Weed Turf. Sci. 4(2):135~143
http://dx.doi.org/10.5660/WTS.2015.4.2.135 Print ISSN 2287-7924, Online ISSN 2288-3312

Research Article Weed & Turfgrass Science

Weed & Turfgrass Science was renamed from formerly both Korean Journal of Weed Science from Volume 32(3), 2012, Korean Journal of Turfgrass Science from Volume 25(1), 2011 and Asian Journal of Turfgrass Science from Volume 26(2), 2012 which were launched by The Korean Society of Weed Science and The Turfgrass Society of Korea founded in 1981 and 1987, respectively.

Genetic Relationships among *Typhula ishikariensis* Varieties from Wisconsin

Seog-Won Chang*

Department of Golf Course Management, Korea Golf University, Hoengseong 225-811, Korea

ABSTRACT. *Typhula ishikariensis* Imai is a causal agent of Typhula snow mold, one of the most important turfgrass diseases in northern regions of the United States. Within Wisconsin isolates, there are three district groups clustered with known isolates of *T. ishikariensis* var. *ishikariensis*, var. *canadensis* and var. *idahoensis* as identified by RAPD markers. To further investigate the genetic relationship among these groups (varieties), monokaryon-monokaryon and dikaryon-monokaryon mating experiments were conducted. Mating types from var. *ishikariensis*, var. *canadensis* and var. *idahoensis* isolates were paired in all possible combinations. Pairings between var. *canadensis* and var. *idahoensis* were highly compatible, while no compatibility was detected between var. *ishikariensis* and either var. *canadensis* or var. *idahoensis*. These results indicate that var. *ishikariensis* is genetically separated from var. *canadensis* and var. *idahoensis*, whereas var. *canadensis* and var. *idahoensis* appeared to be genetically related to each other as a taxonomic unit. In the genetic relationship with the known biological species, var. *ishikariensis* and var. *canadensis* were genetically related to biological species I and II, respectively. However, var. *idahoensis* was not compatible with any of the biological species, suggesting that the pathogen may be in the process of biological speciation from var. *canadensis*.

Key words: Genetic relationship, Mating reaction, Typhula snow mold, *Typhula ishikariensis* varieties

Received on September 5, 2014; Revised on September 15, 2014; Accepted on September 16, 2014

***Corresponding author:** Phone) +82-70-7877-2032, Fax) +82-70-7877-2001; E-mail) changsw802@hanmail.net

Introduction

Typhula ishikariensis Imai is a causal agent of Typhula snow mold, one of the most important winter diseases on turfgrass and winter cereals (Arsvoll and Smith, 1978; Chang et al., 2006a; Smiley et al., 1994), and is common areas with much longer snow cover periods than *T. incarnata* Lasch and *T. phacorrhiza* Reichard (Hsiang et al., 1999; Chang et al., 2006b). After snowmelt, sclerotia are usually visible in infected plants or on soil where they oversummer. In late autumn, sclerotia produce mycelia or basidiospores serve as role as a primary inoculum (Cunfer and Bruehl, 1973; Bruehl and Cunfer, 1975).

Since *T. ishikariensis* was first described by Imai (1930), identification of the fungus mainly relied on morphological descriptions of the sclerotia and fruiting body. However, variation in morphology caused by environmental conditions resulted in taxonomic confusion among researchers, who assigned different names to *Typhula* species and *T. ishikariensis* subspecies level, showing the nomenclature of different species, varieties, biotypes or groups among countries and researchers (Bruehl and Cunfer, 1975; Arsvoll and Smith, 1978; Matsumoto et al., 1982; Matsumoto, 1997). The confusion was solved when morphological characteristics were combined with genetic studies at the species level as a conclusion of Røed (1969) that *T. itoana*, *T. graminum* and *T. incarnata* all were the same species.

Although interfertility experiments have become a valuable tool in the delineation of *Typhula* species, different forms of *T. ishikariensis* have been recognized by researchers. Bruehl et al. (1975) used mating experiments to conclude that *T. ishikariensis* and *T. idahoensis* were different species. However, Arsvoll and Smith (1978) studied *T. ishikariensis* isolates from Canada, USA, Norway, Japan and Switzerland on the basis of morphology, cultural characteristics and mating ability. Based on these studies, they rejected *T. idahoensis* as a separate species and divided *T. ishikariensis* into three varieties: var. *ishikariensis*, var. *canadensis* and var. *idahoensis*.

In Japan, Matsumoto et al. (1982) identified three forms among Japanese isolates of *T. ishikariensis* named biotypes A,

B, and C. From the results of mating experiments as well as culture morphology, biotype A was considered identical to var. *ishikariensis*. Biotype C was similar to var. *canadensis*, while biotype B was considered different from var. *idahoensis* because of the poor mating between them. However, Matsumoto and Tajimi (1991) later concluded that biotypes B and C were a single biological species adapted to different localities based on more extensive studies.

Matsumoto (1997) divided *T. ishikariensis* isolates from around the world into two biological species as defined by Mayr (1963) who described a biological species as "group of interbreeding natural populations that is reproductively isolated from other groups". Matsumoto concluded that biological species I (BSI) included *T. ishikariensis* and *T. ishikariensis* var. *ishikariensis* from North America, biotype A from Japan, and groups I and III from Norway, while Biological species II (BSII) included *T. idahoensis*, *T. ishikariensis* varieties *idahoensis* and *canadensis*, *T. ishikariensis* group II and biotypes B and C.

