Molecular Identification of *Sclerotinia trifoliorum* and *Sclerotinia sclerotiorum* Isolates from the United States and Poland

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Abstract

Symptoms of clover rot caused by *Sclerotinia trifoliorum* or *S. sclerotiorum* are identical, making differentiation and identification of the causal species difficult and time consuming. Polymerase chain reaction (PCR) amplification and nucleotide sequencing were used to examine 40 isolates of *S. trifoliorum* (29 from Poland, 11 from the United States) and 55 isolates of *S. sclerotiorum* (26 from Poland, 29 from the United States). We determined that amplification of the β -tubulin and calmodulin genes with TU1/TU2/TU3 and SscadF1/SscadR1 PCR primers and the presence of introns and single-nucleotide polymorphisms (SNP) within the ribosomal DNA (rDNA) as detected with NS1/NS8 and internal transcribed spacer (ITS)1/ITS4 PCR primers

are effective for rapidly and accurately differentiating between the two species of *Sclerotinia*. In addition, our research revealed a lack of intraspecies variation within *S. sclerotiorum* isolates from the United States and Poland using these same molecular markers. We detected a relatively high degree of intraspecies variability among isolates of *S. trifoliorum* from the United States and Poland using the presence of introns and SNP within the rDNA. SNP and nuclear small-subunit rDNA analyses revealed distinct groups of *S. trifoliorum* among the isolates used in this study. The results of this study provide useful information for clover breeders and pathologists looking to develop clover varieties with durable resistance.

Kura or Caucasian clover (*Trifolium ambiguum* M. Bieb.) is a rhizomatous, persistent legume (Bryant 1974; Speer and Allinson 1985; Taylor and Smith 1997) that tolerates frequent defoliation in monoculture (Peterson et al. 1994), is reported to be adapted to a wide range of soil types (Speer and Allinson 1985), and survives long, cold winters (Sheaffer and Marten 1991). It is native to the Caucasus Region (Bryant 1974; Kannenberg and Elliott 1962; Speer and Allinson 1985) and was introduced outside its center of origin in the first half of the twentieth century (Bryant 1974). It has provided exceptional animal performance in pasture (Mouriño et al. 2003) and as silage (Kammes et al. 2008) when grown in mixture with grasses because of extreme persistence and excellent nutritive value. Commercial use of Kura clover is concentrated in the northern United States, Canada, the South Island of New Zealand, and Australia (Patrick et al. 1994; Peterson et al. 1994; Pryor et al. 1996; Steiner and Snelling 1994; Taylor 2008; Zemenchik et al. 2000). Kura clover has been considered resistant to most virus, root, stem, and foliar pathogens and nematodes that seriously damage red and white clover (Barnett and Gibson 1975; Pederson and McLaughlin 1989; Pederson and Windham 1989). It has also been reported to be highly resistant to *Sclerotinia trifoliorum* Erikss. (Barnett and Diachun 1985, Taylor and Smith 1997), one of the most destructive pathogens of forage legume crops (Kohn 1979). These desirable characteristics have generated interest in using Kura clover as a forage legume in Poland. In 1997 and again in 2009, attempts were made to establish Kura clover from cultivars considered to be resistant to *S. trifoliorum* in the United States. These cultivars, grown in Poland, were nearly decimated due to disease (Baturo-Ciesniewska et al. 2013; Harasimowicz-Hermann and Andrzejewska 1999). When establishment was successful, most plants died within 2 years (Andrzejewska et al. 2016). The damage observed on Kura clover in Poland was attributed to clover rot caused by fungi in the genus *Sclerotinia* (Andrzejewska et al. 2014; Baturo-Ciesniewska et al. 2013).

Clover rot is typically caused by *S. trifoliorum* and *S. sclerotiorum* (Lib.) de Bary (Kohn 1979; Vleugels et al. 2012). Symptoms caused by both species on clover are identical, making the differentiation and identification of species causing clover rot difficult (Pratt and Rowe 1995; Saharan and Mehta 2008; Vleugels et al. 2013). Identification of the two species has historically been based on morphology and biological or cultural characteristics. However, the only unequivocal morphological differentiation between the two species requires observation of ascospore morphology resulting from the induction of carpogenic germination of sclerotia in the laboratory. This process can be very tedious and time consuming, requiring up to several months to complete (Baturo-Ciesniewska et al. 2013; Kohn 1979; Marum et al. 1994; Njambere et al. 2008). Also, some isolates of *S. trifoliorum* are heterothallic and require a compatible mating strain in order to induce carpogenic germination and ascospore formation (Vleugels et al. 2012).

In an effort to increase the efficiency of *Sclerotinia* spp. identification, several different techniques have been applied to facilitate accurate identification: restriction fragment length polymorphisms (Kohn et al. 1988); differential growth rates on agar media (Tariq et al. 1985); sequence divergence within the ribosomal DNA (rDNA) internal transcribed spacer (ITS) (Carbone and Kohn, 1993); presence or absence of group I introns in the nuclear small-subunit (nSSU) of the rDNA (Powers et al. 2001); single-nucleotide polymorphisms (SNP) within the intergenic spacer, calmodulin, and ras protein gene differences (Andrew and Kohn 2009); SNP within the rDNA intergenic spacer region (Njambere et al. 2008); polymerase chain reaction (PCR) amplification of the β -tubulin gene (Vleugels et al. 2012); and discriminatory simplex and multiplex PCR (Abd-Elmagid et al. 2013). Some of these techniques produce reliable results but require specialized equipment and supplies and are time consuming to perform. There are also conflicting reports on the utility of some of the molecular techniques such as the use of rDNA for species identification within *Sclerotinia*. Freeman et al. (2002) reported that species within the Sclerotiniaceae family possess almost identical rDNA sequences and, therefore, rDNA sequences are not useful for species differentiation within the genus *Sclerotinia*. Others have used SNP and group I introns to differentiate species within the genus *Sclerotinia* (Holst-Jensen et al. 1999; Njambere et al. 2008; Powers et al. 2001). During our

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initial studies to identify species of *Sclerotinia* infecting Kura clover, it became apparent that some of the published molecular techniques used were not reliable in distinguishing isolates collected in Poland.

