiles were performed with Bionumerics software version 4.0 (Applied Maths, Kortrijik, Belgium). Genetic similarities were calculated for each pairwise comparison using Pearson's product moment correlation coefficient, taking both band position and intensity into account. Entire profiles of the 24 primer pair combinations were included in the analysis. The similarity matrix was used in an unweighted-pair group method arithmetic average (UPGMA) cluster analysis to generate a dendrogram of the genetic relationship among the genotypes. The cophenetic correlation coefficient (Sokal and Rohlf, 1962) was used as an estimate of the robustness of the pairwise distances in the dendrogram.

Identification of SRAP Polymorphisms Associated with Snow Mold Resistance

The SRAP technique was used for the identification of genetic markers of SM resistance using bulked samples of DNA from five genotypes each ranked according to their level of resistance in the resistant, intermediate, or susceptible groups. For this purpose, an equal amount of DNA (250 ng) extracted from each genotype was pooled within each group for bulk analysis.

Table 3. Sequence of forward and reverse SRAP primers used in this study.

Forward primers		Reverse primers	
F8	5′- GTA GCA CAA GCC GGA AT-3′	R7	5′- GAC ACC GTA CGA ATT TGC-3′
F9	5′- GTA GCA CAA GCC GGA CC-3′	R8	5′- GAC ACC GTA CGA ATT GAC-3′
F10	5′- GTA GCA CAA GCC GGA AG-3′	R9	5′- GAC ACC GTA CGA ATT TGA-3′
F11	5′- CGA ATC TTA GCC GGA TA-3′	R14	5′- CGC ACG TCC GTA ATT AAC-3′
F13	5′- CGA ATC TTA GCC GGC AC-3′	R15	5′- CGC ACG TCC GTA ATT CCA-3′
Me2	5′-TGA GTC CAA ACC GGA GC-3′	Em2	5′-GAC TGC GTA CGA ATT TGC-3′
Me4	5′-TGA GTC CAA ACC GGA CC-3′		

RESULTS

Screening Method and Selection of Genotypes of Contrasting SM Resistance

The results showed that our screening method conducted entirely under controlled conditions gave a reproducible and statistically sound assessment of the genetic potential for resistance to SM (Table 4). The method was sensitive enough to discriminate between levels of SM resistance within our collection of annual bluegrass with percentage of SM injury ranging from ≈20% in the more-resistant up to above 80% in the more-susceptible genotypes. Replicate screening available for a subset of 20 genotypes confirmed the reproducibility of the method as revealed by the significant correlation ($r = 0.57$, $p = 0.01$) between results of both tests (Fig. 2). Most genotypes showed consistent levels of resistance in the two screening assays (Fig. 3). Only a few genotypes (e.g., 11, 22, and 32) differed widely in the two screenings and would require further testing to assess their level of SM resistance accurately.

We used the average SM injury value of the two screening tests, when available, or the one of the second screening for the genotypes that were tested only once, to divide the collection into three broad classes of resistance to SM: resistant (genotypes with less than 35% injury: 5, 14, 19, 20, 27, 30), intermediate (genotypes with injury ranging from 35 to 50%: 3, 6, 7, 8, 9, 15, 17, 18, 21, 22, 28, and 32,), and susceptible (genotypes with 50% injury and more: 4, 10, 11, 12, 13, 16, 23, 24, 25, 26, and 31) (Table 4). This classification was subsequently used in the analysis of the genetic relationship within the annual bluegrass collection.

Geoclimatic Factors and Snow Mold Resistance

Our results show that the relative susceptibility of the genotypes to SM was negatively correlated with the number of days with snow cover during the winter (Table 5) revealing greater SM resistance in genotypes that evolved in regions experiencing long lasting snow cover. Conversely, percentage of SM injury was positively correlated with the maximum average daily maximum temperature in winter months, confirming that genotypes exposed to conditions prone to the development of SM are more likely to be resistant to SM. Except for the minimum daily

Table 4. Percentage of injury assessed using a Horsfall-Barrat modified scale after 1 wk regrowth following incubation at 2°C under 98% humidity with *Microdochium nivale* for both screening tests.

