Chapter 4

Characterization of the complete internal transcribed spacer region of the nuclear ribosomal DNA of *Typhula incarnata*, the *T. ishikariensis* complex and *T. phacorrhiza*

ABSTRACT

Isolates of the *Typhula ishikariensis* complex, *T. incarnata* and *T. phacorrhiza* from around the world were characterized by sequence analyses of the complete internal transcribed spacer region (CITS) of nuclear ribosomal DNA. The hypotheses tested were that the sequence could be used (i) to identify the *Typhula* spp. and (ii) to distinguish the two biological species of the *T. ishikariensis* complex (BSI and BSII). DNA was extracted from the isolates, the CITS region was amplified by PCR and then cloned into a plasmid. Next, the plasmid was transformed into *E. coli* and allowed to multiply, thus amplifying the plasmid containing the CITS region. The plasmid was isolated from the bacteria and then the CITS region was sequenced. Percentage of sequence identity for the 44 sequences from the 30 *Typhula* spp. isolates ranged from 59 to 100% with a 0 to 30% divergence. The 26 sequences from the 20 *T. ishikariensis* isolates were 87 to 100% identical to each other with a 0 to 8% divergence. The 26 sequences from the 20 *T. ishikariensis* isolates were 59 to 78% identical to 11 sequences of five *T. incarnata* isolates, three sequences from two *T. phacorrhiza* isolates and four sequences from three unidentified *Typhula*-like isolates with a 22 to 30% divergence. These results indicated that the CITS region can be used to differentiate the *Typhula* spp. "Signature sequences" were identified that differentiate BSI and BSII of the *T. ishikariensis* complex, however, sequence analyses infer they are the same species undergoing speciation.

INTRODUCTION

Conventional methods for the identification and characterization of fungal plant pathogens sometimes fail when such fungal isolates exhibit atypical phenotypic profiles or morphological characteristics that overlap fungal species concepts. Recent advancements in DNA sequencing technology have greatly enhanced the ability of plant pathologists to identify fungal isolates. DNA sequence information is relatively objective and sequence databases[1] are readily available via the internet (Maidak et al., 1999; Benson et al., 1999). Nucleotide sequences of target DNA genes can be rapidly and accurately obtained from fungal plant pathogens (Hillis, 1996). However, DNA of known genes have not been a primary source of data for *Typhula* species characterization. This study will determine if DNA sequencing of the complete internal transcribed spacer region (CITS) of the nuclear ribosomal DNA (nrDNA) can aid in identifying *Typhula* species.

Rapid and accurate identification of *Typhula* species pathogenic to turfgrasses is an important part of managing the diseases that they cause. The sclerotia of the *Typhula* spp. are the primary diagnostic feature in the taxonomy of the genus (Remsberg, 1940; Corner, 1950; MacDonald, 1961). Unfortunately, at optimal *in vitro* conditions, sclerotia start to form 6 to 16 days for *T. incarnata* and 4 to 20 days for *T. ishikariensis* and both species only grow 2 mm per day (Sweets and Steinstra, 1980). Molecular based identification has the potential to expedite disease diagnosis, improve pathogen characterization and perhaps aid in disease management.

Furthermore, it is not easy to identify the *T. ishikariensis* complex, and the taxonomy of these fungi remains unclear (Smith et al., 1989). The dikaryon-monokaryon mating experiments (Chapter 3) revealed that there are at least two genetically different groups of the *T. ishikariensis* complex in Wisconsin golf courses (Wisconsin group 1 and Wisconsin group 2). Unfortunately, the dikaryon-monokaryon mating experiments are tedious and time

consuming. A rapid and accurate fungal identification technique is needed to expedite identification of the *T. ishikariensis* complex isolates.

Studies of the genotypic differences between *Typhula* species have centered on mating experiments involving compatibility groups (Bruehl et al., 1975; Årsvoll and Smith, 1978; Bruehl and Machtmes, 1979; Matsumoto et al., 1996). However, the only investigations utilizing molecular markers to identify snow mold pathogens are the application of randomly amplified polymorphic DNA (RAPDs) markers to detect genets belonging to *T. ishikariensis* biotype A (Matsumoto et al., 1996) and to analyze *Coprinus psychromorbidus* (sclerotial low temperature basidiomycete, SLTB) strains (Larouche et al., 1995).

An appropriate and effective method for analysis and characterization of closely related species and their boundaries is DNA sequencing of a rapidly evolving nuclear gene target (Hillis et al., 1996). As of yet, no one has characterized DNA sequences of known genes of *Typhula* species. One such target is the complete internal transcribed spacer regions (CITS) of the nuclear ribosomal RNA gene array (nrDNA, Fig. 4.1) which has proven useful for fine scale comparisons (Hillis et al., 1996). Sequences of nrDNA have been used to compare various plant taxa (Zhang et al., 1997; Alice and Campbell, 1999; Steane et al., 1999), bacterial taxa (Lee et al., 1998; Kolbert and Persing, 1999), fungal taxa (Olsen et al., 1986; White et al., 1990; Rafin et al., 1995; Chen et al., 1996), nematode taxa (Al-Banna et al., 1997), fish taxa (Sajdak and Phillips, 1997) and insect taxa (Black et al., 1997).

Also, the characterization of the CITS of nrDNA has been a useful molecular tool in plant pathogen detection and identification. Recently, this technique was used to differentiate *Fusarium* spp. (O'Donnell and Cigelinik, 1997), to detect *Phialophora gregata* in soybean tissues (Chen et al., 1996), to detect *Monosporascus* spp. (Lovic et al., 1995), to identify *Magnaporthe poae* (Bunting et al., 1996) and to characterize *Rhizoctonia solani* (Kanematsu and Naito, 1995; Salazar et al., 1999). The PCR-mediated analysis of the CITS region of nrDNA has illustrated, "a convenient approach to developing 'molecular tools' for detection of

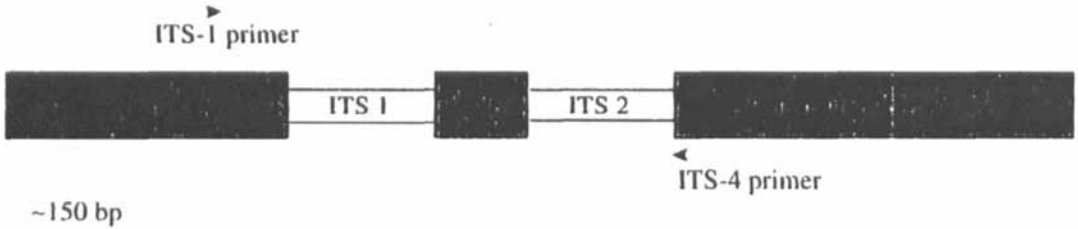


Figure 4.1. Schematic representation of the complete internal transcribed spacer (CITS) region of the nuclear ribosomal DNA (nrDNA). The shadowed boxes represent the ribosomal subunits. The arrows denote the position of the PCR sequencing primers. ITS-1 primer: (TCCGTAGGTGAACCTGCGG) and ITS-4 primer: (TCCTCCGCTTATTGATATG, White et al., 1990).

plant pathogenic fungi without necessarily having any detailed knowledge of their genome organization" (Lovic et al., 1995). Sequence analyses of the CITS region has been particularly useful in species identification (van Herwerden et al., 1999). This molecular method might offer advances in describing the genotypic differences between *T. ishikariensis*, *T. incarnata* and *T. phacorrhiza* while also clarifying the taxonomic classification of the *T. ishikariensis* complex.

The objective of this research was to genetically characterize the *T. ishikariensis* complex, *T. incarnata* and *T. phacorrhiza* isolates by utilizing sequence analysis of the CITS region of the nrDNA. Specifically, the hypotheses being tested were (i) the nucleotide sequence of the CITS of the nrDNA will vary among isolates of *T. incarnata*, the *T. ishikariensis* complex and *T. phacorrhiza* and can be used to infer phylogenetic relationships and (ii) the *T. ishikariensis* complex isolates can be separated into two groups based on nucleotide sequence of the CITS region of the nrDNA.

First, DNA was extracted from *Typhula* isolates from Wisconsin and tester isolates from around the world. The CITS region of the nrDNA was then amplified by the PCR-reaction with the CITS universal primers (Fig. 4.1, White et al., 1990). The PCR product was then inserted into a plasmid and transformed into *E. coli*. The bacteria were allowed to grow thus amplifying the plasmid DNA containing the PCR product insert. The plasmid DNA was then isolated from the bacteria. Finally, the PCR insert within the plasmid DNA was sequenced. Phylogenetic inferences were made based upon the DNA sequence of the CITS region.

Percentage of sequence identity of the CITS for the 44 sequences from the 30 *Typhula* spp. or *Typhula*-like isolates ranged from 59 to 100% with a 0 to 30% divergence. The 26 CITS sequences from the 20 *T. ishikariensis* isolates were 87 to 100% identical with a 0 to 8% divergence. The 26 sequences from the 20 *T. ishikariensis* isolates were 59 to 78% identical to 11 sequences of five *T. incarnata* isolates, three sequences from two *T. phacorrhiza* isolates

and four sequences from three unidentified *Typhula*-like isolates with a 22 to 30% divergence.

The results of the molecular identification experiments indicate that the CITS region of the nrDNA can be used to differentiate *T. incarnata*, *T. ishikariensis* and *T. phacorrhiza*. However, analysis of the CITS and the ITS 1 region of the nrDNA infer that isolates of the two *T. ishikariensis* groups (WIG1-BSI and WIG2-BSII) are the same species but perhaps are undergoing speciation or lineage divergence. Signature sequences were found within the ITS 1 region that could possibly be used to differentiate between biological species I and biological species II of the *T. ishikariensis* complex.

1. A few DNA sequence internet sites include: the DNA Data Bank of Japan (DDBJ), Mishima, Japan at http://www.ddbj.nig.ac.jp/; European Molecular Biology Laboratory, Nucleotide Sequence Database, Hinxton, UK (EMBL/EBI) at http://www.ebi.ac.uk/; GenBank, National Center for Biotechnology Information, Bethesda, Maryland at http://www.ncbi.nlm.nih.gov; and the Ribosomal Database Project II, National Science Foundation, U. S. Department of Energy and Michigan State University at http://www.cme.msu.edu/RDP/.

MATERIALS AND METHODS

Fungal Isolates

The *Typhula* isolates used in this work are listed in Table 4.1. All imported isolates were obtained according to the USDA permit PPQ-526 requirements. The isolates *T. ishikariensis* biotype A (isolates PR9-4-3 and PR7-6-7, Matusmoto and Tajimi, 1993), *T. ishikariensis* Norway group I (isolate 2-5BS-1, Matsumoto et al., 1996), *T. ishikariensis* var. *ishikariensis* (isolate 92-32-MI, Tkachenko et al., 1997) *T. ishikariensis* biotype B (isolate 35-8, Matsumoto and Tajimi, 1991), *T. ishikariensis* Norway group II (isolate 4-3S-5, Matsumoto et al., 1996) and *T. ishikariensis* var. *canadensis* (isolate Can21m, Årsvoll and Smith, 1978) were obtained from N. Matsumoto, National Institute of Agro-Environmental Sciences, Tsukuba, Japan. The isolate *T. ishikariensis* var. *canadensis* (isolate JD13) was obtained from J. D. Smith, Canada, which he collected from North Battleford golf course, Prince Albert, Canada in 1992. The isolates *T. ishikariensis* biotype A (isolate 56263, Matsumoto et al., 1982), *T. ishikariensis* var. *ishikariensis* (isolate 38649, Årsvoll and Smith, 1978), *T. ishikariensis* biotype B (isolate 56264, Matsumoto et al., 1982) and *T. idahoensis* (isolate 32642, Bruehl et al., 1975) were obtained from American Type Culture Collection, Manassas, Virginia (http://www.atcc.org/). Two unidentified *Typhula* isolates, G2 (from *Gaillardia* sp.) and G4 (from *Chenopodium alba*), were collected by C. R. Grau, University of Wisconsin, Madison in the spring of 1998. The other isolates were collected from the survey of Wisconsin golf courses (Chapter 2). Also, the isolates *T. ishikariensis* WIG2-2.136 and WIG2-2.105 were dedikaryotized by growing them on PDA amended with 2% oxgall (Matsumoto and Tronsmo, 1995) to determine if there were differences in DNA sequence of the CITS region between dikaryons and monokaryons of the same isolate.

DNA Extraction

Typhula spp. isolates were grown in 1.5-ml microcentrifuge tubes with 1 ml of potato dextrose broth for one month and the DNA was extracted as described in Protocol #3 (for small

Table 4.1. Subspecies appellations, identification codes and geographical origins of thirty *Typhula* species used in the CITS region of nrDNA characterization experiment.

***Typhula* species**	**Subspecies appellations**	**Identification code**	**Geographical origin**
Typhula ishikariensis	WIG1[a]	1.93	Wisconsin Dells, WI
Typhula ishikariensis	WIG1	2.183	Ogdensburg, WI
Typhula ishikariensis	WIG1	3.120	Woodruff, WI
Typhula ishikariensis	Biotype A	PR9-4-3	Japan
Typhula ishikariensis	Biotype A	PR7-6-7	Japan
Typhula ishikariensis	Biotype A	56263	Japan-ATCC[b]
Typhula ishikariensis	Norway Group I	2-5BS-1	Norway
Typhula ishikariensis	var. *ishikariensis*	38649	Norway-ATCC
Typhula ishikariensis	var. *ishikariensis*	92-32-MI	Russia
Typhula ishikariensis	WIG2[a]	1.31	Jefferson, WI
Typhula ishikariensis	WIG2	2.97	Stevens Point, WI
Typhula ishikariensis	WIG2	2.105	Stevens Point, WI
Typhula ishikariensis	WIG2	3.122	Woodruff, WI
Typhula ishikariensis	Biotype B	35-8	Japan
Typhula ishikariensis	Biotype B	56264	Japan-ATCC
Typhula ishikariensis	Norway Group II	4-3S-5	Norway
Typhula ishikariensis	var. *canadensis*	JD 13	Canada
Typhula ishikariensis	var. *canadensis*	Can21m	Canada
Typhula ishikariensis	WIG2	2.100A	Stevens Point, WI
Typhula idahoensis	NA[c]	32642	Washington-ATCC
Typhula incarnata	NA	1.35	Jefferson, WI
Typhula incarnata	NA	2.100B	Stevens Point, WI
Typhula incarnata	NA	2.136	Wautoma, WI
Typhula incarnata	NA	3.279	Hurley, WI
Typhula incarnata	NA	3.114	Woodruff, WI
Typhula phacorrhiza	NA	3.120B	Woodruff, WI
Typhula phacorrhiza	NA	2.230A	Marshfield, WI
Unidentified *Typhula*	NA	G2	Verona, WI
Unidentified *Typhula*	NA	G4	Arlington, WI
Unidentified *Typhula*	NA	3.129	Woodruff, WI

[a] WIG1 = Wisconsin group 1, WIG2 = Wisconsin group 2.
[b] ATCC = American Type Culture Collection.
[c] NA = not applicable.

amounts of cells, tissues, or plant leaves) of the Easy-DNA™ Kit (Invitrogen®, Carlsbad, CA; Anonymous a, 1998). Briefly, mycelia were crushed in liquid nitrogen, the cells were lysed by the addition of Solution A and subsequent incubation at 65° C. Proteins and lipids were precipitated and extracted by the addition of Solution B and chloroform. The solution was then centrifuged to separate it into two phases with a solid interface separating the two phases. The DNA was in the upper, clear aqueous phase, the proteins and lipids were in the solid interface, and the chloroform formed the lower phase. The DNA was then removed, precipitated with ethanol, and resuspended in TE buffer. In general, the total time for DNA extraction per isolate took approximately 90 minutes. The DNA was then ready for PCR or stored at +4° C for less than 12 hours.