The objective of our research was to develop molecular tools that would quickly and reliably distinguish between *S. sclerotiorum* and *S. trifoliorum* from different continents and host plants. These tools would be particularly useful in identifying *Sclerotinia* spp. that cause clover rot on Kura and other clover species.

Materials and Methods

Isolates of *S. trifoliorum* and *S. sclerotiorum*. In total, 40 isolates of *S. trifoliorum* (29 from Poland, 11 from the United States) and 55 isolates of *S. sclerotiorum* (26 from Poland, 29 from the United States) were obtained from surface-disinfested sclerotia or from host plant parts with symptoms indicative of clover rot (Table 1). Symptomatic plant parts were washed in tap water and surface disinfested for 1 min in 95% ethanol and 1 min in 0.825% sodium hypochlorite (The Clorox Company, Oakland, CA) prior to being plated onto potato dextrose agar (Fisher Scientific, Pittsburgh) and incubated at 15 to 18°C. Isolates were incubated on carrot discs to promote sclerotia formation and were maintained as dry sclerotia in plastic 15-ml tubes at 4°C for long-term storage. An isolate of *S. trifoliorum* (CBS:122377) was obtained from the Centraalbureau voor Schimmelfcultures (CBS) Fungal Biodiversity Centre in The Netherlands and used as a reference isolate for the species in this study.

DNA extraction. Isolates of *Sclerotinia* were grown in potato dextrose broth (EMD Chemicals, Inc., Gibbstown, NJ) for 7 to 10 days and the resulting mycelial culture was vacuum filtered and rinsed in sterile deionized water. DNA was extracted from mycelia using the FASTDNA SPIN Kit and the FastPrep Instrument (Qbiogene, Carlsbad, CA) according to the manufacturer's instructions. DNA quantity was estimated on a NanoDrop UV-Vis Spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA). DNA was then diluted to 25 ng μl^{-1} for use as template for PCR. All DNA was stored at -20°C.

PCR primers and cycling conditions. Primers were used to amplify specific genes or gene regions (Table 2). ITS of the rDNA were amplified using primer pairs ITS1/ITS4 or ITS5/ITS4 (White et al. 1990). nSSU rDNA was amplified using primer pairs NS1/NS8 or NS5/NS6 (White et al. 1990). A portion of the β -tubulin gene was amplified using primer pairs TU1/TU2 (*S. sclerotiorum* isolates) and TU1/TU2/TU3 (*S. trifoliorum* isolates) (Vleugels et al. 2012). The aspartyl protease gene of *S. sclerotiorum* was amplified using the primer pair SSasprF/SSasprR (Abd-Elmagid et al. 2013). The calmodulin gene of *S. trifoliorum* was amplified using the published primer pair STCadF/STCadR (Abd-Elmagid et al. 2013). Primers (SScadF1/SScadR1) were designed for this study (PrimerQuest Software Package; Integrated DNA Technologies, Coralville, IA) to amplify the *S. sclerotiorum* calmodulin gene (Table 2). PCR were performed using GoTaq Green Master Mix (Promega Corp., Madison, WI) containing GoTaq DNA Polymerase in 1× Green GoTaq Reaction Buffer (pH 8.5), 200 μM dNTP, 1.5 mM MgCl_2 , 1 μM each primer, and 2.5 μl of DNA in a total volume of 25 μl /reaction. PCR was performed using conditions specific for each target gene region in an Eppendorf Mastercycler Pro S programmable thermal cycler (Eppendorf AG, Hamburg, Germany). Conditions for amplification of ITS and 18S rDNA were denaturation at 94°C for 5 min; followed by 35 cycles of 1 min of denaturation at 94°C, 1 min of annealing at 52°C, and 1 min of elongation at 72°C; followed by a final elongation step at 72°C for 10 min. Conditions for amplification of the β -tubulin gene were denaturation at 94°C for 5 min; followed by 35 cycles of 30 s of denaturation at 94°C, 30 s of annealing at 55°C, and 1 min of elongation at 72°C; followed by a final elongation step at 72°C for 5 min. Conditions for amplification of the calmodulin and aspartyl protease genes were denaturation at 95°C for 2 min; followed by 35 cycles of 30 s of denaturation at 95°C, 20 s of annealing at 61°C, and 40 s of elongation at 72°C; followed by a final elongation step at 72°C for 3 min.

Proper negative controls (water blanks in place of template DNA) were used in all PCR experiments. PCR fragments were visualized by electrophoresis in 1× Tris-acetate-EDTA (University of Wisconsin-Madison) or 1× Tris-borate-EDTA (Poland) on 1.2 to 1.4% agarose gels containing SYBR Safe DNA gel stain (Life Technologies, Carlsbad, CA) using DNA size markers of either 1-kb ladder (Promega Corp.) or 100-kb ladder (EURx, Gdańsk, Poland) to estimate the size of the fragments.