Screening 1			Screening 2		
Genotype no.	% injury	$P = 0.05$	Genotype no.	% injury	$P = 0.05$
30	20.9	a	27	17.5	a
20	21.4	a	20	18.9	ab
19	27.0	ab	19	21.5	abc
14	34.8	bc	32	23.1	abcd
5	41.2	cd	5	23.9	abcde
7	47.5	cde	7	24.0	abcde
15	50.4	def	15	28.2	bcdef
9	50.6	def	22	30.1	cdefg
23	51.3	def	30	30.7	cdefg
16	51.5	def	11	32.7	cdefgh
3	54.8	defg	14	33.5	defgh
17	56.8	defg	3	34.1	defghi
32	57.8	efgh	8	35.7	efghij
22	61.0	efgh	6	40.5	fghijk
4	69.1	ghi	9	41.4	ghijk
31	70.9	ghi	17	41.5	ghijk
26	71.1	ghi	18	45.8	hijkl
25	72.0	ghi	31	46.7	hijkl
11	85.0	i	21	48.1	ijkl
12	85.3	i	25	48.8	jkl
			28	49.3	jklm
			12	54.1	klmn
			4	54.1	klmn
			16	59.4	lmn
			23	59.7	lmn
			26	60.9	lmn
			13	66.3	mn
			24	68.6	no
			10	87.8	o

temperature in December, the correlation coefficients associated with the other winter climatic factors tested were not significantly different.

Genetic Analysis of Annual Bluegrass Genotypes

Gel images of the SRAP profiles revealed the occurrence of many DNA polymorphisms between the genotypes (Fig. 4). Data from a total of 24 primer set combinations was used to generate a dendrogram of genetic similarity between the genotypes (Fig. 5). Genetic similarity among the 31 genotypes was high with values ranging from 0.80 to 0.93 (Fig. 5). All annual bluegrass genotypes were successfully discriminated by the SRAP markers and were grouped into two major clusters (A and B) with a mean similarity of 0.86. There was a higher frequency of genotypes resistant to SM (4 out of 7) in cluster A than in cluster B (2 out of 24). It is also noteworthy that no susceptible genotype was found in cluster A. Two ecotypes from the same golf course in Southern Ontario along with a genotype from Western Québec were sufficiently distinct from the other genotypes within cluster B to form a sub-cluster (Group III). Regrouping genotypes into robust subclusters reflected in many cases the proximity of their sites of provenance (e.g., Genotypes 23, 25, and 26 or Genotypes 28, 27, and 30). As expected, the highest genetic similarity between two ecotypes occurred with ecotypes 31 and 32 that were collected on two separate greens from the same golf course. High levels of genetic similarity were also found in some genotypes from sites far apart (e.g., Genotypes 7 and 11, Fig. 1).

Identification of SRAP Polymorphisms Associated with Snow Mold Resistance

Bulk samples were used to identify SRAP polymorphisms that occur at different frequencies in the groups of genotypes of contrasting resistance to SM (Fig. 6). There was a clear intensification and/or disappearance of several fragments between bulks of more susceptible and more resistant genotypes. Bulked genotypes with intermediate resistance yielded profiles that resemble those obtained with bulks of more susceptible or more resistant genotypes depending on the primer set combination. For instance, with the primer set F8-R15, four DNA fragments (a, b, d, e) that were undetectable in bulks of SM-susceptible genotypes clearly intensified in bulks regrouping genotypes with intermediate and superior resistance. However, fragment c from the same amplification was detected in bulks of the more resistant genotypes only. Conversely, we noted the disappearance of three fragments (f, g, and h) with the primer set combination F9-R8 in bulks of the more resistant genotypes. Band i obtained with primer set F9-R14 progressively intensified from bulks of more-susceptible to bulks of the more-resistant genotypes. These polymorphic differences obtained with bulks samples indicated that SM resistance is linked to discrete differences in genomic DNA that are shared between genotypes within each group.