DNA amplification

The polymerase chain reaction was performed with some modifications from a standard protocol (Anonymous, 1996). An automated thermal cycler The Rapid Cycler™ (Idaho Technologies Inc., Idaho Falls, ID) was used for the amplification reaction. Primers used for the amplification of the CITS region were ITS-1 and ITS-4, Fig. 4.1 (White et al., 1990). Amplification reactants from The Rapid Cylcer Optimizer Kit #1761 were used to make 26 μl mixtures in individual wells of a microtiter plate. The 26 μl mixtures contained 2.5 μl of Low Mg buffer [10 mM $MgCl_2$; 50 mM Tris; and 250 μg/ml bovine serum albumin (BSA)], 1 μl Ficoll, 2.5 μl dNTPs (200 μM dNTP), 2.5 μl ITS-1 primer (0.5 μM), 2.5 μl ITS-4 primer (0.5 μM), 2.5 μl genomic DNA (5 ng/μl), 2.5 μl diluted *Taq* DNA polymerase (0.4 U/10 μl *Taq* DNA polymerase (Promega, Madison, WI); and 250 μg/ml BSA) and 10 μl of distilled sterilized water (Anonymous, 1992). The 26 μl mixtures were loaded into 30 μl borosilicate capillary tubes (Idaho Technology Inc., Part #1706) and flame sealed at both ends (Anonymous, 1996). The preprogrammed Rapid Cycler™ protocol C57 was used as the cycling parameters. Protocol C57 includes 30 cycles of denaturation for 10 sec. at 94° C,

annealing for 10 sec. at 55° C and elongation for 35 sec. at 72° C (approximately 28 total min.). Each PCR product was visualized by 1.2% agarose gel electrophoresis. The minigel apparatus voltage was set at 110 mV for 20 to 30 min. Ethidium bromide was used to stain the DNA bands and then the DNA fragments were visualized by UV illumination.

Cloning

The TOPO™ TA Cloning® kit (Invitrogen, Carlsbad, CA) was used to directly insert the *Taq* polymerase-amplified PCR products into a plasmid vector. The TOPO TA Cloning Kit uses the pCR® 2.1-TOPO vector with covalently bound topoisomerase I enzyme for fast and efficient PCR cloning (total time is approximately 5 min.) (Anonymous, 1998b). Briefly, a 5 µl cloning reaction was set up containing 1 µl of fresh PCR product (10 ng/µl), 3 µl of sterile distilled water and 1 µl of pCR®-TOPO vector. The reaction was mixed gently and incubated for 5 min. at room temperature. The tube was then placed on ice while vials of TOPO10F' One Shot™ (Invitrogen, Carlsbad, CA) competent *Escherichia coli* cells were thawed on ice.

Transformation

For the transformation reaction, 2 µl of 0.5 M ß-mercaptoethanol was added to each vial of TOPO10F' One Shot™ cells of *E. coli* and gently mixed. Then 2 µl of the TOPO-cloning reaction was added to a vial of TOPO10F' One Shot™ cells and gently mixed. The vial was then incubated on ice for 30 min. The TOPO10F' One Shot™ transformation reaction was then heat shocked for 30 sec. at 42° C without shaking, followed by incubation on ice for 2 min. To grow the competent cells, 250 µl of room temperature SOC medium was placed in the vials and gently mixed. The vials were tightly capped and shaken horizontally at 37° C for 30 min. Then 50, 75 and 100 µl from each transformation was spread evenly on Luria-Bertani (LB) agar plates containing 50 µg/ml ampicillin, 40 µl of 40 mg/ml X-Gal (5-Bromo-4-chloro-3-indolyl-ß-D-galactopyranoside) and 40 µl of 100 mM IPTG (isopropyl-ß-D-thiogalactopyranoside) that had been warmed to 37° C for at least 1 hr. (Anonymous, 1998b). The LB agar plates were inverted and incubated overnight at 37° C. The next day, 10 white

colonies were selected for analysis. The white colonies were cultured in 2 ml of LB broth (Anonymous, 1998b) containing 50 μg/ml ampicillin (LBa) and shaken in a 37° C water bath for 2 to 4 hours. For stored samples of the clones, 100 μl of the culture was placed in 1 ml of LBa and incubated overnight in a 37° C water bath. The next day, 220 μl of sterile glycerol was added to the tube, briefly vortexed and then stored immediately in a -80° C freezer. To multiply the competent cells containing the PCR inserts, 100 μl of each culture was placed in 10 tubes containing 1.5 ml of LBa and incubated and shaken overnight in a 37° C water bath.

Plasmid DNA isolation

The plasmid DNA was isolated from the cultures by using the S. N. A. P.™ Miniprep Kit (Invitrogen, Carlsbad, CA). The S. N. A. P. Miniprep Kit is a plasmid DNA purification system designed to yield ultra-pure plasmid DNA for automated sequencing in 25 min. (Anonymous, 1998c). Briefly, the ten overnight cultures of each clone were centrifuged for 5 min. at 4,000 rpm at room temperature (RT) into two 1.5-ml microcentrifuge tubes (5 culture tubes per microcentrifuge tube). The supernatant was removed and the pellet was resuspended in 150 μl of Resuspension Buffer by briefly vortexing. Then 150 μl of Lysis Buffer was added and the tube was gently inverted 5 to 6 times and incubated for 3 minutes at RT. Next, 150 μl of ice-cold Precipitation Salt was added and then the tube was inverted 6 to 8 times to mix components. The tube was then centrifuged at RT at 14,000 rpm for 5 min. The supernatant from the two microcentrifuge tubes were then pipetted into one sterile microcentrifuge tube while the pellets were discarded. To the supernatant, 600 μl of Binding Buffer was added and the tube inverted 5 to 6 times. The entire solution was then poured onto the S. N. A. P.™ Mini-prep Column/Collection Tube. The S. N. A. P.™ Mini-prep Column/Collection Tube was then centrifuged at RT at 1,000 rpm for 30 sec. The column flow through liquid was discarded. To the column, 500 μl of Wash Buffer was added and again centrifuged RT at 1,000 rpm for 30 sec. Following this step, 900 μl of Final Wash Buffer was added and then centrifuged at RT at 1,000 rpm for 30 sec. The resin in the column

was dried by centrifuging at maximum speed for one min. To elute the plasmid DNA, the resin column was transferred to a sterile microcentrifuge tube and 60 μl of sterile distilled water was added onto the column. The wet resin column was then incubated at RT for at least 3 min. Finally, the plasmid DNA was eluted by centrifugation at maximum speed for 30 sec.

To check the plasmid DNA for the PCR inserts, an *Eco*RI digestion was performed on the S. N. A. P. minipreped DNA. This reaction mixture contained 6.5 μl of sterile distilled water, 1 μl of Dellaporta buffer (Dellaporta et al., 1983), 2 μl of S. N. A. P. Minipreped DNA, and 0.5 μl of *Eco*RI enzyme. The digestion reaction was incubated and shaken at 37° C overnight. Gel electrophoresis (as described previously) was used to visualize the presence of the PCR insert. A TKO 100 Dedicated Mini Fluorometer (Hoefer Scientific Instruments, San Francisco, CA) was used to determine and/or adjust the plasmid DNA concentration to 200 to 250 ng/ml. Automated (fluorescence) DNA sequence analysis was performed on 10 μl of 200 to 250 ng/ml plasmid DNA at the DNA Sequence Laboratory in the Nucleic Acid and Protein Facility at the University of Wisconsin Biotechnology Center (UWBC), University of Wisconsin-Madison, WI. The M13F and M13R primers were used to sequence the 700 to 800 bp PCR product insert.

Sequence Analysis

Sequence files from the UWBC were viewed, evaluated and corrected within the EditView™ Version 1.0 (Automated DNA Sequence Viewer) and SeqEd™ Version 1.03 (DNA Sequence Editor) software (Applied Biosystem Inc., Perkin-Elmer Corp.) according to on-line help and the manuals provided by the UWBC (Anonymous, 1995; Anonymous, 1998d). Corrected SeqEd™ sequences were exported as Genetics Computer Group™ files (GCG, Madison, WI) and the percent identity comparisons were determined by the GAP function within the GCG software. The GCG files were then imported into EditSeq™ software (DNAStar, Madison, WI) and saved as EditSeq™ files. The EditSeq™ files were then imported into the Megalign™ software (DNAStar, Madison, WI). The sequence files

were aligned using the Clustal method and the percentages of sequence divergence were calculated (Table 4.9). The alignment was exported as a NEXUS file and phylogenetic trees were constructed with PAUP Version 4.0 software (Center for Biodiversity, Illinois Natural History Survey, Champaign, IL). Two types of tree-making methods were employed: distance matrix methods and maximum parsimony methods (Felsenstein, 1988). In the distance matrix methods, evolutionary distances (the number of nucleotide substitutions separating two taxonomic units) were computed for all pairs of taxa, and a phylogenetic tree was constructed from the total character differences. In maximum parsimony methods, the nucleotides at each site were used, and the shortest pathway leading to these character states was chosen as the phylogenetic tree.

RESULTS

The CITS region of the nrDNA region of the *Typhula* species ranged in size from 719 to 832 base pairs (bp) in length (Tables 4.2, 4.3 and 4.4). The CITS region length for *T. ishikariensis* ranged from 772-803 bp (Fig. 4.2), for *T. incarnata* 814-832 bp (Fig. 4.3), for *T. phacorrhiza* (Fig. 4.4) and for the unidentified *Typhula*-like fungi 709-798 bp (Fig. 4.4). There was a small difference (2-3 bp) between the dikaryotic isolate *T. ishikariensis* 2.105D (sequence 16 = 772 bp) and the monokaryotic isolates 2.105M1 (sequence 17 = 774 bp) and 2.105M2 (sequence 18 = 775 bp), Fig. 4.2. Also, there was a larger difference (4 to 13 bp) between the dikaryotic *T. incarnata* 2.136D.1 (sequence 33 = 828 bp) and the monokaryotic *T. incarnata* 2.136.2 (sequence 34 = 832 bp) and 2.136.3 (sequence 35 = 815 bp), Fig. 4.3. Furthermore, there were small differences in CITS region bp length between clones of the same isolate (Tables 4.2 and 4.4): sequence 8 and 9 = 3 bp difference; sequence 10 and 11 = 1 bp difference; sequence 12 and 13 = 4 bp difference; and sequence 41 and 42 (3 bp difference). The sequences 39 and 40 were both 721 bp in length (Table 4.4). There were greater differences between different DNA extractions of the same isolate (Tables 4.2 and 4.3): sequences 5 and 6 varied by 13 bp; sequences 16, 17 and 18 varied from 1 to 3 bp; sequences 27, 28 and 29 varied from 1 to 3 bp; and sequences 33, 34 and 35 varied from 4 to 17 bp.

Also, there were bp length differences of the three parts of the CITS region (ITS 1, 5.8 S and ITS 2 regions; Tables 4.5, 4.6 and 4.7). The ITS 1 region bp length ranged from 282 to 307 bp for the *T. ishikariensis* complex sequences, 270 to 286 bp for the *T. incarnata* sequences, 240 to 241 bp for the *T. phacorrhiza* sequences and 246 to 271 bp for the unidentified *Typhula* spp. The 5.8 S region ranged from 147 to 159 bp for the *T. ishikariensis* complex sequences, 149 to 156 bp for the *T. incarnata* sequences, 155 bp for the *T. phacorrhiza* sequences and 142 to 155 bp for the unidentified *Typhula* spp. sequences. The ITS 2 region ranged from 239 to 254 bp for the *T. ishikariensis* complex sequences, 302 to

Table 4.2. Sequence number, karyotic state, sequence length and Genbank accession number of the complete internal transcribed spacer regions (CITS) of the nuclear ribosomal DNA of *Typhula ishikariensis* from Wisconsin, Canada, Japan, Norway and Russia.

Sequence Number	*Typhula ishikariensis* isolate	Identification Code	Sequence length of CITS (bp)	Genbank accession number [a]
01	*T. ishikariensis* WIG1	1.93**[b]	785	AF193347
02	*T. ishikariensis* WIG1	2.183**	789	AF193348
03	*T. ishikariensis* WIG1	3.120**	790	AF193349
04	*T. ishikariensis* Biotype A	PR9-4-3*	793	AF193350
05	*T. ishikariensis* Biotype A	PR7-6-7.1[c]*	790	AF193351
06	*T. ishikariensis* Biotype A	PR7-6-7.2[c]*	803	AF193352
07	*T. ishikariensis* Biotype A	56263**	803	AF193353
08	*T. ishikariensis* Norway group I	2-5BS-1.1[d]*	785	AF193354
09	*T. ishikariensis* Norway group I	2-5BS-1.2[d]*	785	AF193355
10	*T. ishikariensis* var. *ishikariensis*	38649.1[d]*	783	AF193356
11	*T. ishikariensis* var. *ishikariensis*	38649.2[d]*	784	AF193357
12	*T. ishikariensis* var. *ishikariensis*	92-32-MI.1[d]**	789	AF193358
13	*T. ishikariensis* var. *ishikariensis*	92-32-MI.2[d]**	785	AF193359
14	*T. ishikariensis* WIG2	1.31**	769	AF193360
15	*T. ishikariensis* WIG2	2.97**	777	AF193361
16	*T. ishikariensis* WIG2	2.105D[c]**	772	AF193362
17	*T. ishikariensis* WIG2	2.105M1[c]*	774	AF193363
18	*T. ishikariensis* WIG2	2.105M2[c]*	775	AF193364
19	*T. ishikariensis* WIG2	3.122**	776	AF193365
20	*T. ishikariensis* Biotype B	35-8*	794	AF193366
21	*T. ishikariensis* Biotype B	56264**	795	AF193367
22	*T. ishikariensis* Norway group II	4-3S-5*	774	AF193368
23	*T. ishikariensis* var. *canadensis*	JD 13**	774	AF193369
24	*T. ishikariensis* var. *canadensis*	Can21m*	775	AF193370
25	*T. ishikariensis* WIG2	2.100A**	775	AF193371
26	*T. idahoensis*	32642*	784	AF193372

[a] Genbank: http://www.ncbi.nlm.nih.gov.

[b] *= monokaryotic isolate, ** = dikaryotic isolate.

[c] DNA sequence obtained from different DNA extractions of the same isolate.

[d] DNA sequence obtained from different clones of the same DNA extraction.

Table 4.3. Sequence number, karyotic state, sequence length and Genbank accession number of the complete internal transcribed spacer regions (CITS) of the nuclear ribosomal DNA of *Typhula incarnata* isolates from Wisconsin.

Sequence Number	*Typhula incarnata*	Identification Code	Sequence length of CITS (bp)	Genbank accession number[a]
27	*T. incarnata*	1.35.1[b**c]	820	AF193373
28	*T. incarnata*	1.35.2[b**]	821	AF193374
29	*T. incarnata*	1.35.3[b**]	818	AF193375
30	*T. incarnata*	2.100B.1[b**]	821	AF193376
31	*T. incarnata*	2.100B.2[b**]	826	AF193377
32	*T. incarnata*	2.100B.3[b**]	814	AF193378
33	*T. incarnata*	2.136D.1[b**]	828	AF193379
34	*T. incarnata*	2.136M.2[b*]	832	AF193380
35	*T. incarnata*	2.136M.3[b*]	815	AF193381
36	*T. incarnata*	3.279**	818	AF193382
37	*T. incarnata*	3.114**	819	AF193383

[a] Genbank: http://www.ncbi.nlm.nih.gov.

[b] DNA sequence obtained from different DNA extractions of the same isolate.

[c] * = monokaryotic isolate, ** = dikaryotic isolate.

Table 4.4. Sequence number, karyotic state, sequence length and Genbank accession number of the complete internal transcribed spacer regions (CITS) of the nuclear ribosomal DNA of *Typhula phacorrhiza* and unidentified *Typhula* species from Wisconsin.

Sequence Number	*Typhula* isolate	Identification Code[a]	Sequence length of CITS (bp)	Genbank accession numbers[b]
38	*T. phacorrhiza*	3.120B	720	AF1933
39	*T. phacorrhiza*	2.230A.1[c]	721	AF1933
40	*T. phacorrhiza*	2.230A.2[c]	721	AF1933
41	Unidentified *Typhula*	G2.1[c]	798	AF1933
42	Unidentified *Typhula*	G2.2[c]	795	AF1933
43	Unidentified *Typhula*	G4	709	AF1933
44	Unidentified *Typhula*	3.129	719	AF1933

[a] All isolates were dikaryotic.