Sequence analysis and dendrogram construction. In order to obtain sequence data for isolate comparisons and to determine the presence or absence of introns and SNP within the rDNA of the isolates examined, PCR fragments were purified using the Wizard SV Gel and PCR Clean-Up System (Promega Corp.). Sequencing was performed using ABI 3730xl DNA Analyzers (Applied Biosystems, Foster City, CA) at the University of Wisconsin-Madison Biotechnology Center or at Genomed (Warszawa, Poland). Sequence comparisons were carried out using BLASTn on GenBank. Alignments were carried out using ClustalW through the Molecular Evolutionary Genetics Analysis software package, version 6 (MEGA6) (Tamura et al. 2013). A dendrogram was constructed from ITS rDNA sequences using the maximum-likelihood algorithm (MEGA6 software) (Tamura et al. 2013). The Kimura two-parameter model was applied and bootstrap analysis with 1,000 replications was done to assess group support. rDNA sequences obtained from isolates examined in this study were analyzed and compared with reference sequences obtained from the National Center for Biotechnology Information (NCBI) to construct the dendrogram.

Results

SNP in the ITS region. Amplification with primers ITS1 and ITS4 resulted in a 540-bp product with all isolates tested (24 *S. sclerotiorum* and 26 *S. trifoliorum*), regardless of species. Sequencing of both strands revealed SNP at two separate positions (120 and 376). All *S. sclerotiorum* isolates sequenced, regardless of origin, had thymine at both positions, which was previously reported to be typical for the species (Njambere et al. 2008) (Table 1). Five of the Polish *S. trifoliorum* isolates sequenced and the *S. trifoliorum* reference isolate CBS:122377 had guanine (G) at position 120 and cytosine at position 376, which was previously reported to be typical for *S. trifoliorum* (Njambere et al. 2008) (Table 1). In all, 8 of 13 Polish isolates and all 11 *S. trifoliorum* isolates from the United States that were sequenced had thiamine (T) at position 120 and cytosine (C) at position 376 (Table 1). Sequencing of both strands was performed twice to ensure that no errors were made.

Presence of introns in rRNA. Amplification of the isolates with primers ITS5 and ITS4 resulted in an approximately 560-bp product with all isolates of *S. sclerotiorum* (regardless of origin) and with 10 of the Polish and all U.S. isolates of *S. trifoliorum*. Nineteen *S. trifoliorum* isolates from Poland and the reference isolate (CBS:122377) all yielded a product approximately 1,000 bp in size, indicating the presence of at least one intron occurring in the region between the ITS1 and ITS5 primer annealing sites in these *S. trifoliorum* isolates (Powers et al. 2001).

Amplification of the nSSU rDNA with primers NS5 and NS6 revealed the presence of introns in all *S. trifoliorum* isolates tested, with the exception of Polish isolate St1513TR and the *S. trifoliorum* reference isolate CBS:122377 (data not shown).

Amplification of the nSSU rDNA with primers NS1 and NS8 revealed the presence of multiple introns or introns of varying sizes in all *S. trifoliorum* isolates examined, regardless of origin. All of the products obtained from the U.S. isolates tested were of the same size; however, there were at least three different size polymorphisms among the Polish isolates examined (Table 1; Fig. 1). Three polymorphisms of distinct lengths were revealed among the *S. trifoliorum* isolates. In total, 10 of the Polish *S. trifoliorum* and all of the U.S. *S. trifoliorum* isolates examined yielded a product approximately 2,000 bp in size (group I), only 2 isolates of *S. trifoliorum* (St1513TR and CBS 122377) yielded a product approximately 2,200 bp in size (group II), and 18 Polish *S. trifoliorum* isolates

yielded a product approximately 2,500 bp in size (group III). No introns were detected in any of the *S. sclerotiorum* isolates examined.

Amplification of the β -tubulin gene. PCR with primers TU1/TU2/TU3 amplified a single DNA fragment (494 bp) with all *S. sclerotiorum*

isolates examined and two DNA fragments (494 and 390 bp) with all *S. trifoliorum* isolates examined (Table 1; Fig. 2).

Amplification of calmodulin and aspartyl protease genes. PCR with primers STCadF/STCadR and SSasprF/SSasprR resulted in amplicons for all isolates examined, regardless of species. For isolates originating

Table 1. Origin of *Sclerotinia trifoliorum* and *S. sclerotiorum* isolates and results of polymerase chain reaction (PCR) assays and analyses on ribosomal DNA for species identification^a

