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Discovery of a functional *Mycosphaerella* teleomorph in the presumed asexual barley pathogen *Septoria passerinii*

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Abstract

We studied the possibility of a teleomorph associated with the genotypically diverse septoria speckled leaf blotch (SSLB) pathogen of barley, *Septoria passerinii*. A teleomorph in the genus *Mycosphaerella* had been predicted previously based on phylogenetic analyses. This prediction was tested with experiments in the Netherlands and the United States by co-inoculating isolates with opposite mating types onto susceptible barley cultivars and monitoring leaves for sexual structures and for the discharge of ascospores. Characterization of putative hybrid progeny by both molecular (AFLP, RAPD, mating type, and ITS sequencing) and phenotypic analyses confirmed that a *Mycosphaerella* teleomorph of *S. passerinii* has been discovered approximately 125 years after the description of the anamorph. Progeny had recombinant genotypes of the molecular alleles present in the parents, and the identities of representative progeny isolates as *S. passerinii* were confirmed by ITS sequencing. A previously unknown sexual cycle explains the high degree of genetic variation among isolates found in nature. The experimental identification of a predicted teleomorph for *S. passerinii* indicates that cryptic sexual cycles may be common for many other "asexual" fungi with high levels of genotypic diversity.

Keywords: *Septoria passerinii*; Septoria speckled leaf blotch (SSLB); *Hordeum vulgare*; Barley; Teleomorph; Sexual cycle; *Mycosphaerella*; Crossing protocol

1. Introduction

Septoria passerinii Sacc. causes septoria speckled leaf blotch (SSLB) on barley (*Hordeum vulgare*) and was first discovered in Italy in 1879 (Passerini). Since then, SSLB has been reported around the globe in such areas as the Upper Midwest region of the United States, the Prairie Provinces of Canada, Northern Europe, Northern Africa, Western Asia, and Australia (Cunfer and Ueng, 1999; Mathre, 1997). Over the past decade, SSLB epidemics have increased in frequency, and SSLB has become one of the most important, albeit sporadic, foliar diseases of barley in the United States and in Canada (Mathre, 1997; Steffenson, 2003; Toubia-Rahme et al., 2003). Yield losses of up to 38% have been reported in Minnesota and North Dakota, with similar reports of losses up to 20% in Canada (Green and Bendelow, 1961; Toubia-Rahme and Steffenson, 1999). In addition to reductions in yield, SSLB can render the remaining barley grain unacceptable for malting due to reductions in both kernel size and amount of malt extract (Green and Bendelow, 1961).

Many barley cultivars are resistant to *S. passerinii* (Banttari et al., 1975; Buchannon, 1961; Green and Dickson, 1957; Koble et al., 1959; Rasmusson and Rogers,

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1963; Toubia-Rahme and Steffenson, 2004). Green and Dickson (1957) reported that 50 of 126 *H. vulgare* cultivars tested were resistant to this pathogen, but these resistant cultivars were of low malting quality. Extensive breeding programs exist for barley, but there has been little attempt to incorporate resistance to *S. passerinii* into new cultivars (Toubia-Rahme and Steffenson, 2004). This is because breeding programs mainly focus on developing cultivars with high yields and high malting qualities and thus have used parents with little or no resistance to SSLB. Consequently, all of the commercially important cultivars for malt and feed in the Upper Midwest region of the United States grown over the past 25 years have been and still are highly susceptible to this pathogen, even though the major cultivars have changed throughout the years (Helm et al., 2001; Toubia-Rahme and Steffenson, 1999, 2004). Toubia-Rahme and Steffenson (2004) argued that because of the increasing importance of SSLB, there should be more invested in the development of cultivars that incorporate resistance with high yield and malting quality characteristics. They reported that resistance could be found in cultivars from diverse geographical origins, such as North America, South America, Europe, North Africa, and East Asia.

Presently there is evidence of up to six genes controlling resistance to SSLB in barley (Buchannon, 1961; Metcalfe et al., 1970; Rasmusson and Rogers, 1963). These specific resistance genes in the host suggest the presence of avirulence genes in the pathogen. However, such avirulence genes have not yet been identified in *S. passerinii*. Furthermore, formal genetic analysis of the pathogen is not possible due to the fact that only the imperfect stage has been reported (Cunfer and Ueng, 1999). Our previous work, however, provided lines of evidence suggesting the possibility of sexual recombination in this fungus. Despite the fact that *S. passerinii* was generally considered to be an asexual fungus (Cunfer and Ueng, 1999), we used heterologous mating-type probes from the wheat pathogen *Mycosphaerella graminicola* (Waalwijk et al., 2002) to clone the mating-type genes of *S. passerinii* (Goodwin et al., 2003), based on a previously identified close phylogenetic relationship between these two species (Goodwin et al., 2001; Goodwin and Zismann, 2001). In addition, it was shown that both mating-type idiomorphs of *S. passerinii* were found commonly in natural populations on the same leaf among 22 isolates tested, suggesting that sexual recombination under field conditions was possible. This was further substantiated by combined isozyme and RAPD genotyping of these 22 isolates, which yielded 22 unique haplotypes, as expected for sexual, but not asexual, populations (Goodwin et al., 2003).