The difficulty regarding the taxonomy of *T. ishikariensis* has also been evident in Wisconsin, where this fungus is one of the most important pathogens on turfgrass (Chang et al., 2006b). Millet et al. (2001) grouped *T. ishikariensis* samples according to their morphological characteristics into WIG1 and WIG2. From mating experiments with both BS I and II, the authors concluded that WIG1 and WIG2 isolates were genetically related to BSI and BSII, respectively. However, one of the problems observed from Wisconsin samples was the fact that several isolates could not mate or mated spuriously with any of the BS, thus they could not be classified into either known BS.

Using Random Amplified Polymorphic DNA (RAPD) markers, Jung et al. (2000) investigated the genetic relationship among Wisconsin isolates, known isolates classified as *T. ishikariensis* var. *ishikariensis*, var. *idahoensis* and var. *canadensis* as well as some of the tester monokaryons from BSI and BSII. The authors found that the *T. ishikariensis* population in Wisconsin was composed of three genetically distinct groups, which were named groups A, B and C. Interestingly, group A and BSI were clustered with *T. ishikariensis* var. *ishikariensis*. Group B and some isolates of the BSII were clustered with *T. ishikariensis* var. *canadensis*, while group C and some BSII isolates were located within var. *idahoensis*. The objective of this study was to examine the genetic relationship among the three *T. ishikariensis* varieties from Wisconsin isolates as well as the taxonomic relationships with known BS using interfertility experiments.

Materials and Methods

Fungal isolates

A total of 105 isolates of *T. ishikariensis* varieties from Wisconsin were randomly selected from previously identified

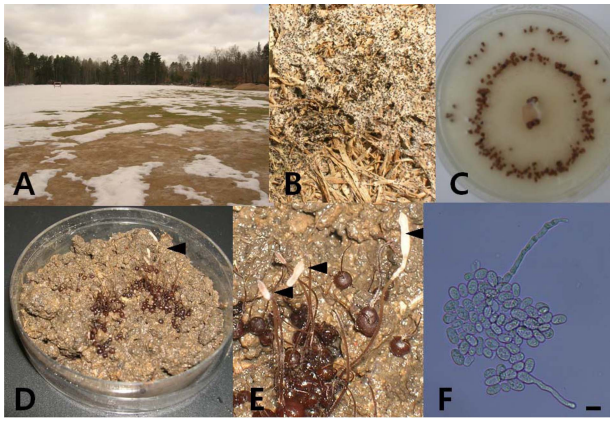

Fig. 1. Snow mold damage by *Typhula ishikariensis* var. *ishikariensis* on creeping bentgrass putting green (A) and the fungus' mycological characteristics (B~F). Old sclerotia of var. ishikariensis in field are black color (B), wheras fresh sclerotia produced on PDA medium after 4 weeks at 10°C are usually brown color (C). The sporocarps were produced after 4 weeks on non-sterilized field soil in a growth chamber at 9/4°C (day/night) with 8 photoperiods. Arrow heads indicate basidiocarp. (D and E). Basiospores and their germination (F). Stained monokaryotic and dikaryotic state of *T. ishikariensis* mycelia are described in Chang and Jung (2008). Scale bar = 2 μm.

Table 1. *T. ishikariensis* varieties isolates used in this study.

Isolates	Classification (stage)	Year and location collected
93-32-MI	var. *ishikariensis* (BS I[a]) (monokaryon)	Unknown, Russia (Matsumoto and Tajimi, 1990)
8-2	Biotype B (BS II) (monokaryon)	Unknown, Japan (Matsumoto et al., 1982, 1997)
4-3, S-5	Group II (BS II) (monokaryon)	1995, Norway (Matsumoto et al., 1996)
NE 9. 4. 5	var. *ishikariensis* (dikaryon)	2001, Big Sand GC, Vilas, WI, U.S.A
NE 10. 5. 1	var. *ishikariensis* (dikaryon)	2001, Big Stone Golf & SB, Oneida, WI, U.S.A
NE 23. 6. 1	var. *ishikariensis* (dikaryon)	2001, DeSmidt's GC & CC, Marinette, WI, U.S.A
NE 63. 11. 1	var. *ishikariensis* (dikaryon)	2001, Merrill GC, Lincoln, WI, U.S.A
NE 73. 17. 2	var. *ishikariensis* (dikaryon)	2001, Peck's Wildwood GC, Vilas, WI, U.S.A
NE 73. 17. 3	var. *ishikariensis* (dikaryon)	2001, Peck's Wildwood GC, Vilas, WI, U.S.A
NE 85. 6. 2	var. *ishikariensis* (dikaryon)	2001, Riveredge CC, Marathon, WI, U. S. A
NW 10. 8. 2	var. *ishikariensis* (dikaryon)	2001, Botten's Green Acres, Douglas, WI, U.S.A
NW 10. 9. 2	var. *ishikariensis* (dikaryon)	2001, Botten's Green Acres, Douglas, WI, U.S.A

Table 1. *T. ishikariensis* varieties isolates used in this study (continued).