[b] Genbank: http://www.ncbi.nlm.nih.gov.

[c] DNA sequence obtained from different clones of the same DNA extraction.

Table 4.5. Base pair length of the complete internal transcribed spacer region (CITS), the internal transcribed spacer region 1 (ITS 1), the 5.8 S region and the internal transcribed spacer region 2 (ITS 2) of the *Typhula ishikariensis* complex sequences.

Sequence number[a]	CITS length	Complete ITS 1 length	Complete 5.8 S length	Complete ITS 2 length
1	785	298	150	246
2	789	298	153	247
3	790	297	155	247
4	793	300	155	247
5	790	298	154	247
6	803	301	159	252
7	803	307	158	247
8	785	297	147	247
9	785	298	154	247
10	783	291	152	249
11	784	294	152	247
12	789	296	155	247
13	785	297	151	246
14	769	283	151	244
15	777	286	157	243
16	772	284	154	243
17	774	282	158	243
18	775	285	156	243
19	776	290	156	239
20	794	294	155	254
21	795	295	155	254
22	774	285	155	243
23	774	285	155	243
24	775	286	155	243
25	775	287	155	242
26	784	296	153	244
range	772-803	282-307	147-159	239-254

[a] See Table 4.2 for specific information about each sequence.

Table 4.6. Base pair length of the complete internal transcribed spacer region (CITS), the internal transcribed spacer region 1 (ITS 1), the 5.8 S region and the internal transcribed spacer region 2 (ITS 2) of the *T. incarnata* isolates (sequences 27 to 37).

Sequence number[a]	CITS length	Complete ITS 1 length	Complete 5.8 S length	Complete ITS 2 length
27	820	271	154	304
28	821	271	155	304
29	818	271	153	303
30	821	271	155	304
31	826	276	156	303
32	814	270	150	303
33	828	277	156	304
34	832	286	152	303
35	815	272	149	303
36	818	271	154	302
37	819	271	154	303
range	814-832	270-286	149-156	302-304

[a] See Table 4.3 for specific information about each sequence.

Table 4.7. Base pair length of the complete internal transcribed spacer region (CITS), the internal transcribed spacer region 1 (ITS 1), the 5.8 S region and the internal transcribed spacer region 2 (ITS 2) of the *T. phacorrhiza* isolates (sequences 38 to 40) and unidentified *Typhula* isolates (sequences 41-44).

Sequence number[a]	CITS length	Complete ITS 1 length	Complete 5.8 S length	Complete ITS 2 length
38	720	240	155	234
39	721	241	155	234
40	721	241	155	234
41	798	271	144	292
42	795	271	142	291
43	709	246	145	229
44	719	246	155	230
range	709-795	240-271	142-155	230-292

[a] See Table 4.4 for specific information about each sequence.

304 bp for the *T. incarnata* sequences, 234 bp for the *T. phacorrhiza* sequences and 229 to 292 bp for the unidentified *Typhula* spp. sequences.

The range of percentage of sequence identities of the CITS between the 44 *Typhula* sequences are presented in Table 4.8 and the matrices are presented in Appendix C (Tables C.1, C.2, C.3 and C.4. The percent identities of the *T. incarnata* sequences for pairwise comparison ranged from 92 to 100% within the species and from 61 to 78% outside the species. Sequences 41 and 42 were 91 to 97% identical to the *T. incarnata* sequences (Table 4.8), this indicates that the unidentified isolate G.2 is (or closely related to) *T. incarnata*. The *T. ishikariensis* sequences were separated into two biological species (TISH I and TISH II) and analyzed. The percent identities of the TISH I and TISH II sequences ranged from 87 to 100% within the species and from 65 to 74% outside the species. The three *T. phacorrhiza* sequences (38, 39 and 40) were 99 to 100% identical for pairwise comparisons within the species and 63 to 72% outside the species. The unidentified *Typhula* sequences (43 and 44) were 59 to 71% percent identical to all the other sequences.

The percent divergence of the sequence pair distances of the *Typhula* spp. using the clustal method with weighted residue weight table is presented in Table 4.9. The percent divergence ranged from 0 to 8% within each species and from 22 to 31% for outside species comparisons. Sequences 41 and 42 were 1 to 2% divergent from the *T. incarnata* sequences (27-37), and thus these unknown *Typhula* spp. are *T. incarnata*.

The sequences of the CITS were aligned by the clustal method within the DNAStar software and are presented in Figs. 4.2 to 4.16. Unique sequence differences between the two biological species of the *T. ishikariensis* complex are apparent within the ITS 1 region and will be discussed later (Figs. 4.4 and 4.5). The partial sequences of the 18 S (Fig. 4.2) and the 28 S region (Figs. 4.15 and 4.16) are identical for all sequences, while the 5.8 S region is highly conserved but not identical (Figs. 4.7 to 4.10). For the species *T. incarnata* and *T. phacorrhiza*, there are some unique sequence areas within the ITS 1 and ITS 2 regions.

Table 4.8. Percentage of sequence identity in the complete internal transcribed spacer region (CITS) between *Typhula ishikariensis* biological species I, *T. ishikariensis* biological species II, *T. incarnata*, *T. phacorrhiza* and the unidentified *Typhula* spp.

Typhula isolates[a]	Sequence numbers	TISH I	TISH II	TIN	TP	T? (41-42)	T? (43-44)
TISH I	1-13, 26	89-100	87-98	61-76	67-72	65-73	60-68
TISH II	14-25	X	95-100	59-78	68-72	68-74	59-67
TIN	27-37	X	X	92-100	65-71	91-97	62-71
TP	38-40	X	X	X	99-100	63-68	63-69
T?	41-42	X	X	X	X	99	64-67
T?	43-44	X	X	X	X	X	92

[a] TISH I = *Typhula ishikariensis* biological species I, TISH II = *T. ishikariensis* biological species II, TIN = *T. incarnata*, TP = *T. phacorrhiza* and T? = unidentified *Typhula* species.

Table 4.9. Percentage of divergence in the complete internal transcribed spacer region (CITS) between *Typhula ishikariensis* biological species I, *T. ishikariensis* biological species II, *T. incarnata*, *T. phacorrhiza* and the unidentified *Typhula* spp.

Typhula isolates[a]	Sequence numbers	TISH I	TISH II	TIN	TP	T? (41-42)	T? (43-44)
TISH I	1-13, 26	0-8	3-8	22-28	22-25	23-27	27-30
TISH II	14-25	X	0-5	22-25	22-24	23-25	27-30
TIN	27-37	X	X	0-2	29-32	1-2	28-31
TP	38-40	X	X	X	0-1	30	27-31
T?	41-42	X	X	X	X	0-1	28-30
T?	43-44	X	X	X	X	X	0-4

[a] TISH I = *Typhula ishikariensis* biological species I, TISH II = *T. ishikariensis* biological species II, TIN = *T. incarnata*, TP = *T. phacorrhiza* and T? = unidentified *Typhula* species.

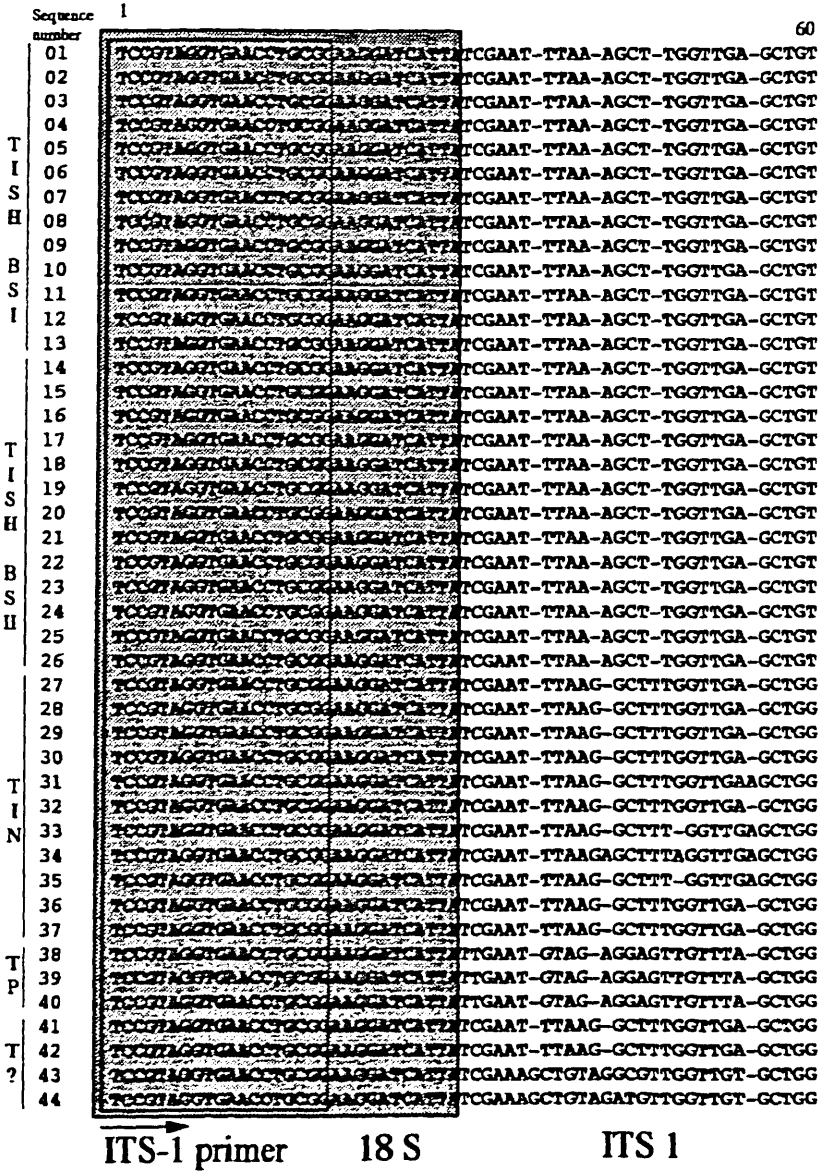
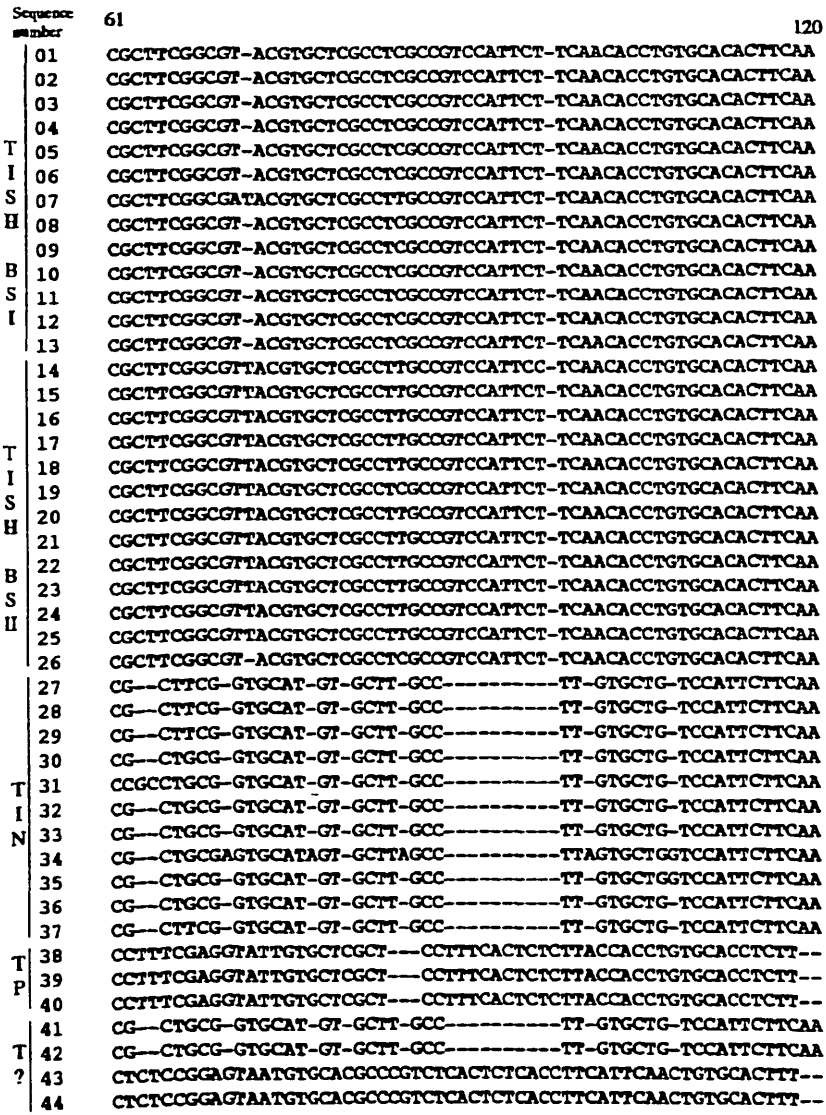


Figure 4.2. Alignment of the complete internal transcribed spacer region (CITS) of the 44 *Typhula* sequences (base pairs 1 to 60) using the Clustal method with weighted residue weight table. TISH BSI and BSII = *T. ishikariensis* biological species I and II, TIN = *T. incarnata*, TP = *T. phacorrhiza* and T? = unknown *Typhula* spp.



ITS 1

Figure 4.3. Alignment of the complete internal transcribed spacer region (CITS) of the 44 *Typhula* sequences (base pairs 61 to 120) using the Clustal method with weighted residue weight table. TISH BSI and BSII = *T. ishikariensis* biological species I and II, TIN = *T. incarnata*, TP = *T. phacorrhiza* and T? = unknown *Typhula* spp.

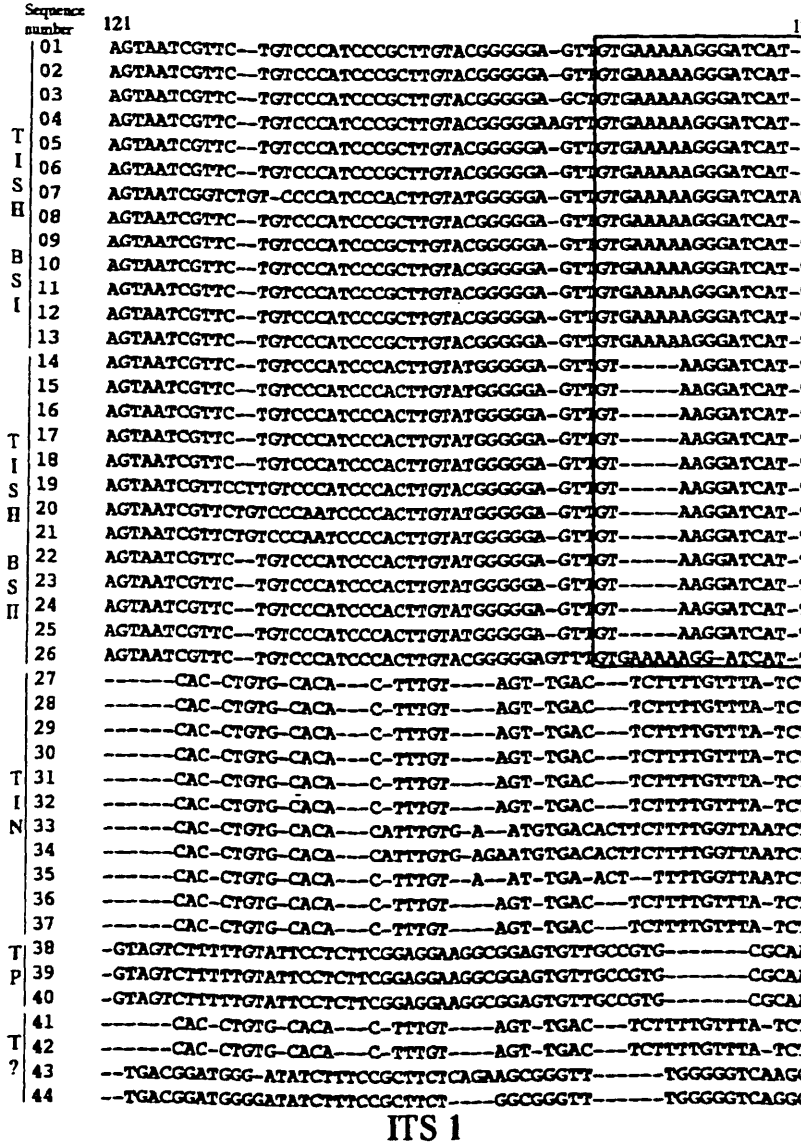


Figure 4.4. Alignment of the complete internal transcribed spacer region (CITS) of the 44 *Typhula* sequences (base pairs 121 to 180) using the Clustal method with weighted residue weight table. *Typhula ishikariensis* "signature sequence" indicated by box. TISH BSI and BSII = *T. ishikariensis* biological species I and II, TIN = *T. incarnata*, TP = *T. phacorrhiza* and T? = unknown *Typhula* spp.