The purpose of this paper was to test the hypothesis that *S. passerinii* has a cryptic teleomorph in the genus *Mycosphaerella*. The relative ease of generating the predicted teleomorph of *S. passerinii*, which has not been noticed in nature over the past 120-plus years, has broad implications for mycology and indicates that many other fungi may be incorrectly classified as asexual.

2. Materials and methods

2.1. Isolates, crossing, and phenotyping procedures

Twelve isolates of *S. passerinii* and two isolates of *M. graminicola* were used in this study (Table 1). Crosses were made both in Wageningen, The Netherlands, and in West Lafayette, IN, USA. Inoculum preparation, inoculations, and crossing procedures were as described previously for *M. graminicola* by Kema et al. (1996c), except that spore suspensions were sprayed onto seedlings instead of being applied with cotton. Environmental conditions for growing seedlings both before and after inoculation were as described previously (Kema et al., 1996a). Isolate combinations for crosses are listed in Table 2. *S. passerinii* crosses were made on 10-day-old seedlings of the barley cvs. Topper 33 and/or Kindred. A cross between *S. passerinii* isolates with the same mating type was included as a negative control to differentiate ascospores generated from environmental contaminants on barley from those generated by *S. passerinii*. *M. graminicola* test crosses were made on the wheat cv. Taichung 29 and served as a positive control for the crossing procedure, as a negative control to differentiate ascospores generated from environmental contaminants on wheat, and as a reference for diagnostic comparison of *M. graminicola* ascospores with those potentially produced by the *S. passerinii* teleomorph, since we speculated earlier that ascospores from these species were likely to be similar morphologically (Goodwin et al.,

Table 1
Summary information about the isolates of *Septoria passerinii* and *Mycosphaerella graminicola* used in this study

Species	Isolate	Collection location	Mating type
S. passerinii[a]	P62	North Dakota, USA	*mat 1-1*
	P63	North Dakota, USA	*mat 1-1*
	P64	North Dakota, USA	*mat 1-1*
	P65	North Dakota, USA	*mat 1-1*
	P66	North Dakota, USA	*mat 1-2*
	P67	North Dakota, USA	*mat 1-2*
	P68	North Dakota, USA	*mat 1-1*
	P71[b]	North Dakota, USA	*mat 1-1*
	P75	North Dakota, USA	*mat 1-1*
	P78	Minnesota, USA	*mat 1-2*
	P81	Minnesota, USA	*mat 1-2*
	P83[b]	North Dakota, USA	*mat 1-2*
M. graminicola	IPO323	The Netherlands	*mat 1-1*
	IPO94269	The Netherlands	*mat 1-2*

[a] The isolates of *S. passerinii* were as reported previously by Goodwin et al. (2003).

[b] Cultures of these isolates have been deposited into the collection of the Fungal Biodiversity Center (Centraalbureau voor Schimmelcultures) in Utrecht, the Netherlands, under accession numbers: P71 = CBS 120383 and P83 = CBS 120382. Progeny isolates P71 × P83 A = CBS 120384 and P71 × P83 B = CBS 120385 also were deposited.

Table 2
In planta crosses between and among isolates of *Septoria passerinii* and *Mycosphaerella graminicola*

Isolates	Cultivars for crossing	Locations for crossing
P71 × P83	Topper 33 and Kindred	Wageningen and West Lafayette
P78 × P83	Topper 33	Wageningen
P62 × P81	Kindred	West Lafayette
P62 × P83	Kindred	West Lafayette
P62 × P81	Kindred	West Lafayette
P63 × P78	Kindred	West Lafayette
P64 × P81	Kindred	West Lafayette
P65 × P66	Kindred	West Lafayette
P68 × P67	Kindred	West Lafayette
P71 × P81	Kindred	West Lafayette
P71 × P83	Kindred	West Lafayette
P75 × P78	Kindred	West Lafayette
P63 × P67	Topper 33 and Kindred	Wageningen and West Lafayette
IPO323 × IPO94269	Taichung 29	Wageningen
P71 × IPO94269	Topper 33 and Taichung 29	Wageningen
IPO323 × P83	Topper 33 and Taichung 29	Wageningen