DISCUSSION

Annual bluegrass has long been considered an unwanted weed invading golf courses. However, when intensively managed, its playing qualities are equal to or better than those of planted species (Johnson et al., 1993). Its susceptibility to SM leading to poor winter survival is however a disincentive to the development of improved cultivars of this species. Pink SM is the most prevalent and destructive winter disease on cool-season turfgrass in the northern Canada and United States, particularly where snow cover persists for a long period (Johnston and Golob, 2004). The current method of control relies on preventive fall application

of expensive fungicides before greens covers or the establishment of an insulating snow cover.

Genetic variability in resistance toward SM diseases has been shown in many grasses species (Casler et al., 2006; Tronsmo, 1992; Zhao et al., 2005) and could be used to identify genotypes more broadly adapted to winter stresses. Field selection for resistance to low temperature fungi is generally difficult due to the variable nature of disease development and the complex environmental conditions that occur in the field. Environmental interactions often cause erratic SM development in the field and make the interpretation of screening trials difficult. Selection under controlled environment is frequently used to increase the accuracy of the screening but the selection procedures have to be standardized (Nakajima and Abe, 1990).

To validate our screening method under controlled conditions, two screenings were made sequentially, and these confirmed the method to be reliable and reproducible. Our screening method was also very sensitive and allowed resolution of different levels of SM resistance of more than 20 genotypes of annual bluegrass simultaneously. Our results showed that although annual bluegrass is considered very susceptible to SM, genotypes resistant to SM can be found and identified. With the exception of creeping bentgrass (Vergara and Bughrara, 2003), most research efforts on SM resistance has thus far been limited to the improvement of resistance in cereal crops (Iriki et al., 2001). Our results indicate that similar improvement could be successfully achieved in annual bluegrass.

Molecular marker approaches for the classification of genotypes are abundant and have been previously applied to several turfgrass species (Budak et al., 2004b; Posselt et al., 2006; Vergara and Bughrara, 2003). DNA markers are unaffected by the environment and thereby can be associated with agronomic traits to develop marker-assisted approaches in breeding programs. Various molecular techniques, including RAPD, ISSR, and AFLP have been successfully applied to uncover DNA polymorphisms in *P. annua* (Carson et al., 2007; Chwedorzewska, 2008; Mengistu et al., 2000; Sweeney and Danneberger, 1995). In the current study, SRAP markers were used to assess the diversity and relatedness among genotypes collected over a wide geographic range. In agreement with previous reports (Carson et al., 2007; Chwedorzewska, 2008), we observed high genetic similarity among genotypes of various origins. Limited genetic variability over a large territory could be due in part to the reproductive biology of annual bluegrass which is mostly achieved through self pollination. Nevertheless, SRAP markers were able to distinguish all the genotypes within our collection.

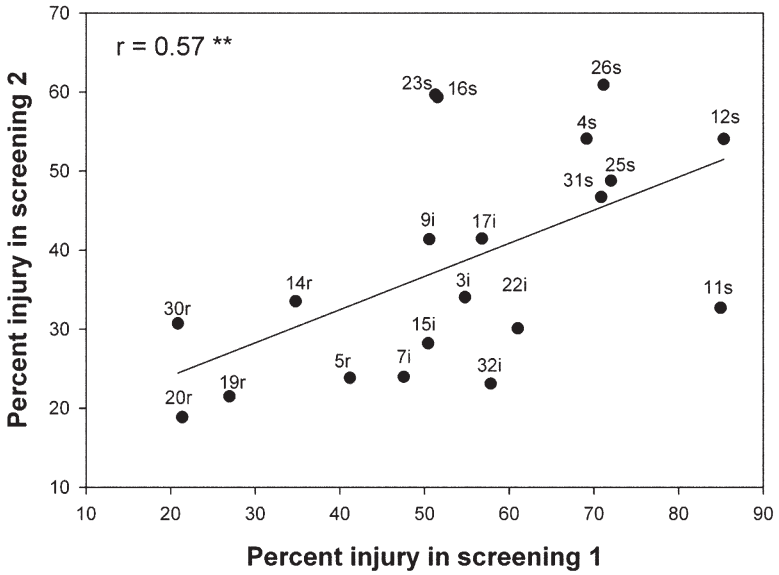

Figure 2. Relationship between the percentage of injury caused by SM in the two screening assays. A linear regression curve was adjusted to the data set and a correlation coefficient "*r*" was calculated. Genotypes are identified by numbers above the dots with respective level of resistance to SM: (s) susceptible, (i) intermediate, and (r) resistant. ***P* = 0.01.