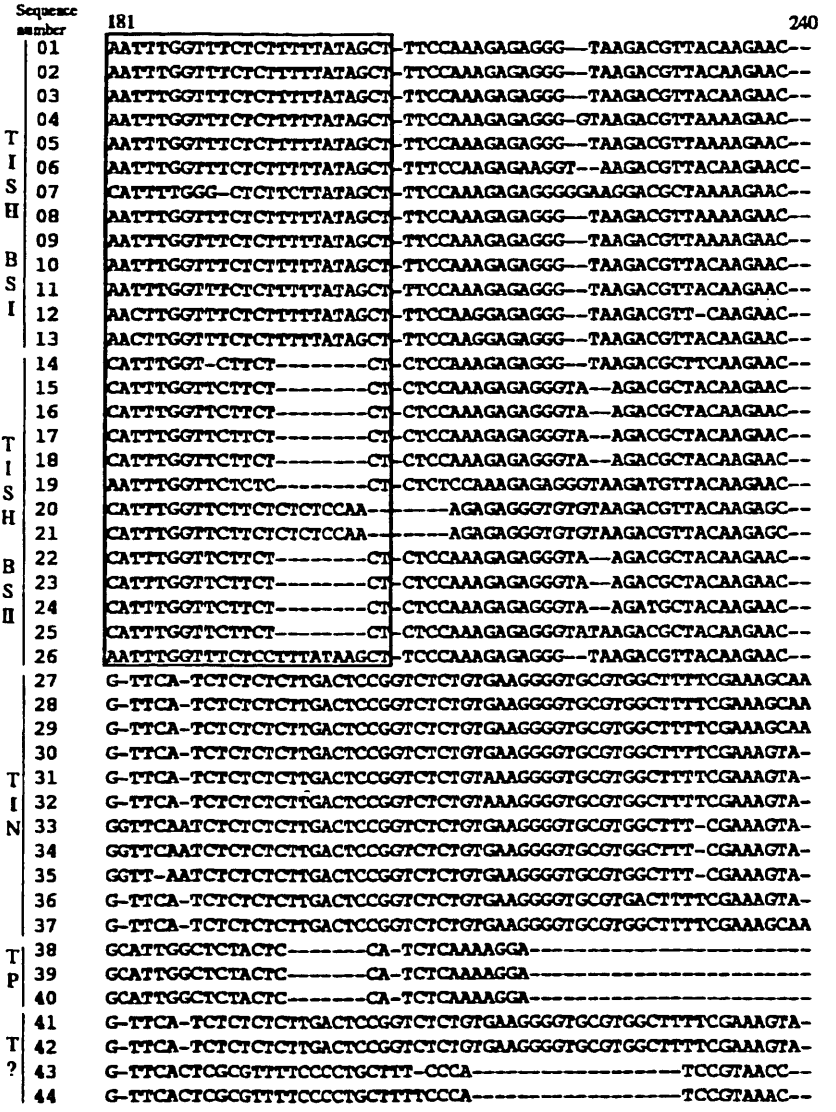
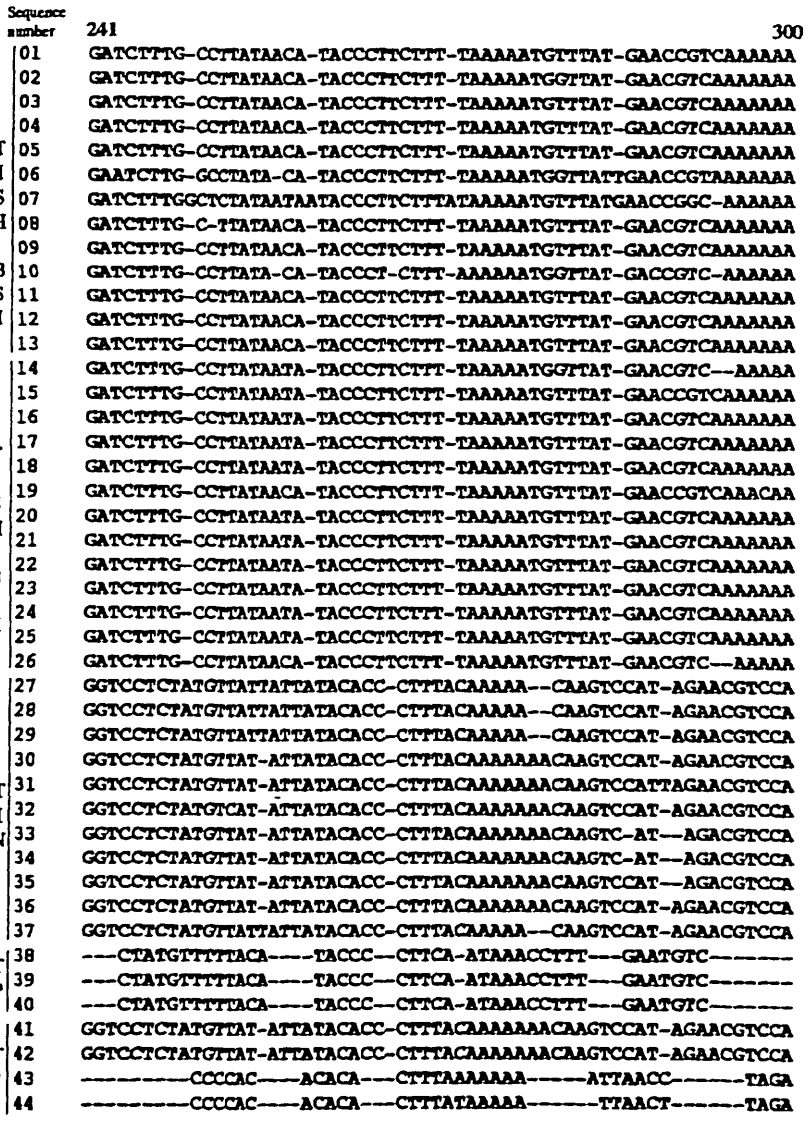


Figure 4.5. Alignment of the complete internal transcribed spacer region (CITS) of the 44 *Typhula* sequences (base pairs 181 to 240) using the Clustal method with weighted residue weight table. *Typhula ishikariensis* "signature sequence" indicated by box. TISH BSI and BSII = *T. ishikariensis* biological species I and II, TIN = *T. incarnata*, TP = *T. phacorrhiza* and T? = unknown *Typhula* spp.



ITS 1

Figure 4.6 Alignment of the complete internal transcribed spacer region (CITS) of the 44 *Typhula* sequences (base pairs 241 to 300) using the Clustal method with weighted residue weight table. TISH BSI and BSII = *T. ishikariensis* biological species I and II, TIN = *T. incarnata*, TP = *T. phacorrhiza* and T? = unknown *Typhula* spp.

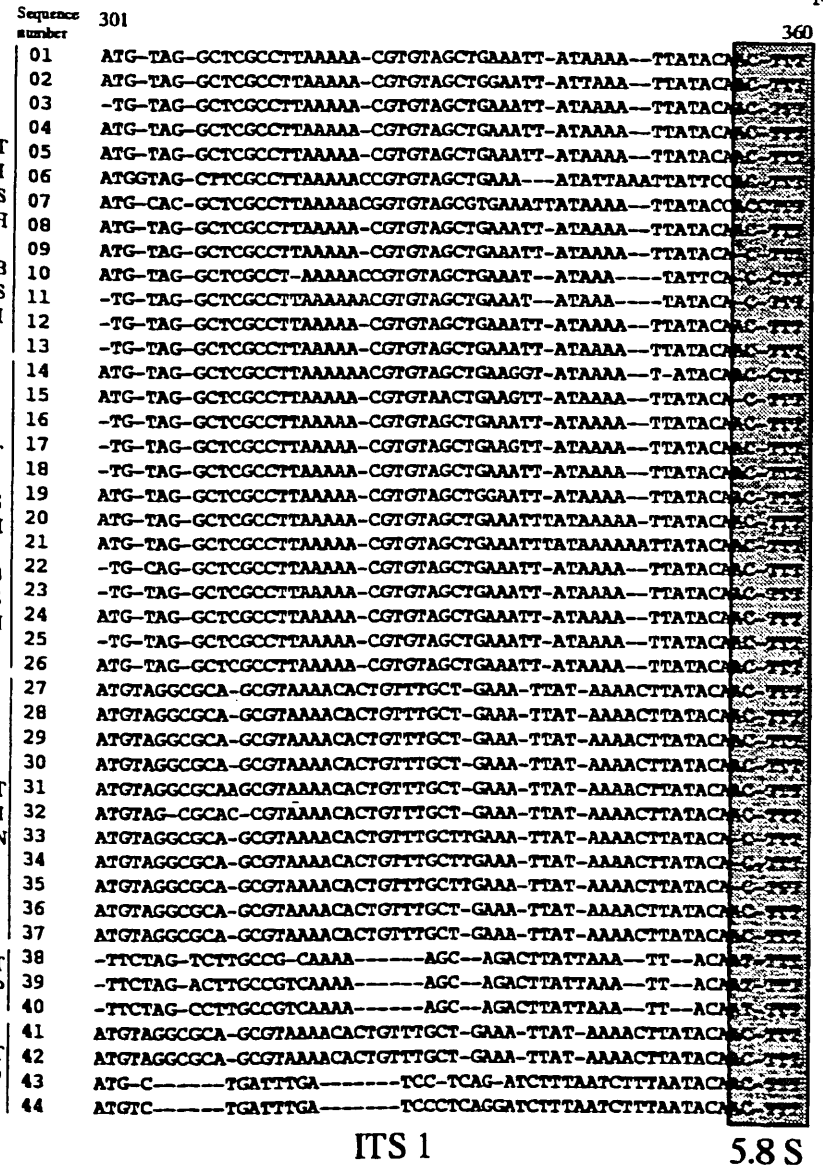


Figure 4.7. Alignment of the complete internal transcribed spacer region (CITS) of the 44 *Typhula* sequences (base pairs 301 to 360) using the Clustal method with weighted residue weight table. TISH BSI and BSII = *T. ishikariensis* biological species I and II, TIN = *T. incarnata*, TP = *T. phacorrhiza* and T? = unknown *Typhula* spp.

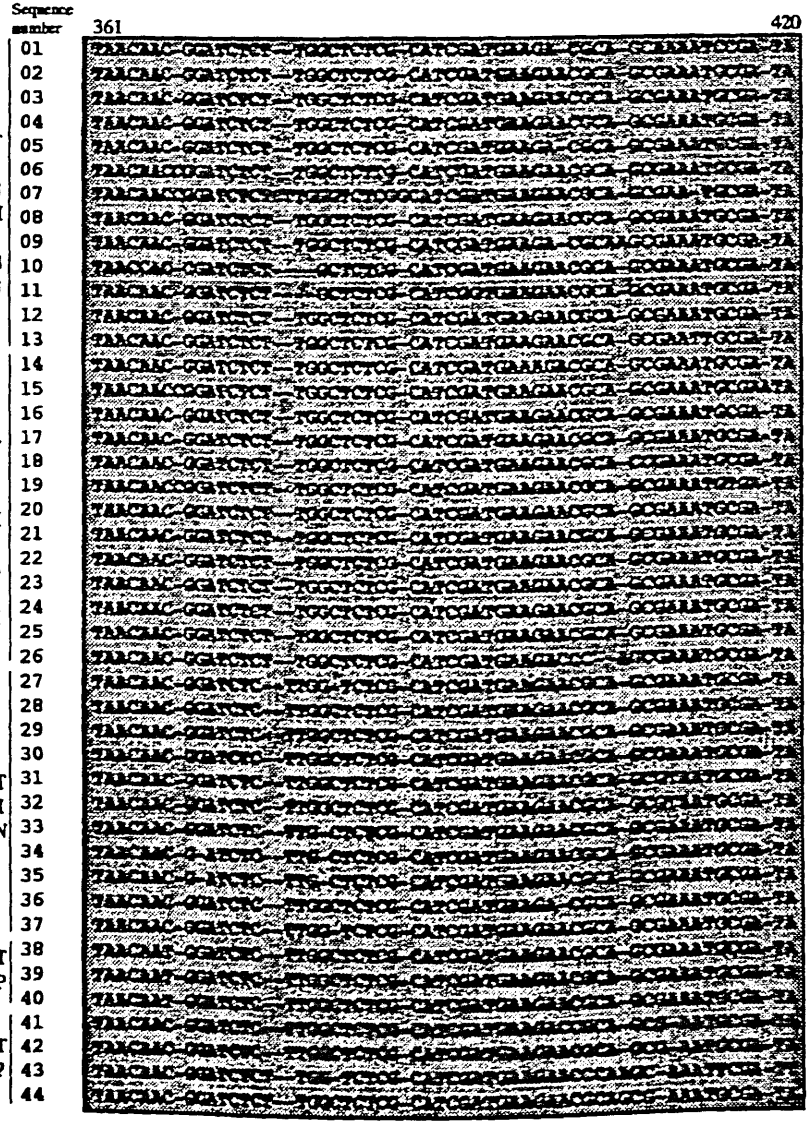


Figure 4.8. Alignment of the complete internal transcribed spacer region (CITS) of the 44 *Typhula* sequences (base pairs 361 to 420) using the Clustal method with weighted residue weight table. TISH BSI and BSII = *T. ishikariensis* biological species I and II, TIN = *T. incarnata*, TP = *T. phacorrhiza* and T? = unknown *Typhula* spp.

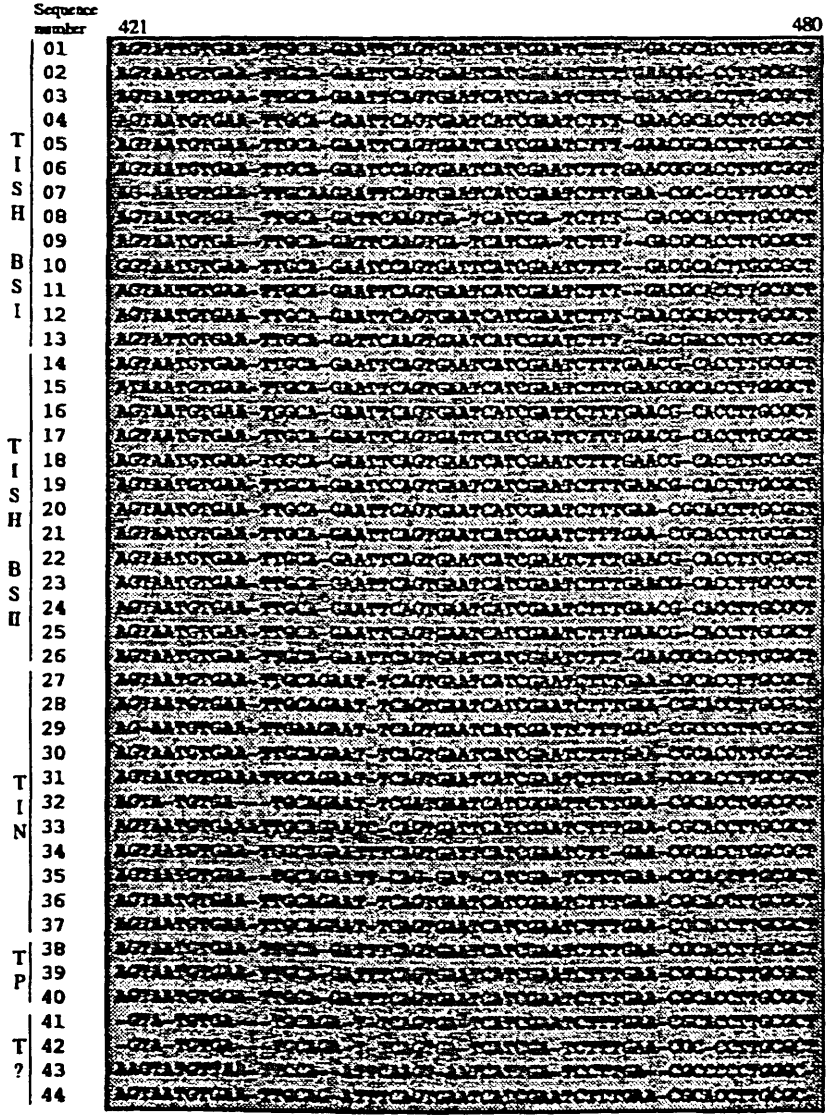


Figure 4.9. Alignment of the complete internal transcribed spacer region (CITS) of the 44 *Typhula* sequences (base pairs 421 to 480) using the Clustal method with weighted residue weight table. TISH BSI and BSII = *T. ishikariensis* biological species I and II, TIN = *T. incarnata*, TP = *T. phacorrhiza* and T? = unknown *Typhula* spp.