2003). Finally, we also performed interspecific crosses between *S. passerinii* and *M. graminicola* because of the suggested close phylogenetic relationship between these species (Goodwin and Zismann, 2001). Plants were placed on a rotating table in an inoculation cabinet, and spore suspensions (at concentrations of 10^7 per ml in a total of 30, 15 ml per parental isolate) were sprayed until run-off. Incubations in Wageningen and West Lafayette were conducted as described by Kema et al. (1996c) and Adhikari et al. (2003), respectively. After symptoms developed during incubation in the greenhouse (22 °C, >85% RH), seedlings were placed outside in large plastic pots covered with 1.5-mm-mesh plastic screens. Crosses were attempted seven times between September 2002 and May 2005. Leaf samples were collected once per week from 7 to 12 weeks after inoculation in the Netherlands and from 4 to 10 weeks in the U.S. for discharge of ascospores onto 2% water agar and for microscopical identification of the sexual structure. Proposed parental isolates and the resulting progeny were inoculated onto the susceptible barley cv. Topper 33 for phenotypic comparisons.

2.2. Comparative taxonomical analyses of discharged ascospores and of sexual structures

Because plants were outside for up to 10 weeks, they were exposed to sexual and asexual spores of naturally occurring contaminant fungal species. In addition, it was impossible to know for certain what type of ascospores to expect for *S. passerinii* because they had not been described previously. Therefore, all discharged ascospores were meticulously categorized for size, shape, number of cells, pigmentation, and germination pattern on 2% water agar. All non-*M. graminicola* ascospore types discharged from leaves inoculated with *M. graminicola*, as well as all ascospore types discharged from leaves that were co-inoculated with isolates of *S. passerinii* with the same mating type, were considered to be environmental contaminants. Examples of the different types of discharged ascospores were transferred as single spores to yeast-glucose broth (YGB) and then onto potato dextrose agar (PDA) for comparisons of growth with that of *S. passerinii*. Infected leaf samples that were co-inoculated with isolates of *S. passerinii* with opposite mating types were also screened microscopically to find the associated sexual structure.

2.3. DNA extraction and analyses

In preparation for DNA extraction, isolates were grown in YGB for 10 days, at which time spores were pelleted and subsequently lyophilized. Total genomic DNA was extracted from 10 mg of lyophilized spores using the Puregene DNA isolation kit (Gentra System Inc., Minneapolis, MN, USA) and eluted with 50 µl of TE buffer (pH 8.0). All PCRs were performed in either an MJ PTC-200 Peltier (MJ Research, Watertown, MA, USA) or a Perkin-Elmer 9600 (Perkin-Elmer, Foster City, CA, USA) thermal cycler. Primers and adapters used in this study are listed in Table 3.

To confirm ascospores as progeny from *S. passerinii* crosses and to determine allelic segregation ratios, parental isolates and presumed progeny were screened using mating-type PCR, Random Amplification of Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP), and Internal Transcribed Spacer (ITS) analysis. For the mating-type analysis, primers developed by Goodwin et al. (2003) were used. PCRs were done in 25-µl reactions, each containing 2.5 µl of 10 mM dNTPs, 2.5 µl of 10× PCR buffer, 1.5 µl of 25 mM $MgCl_2$, 0.1 µl of 5 U/µl AmpliTaq DNA polymerase (Applied Biosystems), 2.5 µl of 0.01% G-2500 Gelatin (Sigma), 1.33 µl each of 4 µM

Table 3
Primers and adapters for *Septoria passerinii* used in this study

Name	Sequence (5′ to 3′)	Purpose
MT-F	CTTCTTGCCTGCGCCACAGG	*mat 1-1* and *mat 1-2* PCR
Alpha(1594)R	CGGTATGTGGATGGAAGAAAGG	*mat 1-1* PCR
HMG(849)R	TAGTCGGGACCTGAAGGAGTG	*mat 1-2* PCR
OPA-9	GGGTAACGCC	RAPD
*Eco*RI adapter	CTCGTAGACTGCGTACC AATTGGTACGCAGTC	AFLP
*Mse*I adapter	GACGATGAGTCCTGAG TACTCAGGACTCAT	AFLP
E00	GACTGCGTACCAATTC	AFLP
M00	GATGAGTCCTGAGTAA	AFLP
E19	GACTGCGTACCAATTCGA	AFLP
M16	GATGAGTCCTGAGTAACC	AFLP
ITS4	TCCTCCGCTTATTGATATGC	ITS sequencing
ITS5	GGAAGTAAAAGTCGTAACAAGG	ITS sequencing