Code ^f	Host ^g	Year ^h	Loc ⁱ	SNP ^b		ITS ^j	SSU ^c		Bands ^k	Product ^d			Acc. number ^e		
				120	376		NS5/6	NS1/8		STC	SSas	Ssca	NCBI	CBS	
Polish															
<i>S. trifoliorum</i>															
St 0111 TA	<i>Trifolium ambiguum</i>	2011	KP	T	C	–	+	I	+	+	+	–	...	...	
St 0211 TA	<i>T. ambiguum</i>	2011	KP	T	C	–	+	I	+	+	+	–	JQ743329	...	
St 0411 TA	<i>T. ambiguum</i>	2011	KP	T	C	–	+	I	+	+	+	–	...	...	
St 0511 TA	<i>T. ambiguum</i>	2011	KP	T	C	–	+	I	+	+	+	–	KT970795	133234	
St 0612 TA	<i>T. ambiguum</i>	2012	KP	G	C	+	+	III	+	+	+	–	...	...	
St 0712 TA	<i>T. ambiguum</i>	2012	KP	T	C	–	+	I	+	+	+	–	...	...	
St 0812 TA	<i>T. ambiguum</i>	2012	KP	T	C	–	+	I	+	+	+	–	KT224641	...	
St 0912 TA	<i>T. ambiguum</i>	2012	KP	G	C	+	+	III	+	+	+	–	...	...	
St 1412 TA	<i>T. ambiguum</i>	2012	KP	G	C	+	+	III	+	+	+	–	KT224642	...	
St 1513 TR	<i>T. repens</i>	2013	KP	T	C	+	–	II	+	+	+	–	KT224643	140899	
St 1613 TR	<i>T. repens</i>	2013	KP	/	/	+	+	III	+	+	+	–	...	...	
St 1713 TR	<i>T. repens</i>	2013	KP	/	/	+	+	III	+	+	+	–	...	...	
St 1813 TP	<i>T. pratense</i>	2013	KP	T	C	–	+	I	+	+	+	–	KT986229	140900	
St 1913 TP	<i>T. pratense</i>	2013	KP	/	/	–	+	I	+	+	+	–	...	...	
St 2013 TP	<i>T. pratense</i>	2013	KP	/	/	+	+	III	+	+	+	–	...	...	
St 2113 TR ^e	<i>T. resupinatum</i>	2013	KP	G	C	+	+	III	+	+	+	–	KT224644	140901	
St 2213 TR	<i>T. repens</i>	2013	KP	/	/	+	+	III	+	+	+	–	...	...	
St 2313 TR	<i>T. repens</i>	2013	KP	/	/	+	+	III	+	+	+	–	...	...	
St 2413 TA	<i>T. ambiguum</i>	2013	P	G	C	+	+	III	+	+	+	–	KT986230	140902	
St 2513 TA	<i>T. ambiguum</i>	2013	P	/	/	+	+	III	+	+	+	–	...	...	
St 2613 TA	<i>T. ambiguum</i>	2013	P	/	/	–	+	I	+	+	+	–	...	...	
St 2713 TA	<i>T. ambiguum</i>	2013	P	/	/	+	+	III	+	+	+	–	...	...	
St 2813 TR	<i>T. repens</i>	2013	P	/	/	+	+	III	+	+	+	–	...	...	
St 2913 TR	<i>T. repens</i>	2013	P	/	/	+	+	III	+	+	+	–	...	...	
St 3013 TR	<i>T. repens</i>	2013	P	/	/	+	+	III	+	+	+	–	...	...	
St 3113 TP	<i>T. pratense</i>	2013	P	/	/	+	+	III	+	+	+	–	...	...	
St 3213 TP	<i>T. pratense</i>	2013	P	/	/	+	+	III	+	+	+	–	...	...	
St 3313 TR	<i>T. repens</i>	2013	P	/	/	–	+	I	+	+	+	–	...	...	
St 3413 TR	<i>T. repens</i>	2013	P	/	/	+	+	III	+	+	+	–	...	...	
<i>S. sclerotiorum</i>															
Ss 0311 BN	<i>Brassica napus</i>	2011	KP	T	T	–	–	0	–	+	+	+	KT970791	...	
Ss 1012 HA	<i>Helichrysum arenarium</i>	2012	KP	T	T	–	–	0	–	+	+	+	...	...	
Ss 1112 HA	<i>H. arenarium</i>	2012	KP	T	T	–	–	0	–	+	+	+	...	...	
Ss 1212 HA	<i>H. arenarium</i>	2012	KP	T	T	–	–	0	–	+	+	+	KT224645	140893	
Ss 1312 DC	<i>D. carota</i>	2012	KP	T	T	–	–	0	–	+	+	+	KT224646	...	
Ss 3513 BN	<i>B. napus</i>	2013	KP	T	T	–	–	0	–	+	+	+	KT224647	140894	
Ss 3613 BN	<i>B. napus</i>	2013	KP	/	/	–	–	0	–	+	+	+	...	...	
Ss 3713 BN	<i>B. napus</i>	2013	KP	/	/	–	–	0	–	+	+	+	...	...	
Ss 3813 BN	<i>B. napus</i>	2013	KP	/	/	–	–	0	–	+	+	+	...	...	
Ss 3913 BN	<i>B. napus</i>	2013	KP	/	/	–	–	0	–	+	+	+	...	...	
Ss 4013 BN	<i>B. napus</i>	2013	KP	/	/	–	–	0	–	+	+	+	...	...	
Ss 4113 BN	<i>B. napus</i>	2013	KP	/	/	–	–	0	–	+	+	+	...	...	
Ss 4213 BN	<i>B. napus</i>	2013	KP	/	/	–	–	0	–	+	+	+	...	...	

(continued on next page)

^a Symbols: / indicates not analyzed and + or – indicate the presence or absence, respectively, of the PCR product.

^b Presence of single-nucleotide polymorphism (SNP) reaction with internal transcribed spacer (ITS)1/ITS4 at positions 120 or 376.

^c Presence of introns in small subunit (SSU) reaction with NS5/6 and NS1/8. Length of product with NS1/NS8: 0 indicates no introns and I to III indicate groups of isolates with introns of different size.

^d Product with STCadF/STCadR (STC), SSasprF/SSasprR (SSas), and SscadF1/SscadR1 (Ssca).

^e Accession numbers in the Centraalbureau voor Schimmelcultures (CBS) and National Center for Biotechnology Information (NCBI).

^f Codes for Polish and U.S. isolates of *S. trifoliorum* and *S. sclerotiorum* and control samples (Control).

^g Host plant.

^h Year of collection.

ⁱ Location of collection in Polish provinces (KP = Kuyavia-Pomerania, P = Pomerania, and LS = Lower Silesia), and states of the United States (IL = Illinois, MI = Michigan, MN = Minnesota, and WI = Wisconsin), and The Netherlands (NL).

^j Presence of introns in reaction with ITS5/4.

^k Presence of two bands in reaction with TU1/TU2/TU3.

^l *S. trifoliorum* from the soil under *Trifolium* sp.