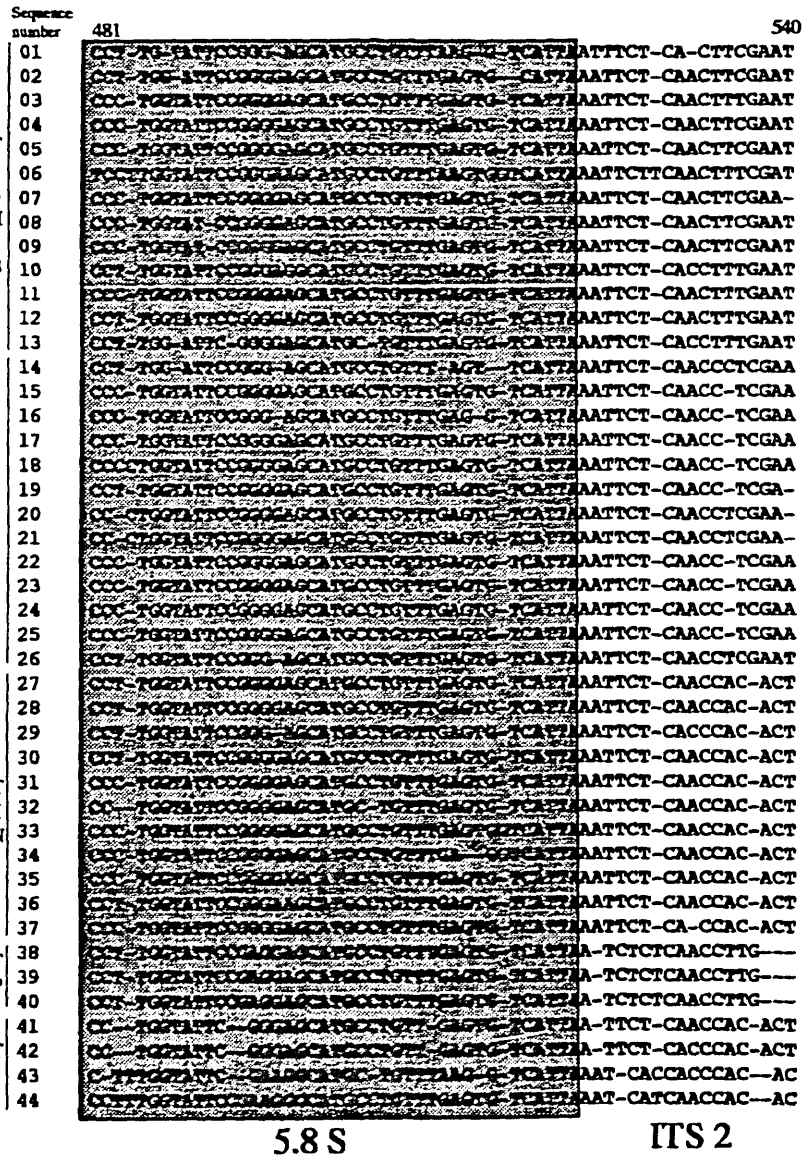
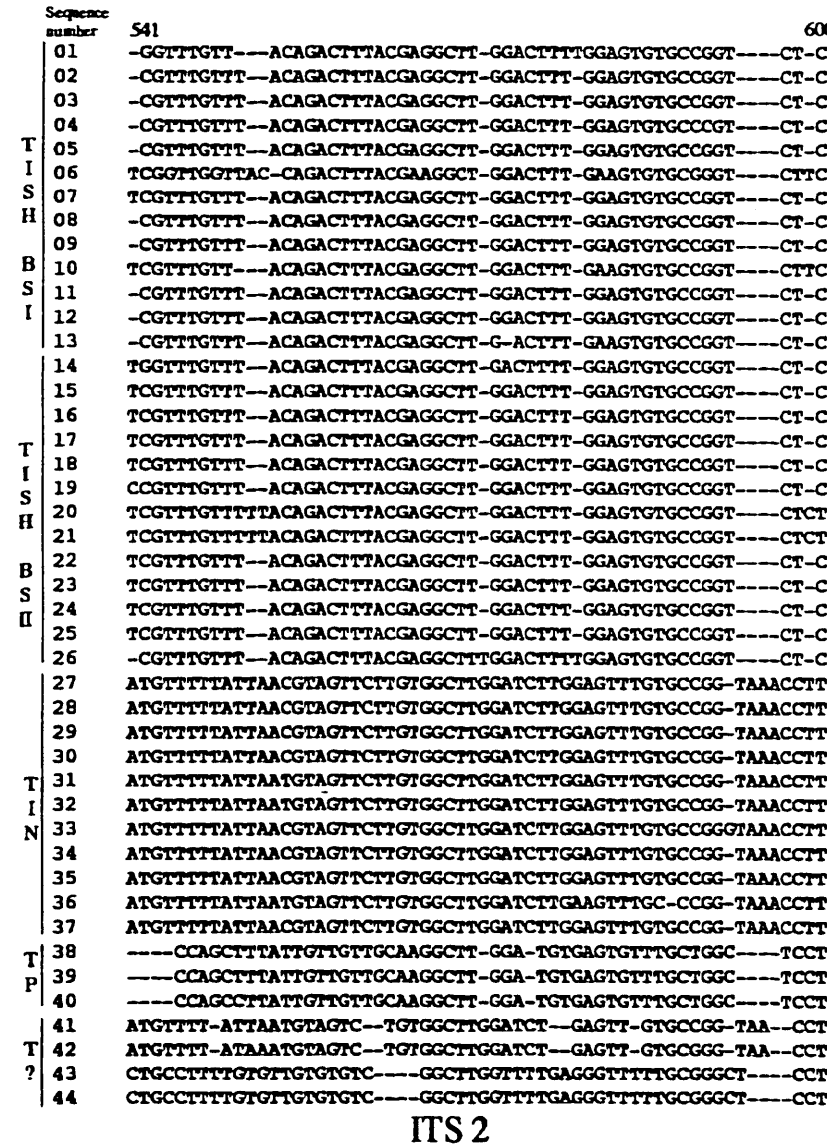


Figure 4.10. Alignment of the complete internal transcribed spacer region (CITS) of the 44 *Typhula* sequences (base pairs 481 to 540) using the Clustal method with weighted residue weight table. TISH BSI and BSII = *T. ishikariensis* biological species I and II, TIN = *T. incarnata*, TP = *T. phacorrhiza* and T? = unknown *Typhula* spp.



ITS 2

Figure 4.11. Alignment of the complete internal transcribed spacer region (CITS) of the 44 *Typhula* sequences (base pairs 541 to 600) using the Clustal method with weighted residue weight table. TISH BSI and BSII = *T. ishikariensis* biological species I and II, TIN = *T. incarnata*, TP = *T. phacorrhiza* and T? = unknown *Typhula* spp.

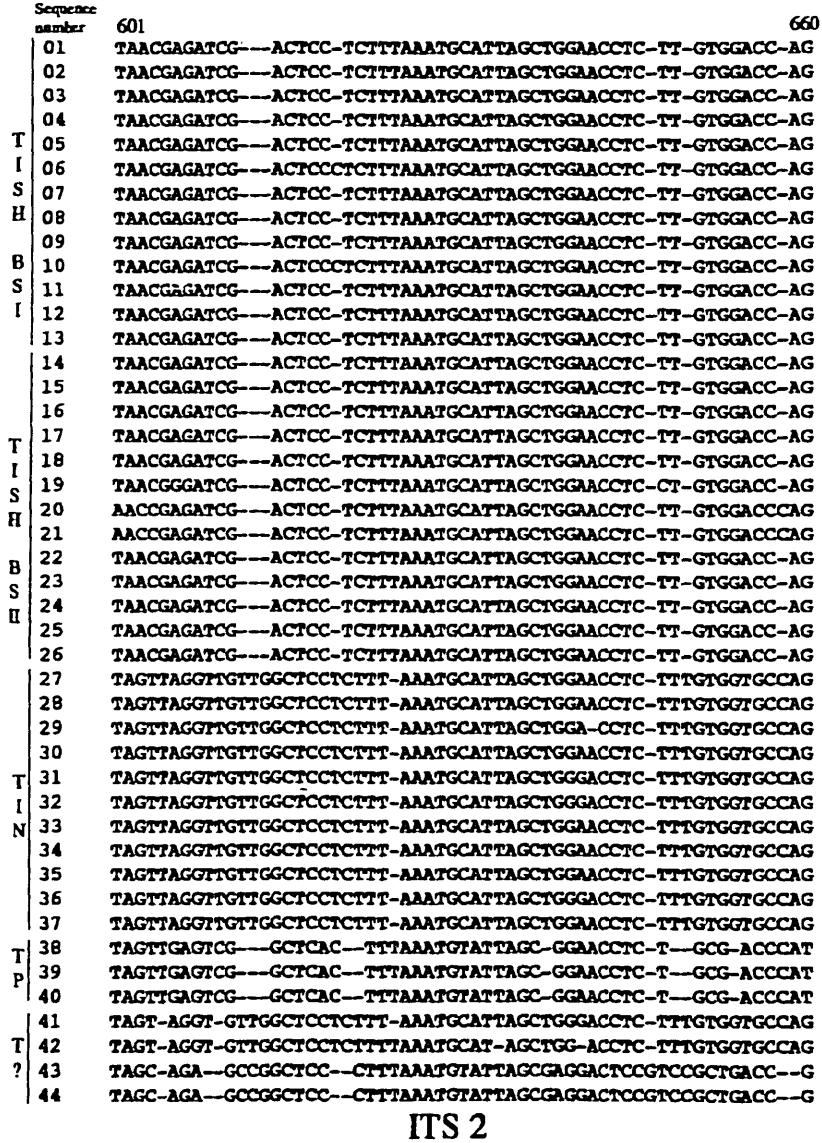


Figure 4.12. Alignment of the complete internal transcribed spacer region (CITS) of the 44 *Typhula* sequences (base pairs 601 to 660) using the Clustal method with weighted residue weight table. TISH BSI and BSII = *T. ishikariensis* biological species I and II, TIN = *T. incarnata*, TP = *T. phacorrhiza* and T? = unknown *Typhula* spp.

	Sequence number	661	720
	01	ACTACGGTGTGATAAT-TATCTACGCTGTTGGTC-TTGTGAAGCA-CTTTATTGTTACGA	
	02	ACTACGGTGTGATAAT-TATCTACGCTGTTGGTC-TTGTGAAGCA-CTTTATTGTTACGA	
	03	ACTACGGTGTGATAAT-TATCTACGCTGTTGGTC-TTGTGAAGCA-CTTTATTGTTACGA	
	04	ACTACGGTGTGATAAT-TATCTACGCTGTTGGTC-TTGTGAAGCA-CTTTATTGTTACGA	
T	05	ACTACGGTGTGATAAT-TATCTACGCTGTTGGTC-TTGTGAAGCA-CTTTATTGTTACGA	
I	06	ACTACGGTGTGATAAT-TATCTACGCTGTTGGTC-CTGTGAAGCA-CTTTATTGTTACGA	
S	07	ACTACGGTGTGATAAT-TATCTACGCTGTTGGTC-TTGTGAAGCA-CTTTATTGTTACGA	
H	08	ACTACGGTGTGATAAT-TATCTACGCTGTTGGTC-TTGTGAAGCA-CTTTATTGTTACGA	
	09	ACTACGGTGTGATAAT-TATCTACGCTGTTGGTC-TTGTGAAGCA-CTTTATTGTTACGA	
B	10	ACTACGGTGTGATAAT-TATCTACGCTGTTGGTC-TTGTGAAGCA-CTTTATTGTTACGA	
S	11	ACTACGGTGTGGTAAT-TATCTACGCTGTTGGTC-TTGTGAAGCA-CTTTATTGTTACGA	
I	12	ACTACGGTGTGATAAT-TATCTACGCTGTTGGTC-TTGTGAAGCA-CTTTATTGTTACGA	
	13	ACTACGGTGTGATAAT-TATCTACGCTGTTGGTC-TTGTGAAGCA-CTTTATTGTTACGA	
	14	ACTACGGTGTGATAAT-TATCTACGCTGTTGGTC-TTGTGAAGCA-CTTTATTGTTACGA	
	15	ACTACGGTGTGATAAT-TATCTACGCTGTTGGTC-TTGTGAAGCA-CTTTATTGTTACGA	
	16	ACTACGGTGTGATAAT-TATCTACGCTGTTGGTC-TTGTGAAGCA-CTTTATTGTTACGA	
T	17	ACTACGGTGTGATAAT-TATCTACGCTGTTGGTC-TTGTGAAGCA-CTTTATTGTTACGA	
I	18	ACTACGGTGTGATAAT-TATCTACGCTGTTGGTC-TTGTGAAGCA-CTTTATTGTTACGA	
S	19	ACTACGGTGTGATAAT-TATCTACGCTGTTGGTC-TTGTGAAGCA-CTTTATTGTTACGA	
H	20	ACTACGGTGTGATAAT-TATCTACGCTGTTGGTC-TTGTGAAGCA-CTTTATTGTTACGA	
	21	ACTACGGTGTGATAAT-TATCTACGCTGTTGGTC-TTGTGAAGCA-CTTTATTGTTACGA	
B	22	ACTACGGTGTGATAAT-TATCTACGCTGTTGGTC-TTGTGAAGCA-TTTTATTGTTACGA	
S	23	ACTACGGTGTGATAAT-TATCTACGCTGTTGGTC-TTGTGAAGCA-CTTTATTGTTACGA	
II	24	ACTACGGTGTGATAAT-TATCTACGCTGTTGGTC-TTGTGAAGCA-CTTTATTGTTACGA	
	25	ACTACGGTGTGATAAT--ATCTACGCTGTTGGTC-TTGTGAAGCA-CTTTATTGTTACGA	
	26	ACTACGGTGTAATAAT-TATCTACGCTGTTGGTC-TTGTGAAGCA-CTTTATTGTTACGA	
	27	ACTATGGTGTGATAAT-TATCTACGCTGTGGTTTGTTGCGCTGCGAATTTA--ACTATGG	
	28	ACTATGGTGTGATAAT-TATCTACGCTGTGGTTTGTTGCGCTGCGAATTTA--ACTATGG	
	29	ACTATGGTGTGATAAT-TATCTACGCTGTGGTTTGTTGCGCTGCGAATTTA--ACTATGG	
	30	ACTATGGTGTGATAAT-TATCTACGCTGTGGTTTGTTGCGCTGCGAATTTA--ACTATGG	
T	31	ACTATGGTGTGATAAT-TATCTACGCTGTGGTTTGTTGCGCTGCGAATTTA--ACTATAG	
I	32	ACTATGGTGTGATAAT-TATCTACGCTGTGGTTTGTTGCGCTGCGAATTTA--ACTATAG	
N	33	ACTATGGTGTGATAAT-TATCTACGCTGTGGTTTGTTGCGCTGCGAATTTA--ACTATGG	
	34	ACTATGGTGTGATAAT-TATCTACGCTGTGGTTTGTTGTGCTGCGAATTTA--ACTATGG	
	35	ACTATGGTGTGATAAT-TATCTACGCTGTGGTTTGTTGCGCTGCGAATTTA--ACTATGG	
	36	ACTATGGTGTGATAAT-TATCTACGCTGTGGTTTGTTGCGCTGCGAATTTA--ACTATGG	
	37	ACTATGGTGTGATAAT-TATCTACGCTGTGGTTTGTTGCGCTGCGAATTTA--ACTATGG	
T	38	-CATTGGTGTGATAATCTATCTACGCTATTGGT----GCAAGCCTTCTTTATTGAAGTTG	
P	39	-CATTGGTGTGATAATCTATCTACGCTATTGGT----GCAAGCCTTCTTTATTGAAGTTG	
	40	-CATTGGTGTGATAATCTATCTACGCTATTGGT----GCAAGCCTTCTTTATTGAAGTTG	
	41	ACTATGGTGTGATAAT-TATCTACGCTGTGGTTTGTTGCGCTGCGAATTTA--ACTATGG	
T	42	ACTATGGTGTGATAAT-TATCTACGCTGTGGTTTGTTGCGCTGCGAATTTA--ACTATGG	
?	43	TCTCTGGCGTGATAAT-TATCTA----------GTCA-AAGGCGAG---AATGTGATGG	
	44	TCTCTGGCGTGATAAT-TATCTA----------GTCA-AAGGCGAG---AATGTGATGG	

ITS 2

Figure 4.13. Alignment of the complete internal transcribed spacer region (CITS) of the 44 *Typhula* sequences (base pairs 661 to 720) using the Clustal method with weighted residue weight table. TISH BSI and BSII = *T. ishikariensis* biological species I and II, TIN = *T. incarnata*, TP = *T. phacorrhiza* and T? = unknown *Typhula* spp.