Carson et al. (2007) were also able to differentiate several closely related genotypes of perennial annual bluegrass (labeled creeping bluegrass) with only a few exceptions using ISSR markers. Our results agree with the conclusion of Budak et al. (2004a) that SRAP markers are highly polymorphic and very efficient in estimating genetic variability among turfgrass species.

Study of the genetic variability of annual bluegrass by the SRAP technique further demonstrates the powerful selection pressure that SM diseases exert. The UPGMA dendrogram yielded two main clusters that differed markedly in their proportion of SM-resistant genotypes, showing that the genetic dissimilarity among genotypes was associated to a large extent with SM resistance. The annual bluegrass genotypes compared in our study were divided in two main clusters that differed in SM resistance. Although cluster A was much smaller than cluster B, it contained most of the SM resistant genotypes and none of the susceptible genotypes. Analysis of the relationships between SM resistance and climatic factors

Table 5. Spearman correlation coefficient of various climatic factors with the percentage of injury assessed after 1 wk regrowth of the second screening of the genotypes. *n* = 29.

	Days with snow cover	Max. daily temp.	Min. daily temp.	Rain precipitation	Snow precipitation
				mm	cm
December	–0.714*	0.714*	0.738*	0.690 ns†	–0.142 ns
January	–0.738*	0.738*	0.642 ns	0.666 ns	–0.214 ns
February	–0.778*	0.762*	0.642 ns	0.571 ns	–0.238 ns
March	–0.714*	0.762*	0.642 ns	0.667 ns	–0.333 ns

*Significant at the 0.05 probability level.

†ns, not significant.

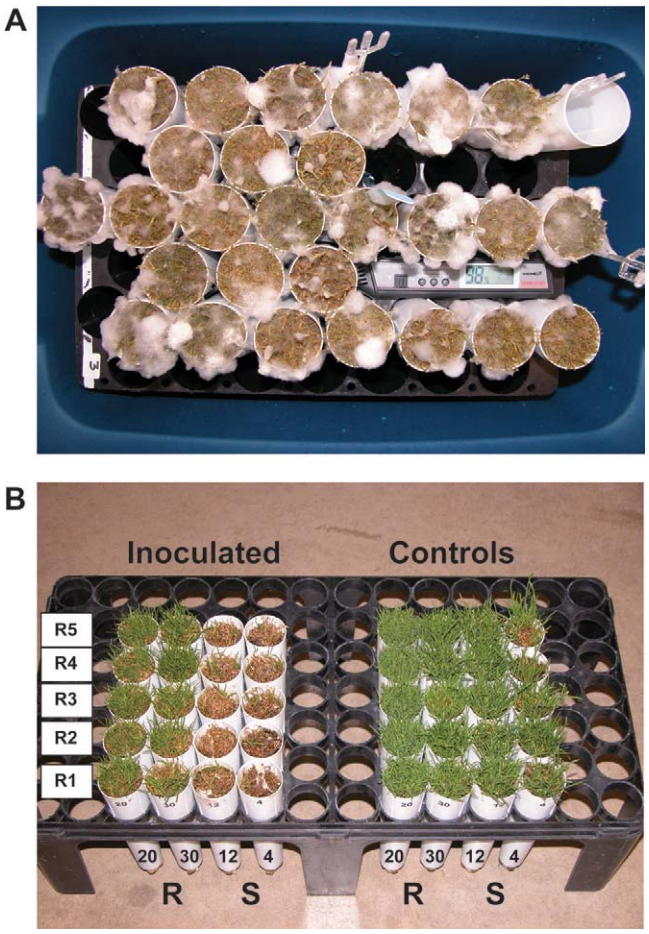

Figure 3. (A) Visual assessment of mycelium development on 27 individual genotypes after 7 wk of incubation with *Microdochium nivale* inoculum at 2°C. (B) Visual assessment after 1 wk regrowth of 4 genotypes previously incubated 7 wk, with or without *M. nivale inoculum.* Genotypes 20 and 30 were classified as SM resistant while Genotypes 12 and 4 were susceptible. R1 to R5 represent five repetitions within the screening test.