Isolates	Classification (stage)	Year and location collected
NW 26. 9. 3	var. *ishikariensis* (dikaryon)	2001, Fox Run GC, Burnett, WI, U.S.A
NW 33. 2. 3	var. *ishikariensis* (dikaryon)	2001, Hayward G&TC, Sawyer, WI, U.S.A
NW 69. 8. 5	var. *ishikariensis* (dikaryon)	2001, Spider Lake Golf Resort, Sawyer, WI, U.S.A
NE 27. 4. 1	var. *canadensis* (dikaryon)	2001, Edgewater GC, Lincoln, WI, U. S. A
NE 31. 5. 5	var. *canadensis* (dikaryon)	2001, Four Season GC, Marinette, WI, U.S.A
NE 42. 13. 1	var. *canadensis* (dikaryon)	2001, High Cliff GC, Calumet, WI, U.S.A
NE 57. 2. 4	var. *canadensis* (dikaryon)	2001, Maple Hills GC, Shawano, WI, U.S.A
NE 68. 17. 2	var. *canadensis* (dikaryon)	2001, Nicolet GC, Forest, WI, U.S.A
NE 90. 11. 3	var. *canadensis* (dikaryon)	2001, Sandalwood GC, Oconto, WI, U.S.A
NE 95. 6. 5	var. *canadensis* (dikaryon)	2001, Spread Eagle GC, Oconto, WI, U.S.A
NE 104. 14. 4	var. *canadensis* (dikaryon)	2001, Trout Lake G & CC, Vilas, WI, U.S.A
NE 110. 1. 3	var. *canadensis* (dikaryon)	2001, Wander Springs, Outagamie, WI, U.S.A
NW 7. 6. 1	var. *canadensis* (dikaryon)	2001, Barronett Hills GC, Barron, WI, U.S.A
NW 10. 6. 5	var. *canadensis* (dikaryon)	2001, Botten's Green Acres, Douglas, WI, U.S.A
NW 14. 13. 5	var. *canadensis* (dikaryon)	2001, Chippewa Valley GC, Dunn, WI, U.S.A
NW 48. 1. 1	var. *canadensis* (dikaryon)	2001, Neillsville GC, Clark, WI, U.S.A
NW 57. 4. 1	var. *canadensis* (dikaryon)	2001, Pine Meadow GC, Douglas, WI, U.S.A
NW 73. 18. 1	var. *canadensis* (dikaryon)	2001, Tagalong, Washburn, WI, U.S.A
SE 79. 12. 4	var. *canadensis* (dikaryon)	2001, Quit Qui Oc GC, Sheboygan, WI, U.S.A
SE 86. 27. 3	var. *canadensis* (dikaryon)	2001, Rolling Meadows GC, Fond Du Lac, WI, U.S.A
SW 8. 10. 4	var. *canadensis* (dikaryon)	2001, Castle Rock GC, Juneau, WI, U.S.A
NE 4. 10. 5	var. *idahoensis* (dikaryon)	2001, Antigo Bass Lake, Langlade, WI, U.S.A
NE 31. 2. 3	var. *idahoensis* (dikaryon)	2001, Four Season GC, Marinette, WI, U.S.A
NE 34. 4. 10	var. *idahoensis* (dikaryon)	2001, Gateway, Vilas, WI, U.S.A

Table 1. *T. ishikariensis* varieties isolates used in this study (continued).

Isolates	Classification (stage)	Year and location collected
NE 45. 6. 5	var. *idahoensis* (dikaryon)	2001, Homestead GC, Wood, WI, U.S.A
NE 95. 3. 5	var. *idahoensis* (dikaryon)	2001, Spread Eagle GC, Oconto, WI, U.S.A
NW 7. 2. 1	var. *idahoensis* (dikaryon)	2001, Barronett Hills GC, Barron, WI, U.S.A
NW 9. 7. 2	var. *idahoensis* (dikaryon)	2001, Bloomer Memorial GC, Chippewa, WI, U.S.A
NW 33. 18. 5	var. *idahoensis* (dikaryon)	2001, Hayward G&TC, Sawyer, WI, U.S.A
NW 45. 16. 4	var. *idahoensis* (dikaryon)	2001, Mellen CC, Ashland, WI, U.S.A

[a]Classification given by the author who described the isolate.

collections (Fig. 1 A and B) (Chang et al., 2006b) and 39 isolates are used for mating are listed in Table 1. Based on morphological and DNA fingerprinting data (Chang et al., 2006b), all isolates selected out of 39 isolates were pathogenic on three bentgrass species (*Agrostis* sp.) (Chang et al., 2006a). In order to test mating compatibility with Wisconsin isolates, three tester monokaryons representing *T. ishikariensis* Biological Species (BS) I and BS II were obtained from Dr. Naoyuki Matsumoto, National Institute of Agro-Environmental Sciences, Tsukuba, Japan (Table 1).

Determination of mating types

Fifteen dikaryotic isolates from *T. ishikariensis* var. *ishikariensis*, 15 from var. *canadensis* and 45 from var. *idahoensis* were used to obtain monokaryons from basidiospores. A larger number of isolates from *T. ishikariensis* var. *idahoensis* were chosen due to the failure of this variety to produce sporocarps in preliminary experiments. To obtain monokaryotic isolates the procedures used by Bruehl and Cunfer (1975) were followed. At least 20 sclerotia per isolate were placed on non-sterilized soil in pots (Fig. 1 C, D, and E). The pots were placed into plastic trays containing water to assure constant moisture and maintained in a growth chamber at 5 C with 8 h photoperiods at 3.45 $mEm^{-2}s^{-1}$ (ca. 300 lux).

Once the most sporocarps arose and reached their mature stage (about one week after emerging), they were collected. Each was individually placed on the lid of a petri dish containing 1.5% water agar (WA) medium, and incubated at 10 C. After 4-5 days, the basidiospores showered onto the WA were suspended in distilled-sterilized water and spread out onto the media surface. The plates were incubated at 10 C for 4-7 days or until germinating spores were observed. Following that, a single germinated basidiospore was transferred from

WA to PDA (Potato Dextrose Agar) medium and incubated at 10 C until colony formation (10-15 days) (Fig. 1 F). The colonies were then examined for the presence or absence of clamp connections, the absence of which is an indication of the monokaryotic state.