^m ITS sequences of *S. trifoliorum* and *S. sclerotiorum*.

from Poland, the intensity of the band visualized on the gel with primers STCadF/STCadR was greater for all *S. trifoliorum* isolates in comparison with the *S. sclerotiorum* isolates (Fig. 3). In contrast, the intensity of the band visualized on the gel with primers SSasprF/SSasprR was greater for all *S. sclerotiorum* isolates in comparison

with the *S. trifoliorum* isolates. The primer pair SScadF1/SScadR1 amplified a single fragment (100 bp) in all of the *S. sclerotiorum* isolates tested but in none of the *S. trifoliorum* isolates tested (Fig. 4).

Dendrogram based on rDNA sequences. DNA sequences obtained during the course of this research from fragments of the

Table 1. (continued from preceding page)

Code ^f	Host ^g	Year ^h	Loc ⁱ	SNP ^b		ITS ^j	SSU ^c		Bands ^k	Product ^d			Acc. number ^e	
				120	376		NS5/6	NS1/8		STC	SSas	Ssca	NCBI	CBS
Ss 4313 BN	<i>B. napus</i>	2013	KP	/	/	–	–	0	–	+	+	+	...	...
Ss 4413 BN	<i>B. napus</i>	2013	KP	/	/	–	–	0	–	+	+	+	...	...
Ss 4513 BN	<i>B. napus</i>	2013	KP	/	/	–	–	0	–	+	+	+	...	...
Ss 4613 BN	<i>B. napus</i>	2013	KP	/	/	–	–	0	–	+	+	+	...	...
Ss 4713 DC	<i>D. carota</i>	2013	KP	T	T	–	–	0	–	+	+	+	...	...
Ss 4812 AG	<i>Apium graveolens</i>	2012	LS	T	T	–	–	0	–	+	+	+	KT224648	140895
Ss 4912 DC	<i>D. carota</i>	2012	LS	/	/	–	–	0	–	+	+	+	...	...
Ss 5012 DC	<i>D. carota</i>	2012	LS	/	/	–	–	0	–	+	+	+	...	...
Ss 5112 PC	<i>Petroselinum crispum</i>	2012	LS	T	T	–	–	0	–	+	+	+	KT224649	140896
Ss 5213 LS	<i>Lactuca sativa</i>	2013	LS	T	T	–	–	0	–	+	+	+	KT224650	140897
Ss 5314 DC	<i>D. carota</i>	2014	KP	T	T	–	–	0	–	+	+	+	KT970792	141019
Ss 5415 BO	<i>B. oleracea</i> var. <i>italica</i>	2015	KP	T	T	–	–	0	–	+	+	+	KT970793	140898
Ss 5515 BO	<i>B. oleracea</i> var. <i>italica</i>	2015	KP	/	/	–	–	0	–	+	+	+	...	...
U.S.														
<i>S. trifoliorum</i>														
St 01 TP	<i>T. pratense</i>	1996	WI	T	C	–	+	I	+	+	+	–	...	...
St 02 TP	<i>T. pratense</i>	1997	WI	T	C	–	+	I	+	+	+	–	KT224651	...
St 03 TP	<i>T. pratense</i>	1992	WI	T	C	–	+	I	+	+	+	–	KT224652	...
St 04 TP	<i>T. pratense</i>	1996	WI	T	C	–	+	I	+	+	+	–	...	...
St 05 TP	<i>T. pratense</i>	1995	WI	T	C	–	+	I	+	+	+	–	...	...
St 06 TP	<i>T. pratense</i>	1992	WI	T	C	–	+	I	+	+	+	–	...	...
St 07 TP	<i>T. pratense</i>	1992	WI	T	C	–	+	I	+	+	+	–	...	...
St 08 TP	<i>T. pratense</i>	1994	WI	T	C	–	+	I	+	+	+	–	...	...
St 09 TP	<i>T. pratense</i>	1997	WI	T	C	–	+	I	+	+	+	–	...	...
St 10 TP	<i>T. pratense</i>	1993	WI	T	C	–	+	I	+	+	+	–	...	...
St 11 TP	<i>T. pratense</i>	1994	WI	T	C	–	+	I	+	+	+	–	...	...
<i>S. sclerotiorum</i>														
Ss 12 GM	<i>Glycine max</i>	2012	WI	T	T	–	–	0	–	+	+	+	KT224653	...
Ss 13 GM	<i>G. max</i>	2012	WI	/	/	–	–	0	–	+	+	+	...	...
Ss 14 BN	<i>B. napus</i>	Unknown	IL	T	T	–	–	0	–	+	+	+	KT224654	...
Ss 15 GM	<i>G. max</i>	Unknown	IL	T	T	–	–	0	–	+	+	+	KT224655	...
Ss 16 AT	<i>Abutilon theophrasti</i>	Unknown	IL	T	T	–	–	0	–	+	+	+	KT224656	...
Ss 17 GM	<i>G. max</i>	Unknown	MI	T	T	–	–	0	–	+	+	+	...	...
Ss 18 GM	<i>G. max</i>	Unknown	MI	/	/	–	–	0	–	+	+	+	...	...
Ss 19 GM	<i>G. max</i>	2000	WI	T	T	–	–	0	–	+	+	+	...	...
Ss 20 GM	<i>G. max</i>	Unknown	WI	/	/	–	–	0	–	+	+	+	...	...
Ss 21 GM	<i>G. max</i>	Unknown	WI	/	/	–	–	0	–	+	+	+	...	...
Ss 22 GM	<i>G. max</i>	2012	WI	/	/	–	–	0	–	+	+	+	...	...
Ss 23 GM	<i>G. max</i>	2012	WI	/	/	–	–	0	–	+	+	+	...	...
Ss 24 GM	<i>G. max</i>	2012	WI	/	/	–	–	0	–	+	+	+	...	...
Ss 25 GM	<i>G. max</i>	Unknown	WI	/	/	–	–	0	–	+	+	+	...	...
Ss 26 GM	<i>G. max</i>	2003	WI	T	T	–	–	0	–	+	+	+	...	...
Ss 27 GM	<i>G. max</i>	2012	WI	/	/	–	–	0	–	+	+	+	...	...
Ss 28 GM	<i>G. max</i>	2012	WI	/	/	–	–	0	–	+	+	+	...	...
Ss 29 GM	<i>G. max</i>	2000	WI	T	T	–	–	0	–	+	+	+	KT224657	...
Ss 30 GM	<i>G. max</i>	Unknown	WI	/	/	–	–	0	–	+	+	+	...	...
Ss 31 GM	<i>G. max</i>	2012	WI	/	/	–	–	0	–	+	+	+	...	...
Ss 32 GM	<i>G. max</i>	2012	WI	/	/	–	–	0	–	+	+	+	...	...
Ss 33 GM	<i>G. max</i>	Unknown	WI	/	/	–	–	0	–	+	+	+	...	...
Ss 34 GM	<i>G. max</i>	2012	WI	/	/	–	–	0	–	+	+	+	...	...
Ss 35 GM	<i>G. max</i>	Unknown	WI	/	/	–	–	0	–	+	+	+	...	...
Ss 36 GM	<i>G. max</i>	2012	WI	/	/	–	–	0	–	+	+	+	...	...
Ss 37 PV	<i>Phaseolus vulgaris</i>	2009	WI	T	T	–	–	0	–	+	+	+	KT224658	...
Ss 38 P	<i>Petunia</i> sp.	2009	MN	T	T	–	–	0	–	+	+	+	KT224659	...
Ss 39 GM	<i>G. max</i>	2013	MN	T	T	–	–	0	–	+	+	+	KT224660	...
Ss 40 GM	<i>G. max</i>	2013	MN	/	/	–	–	0	–	+	+	+	...	...
Control														
CBS:122377	Soil ^l	2007	NL	G	C	+	–	II	+	+	+	–	KT970794	122377
EU082464	ITS <i>S. trif</i> ^m	/	/	G	C	+	+	/	/	/	/	/	EU082464	...
EU082466	ITS <i>S. scler</i> ^m	/	/	T	T	–	–	/	/	/	/	/	EU082466	...