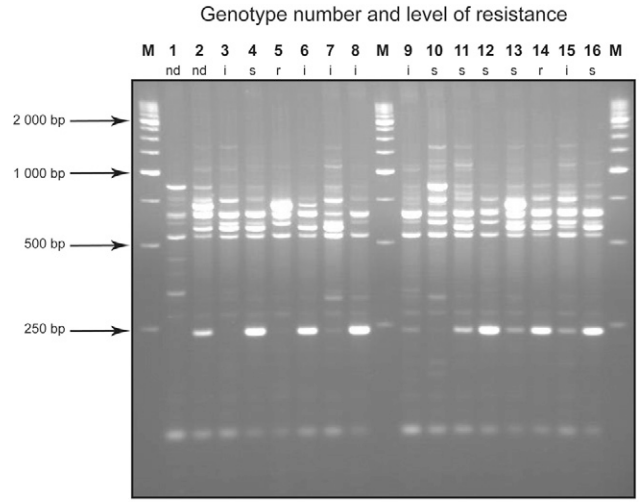

Figure 4. SRAP profiles of DNA from 16 genotypes of annual bluegrass. Lane identification by numbers and letters respectively refer to ecotype number and relative SM resistance including s: susceptible, i: intermediate, r: resistant, and nd: not determined. Lanes identified as "M" contain a 250 bp size marker (Roche diagnostic). Primer combination F13-R9 was used. PCR products were fractionated on a 2% (w/v) agarose gel in 1X Tris-Borate-EDTA running buffer pH 8.0 (75V for 3 hours). Ethidium bromide (1.0 μg mL^{-1}) was added to the gel before electrophoresis.

revealed that duration of snow cover and temperature in winter play an important role in the evolution of genetic resistance of annual bluegrass. The severity of SM damage to cereals and turfgrasses is generally governed by environmental conditions such as temperature, humidity, snow cover duration, and availability of inoculum (Gaudet, 1994; Hsiang et al., 1999). In addition, there is variation among isolates of *M. nivale* in terms of virulence (Hofgaard et al., 2006), and there is some indirect evidence for host-specialization among turfgrass isolates (Mahuku et al., 1998).

Our results confirm that conditions that prevail under extended snow cover exert a strong selective pressure that can cause variation in resistance to SM. Several mechanisms can create genomic variations in response to environmental pressure, including mutations, polyploïdization, and outcrossing. It has been reported that the mode of reproduction of annual bluegrass is strongly determined by the environmental conditions to which it is exposed (Koshy, 1969). Under stress conditions, the rate of outcrossing, which is more conducive to genetic variability, may increase and influence the genetic structure of the population (Ellis, 1973). High rates of outcrossing have been observed in some populations of annual bluegrass from western Oregon under the selective pressure of herbicide applications (Mengistu et al., 2000). In the current study, several subclusters within the dendrogram generally reflected the proximity of the sites of origin. There were, however, noticeable instances where genotypes from distant sites were grouped on the basis of their resistance to SM or other unknown characteristics. Carson et al. (2007) also observed that *P. annua* accessions collected from distinct geographic origins may possess genetic similarities.