At least 12 monokaryons per dikaryotic isolate were selected. These monokaryons were paired in all combinations in order to identify the four mating types. Pairings were made following the methodology used by Bruehl et al. (1975) with slight modifications. Plugs of mycelia from two monokaryons were placed 1 cm apart in a petri dish containing PDA. After 15 days at 10 C, hyphae from the junction zone of both colonies were examined under the microscope. Presence with numerous and normal clamps connections indicated a compatible pairing as a positive reaction '+', meaning two isolates were considered to have a closer relationship. When the pairing was incompatible no clamp connections were observed and the reaction was noted as negative '-'. When no compatible reaction was noticed between two isolates, the isolates were considered to be not genetically related. An uncertain relationship with very few clamp connections or abnormal growth of mycelium was noted as '±'.

The assignment of the mating type within each isolate was arbitrary, based on the tetrapolar incompatibility system present in *Typhula* spp. (Bruehl et al., 1975; Chang and Jung, 2008). Positive or compatible reactions can only be possible between two monokaryons carrying different alleles in both A and B factors (i.e. $A_1B_1 + A_2B_2$). On the contrary, mating cannot occur when there is at least one allele in common (i.e. $A_1B_1 + A_1B_2$).

Monokaryon-monokaryon pairing

Four monokaryons representing the four mating types from each dikaryon were selected. Mating experiments were made by taking the four mating types from each isolate and pairing them in all combinations. Pairings and identification of mating reactions were done per the methods of Bruehl et al. (1975) explained in the previous section.

To investigate the compatibility between Wisconsin monokaryotic isolates and known tester monokaryons, four mating types of each variety were paired with the monokaryons representing *T. ishikariensis* BS I and BS II. Compatible reactions meant the isolates were genetically compatible and considered as the same BS.

Dikaryon-monokaryon pairing

Thirty dikaryons from three varieties were selected and mated with four mating types of each variety. Pairings and identification of mating reactions were done per the methods of Bruehl et al. (1975) with slight modifications. Five mm diameter agar discs with mycelia were cut from the margin of actively growing PDA cultures of both monokaryotic testers and dikaryotic isolates and placed approximately 1 cm apart on PDA plates. After 3 to 4 days of colony contact (ca. 15 days incubation at 10 C), a 5 mm agar disc was cut from the monokaryon colony 1 cm behind the colony junction and transferred to a new PDA plate. The mycelia growing from the junction piece was microscopically examined for the presence of clamp connections one week later. Identification of mating reactions was done as the methods of Bruehl et al. (1975) explained in the previous section.

Results

Determination of mating types

Seventy-five isolates from three *T. ishikariensis* varieties were examined to obtain monokaryons. From *T. ishikariensis* var. *ishikariensis*, five out of fifteen isolates (33%) produced sporocarps, but only two of them (13.3%) produced monokaryons. Seven isolates out of fifteen (46.6%) from *T. ishikariensis* var. *canadensis* produced sporocarps. From them, five isolates (33%) produced basidiospores that developed into monokaryotic colonies. Five isolates out of forty-five (11%) from *T. ishikariensis* var. *idahoensis* produced sporocarps, but only two (4.4%) produced basidiospores that developed into

Table 2. Production of sporocarps and monokaryons from *T. ishikariensis* varieties isolated in Wisconsin.

Variety	Number of dikaryons	Number of dikaryons producing sporocarps	Number of dikaryons that produced monokaryons
Var. *ishikariensis*	15	5	2
Var. *canadensis*	15	7	5
Var. *idahoensis*	45	5	2

Table 3. Mating types of each T. ishikariensis variety identified from dikaryon isolates.

Isolate	Variety	Number of monokaryons mated	Number of mating types identified
NE63.11.1	var. *ishikariensis*	12	3
NE85.6.2	var. *ishikariensis*	12	4
NE27.4.1	var. *canadensis*	12	4
NW14.13.5	var. *canadensis*	12	4
NW48.1.1	var. *canadensis*	14	0
NW57.4.1	var. *canadensis*	12	4
NW73.18.1	var. *canadensis*	12	4
NE34.4.10	var. *idahoensis*	13	4
NW33.18.5	var. *idahoensis*	13	4

Table 4. Pairings among four mating types of Wisconsin dikaryons representing *T. ishikariensis* varieties.

Isolate[a]		NE63.11.1 (var. i*shikariensis*)				NW14.13.5 (var. *canadensis*)				NW57.4.1 (var. *canadensis*)				NW73.18.1 (var. *canadensis*)				NE34.4.10 (var. *idahoensis*)			
	Mating type	A_1 B_1	A_1 B_2	A_2 B_1	A_2 B_2	A_1 B_1	A_1 B_2	A_2 B_1	A_2 B_2	A_1 B_1	A_1 B_2	A_2 B_1	A_2 B_2	A_1 B_1	A_1 B_2	A_2 B_1	A_2 B_2	A_1 B_1	A_1 B_2	A_2 B_1	A_2 B_2
NE63.11.1 (var.*ishikariensis*)	A_1B_1[b]	-	-	-	+																
	A_1B_2	-	+	+	-																
	A_2B_1	-	+	+	-																
	A_2B_2	+	-	-	-																
NW14.13.5 (var. *canadensis*)	A_1B_1	-[c]	-	-	-	-	-	-	+												
	A_1B_2	-	-	-	-	-	-	+	-												
	A_2B_1	-	-	-	-	-	+	-	-												
	A_2B_2	-	-	-	-	+	-	-	-												
NW57.4.1 (var. *canadensis*)	A_1B_1	-	-	-	-	+	+	+	+	-	-	-	+								
	A_1B_2	-	-	-	-	+	+	+	+	-	-	+	-								
	A_2B_1	-	-	-	-	+	+	+	+	-	+	-	-								
	A_2B_2	-	-	-	-	+	+	+	+	+	-	-	-								
NW73.18.1 (var. *canadensis*)	A_1B_1	-	-	-	-	+	+	+	±	-	-	+	-	-	-	-	+				
	A_1B_2	-	-	-	-	+	-	+	+	-	-	+	+	-	-	+	-				
	A_2B_1	-	-	-	-	+	+	+	+	+	-	-	-	-	+	-	-				
	A_2B_2	-	-	-	-	+	-	+	-	+	+	-	-	+	-	-	-				
NE34.4.10 (var. *idahoensis*)	A_1B_1	-	-	-	-	+	+	-	-	+	+	+	+	+	+	+	+	-	-	-	+
	A_1B_2	-	-	-	-	+	+	+	+	+	+	+	+	+	+	±	+	-	-	+	-
	A_2B_1	-	-	-	-	+	+	+	+	+	+	+	±	+	+	-	±	-	+	-	-
	A_2B_2	-	-	-	-	+	-	+	-	+	+	+	±	+	+	-	+	+	-	-	-