ITS rDNA (amplicons from primers ITS1 and ITS4) from a subset of isolates (25 *S. trifoliorum* isolates, including CBS 122377, and 23 *S. sclerotiorum* isolates) were used to construct a dendrogram using the maximum-likelihood algorithm. Sequences obtained from NCBI for six *S. trifoliorum* and six *S. sclerotiorum* isolates were used for reference and comparison purposes (Fig. 5, accession numbers). In addition, sequences from *S. glacialis*, *S. homoeocarpa*, *S. minor*, *S. tetraspora*, *Dumontinia tuberosa*, and *Botrytis cinerea* were included for additional comparison (Fig. 5, accession numbers). The resulting dendrogram demonstrated that *S. sclerotiorum*, *S. minor*, and *S. trifoliorum* are closely related and all grouped into a single clade; however, there was a clear grouping within the clade by species (Fig. 5). All *S. sclerotiorum* isolates fell into a single group. Isolates of *S. trifoliorum* grouped into a large subclade comprising three distinct groups of isolates. The first group comprised five isolates (all Polish) and four of the NCBI reference isolates, all of which possess G/C at positions 120/376 in the ITS sequence. A second, larger group comprised 19 isolates (11 U.S. isolates and 9 Polish isolates) and 2 of the NCBI reference isolates, all of which possess T/C at positions 120/376 in the ITS sequence. Isolate CBS:122377, the sole member of the third group, possesses G/C at positions 120/376, which is typical for *S. trifoliorum* (Njambere et al. 2008) but differs from the other *S. trifoliorum* isolates by five other nucleotide substitutions (sequence data deposited in NCBI; Table 1). All isolates were sequenced twice to ensure the validity of the data. The overall mean distance of the rDNA ITS sequences was computed using the Mega 6 software package. The diversity of the 70 analyzed sequences (representing three genera and eight distinct species) was 1.6%, whereas the diversity of the rDNA ITS sequences of only the Polish and U.S. isolates of *S. trifoliorum* and *S. sclerotiorum* was 0.2%, indicating a high degree of similarity.

Discussion

The use of SNP and the presence of introns within the rDNA were useful to differentiate between *S. sclerotiorum* and *S. trifoliorum*. Using SNP or intron analyses techniques, no variation in the rDNA of *S. sclerotiorum* was identified in the collection of isolates in this study. However, using SNP combined with the presence or absence of introns, we detected intraspecies variation within *S. trifoliorum*. SNP analyses revealed two groups of *S. trifoliorum* isolates among the isolates included in our study. One group of five Polish isolates and the *S. trifoliorum* reference isolate (CBS:122377) all possessed the SNP G/C at positions 120/376 within the ITS region of the rDNA sequence, which has been reported to be typical for *S. trifoliorum* (Njambere et al. 2008). Interestingly, these same isolates were placed into either group II or III based on product size and presence of

introns within nSSU rDNA (amplification with primers NS1/NS8). A second group of *S. trifoliorum* isolates in our study, comprising 8 Polish isolates and all 11 U.S. isolates, possessed the SNP T/C at positions 120/376. All of these isolates, with one exception (St1513TR), were placed into group I based on product size and presence of introns within nSSU rDNA (amplification with primers NS1/NS8). Njambere et al. (2008) previously reported that all of the *S. trifoliorum* isolates from chickpea in California used in their study possessed G/C at positions 120/376. Yli-Mattila et al. (2010) examined a small number of *S. trifoliorum* isolates from Finland and detected the SNP T/C at positions 120/376 in the ITS rDNA region in at least two of their isolates from red clover. All 11 of the isolates used in our study from red clover from Wisconsin possessed T/C at the same positions within the ITS rDNA region analyzed.