MT-F, Alpha(1594)R, and HMG(849)R primers, 3 μl of 1 ng/μl target DNA, and 8.9 μl of sterile double-distilled (sdd) water. Thermal cycler conditions were as described previously (Goodwin et al., 2003), and the annealing temperature was 55 °C. For the RAPD analysis, PCRs were done in 25-μl reactions, each containing 2.5 μl of 2 mM dNTPs, 2.5 μl of 10× PCR + $MgCl_2$ buffer, 0.25 μl of 50 mM $MgCl_2$, 0.06 μl of 5 U/μl Taq DNA polymerase (Roche), 2.5 μl of 10 ng/μl OPA9 primer (Operon Technologies), 1.5 μl of 0.5 ng/μl DNA, and 15.69 μl of sdd water. Cycling parameters were as described previously by Kema et al. (1996c). Amplicons from both RAPD and mating-type PCRs were run on 1.2% agarose gels for visualization. Fluorescent AFLP analysis was done according to the protocol described previously by Flier et al. (2003). DNA was digested using enzymes *Eco*RI and *Mse*I with primers E00 and M00 and then ligated with *Eco*RI and *Mse*I adapters. Primary amplification was with primers E00 and M00, while secondary amplification was with primers E19 (fluorescent, Cy5-labeled) and M16, each with two selective nucleotides. Amplified bands were viewed using ALFwin Evaluation software (Amersham Pharmacia Biotech, Roosendaal, The Netherlands). For ITS sequencing, PCRs were done in 25-μl reactions, each containing 2.5 μl of 10 mM dNTPs, 2.5 μl of 10× Mango PCR buffer, 1.5 μl of 25 mM $MgCl_2$, 0.5 μl of 1U/μl Mango Taq DNA polymerase (Bioline), 2.5 μl each of 2 μM primers ITS4 and ITS5, 1 μl of 10 ng/μl target DNA, and 12 μl of sdd water. Cycling parameters were as described previously by Goodwin and Zismann (2001). Sequencing was done with the ThermoSequenase fluorescence-labeled primer cycle sequencing kit on an ALFexpress automated DNA sequencer (Amersham Pharmacia Biotech, Piscataway, NJ, USA) as described previously (Goodwin and Zismann, 2001). Digestions of ITS regions were done with the enzyme *Sau*3AI as described previously (Goodwin and Zismann, 2001).

3. Results

3.1. Comparative taxonomical analyses of discharged ascospores and of sexual structures

Routine test crosses between *M. graminicola* isolates IPO323 and IPO94269 that were used as positive controls for the crossing procedure discharged ascospores from eight through 12 weeks after inoculation. During weeks 11 and 12 (November 2002), we also identified a substantial number of two-celled ascospores (~80) from plants that were co-inoculated with *S. passerinii* isolates P71 and P83 that closely resembled those from *M. graminicola* in morphology and early growth development. Ascospores of the two species were similar in their germination patterns. Initially, two germ tubes arose from the polar ends and grew parallel to the long axis of the spore. Additional secondary germ tubes (1–2) arose at the ascospore septum and grew perpendicular to the long axis of the ascospore. Ascospores remained hyaline and did not develop additional septa during the initial phase of germination. We were able to isolate 17 of those as single-ascospore cultures for further analyses. Repeated attempts to cross *S. passerinii* resulted in a positive discharge of eight ascospores of the same type as mentioned above during May 2005 in West Lafayette, this time from cv. Kindred that was co-inoculated with *S. passerinii* isolates P63 and P67. One of these was isolated as a single-ascospore culture. The colonies developing from all 18 proposed progeny on PDA plates, as well as their morphology and growth rate in YGB cultures (not shown), were identical to those of the parental isolates.

In addition, numerous different types of ascospores were discharged from barley leaves that were co-inoculated with two *S. passerinii* isolates, including the control crosses between isolates of the same mating type, during this same time period. We monitored thousands of ascospore contaminants on barley, some of which could be identified. One species of *Didymella* with an *Ascochyta* anamorph, one species of *Leptosphaerulina*, and four species of *Paraphaeosphaeria* (including *P. michotii*) were isolated commonly. In addition, two-celled ascospores of *Davidiella tassiana*, the teleomorph of *Cladosporium herbarum*, also were encountered regularly on older leaf material. Single-spore isolates from a sampling of these contaminants did not show any similarity to *S. passerinii* in *in vitro* growth tests on PDA or in YGB (not shown).