[a]Name of dikaryon from which the mating type was selected.
[b]Hypothetical assigned alleles. Numbers 1 and 2 are used to refer to as different alleles but are not intended to represent any specific genotype.
[c]+ indicates compatible reaction, -indicates no compatible reaction, ± indicates an uncertain reaction where few clamp connections were observed.

Table 5. Pairings among mating types of Wisconsin dikaryons representing *T. ishikariensis* varieties and tester monokaryons representing the biological species I and II.

Tester monokaryon	Biological species, mating type	NE63.11.1 (var. *ishikariensis*)				NW14.13.5 (var. *canadensis*)				NW57.4.1 (var. *canadensis*)				NE 34.4.10 (var. *idahoensis*)			
		A_1B_1[a]	A_1B_2	A_2B_1	A_2B_2	A_1B_1	A_1B_2	A_2B_1	A_2B_2	A_1B_1	A_1B_2	A_2B_1	A_2B_2	A_1B_1	A_1B_2	A_2B_1	A_2B_2
92-32-MI	I, A_1B_1	+[b]	+	+	+	±	-	-	-	-	-	-	-	-	-	-	-
8-2	II, A_2B_1	-	-	-	-	+	-	-	+	+	-	+	-	-	-	-	-
4.3,5.2	II, A_2B_2	-	-	±	-	+	-	+	+	+	+	+	+	-	-	-	-

[a]Hypothetical assigned alleles. Numbers 1 and 2 are used to refer to as different alleles but are not intended to represent any specific genotype.
[b]+ indicates compatible reaction, - indicates no compatible reaction, ± indicates an uncertain reaction where few clamp connections were observed.

monokaryons (Table 2).

In all cases, more than 30 monokaryons were obtained from each isolate that produced the monokaryotic colony. The sets of four mating types with the exception of two isolates were obtained from one dikaryon of *T. ishikariensis* var. *ishikariensis*, from four dikaryons of var. *canadensis*, and from two dikaryons of var. *idahoensis*. The result demonstrates that the most isolates represented the tetrapolar incompatibility mating system (Table 3). However, *T. ishikariensis* var. *ishikariensis* NE 63.11.1 yielded three mating types and monokaryons from var. *canadensis* NW48.1.1 could not be assigned to any of the mating type because several monokaryons failed to mate with

Table 6. Dikaryon-monokaryon mating reactions of dikaryotic *T. ishikariensis* varieties isolates with monokaryotic tester isolates of Wisconsin.

Variety [a]	Isolate	NE 63.11.1 (var. *ishikariensis*)[a]				NW 14.13.5 (var. *canadensis*)				NE 34.4.10 (var. *idahoensis*)			
		A_1B_1[b]	A_1B_2	A_2B_1	A_2B_2	A_1B_1	A_1B_2	A_2B_1	A_2B_2	A_1B_1	A_1B_2	A_2B_1	A_2B_2
Var. *ishikariensis*	NE 9.4.5	+[c]	±	-	+	-	-	-	-	-	-	-	-
	NE 10.5.1	+	+	+	+	-	-	-	-	-	-	-	-
	NE 23.6.1	+	-	-	+	-	-	-	-	-	-	-	-
	NE 73.17.2	-	+	+	±	-	-	-	-	-	-	-	-
	NE 73.17.3	-	-	+	+	-	-	-	-	-	-	-	-
	NW 10.8.2	+	+	+	+	-	-	-	-	-	-	-	-
	NW 10.9.2	+	±	-	+	-	-	-	-	-	-	-	-
	NW 26.9.3	+	+	+	+	-	-	-	-	-	-	-	-
	NW 33.2.3	+	-	-	+	-	-	-	-	-	-	-	-
	NW 69.8.5	+	-	±	+	-	-	-	-	-	-	-	-
Var. *canadensis*	NE 31.5.5	-	-	-	-	+	-	±	+	-	-	-	-
	NE 42.13.1	-	-	-	-	-	+	+	-	+	-	-	+
	NE 57.2.4	-	-	-	-	±	+	+	-	-	-	-	-
	NE 68.17.2	-	-	-	-	-	+	+	-	-	-	-	-
	NE 90.11.3	-	-	-	-	+	+	+	+	-	-	-	-
	NE 95.6.5	-	-	-	-	-	+	+	±	-	-	-	-
	NE 104.14.4	-	-	-	-	+	+	+	+	-	-	-	-
	NE 110.1.3	-	-	-	-	+	-	-	+	+	-	-	+
	NW 7.6.1	-	-	-	-	-	+	+	-	+	±	-	+
	NW 10.6.5	-	-	-	-	-	+	+	±	+	-	-	+
	SE 79.12.4	-	-	-	-	+	-	-	+	-	-	-	-
	SE 86.27.3	-	-	-	-	+	+	+	+	-	-	-	-
	SW 8.10.4	-	-	-	-	+	+	+	+	-	-	-	-
Var. *idahoensis*	NE 4.10.5	-	-	-	-	+	-	-	+	-	-	-	-
	NE 31.2.3	-	-	-	-	+	-	+	+	+	±	-	+
	NE 45.6.5	-	-	-	-	+	+	+	+	-	-	-	-
	NE 95.3.5	-	-	-	-	+	+	+	+	-	-	-	-
	NW 7.2.1	-	-	-	-	-	+	+	-	+	±	-	+
	NW 9.7.2	-	-	-	-	-	+	+	-	+	+	+	+
	NW 45.16.4	-	-	-	-	-	-	-	-	-	-	-	-