Breeding programs can greatly benefit from the identification of DNA polymorphisms linked to traits of interest for the development of marker-assisted breeding (Morgante, 2006; Weising et al., 2005; Xu and Crouch, 2008). Bulk segregant analysis has been successfully applied for the identification of disease resistance genes (Michelmore et al., 1991). Our results indicate that the identification of molecular markers using bulk segregant analysis of SM resistance in annual bluegrass is worth pursuing, although any marker linkage would need subsequent confirmation with segregating populations. Distinct polymorphisms were clearly identified between groups of genotypes with high, intermediate, and low levels of resistance. These results highlight the fact that resistance to SM relies on both the increase in the frequency of favorable genes and the elimination of unfavorable ones. Sequencing of

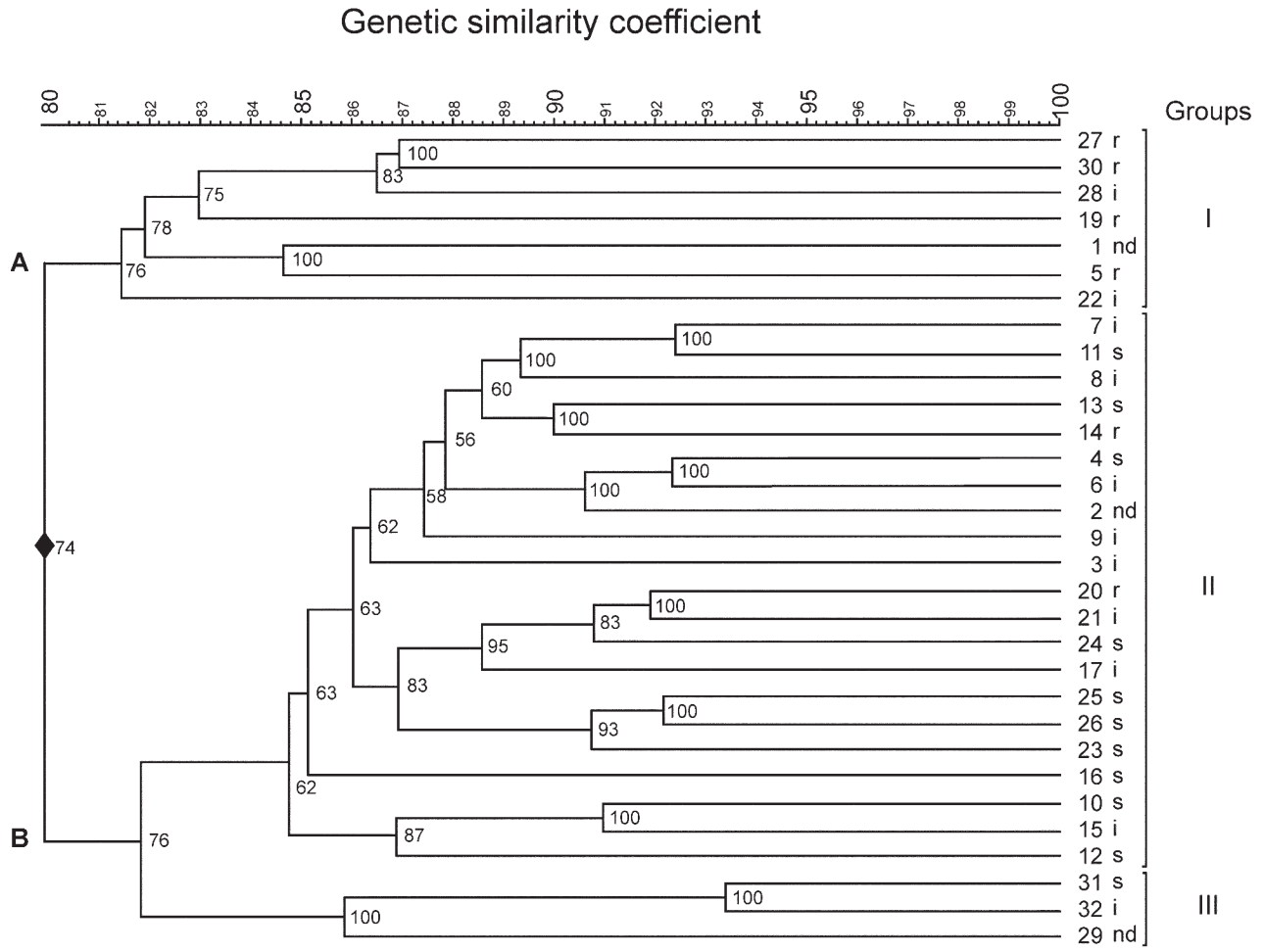

Figure 5. Dendrogram of 31 annual bluegrass genotypes based on a composite analyses matrix from 24 SRAP primer combinations. Hierarchical clustering of genotypes was produced by unweighted pair-group method with arithmetic average (UPGMA) of genetic distances. Genotypes and their relative SM resistance are identified at the right of the dendrogram. Cophenetic correlations are provided at each subcluster root as an estimate of robustness.