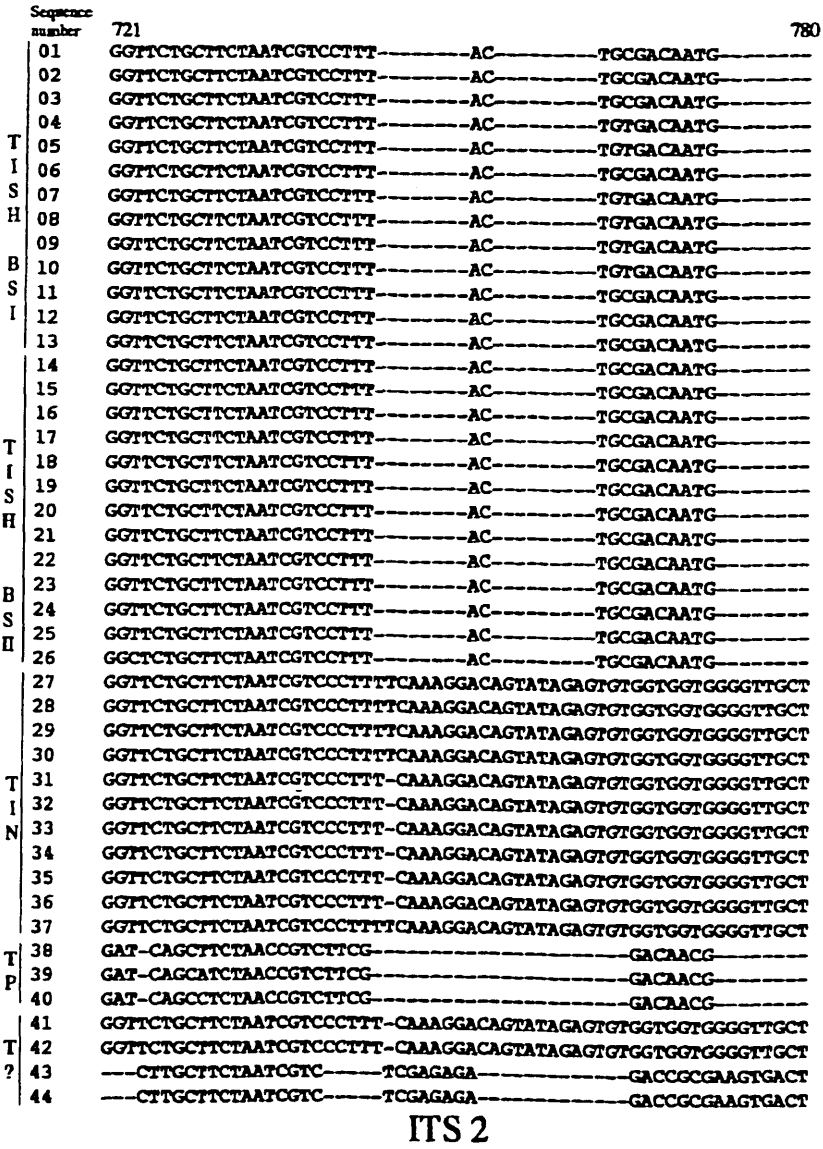


Figure 4.14. Alignment of the complete internal transcribed spacer region (CITS) of the 44 *Typhula* sequences (base pairs 721 to 780) using the Clustal method with weighted residue weight table. TISH BSI and BSII = *T. ishikariensis* biological species I and II, TIN = *T. incarnata*, TP = *T. phacorrhiza* and T? = unknown *Typhula* spp.

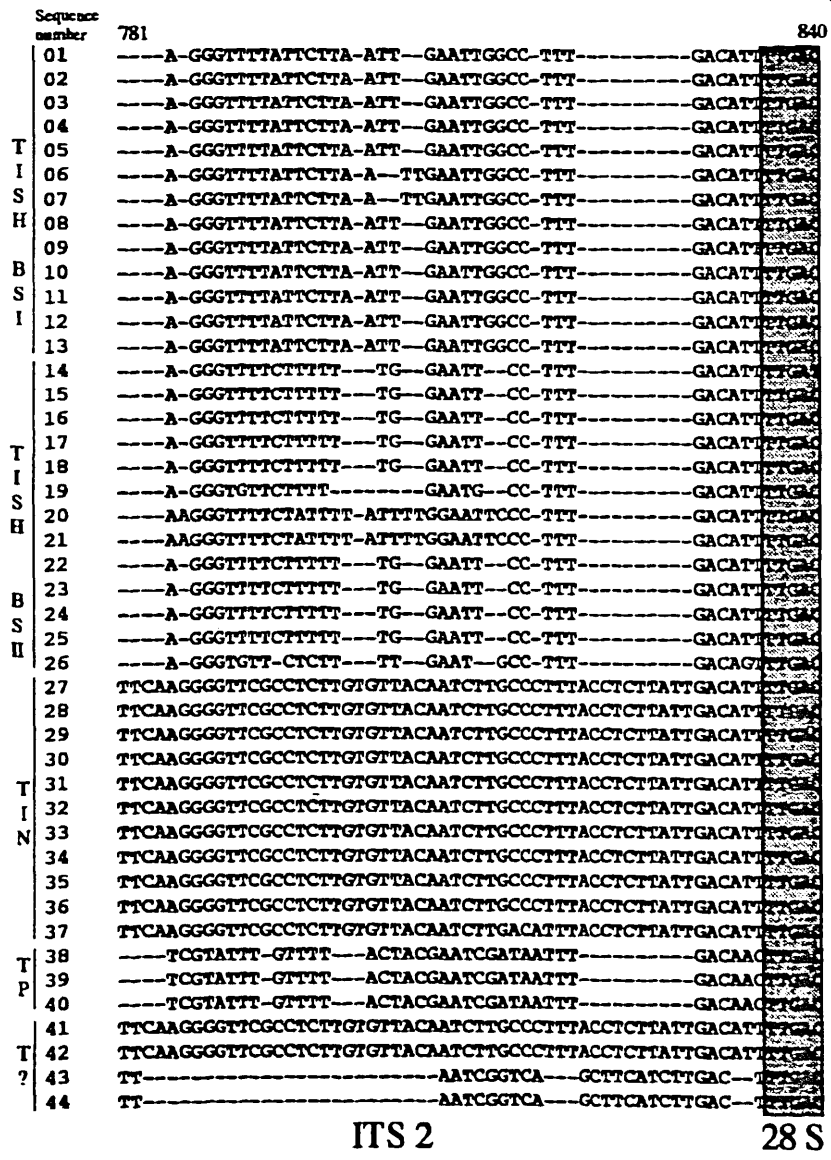


Figure 4.15. Alignment of the complete internal transcribed spacer region (CITS) of the 44 *Typhula* sequences (base pairs 781 to 840) using the Clustal method with weighted residue weight table. TISH BSI and BSII = *T. ishikariensis* biological species I and II, TIN = *T. incarnata*, TP = *T. phacorrhiza* and T? = unknown *Typhula* spp.

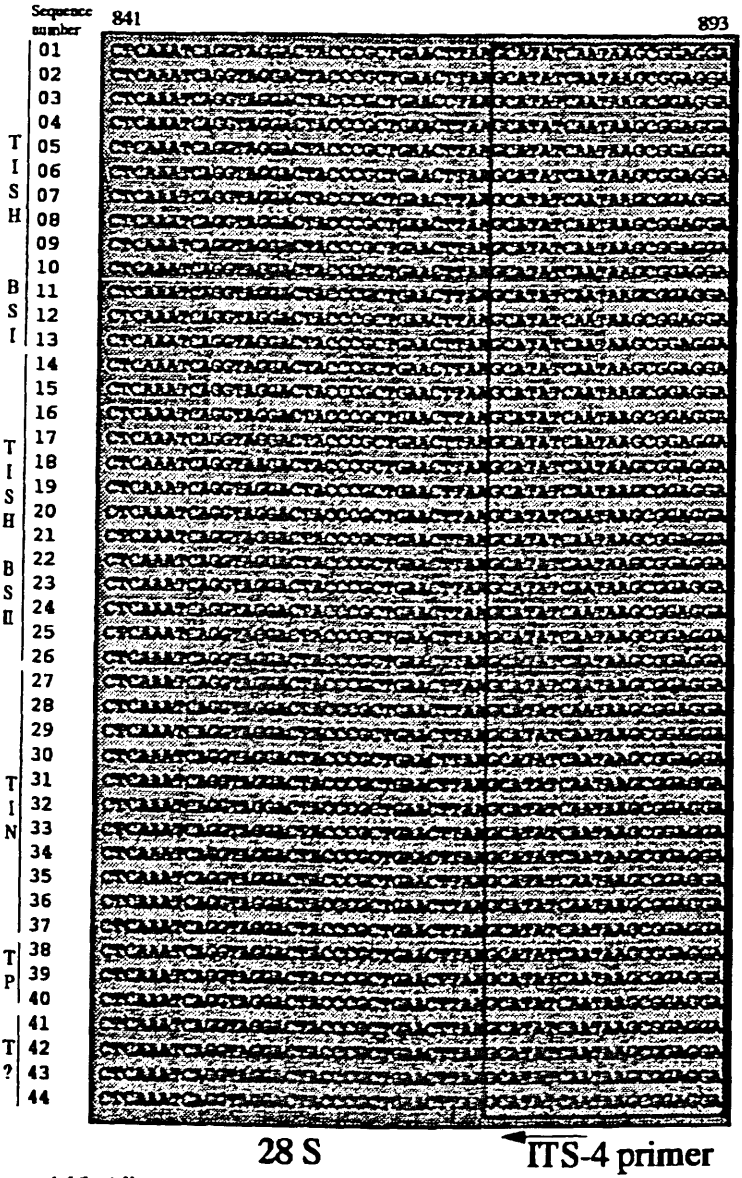


Figure 4.16. Alignment of the complete internal transcribed spacer region (CITS) of the 44 *Typhula* sequences (base pairs 841 to 893) using the Clustal method with weighted residue weight table. TISH BSI and BSII = *T. ishikariensis* biological species I and II, TIN = *T. incarnata*, TP = *T. phacorrhiza* and T? = unknown *Typhula* spp.

Two phylogenetic trees were constructed from the CITS of forty-four aligned sequences of the *Typhula* species. First, a slanted cladogram of the distance measure analysis showing the relationships of the *Typhula* sequences is presented in Fig. 4.17. The data were resampled with 5,000 bootstrap replicates (Felenstein, 1985) by using the heuristic search option of PAUP. The percentage of bootstrap replicates that yielded each grouping (group frequencies) was used as a measure of statistical confidence and are indicated above the cladogram branches (see Fig. 4.17). Three *T. phacorrhiza* sequences (38, 39 and 40) were set as the outgroup. The slanted cladogram clustered the sequences into three groups: the unidentified *Typhula* sequences (43, 44), *T. incarnata*, and the *T. ishikariensis* complex. The *T. ishikariensis* complex formed a topology that displayed a tendency to form two groups (BSI = sequences 1 to 13 and 26, and BSII = sequences 14 to 25) but the group frequencies ranged from 70 to 100%, which indicated variability between the two groups (BSI and BSII) but not enough to infer that they are different species.

The second tree constructed from the CITS of the *Typhula* species was based on parsimony analysis. A strict consensus phylogram of the CITS parsimony analysis showing the relationships among the *Typhula* species is presented in Fig. 4.18. The data were resampled with 10,000 jackknife replicates (Felenstein, 1985) by using the heuristic search option of PAUP. The percentage of jackknife replicates that yielded each grouping (jackknife percentages) are indicated above the phylogram branches (see Fig. 4.18). The three *T. phacorrhiza* sequences (38, 39 and 40) were again used as an outgroup. The branch lengths of the phylogram represent genetic distances between groups. The *T. incarnata*, *T. ishikariensis* and the unidentified *Typhula* sequences were separated into three groups with no clear separation within the *T. ishikariensis* complex.

The alignment of the forty-four *Typhula* CITS sequences indicated the presence of unique sequence differences between the two biological species of the *T. ishikariensis* complex within the ITS 1 region (Figs. 4.4 and 4.5). The sequences of the ITS 1 region of the *T.*

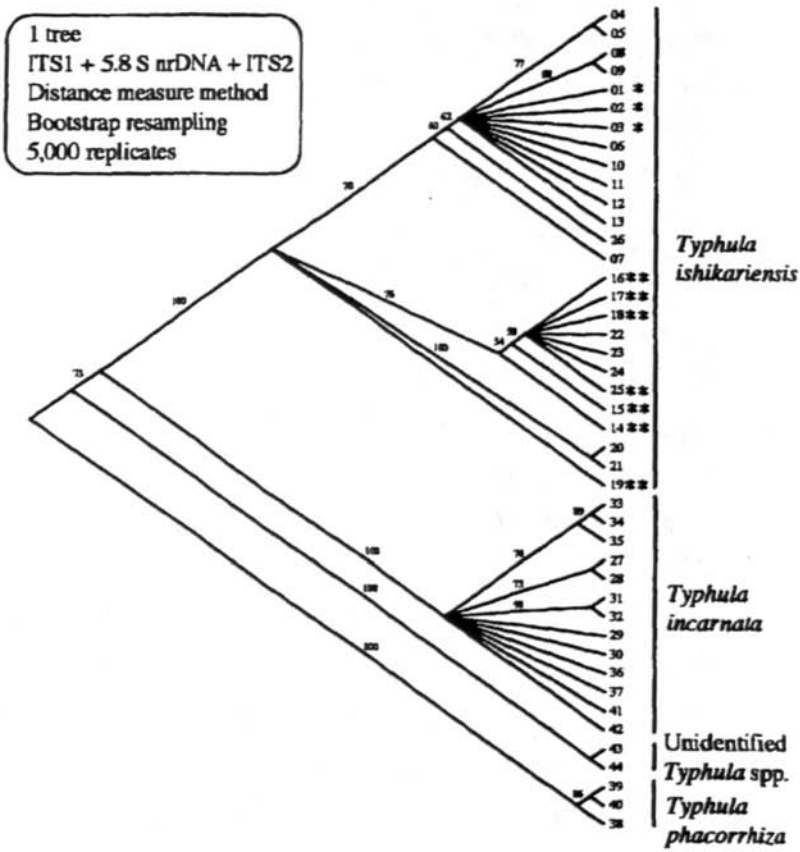


Figure 4.17. Slanted cladogram of distance measure analysis showing relationships of twenty-six *Typhula ishikariensis*, thirteen *T. incarnata*, two unidentified *Typhula* spp. and three *T. phacorrhiza* sequences. The cladogram was derived from the nucleotide sequences of the complete internal transcribed (CITS) region of the nuclear ribosomal DNA (nrDNA). The sequences were aligned by DNAStar Clustal alignment and the cladogram was generated by PAUP 4.0. Three *T. phacorrhiza* sequences (38, 39 and 40) were set as the outgroup. The numbers above branches indicate group frequencies from 5,000 bootstrap replicates. Sequences are identified by numbers as described in Tables 4.2, 4.3 and 4.4. The species are marked on the right. * = *T. ishikariensis* WIG1; ** = *T. ishikariensis* WIG2.