[a]Name of dikaryon from which the mating type was selected.
[b]Hypothetical assigned alleles. Numbers 1 and 2 are used to refer to as different alleles but are not intended to represent any specific genotype.
[c]+ indicates compatible reaction, -indicates no compatible reaction, ± indicates an uncertain reaction where few clamp connections were observed.

any other. In each case, four monokaryons representing the four mating types were selected. For those isolates whose compatibility groups could not be assigned, four monokaryons were chosen based on their ability to mate (i.e. two monokaryons unable to mate with any other monokaryon and two monokaryons able to mate with the rest). The reason monokaryons were selected in this way is that it permitted us to know whether those isolates incapable to mate with monokaryons coming from the same dikaryon, were also unable to mate with monokaryons from other dikaryons. The inability of mating could suggest the presence of infertility factors.

Genetic relationships among *T. ishikariensis* varieties by monokaryon-monokaryon pairing

Mating types from five isolates were selected to mate among

varieties (Table 4). All four mating types with the exception of NE 63.11.1 were compatible with mating type carrying different alleles of same isolates. Among *T. ishikariensis* var. *canadensis*, 34 positive reactions, 12 negative reactions, and 1 uncertain reaction were found. In pairing among three varieties, no compatible reactions were observed between monokaryons from *T. ishikariensis* var. *ishikariensis* and var. *canadensis* or var. *idahoensis*, while monokaryons of var. *canadensis* and var. *idahoensis* were compatible with one or more monokaryons from the both variety. The pairing of *T. ishikariensis* var. *idahoensis* with var. *canadensis* produced 38 positive reactions, 6 negative reactions and 4 uncertain reactions.

Mating types from four isolates were selected to mate with the known BS testers (Table 5). The four mating types from *T. ishikariensis* var. *ishikariensis* isolate NE63.11.1 were compatible with the tester monokaryon of BSI, but not compatible with most of the BSII testers. Only one monokaryon produced an uncertain reaction '±' with 4.3,5.2, forming very few clamp connections and rhizomorphous hyphae. Two or more mating types from the two isolates of *T. ishikariensis* var. *canadensis* successfully mated with the two BS II testers. *T. ishikariensis* var. *canadensis* isolates were not compatible with BS I, with the exception of one monokaryon from isolate NW14.13.5, which paired uncertainly with the BS I tester 92-32-MI. Monokaryons from *T. ishikariensis* var. *idahoensis* did not mate with any of the BS testers.

Genetic relationships among *T. ishikariensis* varieties by dikaryon-monokaryon pairing

Thirty dikaryotic isolates of *T. ishikariensis* varieties were paired with four monokaryotic isolates of each variety (Table 6). All *T. ishikariensis* var. *ishikariensis* mated positively with at least two mating types of var. *ishikariensis* testers. No compatible reactions, however, were observed between dikaryons from *T. ishikariensis* var. *ishikariensis* and monokaryons of var. *canadensis* or var. *idahoensis*. In addition, monokaryons of *T. ishikariensis* var. *ishikariensis* were not compatible with dikaryons of var. *canadensis* or var. *idahoensis*. All dikaryons of the *T. ishikariensis* var. *canadensis* mated positively with at least two mating types of var. *canadensis* testers. In contrast with *T. ishikariensis* var. *ishikariensis*, four dikaryons of var. *canadensis* showed compatible reactions with two or more tester monokaryons from var. *idahoensis*. In the case of *T. ishikariensis* var. *canadensis* monokaryons, all mating types were mated with four var. *idahoensis* dikaryons with the exception of NW 45.16.4. Three out of seven dikaryotic isolates from *T. ishikariensis* var. *idahoensis* gave compatible reactions with at least two var. *idahoensis* testers. Six of *T. ishikariensis* var. *idahoensis* dikaryotic isolates showed the compatible reactions with two more tester monokaryons from var. *canadensis*.

Discussion

Incompatibility between monokaryons derived from basidiospores of *Typhuls* species is determined by complementation of multiple alleles at two loci (Cunfer, 1974). The incompatibility alleles have been used as convenient genetic markers for taxonomy of *Typhula* spp. (Kiyomoto and Bruehl, 1976). Within *T. ishikariensis* varieties, the incompatibility system using monokaryons or dikaryons are an important key to solve the taxonomic confusion as well as genetic relationships within and among the fungi (Bruehl et al., 1975; Matsumoto, 1997; Matsumoto et al., 1982; Matsumoto and Tajimi, 1990).