Using the primers NS1 and NS8 to amplify the nSSU rDNA, we were able to differentiate between *S. sclerotiorum* and *S. trifoliorum* isolates based on amplicon sizes. All isolates of *S. sclerotiorum* used in this study produced a single amplicon with no length variations, whereas multiple introns or introns of varying sizes were found in all *S. trifoliorum* isolates examined, resulting in amplicons of varying

Fig. 1. Products of polymerase chain reaction amplification of *Sclerotinia trifoliorum* and *S. sclerotiorum* isolates using the primers NS1 and NS8. A product of approximately 1,750 bp in size (no introns) was identified in *S. sclerotiorum* isolate Ss5314 DC (lane 1) and larger products of different sizes in *S. trifoliorum* St 1813 TP with introns group I (lane 2), St 1513 TR with introns group II (lane 3), and St 0912 TA with introns group III (lane 4).

Table 2. Targets, primer code, primer sequence, and expected size of polymerase chain reaction products amplified in this study

Target ^a	Primer code	Sequence 5'→3'	Expected size (bp)
ITS region ^b	ITS1/ITS4	TCCGTAGGTGAACCTGCGG TCCTCCGCTTATTGATATGC	540
ITS region ^b	ITS5/ITS4	TCCTCCGCTTATTGATATGC GGAAAGTAAAGTCGTAACAAGG	560 (<i>S. sclerotiorum</i>), 1,000 (<i>S. trifoliorum</i>)
18S ^b	NS5/NS6	AACTTAAAGGAATTGACGGAAG GCATCACAGACCTGTTATTGCCTC	250 (<i>S. sclerotiorum</i>), 600 (<i>S. trifoliorum</i>)
18S ^c	NS1/NS8	GTAGTCATATGCTTGTCTC TCCGACGGTTCACCTACGGA	1,750 (<i>S. sclerotiorum</i>), >1,750 (<i>S. trifoliorum</i>)
Gene			
β-Tubulin ^d	TU1/TU2/TU3	CCTGAAAAGCACCCCACTAT ACGGCACGAGGAACATACTT AACTCAACTCGACCGATGCT	494 (<i>S. sclerotiorum</i> and <i>S. trifoliorum</i>), 390 (<i>S. trifoliorum</i>)
Calmodulin ^e	STCadF/STCadR	TCCTAGATCGACTCTCCTCCTTT CGTGTATTGCCTCCTTGTGTG	97
Aspartyl protease ^e	SSasprF/SSasprR	CATTGGAAGTCTCGTCGTC TCAAACGCCAAAGCTGTATG	171
Calmodulin ^f	SscadF1/SscadR1	TGTCACAGTTCGACTCTCCTCT TGTTATTGCCCCCTTGTGTGGT	100

^a ITS = internal transcribed spacer.

^b Njambere et al. (2008) and White et al. (1990).

^c Powers et al. (2001) and White et al. (1990).

^d Vleugels et al. (2012).

^e Abd-Elmagid et al. (2013).

^f New primers designed in this research.

sizes. We detected that there are polymorphisms of at least three distinct lengths among isolates within *S. trifoliorum*, with the most variability within the Polish isolates. All U.S. *S. trifoliorum* isolates produced the same-sized amplicon when gene targets were amplified with the primer pairs NS1/NS8 and NS5/NS6. However, at least three polymorphisms of distinct lengths were revealed with amplification of the Polish isolates with primers NS1/NS8. The high level of intraspecific variation among isolates of *S. trifoliorum* detected by the presence or absence of nSSU rDNA introns was previously reported (Njambere et al. 2008; Powers et al. 2001). Our results support the previous findings. Interestingly, most of the variation among isolates was detected within the Polish isolates we tested. These isolates came from at least four host species and two locations collected over a period of 3 years. There was no apparent variation among the U.S. isolates tested in this study, which could be attributed to the fact that all of the U.S. isolates were collected from the same host species (red clover) and same location (Wisconsin) over a span of several years. A more thorough testing of U.S. populations of *S. trifoliorum* utilizing isolates from a broader host range and from various geographical locations within the United States would be necessary to make a more accurate description of the intraspecific variability among isolates of *S. trifoliorum* present there.

S. trifoliorum is a heterothallic fungus (F and S mating types) and, therefore, is capable of sexual reproduction and outcrossing as well as vegetative reproduction (Vleugels et al. 2012). The genetic variation we observed within the Polish isolates in our study may be an indication of genetic mixing occurring in populations of

S. trifoliorum in Poland. *S. sclerotiorum*, on the other hand, is a homothallic fungus capable of self-fertilization, outcrossing, and vegetative reproduction (Vleugels et al. 2012). It is likely that self-fertilization or vegetative reproduction are the primary means of reproduction in *S. sclerotiorum*, because outcrossing would require close proximity of genetically different isolates. The differences in reproductive mechanisms between species of *Sclerotinia* likely explain the intraspecies variation documented within the Polish *S. trifoliorum* isolates used in our study and the lack of intraspecies variation observed within the rDNA with the *S. sclerotiorum* isolates.

The dendrogram based on rDNA ITS sequences demonstrates that *S. sclerotiorum* and *S. trifoliorum* are closely related species, with both species grouping into a single clade. The dendrogram further demonstrates the lack of intraspecies variability within the rDNA of the *S. sclerotiorum* isolates used in our study and the presence of intraspecies variation with *S. trifoliorum*. Isolates of *S. sclerotiorum* from at least nine different host plants from the United States and Poland were examined and all isolates tested aggregated into one large group. Isolates of *S. trifoliorum* aggregated into a large subclade comprising three distinct groups, corresponding to the SNP at locations 120/376 in the ITS rDNA region sequenced. The results of our work and that of previous studies demonstrate the intraspecies variation within *S. trifoliorum*. Additional studies that include isolates from various geographic locations and additional host plants are required to fully exploit the utility of ITS rDNA for molecular characterization of *S. trifoliorum*.