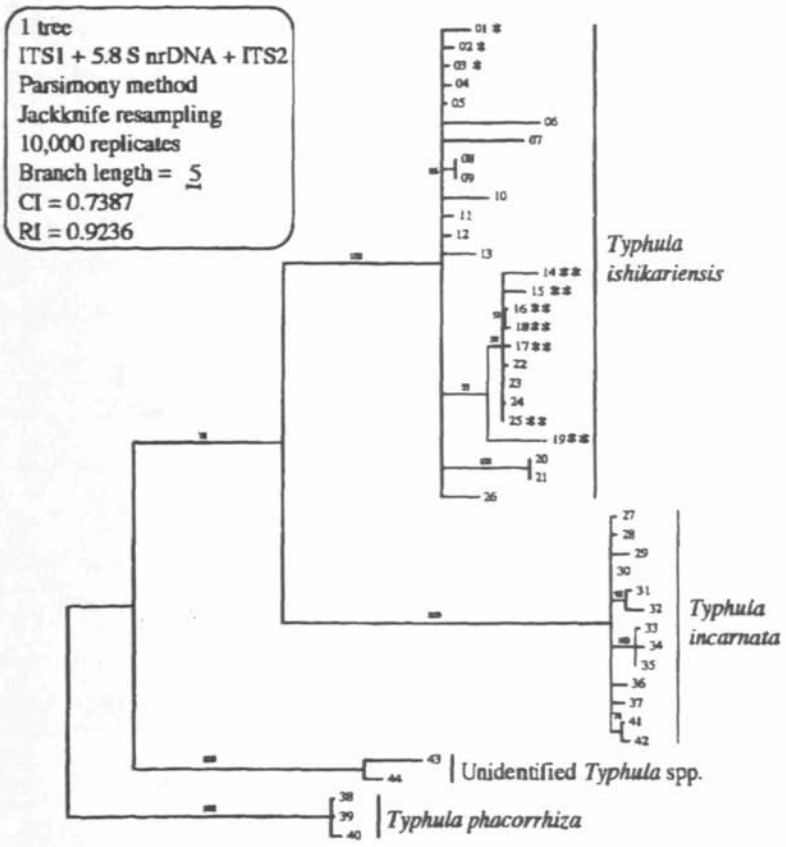


Figure 4.18. Strict consensus phylogram of parsimony analysis showing relationships among twenty-six *Typhula ishikariensis*, thirteen *T. incarnata*, two unidentified *Typhula* spp. and three *T. phacorrhiza* sequences. The nucleotide sequences of the complete internal transcribed spacer (CITS) regions of the nuclear ribosomal DNA (nrDNA) were aligned by DNAStar Clustal alignment and the phylogram was generated by PAUP 4.0. Three *T. phacorrhiza* sequences (38, 39 and 40) were set as the outgroup. The numbers above branches indicate the percentages from 10,000 jackknife replicates. Horizontal lengths represent genetic distances and the scale for branch lengths is indicated. Sequences are identified by numbers as described in Tables 4.2, 4.3 and 4.4. The species are marked on the right. * = *T. ishikariensis* WIG1; ** = *T. ishikariensis* WIG2.

ishikariensis complex were aligned by the Clustal method within the DNAStar software program and the "signature sequence" where the biological species can be differentiated is presented in Fig. 4.19. This signature sequence is approximately forty-four bp long and ranges from approximately 145 bp to 189 bp within the ITS 1 region.

Also, from the *T. ishikariensis* complex ITS 1 region alignment, two phylogenetic trees were constructed. First, a slanted cladogram of the distance measure analysis showing the relationships of the *T. ishikariensis* complex ITS 1 sequences is presented in Fig. 4.20. The data were resampled with 5,000 bootstrap replicates (Felenstein, 1985) by using the heuristic search option of PAUP. The percentage of bootstrap replicates that yielded each grouping (group frequencies) was used as a measure of statistical confidence and are indicated above the cladogram branches (see Fig. 4.20). The *T. incarnata* sequence 27 was set as the outgroup. The slanted cladogram clustered the *T. ishikariensis* complex sequences into two groups: biological species I (1 to 13 and 26 and biological species II (14 to 25). However, the *T. ishikariensis* complex formed a topology that displayed a tendency to form two groups (BSI = sequences 1 to 13 and 26, and BSII = sequences 14 to 25) but the group frequencies were 58 and 67%, which indicated variability between the two groups (BSI and BSII) but not enough to infer that they are different species.

The second tree constructed from the ITS 1 region of the *T. ishikariensis* complex was based on parsimony analysis. A strict consensus phylogram showing the relationships among the *T. ishikariensis* complex is presented in Fig. 4.21. The data were resampled with 10,000 jackknife replicates (Felenstein, 1985) using the heuristic search of PAUP. The percentage of jackknife replicates that yielded each grouping are indicated above the phylogram branches (see Fig. 4.21). The *T. incarnata* sequence 27 was set as the outgroup. The branch lengths represent genetic distances between groups. The *T. ishikariensis* sequences were separated into three groups with only 78, 67 and 76% jackknife percentages, which indicates variation between the two biological species but not enough to infer that they are different species.

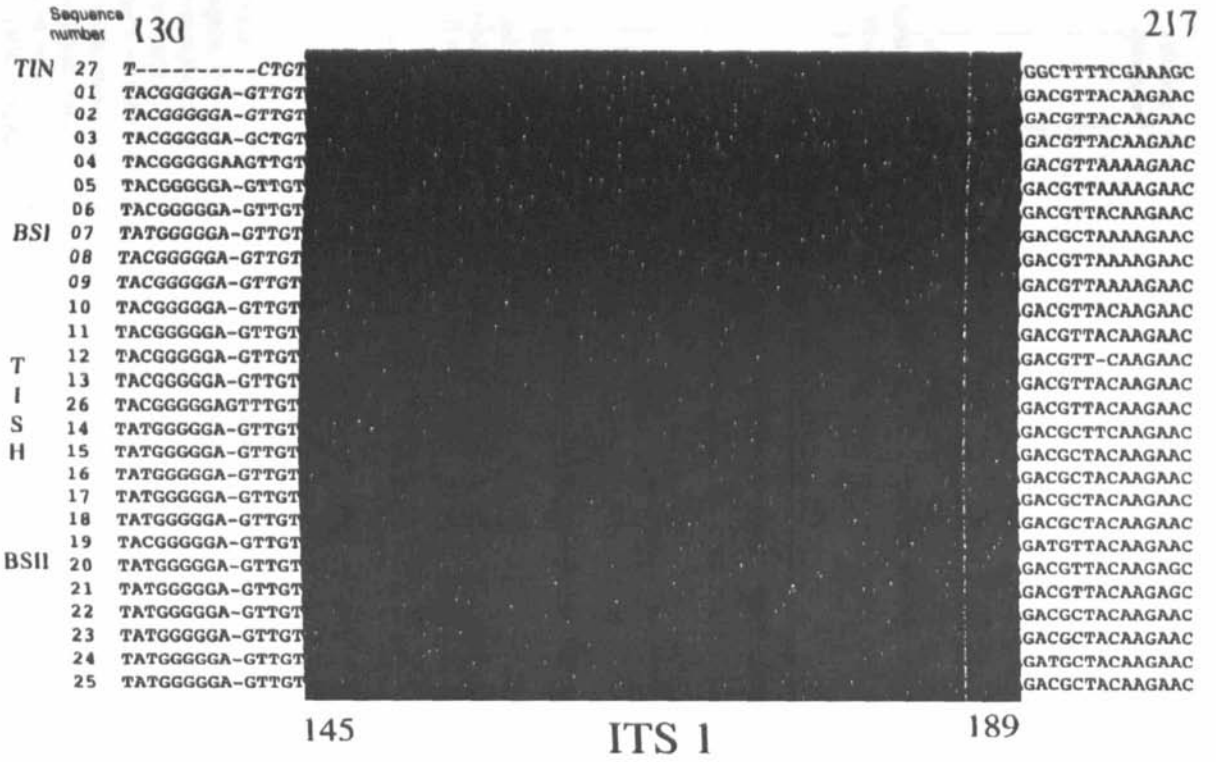


Figure 4.19. Signature sequence within the ITS 1 region of the nrDNA that differentiates the biological species of the *Typhula ishikariensis* complex. The sequence is highlighted and the approximate range and distance within the ITS 1 region are shown.

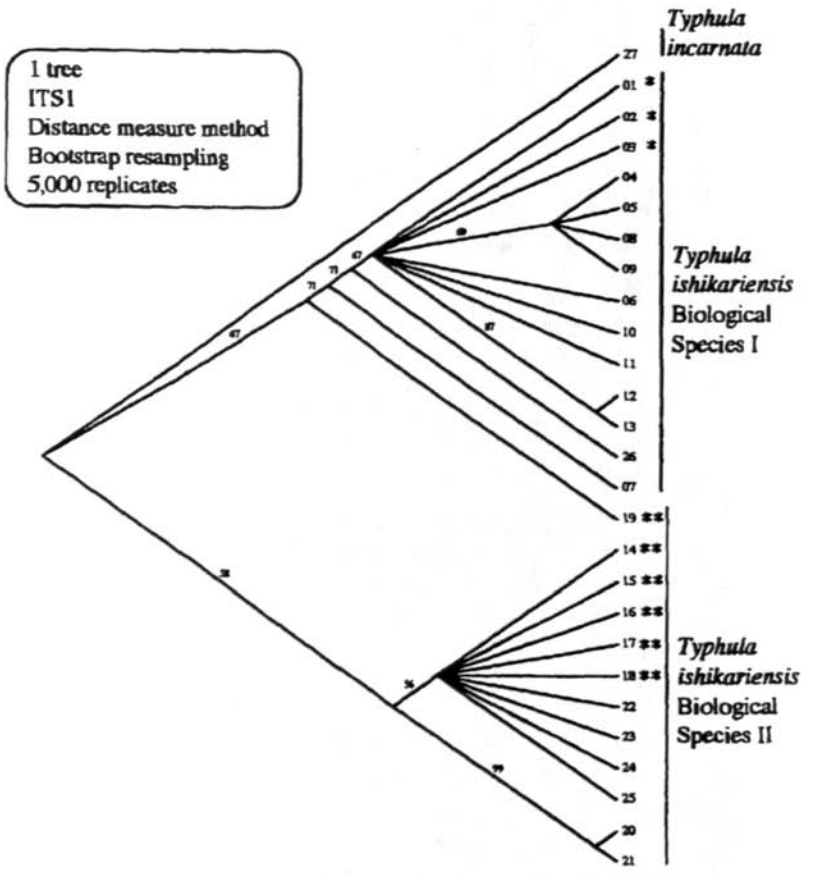


Figure 4.20. Slanted cladogram of distance measure analysis showing relationships of twenty-six *Typhula ishikariensis* and one *T. incarnata* sequences. The cladogram was derived from the nucleotide sequences of the internal transcribed region 1 (ITS1) of the nuclear ribosomal DNA (nrDNA). The sequences were aligned by DNAStar Clustal alignment and the cladogram was generated by PAUP 4.0. One *T. incarnata* sequence (27) was set as the outgroup. The numbers above branches indicate group frequencies from 5,000 bootstrap replicates. Sequences are identified by numbers as described in Tables 4.2 and 4.3. The species are marked on the right. * = *T. ishikariensis* WIG1; ** = *T. ishikariensis* WIG2.

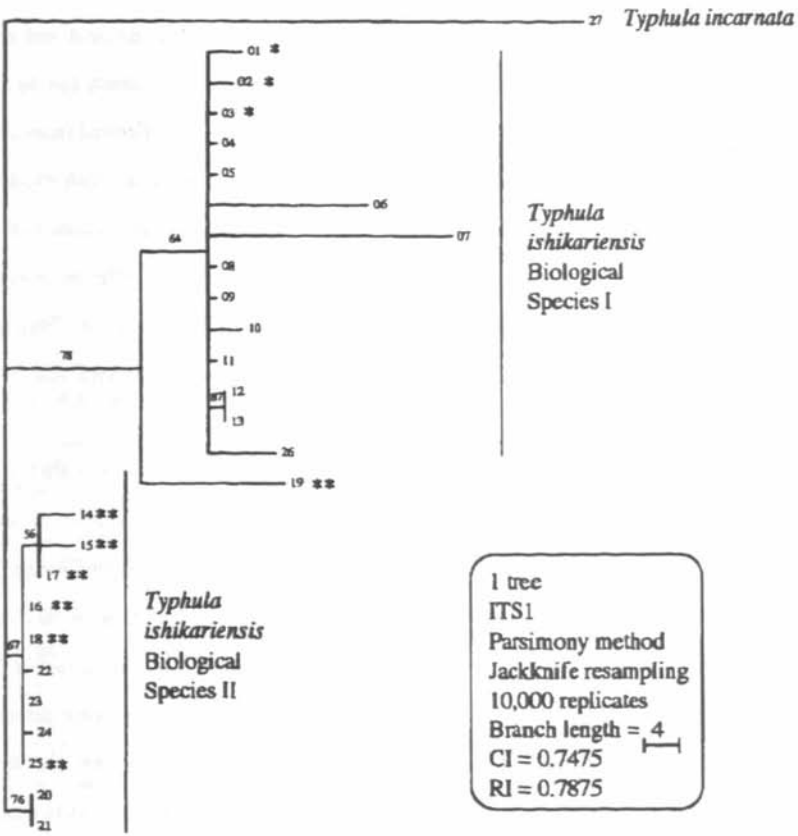


Figure 4.21. Strict consensus phylogram of parsimony analysis showing relationships among twenty-six *Typhula ishikariensis* and one *T. incarnata* sequences. The nucleotide sequences of the internal transcribed spacer region 1 (ITS1) of the nuclear ribosomal DNA (nrDNA) were aligned by DNAStar Clustal alignment and the phylogram was generated by PAUP 4.0. One *T. incarnata* sequence (27) was set as the outgroup. The numbers above branches indicate the percentages from 10,000 jackknife replicates. Horizontal lengths represent genetic distances and the scale for branch lengths is indicated. Sequences are identified by numbers as described in Tables 4.2 and 4.3. The species are marked on the right. * = *T. ishikariensis* WIG1; ** = *T. ishikariensis* WIG2.

DISCUSSION

Sequence analyses of the CITS region of the nrDNA is a consistent and accurate method for fungal plant pathogen identification. The technique consistency can be roughly estimated by calculating the percent identities of sequences that were derived from different DNA extractions of the same isolate and from different clones of the same DNA extraction of the same isolate (Table 4.10). The percent identities of different DNA extractions of the same isolate ranged from 94 to 100% identical, while sequences derived from different clones of the same DNA extraction of the same isolate ranged from 96 to 100% identical. This level of technique consistency supports further use of this technique as a rapid and accurate identification tool.

Sequences derived from different DNA extractions and different clones are consistent (94 to 100% identical, Table 4.8), but are there sequence differences between monokaryons and dikaryons of the same isolate? Sequence 16 (dikaryotic *T. ishikariensis* isolate 2.105) versus sequences 17 and 18 (monokaryotic *T. ishikariensis* isolate 2.105) were 99 to 100 % identical (Appendix C, Table C.1). Also, sequence 33 (dikaryotic *T. incarnata* isolate 2.136) versus sequences 34 and 35 (monokaryotic *T. incarnata* isolate 2.136) were 96 to 98% identical (Appendix C, Table C.3). The percent identities for dikaryons versus monokaryons of the same isolate ranged from 96 to 100%. However, this is not enough evidence to reject the theory that there are differences between monokaryons and dikaryons of the same isolate. Future experiments could be designed to determine sequence differences between monokaryotic and dikaryotic states of the same isolate by increasing the number of sequence comparisons. Also, it would be interesting to determine if dikaryotic hybrids from a monokaryotic BSI (or WIG1) isolate with a monokaryotic BSII (or WIG2) isolate mating would produce a "new hybrid" CITS nrDNA sequence.