The production of monokaryons from dikaryotic isolates of *T. ishikariensis* in this study was low (Table 2). Some isolates never produced sporocarps, some formed sporocarps but not basidiospores and others produced basidiospores that never germinated or germinated but did not develop into a colony. In total, only 33% of the dikaryons from *T. ishikariensis* var. *canadensis* produced monokaryons, but this amount was higher than for var. *ishikariensis* and var. *idahoensis*, with only 11% and 4.4% monokaryon production from fertile dikaryons, respectively. This phenomenon was similar to a report by Bruehl et al. (1975) that many dikaryons did not produce sporocarps, produced few basidiospores or mostly spores that did not germinate, or produced spores that germinate, but failed to develop further. In addition, these relative proportions were observed by Bruehl et al. (1978), who explained that sterility was common in *T. idahoensis* and less common in *T. ishikariensis*. The authors also found that 90% of a collection of 2000 *T. idahoensis* isolates from Idaho, Utah, Montana and Wyoming were infertile, concluding that *T. idahoensis* from those regions were nearly asexual.

Even though in our study *T. ishikariensis* var. *canadensis* appears to be more fertile than var. *ishikariensis* and var. *idahoensis* (Table 2), this may be due to environmental conditions rather than intrinsic fertility of the fungi. Each of the varieties favors different habitats (Chang et al., 2006b; Matsumoto et al., 1982). Although we used a constant condition (5 C with 8 h daylength) for the incubation of all three varieties, a specific environment may be necessary for the production and development of the sexual stage in basidiomycetes (Bruehl and Cunfer, 1975; Dahlberg and Van Etten, 1982; Kawakami et al., 2004). Kawakami et al. (2004) also reported that environmental factors including fluctuating temperature (10/5 C; day/night), high humidity, and high intensity are conductive to sporocarp formation in *T. ishikariensis* biotype A and B.

The mating experiments were successful in characterizing the *T. ishikariensis* subspecies at the variety level (Table 4 and 6). Most mating types mated monokaryons carrying different alleles obtained from the same isolate, demonstrating that

most isolates have the typical tetrapolar incompatibility. In particular, at least 10 alleles of A and 10 of the B locus were identified within *T. ishikariensis* var. *canadensis*, suggesting that this population exists as an active interbreeding unit (Kiyomoto and Bruehl, 1976; Bruehl and Machtmes, 1979). Upon examining the genetic relationships among *T. ishikariensis* varieties, var. *canadensis* and var. *idahoensis* were found to be closely related to each other. All *T. ishikariensis* var. *canadensis* monokaryons mated with mating types of var. *idahoensis* and the resulting dikaryons had normal and numerous clamp connections, indicating that the varieties were genetically compatible as a taxonomic unit. These trends were also shown in the data from dikaryon-monokaryon mating. The two varieties in the pairing mated with each other, although the recovery of parental allele was different among isolates. This may be associated with recovery of both alleles of a parental haploid nucleus, recovery of one parental allele, and recovery of one or two different alleles from the other nucleus (Bruehl et al., 1975).

Monokaryons from *T. ishikariensis* var. *ishikariensis* did not mate with those of var. *canadensis* or var. *idahoensis* at all, although the sample size was very small. These trends were confirmed with the data from dikaryon-monokaryon mating. Di- or mono-karyons from *T. ishikariensis* var. *ishikariensis* did not mate with mono- or di-karyons of var. *canadensis* or var. *idahoensis* at all. These results indicate that *T. ishikariensis* var. *ishikariensis* is incompatible with both varieties, which is a sign that a reproductive barrier might be present. Similar results have been found in interactions among isolates of *T. ishikariensis* varieties with different origins (Arsvoll and Smith, 1978; Bruehl et al., 1978; Bruehl and Machtmes, 1980; Matsumoto, 1997). Interestingly, however, our result was very different with that of Jung et al. (2000), which found that the genetic relationship between *T. ishikariensis* var. *ishikariensis* and var. *idahoensis* appears to be closer than that of var. *canadensis* and var. *idahoensis*. A possible interpretation of this discrepancy is that their genetic map was constructed using RAPD markers, which are random and short primers and therefore may not be linked to mating factors.

Our results support the hypothesis that *T. ishikariensis* var. *ishikariensis* is distinctly separated from the other two varieties. The concept of a biological species in general establishes "speciation" as the process by which a genetically cohesive group of interbreeding individuals diverges into two or more genetically distinct groups of individuals (Bock, 1986), in spite of its other characteristics. If this concept is applied to *T. ishikariensis* varieties, it is possible to say that *T. ishikariensis* var. *ishikariensis* and other varieties are different species, or the two groups might be undergoing speciation.

As stated by Petersen and Hughes (1999), interbreeding populations may diverge genetically over time until speciation occurs. During that process, strong reproductive barriers often accompany the differentiation into species, with many factors such as ecological and geographical separation contributing to those barriers. In this sense, the habitat of *T. ishikariensis* varieties is very important. Based on ecological habitats of three varieties, *T. ishikariensis* var. *canadensis* and var. *idahoensis* favored the more unstable environment (Matsumoto et al., 1982; Chang et al., 2006b), have low growth ability (Bruehl and Machtmes, 1980), and have a limited host range (Matsumoto and Tajimi, 1993) compared with var. *ishikariensis*. Thus, even though the three varieties are able to grow in the same area, *T. ishikariensis* var. *canadensis* and var. *idahoensis* might not occur at the same time as var. *ishikariensis* which could be an ecological barrier for interbreeding. If this barrier has been present for a long time, reproductive isolation might have occurred leading to genetic divergence.