fragments of interest could provide insights into the nature of the genes underlying SM resistance. Li and Quiros (2001) have shown that up to 60% of the SRAP markers were located within coding regions of genes. In addition to their application in breeding programs, polymorphisms associated with SM could also be applied into survey of the diversity of SM resistance on a given golf course or over a broader region. This information could help managers to adapt their control methods to the specific level of resistance of their courses in accordance to the requirements of integrated pest management programs.

The development of seed sources more resistant to SM is a priority for the turfgrass industry and would clearly offer major environmental benefits through the reduction of fungicide use. To optimize a screening method for the selection of resistant genotypes of annual bluegrass, we used material from golf courses that have been through natural selection pressures for biotic and abiotic stresses which make them excellent genetic stock for turf breeding programs (Vergara and Bughrara, 2004). Among the 29 genotypes of annual bluegrass tested, we observed large genetic variability for SM resistance. Other studies with grasses and cereals also observed large genetic variability for SM resistance and successfully integrated this feature into breeding programs to develop resistant cultivars.

Comparative analysis of genotypes of contrasting resistance to SM will allow a more refined search for the molecular and genetic bases of SM resistance in annual bluegrass. In addition, a search can be made for genotypes that possess cross-adaptation to other winter stresses typically encountered on golf courses located in northern climates, including low temperature anoxia and freezing stress. Along with SM resistance, these are winter survival attributes that are highly desired by superintendents for reliable spring regrowth of golf greens. The identification of DNA polymorphisms associated with SM resistance offers future opportunities with regard to the development of marker-assisted breeding and the assessment of SM resistance on golf greens. Evaluation of performance of selected genotypes with contrasted SM resistance is currently underway under field conditions.

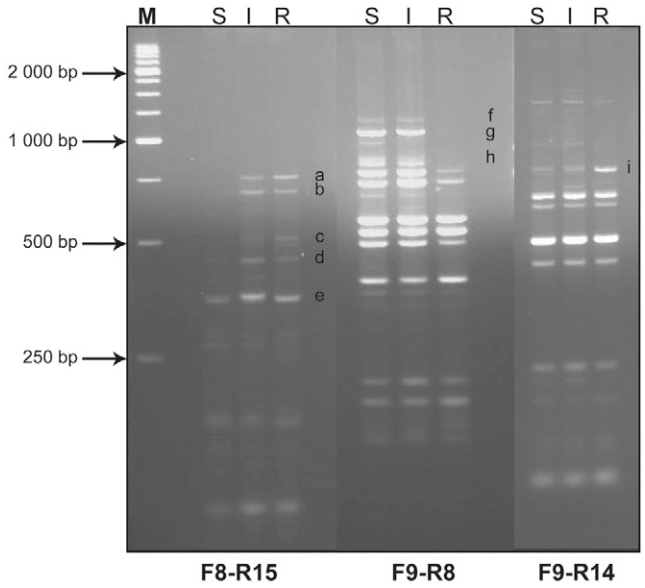

Figure 6. SRAPs of bulked genotypes with various levels of resistance to SM: (S) susceptible, (I) intermediate, (R) resistant. Lane M contains a 250-bp size marker (Roche diagnostic). Amplicons were separated on a 2% (w/v) agarose gel in 1X Tris-Borate-EDTA running buffer pH 8.0 (75 V for 3 h). Ethidium bromide (1.0 μg mL^{-1}) was added to the gel before electrophoresis. Primer set combinations (forward:reverse) are indicated below the panels.

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