Fig. 2. Products of polymerase chain reaction amplification of *Sclerotinia trifoliorum* and *S. sclerotiorum* isolates using the primers TU1, TU2, and TU3. Products of 494 and 390 bp in size were identified in *S. trifoliorum* isolates St 0211 TA, St 0511 TA, St 2113 TRe, St 01 TP, and St 09 TP (lanes 1 to 5, respectively). A single product of 494 bp was identified in *S. sclerotiorum* isolates Ss0311 BN, Ss 1212 HA, Ss5314 DC, Ss 12 GM, and Ss 39 GM (lanes 6 to 10, respectively).

Fig. 3. Products of polymerase chain reaction amplification of *Sclerotinia trifoliorum* and *S. sclerotiorum* isolates originating from Poland using (row a) primers SSasprF and SSasprR and (row b) STCadF and STCadR. Products of appropriate size were identified using both primer sets for *S. trifoliorum* isolates St 0211 TA, St 0511 TA, St 1813 TP, St 2113 TRe, St 2413 TA, St 2813 TR, St 3313 TR, and CBS:122377 (lanes 1 to 8, respectively) and *S. sclerotiorum* isolates Ss 0311 BN, Ss 1212 HA, Ss 3813 BN, Ss 4812 AG, Ss 5112 PC, Ss 5213 LS, and Ss 5314 DC (lanes 9 to 15, respectively).

Fig. 4. Products of polymerase chain reaction amplification of *Sclerotinia trifoliorum* and *S. sclerotiorum* isolates using the primers SscadF1 and SscadR1. A product of 100 bp in size was identified only in *S. sclerotiorum* isolates Ss0311 BN, Ss 1212 HA, Ss 3813 BN, Ss 4812 AG, Ss 5112 PC, Ss 5213 LS, Ss5314 DC, Ss 12 GM, Ss 37 PV, and Ss 39 GM (lanes 12 to 21, respectively) whereas no PCR product was identified in *S. trifoliorum* isolates St 0211 TA, St 0511 TA, St 1813 TP, St 2113 TRe, St 2413 TA, St 2813 TR, St 3313 TR, St 01 TP, St 03 TP, St 09 TP, and CBS:122377 (lanes 1 to 11, respectively).

Fig. 5. Dendrogram constructed based on DNA sequences obtained from fragments of the ITS rDNA (amplicons from primers ITS1 and ITS4) from a subset of isolates (25 *Sclerotinia trifoliorum* and 23 *S. sclerotiorum* isolates) using the unweighted pair group method with arithmetic mean algorithm. Ribosomal DNA sequences obtained from isolates examined were analyzed and compared with reference sequences obtained from the National Center for Biotechnology Information for six *S. trifoliorum* and six *S. sclerotiorum* isolates. Additional sequences from *S. glaucialis*, *S. homoeocarpa*, *S. minor*, *S. tetraspora*, *Dumontinia tuberosa*, and *Botrytis cinerea* were included for additional comparison.

Amplification of *Sclerotinia* DNA with primers STcadF/STcadR and SSasprF/SSasprR was reported to be species specific in simplex and multiplex PCR (Abd-Elmagid et al. 2013). We found that amplification with both primer sets resulted in amplicons for all isolates tested, regardless of species, although the intensity of the band differed based on the specificity of the primer pair. A more intense band was obtained with isolates of *S. sclerotiorum* and primers SSasprF/SSasprR than was obtained with isolates of *S. trifoliorum*. Similarly, a more intense band was obtained with isolates of *S. trifoliorum* and primers STcadF/STcadR than was obtained with isolates of *S. sclerotiorum*. Many factors can affect the intensity of bands obtained from PCR amplification; thus, basing species identification on band intensities is not possible. The presence of multiple amplicons, even of varying intensities, would confound the interpretation of multiplex PCR.

Primers designed to amplify the β -tubulin gene were effective in differentiating *S. sclerotiorum* and *S. trifoliorum* in this study. In addition, the primer pair developed for this study, SScadF1/SScadR1, amplified a single amplicon with *S. sclerotiorum* isolates but did not yield an amplification product with *S. trifoliorum* isolates. Based on the work presented here, PCR targeting the β -tubulin and calmodulin genes of the genus *Sclerotinia* is useful for rapid species identification. PCR analysis with the β -tubulin and calmodulin primer pairs is a relatively quick procedure utilizing supplies and equipment readily available to most laboratories. No extra step of DNA sequencing is necessary to differentiate between *Sclerotinia* spp. These PCR tools will be useful for differentiating between *Sclerotinia* spp. infecting clover and other plant hosts.

Finally, the genetic variation observed among isolates of *S. trifoliorum* is especially useful information for clover breeders and pathologists looking to develop resistant clover populations. The lack of knowledge about the genetic diversity of *S. trifoliorum* has been a hindrance to clover breeding programs in Europe (Vleugels et al. 2012). The information presented from our research suggests that, during selection of resistant lines, plants should be challenged by an array of *S. trifoliorum* isolates, not just one putatively aggressive isolate. The variation among these isolates may result in a range of aggressiveness levels or a differential pathogenicity signature. This was evident when Kura clover cultivars were sent from the United States to Poland. These cultivars were considered resistant to *S. trifoliorum* in the United States but were nearly decimated when planted in Poland.

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