Interestingly, pairings with the known BS also provided a possibility that there are three biologically different groups in Wisconsin. *T. ishikariensis* var. *ishikariensis* and var. *canadensis* were genetically related to BS I and BS II, but var. *idahoensis* was not related to either BS I or BS II isolates. Similarly, Millet et al. (2001) found that several isolates from Wisconsin could not mate or mated spuriously with any of the BS. This phenomenon suggests that *T. ishikariensis* var. *idahoensis* may represent a third group with genetically different traits from var. *canadensis* in only North America as described by Arsvoll and Smith (1978). Otherwise, the fungi may be in the process of biological speciation from var. *canadensis*. Further experiments need to be done to understand more about three varieties.

References

Arsvoll, K. and Smith, J.D. 1978. *Typhula ishikariensis* and its varieties, var. *idahoensis* comb. Nov. and var. *canadensis* var. nov. Can. J. Bot. 56:348-364.

Bock, W.J. 1986. Species concept, speciation, and macroevolution. In: Iwatsuki et al. (Eds.). Modern aspects of species. Univ. of Tokyo Press, pp. 31- 57.

Bruehl, G.W. and Cunfer, B.M. 1975. *Typhula* species pathogenic to wheat in the Pacific Northwest. Phytopathology 65:755-760.

Bruehl, G.W. and Machtmes, R. 1979. Alleles of the incompatibility factors of *Typhula ishikariensis*. Can. J. Bot. 57:1252-1254.

Bruehl, G.W. and Machtmes, R. 1980. Cultural variation within *Typhula idahoensis* and *T. ishikariensis* and the species concept. Phytopathology 70:867-871.

Bruehl, G.W. Machtmes, R. and Kiyomoto, R. 1975. Taxonomic relationships among *Typhula* species as revealed by mating experiments. Phytopathology 65:1108-114.

Bruehl, G.W. Machtmes, R., Kiyomoto, R. and Christen, A. 1978. Fertility and incompatibility alleles of *Typhula idahoensis*. Phytopathology 68:1307-1310.

Chang, S.W., Chang, T.H., Tredway, L. and Jung, G. 2006a. Aggressiveness of *Typhula ishikariensis* isolates to cultivars of bentgrass

species (*Agrostis* spp.) under controlled environment conditions. Plant Dis. 90:951-956.

Chang, S.W. and Jung, G. 2008. The first linkage map of the plant-pathogenic basidiomycete *Typhula ishikariensis*. Genome 51:128-136.

Chang, S.W., Scheef, E., Abler, R.A.B., Clayton, M.K., Thomson, P., et al. 2006b. Distribution of *Typhula* species and *T. ishikariensis* varieties in Wisconsin, Utah, Michigan and Minnesota states. Phytopathology 96:926-933.

Cunfer, B.M. 1974. Sexual incompatibility and aspects of the mono- and dikaryotic phases of *Typhula idahoensis*. Phytopathology 64:123-127.

Cunfer, B.M. and Bruehl, G.W. 1973. Role of basidiospores as propagules and observations of sporophores of *Typhula idahoensis*. Phytopathology 63:115-20.

Dahlberg, K.R. and Van Etten, J.L. 1982. Physiology and biochemistry of fungal sporulation. Annu. Rev. Phytopathol. 20:281-301.

Hsiang, T., Matsumoto, N. and Millet, S. 1999. Biology and management of Typhula snow molds of turfgrasses. Plant Dis. 83:788-798.

Imai, S. 1930. On the Clavariaceae of Japan II. Transactions of the Sapporo Natural History Society-Sapporo Hadubutsu Gakkai Kaihao 11:70-77.

Jung, G., Hong, S. and Maxwell, D. 2000. Genetic characterization and detection of *Typhula* species and *Typhula ishikariensis* biological species by DNA markers and sequences of transcribed ribosomal DNA. Phytopathology 91:S45.

Kawakami, A., Matsumoto, N. and Naito, S. 2004. Environmental factors influencing sporocarp formation in *T. ishikariensis*. J. Gen. Plant Pathol. 70:1-6.

Kiyomoto, R.K. and Bruehl, G.W. 1976. Sexual incompatibility and virulence in *Typhula idahoensis*. Phytopathology 66:1001-1006.

Matsumoto, N. 1997. Evolution and adaptation in snow mold fungi. Soil Microorganisms 50:13-19 (In Japanese).

Matsumoto, N. and Tajimi, A. 1990. Continuous variation within isolates of *Typhula ishikariensis* biotypes B and C associated with habitat differences. Can. J. Bot. 68:1768-1773.

Matsumoto, N. and Tajimi, A. 1991. *Typhula ishikariensis* biotypes B and C, a single biological species. Trans. Mycol. Soc. Jpn. 32:273-281.

Matsumoto, N. and Tajimi, A. 1993. Interfertility in *Typhula ishikariensis* biotypes A. Trans. Mycol. Soc. Jpn. 34:209-213.

Matsumoto, N., Sato, T. and Araki, T. 1982. Biotype differentiation in the *Typhula ishikariensis* complex and their allopathy in Hokkaido. Ann. Phytopath. Soc. Jpn. 48:275-280.

Mayr, E. 1963. Populations, species and evolution. Harvard Univ. Press, Cambridge, MA, USA.

Millet, S.M., Matsumoto, N. and Maxwell, D.P. 2001. Mating reactions of *Typhula ishikariensis* Imai complex isolates from Wisconsin golf courses. International Turfgrass Society Research Journal 9:695-700.

Petersen, R.H. and Hughes, K.W. 1999. Species and speciation in mushrooms. Development of a species concept poses difficulties. Biosci. 49:440-452.

Røed, H. 1969. On the relationship between *Typhula graminun* Karst. and *Typhula incarnata* Lasch ex. Fr. Friesia 9:219-225.

Smiley, R.W., Denoeden, P.H. and Clarke, B.B. 1994. Compendium of turfgrass diseases, 2nd ed. APS Press, St. Paul, MN, USA.