The results of the molecular identification experiments indicate that the CITS region of the nrDNA can be used to differentiate *T. incarnata*, *T. ishikariensis* and *T. phacorrhiza*. The

Table 4.10. Percent identities of different DNA extractions of the same isolate and of different clones of the same DNA extraction of the same isolate.

Sequences of Different DNA extractions[a]	*Typhula* spp.	Percent identity	Sequences of Different clones[b]	*Typhula* spp.	Percent identity
5, 6	*T. ishikariensis*	94	8, 9	*T. ishikariensis*	100
16, 17, 18	*T. ishikariensis*	99-100	10, 11	*T. ishikariensis*	96
27, 28, 29	*T. incarnata*	99-100	12, 13	*T. ishikariensis*	99
30, 31, 32	*T. incarnata*	97-100	39, 40	*T. phacorrhiza*	100
33, 34, 35	*T. incarnata*	96-98	41, 42	*T. phacorrhiza*	99

[a] Different DNA extractions of the same isolate.
[b] Different clones of the same isolate.

percent identities, cladogram and phylograms of the CITS region of the nrDNA clearly differentiate the three *Typhula* species.

Also, analyses of the CITS and the ITS1 regions of the nrDNA infer that isolates of the two *T. ishikariensis* groups (WIG1-BSI and WIG2-BSII) are the same species but perhaps are undergoing speciation or lineage divergence. The 26 CITS sequences from the 20 *T. ishikariensis* isolates were 87 to 100% identical with a 0 to 8% divergence. Percentage of sequence identity of the CITS for the 44 sequences from the 30 *Typhula* spp. or *Typhula*-like isolates ranged from 59 to 100% with a 0 to 30% divergence. The 26 sequences from the 20 *T. ishikariensis* isolates were 59 to 78% identical to the eleven sequences of five *T. incarnata* isolates, three sequences from two *T. phacorrhiza* isolates and four sequences from 3 unidentified *Typhula*-like isolates and had a 22 to 30% divergence.

More work on the ITS 1 region "signature sequence" is needed as conflicting sequence data has been reported (Hsiang and Wu, 1999). The percentage of sequence identities of Hsiang and Wu's sequences as compared to the 26 *T. ishikariensis* sequences reported here are presented in Table 4.11. The percentage of sequence identities among the three sequences ranged from 93 to 95%. These three sequences along with the 26 *T. ishikariensis* sequences from this study were aligned using the Clustal method option of DNAStar and the signature sequence area within the ITS 1 region is presented in Fig. 4.22. Both *Typhula ishikariensis* varieties *canadensis* and *ishikariensis* sequences from (Hsiang and Wu, 1999) have the BSII signature sequence (Fig. 4.22). This conflicts with sequences 10, 11, 12 and 13 (*T. ishikariensis* var. *ishikariensis*) that have the signature sequence of BSI not BSII. The *T. ishikariensis* var. *idahoensis* sequence (Hsiang and Wu, 1999) does have the *T. idahoensis* signature as in this study.

The *T. ishikariensis* phylogenetic trees constructed from the ITS1 region of the CITS of nrDNA (Figs. 4.20 and 4.21) support the grouping of the *T. ishikariensis* complex into two diverging groups (biological species I and biological species II). The only exception is the

Table 4.11. Percentage of sequence identity in the complete internal transcribed spacer region between the University of Wisconsin study *Typhula ishikariensis* sequences (1 to 26), one *T. incarnata* sequence (27) and three sequences downloaded from GenBank, *T. ishikariensis* var. *canadensis* (AF134711), *T. ishikariensis* var. *idahoensis* (AF134712) and *T. ishikariensis* var. *ishikariensis* (AF134713) (Hsiang and Wu, 1999).

Sequence number	*T. ishikariensis* var. *ishikariensis*	*T. ishikariensis* var. *idahoensis*	*T. ishikariensis* var. *canadensis*
1	95	93	92
2	95	93	92
3	96	95	93
4	95	95	93
5	96	95	93
6	90	90	86
7	89	87	86
8	95	94	92
9	94	93	92
10	92	89	88
11	95	94	92
12	96	94	93
13	94	93	91
14	96	92	93
15	97	93	94
16	98	95	95
17	98	94	95
18	98	95	95
19	96	94	93
20	96	92	93
21	97	92	93
22	98	95	95
23	98	95	96
24	98	95	95
25	98	95	96
26	95	97	92
27	72	72	67

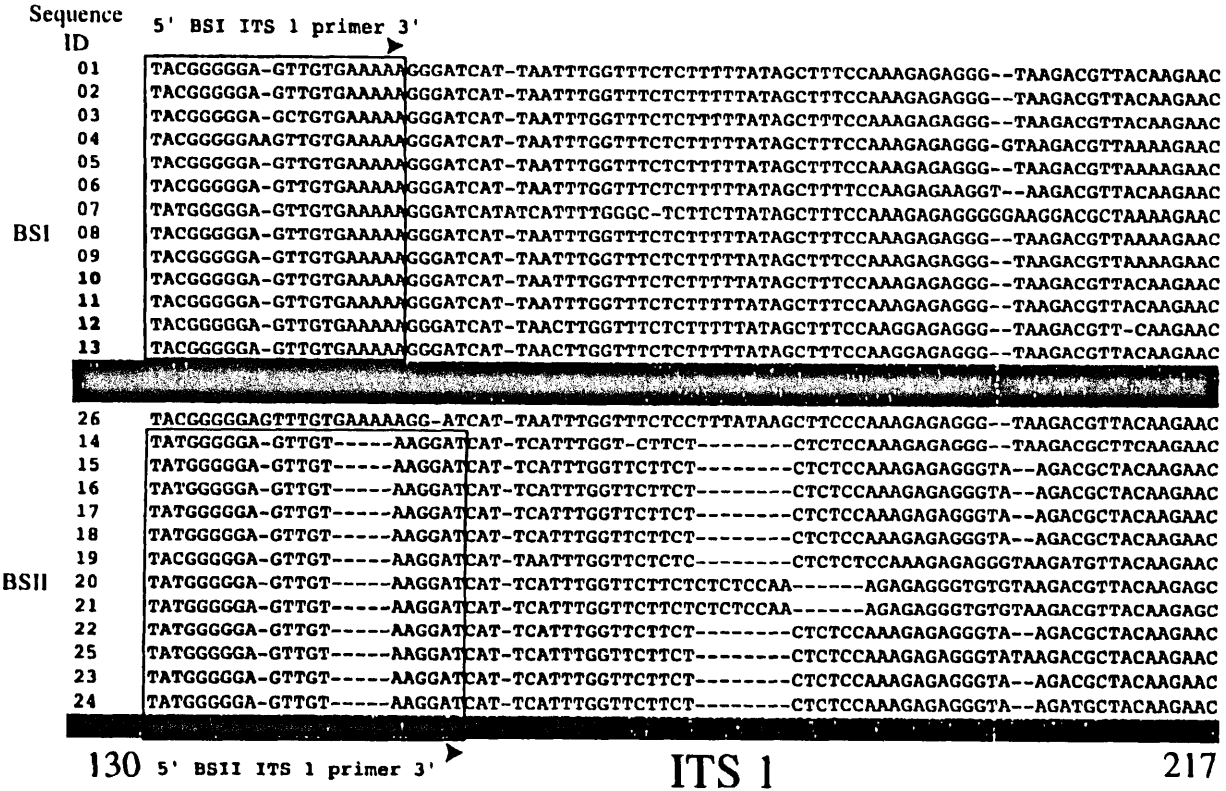


Figure 4.22. Signature sequence alignment within the ITS 1 region of the nrDNA that differentiates the biological species of the *Typhula ishikariensis* complex. Hsiang and Wu's (1999) sequences* are highlighted. Potential primer sites are indicated by arrows. Sequences 10, 11, 12 and 13 are from *T. ishikariensis* var. *ishikariensis* which is the same as Hsiang and Wu's "ish*".

placement of sequence 19 (*T. ishikariensis* WIG2 isolate 3.122). Sequence 19 contains the "signature sequence" that distinguishes it as biological species II, Fig. 4.19. However, sequence 19 is placed within the biological species I clade of the phylogenetic trees, Figs. 4.20 and 4.21. One possible explanation for the placement of sequence 19 in the biological species I clade is that it is a hybrid of the two biological species. Christen and Bruehl (1978) reported that hybrids of *T. ishikariensis* X *T. idahoensis* can exist in nature and maybe *T. ishikariensis* WIG2 isolate 3.122 is a hybrid of *T. ishikariensis* biological species I X *T. ishikariensis* biological species II. It is possible that a *T. ishikariensis* biological species I X *T. ishikariensis* biological species II hybrid could possibly give conflicting CITS sequence data, intermediate dikaryon-monokaryon mating reactions and atypical morphological characteristics. Isolate 3.122 was also one of the six isolates (out of 46 total) that had a positive or intermediate mating reaction with *T. ishikariensis* Norway group III isolate 6-IBS-4 (Tables 3.3 and 3.4). Four of these six isolates were collected from the same golf course: Trout Lake Golf and Country Club, Woodruff, WI. It was estimated that 45% of the sampled fairways of this golf course were damaged by Typhula blight and that *T. incarnata*, *T. ishikariensis*, *T. phacorrhiza* and an unidenitified *Typhula* species were collected (Table 2.22).

Minor support of the grouping of the *T. ishikariensis* isolates into two diverging groups was demonstrated by comparison of the results of the dikaryon-monokaryon pairings with the CITS results (Tables 4.12 and 4.13). The average percent identities between 3 WIG1 sequences X 7 BSI sequences and 6 WIG2 sequences X 3 BSII sequences were both 98% (Tables 4.12 and 4.13). On the other hand, the average percent identities for the WIG1XBSII comparison was 96% and for the WIG2XBSI comparison it was 95%. These differences may or may not be significant, but when these average % identities are connected with the percent dikaryotization (WIG1XBSI = 98%, WIG1XBSII = 0%, WIG2XBSI = 0% and WIG2XBSII = 56%; Table 4.15), two separate groups are apparent.

The potential use of this technique as a rapid and accurate diagnostic tool is

Table 4.12. Dikaryon-monokaryon mating reactions and percentage sequence identities between Wisconsin group 1 and Wisconsin group 2 isolates versus biological species I, biological species II and Norway group III isolates.

Wisconsin grouping / sequence number	Isolate number	Dikaryon-monokaryon reaction / % sequence identity										
		Biological species I sequence number							Biological species II sequence number			Norway group III
		Japan Biotype A PR9-4-3	Japan Biotype A PR7-6-7		Russia var. *ishikariensis* 92-32-MI		Norway Group I 2-5BS-1		Japan Biotype B 35-8	Norway Group II 4-3,S-5	Canada var. *canadensis* Can21m	Norway Group III 6-IBS-4
		4	5	6	12	13	8	9	20	22	24	NA[a]
WIG1/1	1.93	+ / 97	±/+ / 97	±/+ / 96	+ / 98	+ / 96	+ / 98	+ / 98	- / 94	- / 97	- / 96	-
WIG1/2	2.183	+ / 98	+ / 99	+ / 93	+ / 99	+ / 97	+ / 98	+ / 97	- / 95	- / 97	- / 97	-
WIG1/3	3.120	+ / 99	+ / 99	+ / 94	+ / 99	+ / 98	+ / 99	+ / 98	- / 95	- / 98	±/- / 98	±
WIG2/14	1.31	- / 95	- / 95	- / 89	- / 95	- / 93	- / 94	- / 94	- / 96	- / 96	+ / 97	-
WIG2/15	2.97	- / 95	- / 96	- / 93	- / 96	- / 95	- / 94	- / 94	±/+ / 96	- / 98	+ / 98	-
WIG2/16	2.105	- / 97	- / 98	- / 92	- / 97	- / 96	- / 96	- / 96	- / 98	+ / 100	+ / 100	-
WIG2/17	2.105	- / 97	- / 98	- / 92	- / 97	- / 96	- / 97	- / 97	- / 98	+ / 99	+ / 100	-
WIG2/18	2.105	- / 97	- / 98	- / 92	- / 97	- / 96	- / 96	- / 95	- / 98	+ / 99	+ / 100	-
WIG2/19	3.122	- / 96	- / 92	- / 92	- / 97	- / 96	- / 96	- / 94	+ / 94	-/+ / 98	+ / 97	+/ ±

[a] NA = Norway group III isolate was not sequenced.

Table 4.13. Percent dikaryotization and average percent identity between Wisconsin group 1 and 2 versus biological species I and II.

Wisconsin grouping	BSI % dikaryotization[a]	BSI average % identity	BSII % dikaryotization[a]	BSII average % identity
WIG1	98%	98%	0%	96%
WIG2	0%	95%	56%	98%

[a] WIG1 vs. BSI: dikaryon-monokaryon pairings 19 (+), 2 (±/+) = 98% dikaryotization; WIG1 vs. BSII: dikaryon-monokaryon pairings 8 (-), 1 (±/-) = 0% dikaryotization; WIG2 vs. BSI: dikaryon-monokaryon pairings 42 (-) = 0% dikaryotization; and WIG2 vs. BSII: dikaryon-monokaryon pairings 10 (+), 6 (-), 1 (±/+), 1 (-/+) = 56% dikaryotization.

demonstrated by the molecular identification of the unidentified *Typhula* species isolate G2 (collected from *Gaillardia* spp. by C. R. Grau). *In vitro* the G2 isolate had morphological characteristics that resembled both *T. phacorrhiza* and *T. incarnata*. The sclerotia were large and appeared to have a small stipe attaching it to the plant debris. However, the color and rind cell patterns were intermediate between the two species. The sequences (41 and 42) of this isolate were 91 to 97% identical to the *T. incarnata* isolates, Table B.4. This high level of % identity indicates that the G2 isolate is *T. incarnata*.

However, a disadvantage of this technique is that tester isolates or reported sequence data is needed before unknown isolates are positively identified. The failure to identify the isolates G4 (sequence 43) and T? 3.129 (sequence 44) illustrates this disadvantage. These two isolates were 92% identical to each other but their morphological characteristics were vastly different. Isolate T? 3.129 had large dark brown to almost black sclerotia with light cream colored outer edges on culture media. The T? 3.129 sclerotia coalesced into linear bands on the surface of the PDA. On the other hand, isolate G4 had large brown stipullate sclerotia that were singular on the surface of PDA. It may be that isolate G4 and T? 3.129 are closely related. However, further characterization of these isolates is needed before their identity is revealed.

Future studies centered on the CITS region should include more *T. idahoensis* and *T. ishikariensis* var. *idahoensis* isolates from around the world. In this study only one *T. idahoensis* isolate was sequenced (sequence 26). The *T. idahoensis* sequence was grouped within the biological I species, Figs. 4.17 and 4.18. However, the biological I species concept considers *T. idahoensis* to be within the biological II species concept, Fig. 3.1. This conflict could be clarified if more *T. idahoensis* and *T. ishikariensis* var. *idahoensis* isolates are sequenced.

Further sequencing of the ITS 1 region of *T. ishikariensis* complex isolates should be conducted to investigate the potential use of the "signature sequence" as an identification tool.

After more sequences are compared and if the "signature sequences" are consistently found, then primers can be designed to readily identify an unknown isolate as being either similar to biological species I or biological species II. Fig. 4.22 illustrates the 5' to 3' primers (BSI primer and BSII primer) that could be created and paired with the ITS-2 primer (White et al., 1990; which is a 3' to 5' primer located within the 5.8 S region). The design of these primers would then eliminate the need to sequence the whole CITS region in order to make a molecular identification. If the implementation of these primers is successful, molecular identification of the biological species could then be completed within one day, or even hours.

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