The interspecies crosses between *S. passerinii* and *M. graminicola* resulted in numerous ascospores (two to four celled), but their growth on PDA and in YGB did not resemble that of either *S. passerinii* or *M. graminicola*. Subsequent RAPD characterization (data not shown) excluded them as interspecies hybrids, so they were considered to be contaminants.

Infected leaf samples inoculated with isolates of *S. passerinii* with opposite mating types from which *Mycosphaerella* ascospores were successfully harvested were examined microscopically to locate ripe ascomata. Despite numerous attempts over several years, only a single, partly decayed ascoma was found. Ascospores were observed to be hyaline, thin-walled, obovoid, and 10–15 × 3–4 μm. Due to the poor state of the material, the sexual stage could not be officially named, although it clearly resembled *M. graminicola* in general morphology. We therefore propose that the *S. passerinii* teleomorph belongs to the genus *Mycosphaerella*, as is indicated by its DNA phylogeny (Goodwin et al., 2001).

3.2. Genotyping

The 17 proposed progeny from the cross between *S. passerinii* isolates P71 and P83 were genotyped based on mating-type PCR, RAPD and AFLP markers, and by ITS analyses. The mating-type PCRs were positive and matched the expected 1:1 segregation ratio (*mat1-1*:*mat1-2* = 10:7; $\chi^2 = 0.53$; $P = 0.05$) typical for an organism with a heterothallic, bipolar mating system (data not shown).

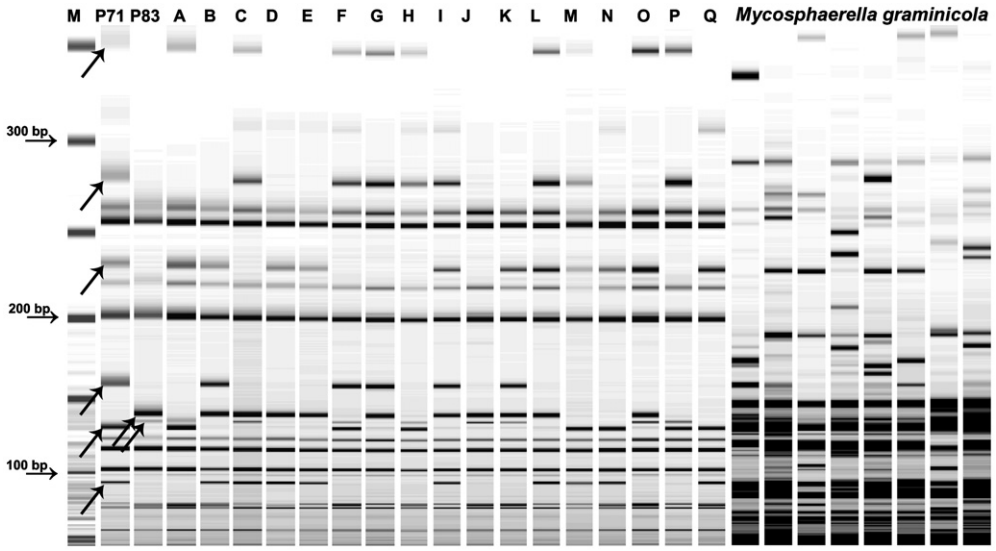

Fig. 1. Genotypes of the parental *Septoria passerinii* isolates P71 and P83 compared to 17 ascospore progeny (isolates A through Q) and seven single-ascospore isolates of *Mycosphaerella graminicola* using AFLP markers. Primers *Eco*RI-GA and *Mse*I-CC were used for all isolates. Diagonal arrows indicate polymorphic bands between the *S. passerinii* parental isolates (six from P71 and two from P83). Horizontal arrows indicate reference size markers.

Furthermore, the RAPD analysis showed that the majority of these progeny had recombinant genotypes based on just three markers (not shown), indicating that these *S. passerinii* isolates were the parents of the collected offspring. Genotyping of the ascospore set using AFLP confirmed this conclusion (Fig. 1). Results of the AFLP analysis with the primers *Eco*RI-GA and *Mse*I-CC showed that the parental isolates P71 and P83 had six and two unique bands, respectively, and had an additional 10 bands in common. All putative progeny isolates possessed these 10 shared bands and additionally displayed at least two of the eight unique bands observed for the parental isolates P71 and/or P83. All 17 proposed progeny had recombinant genotypes except for one that had the same genotype as P83, but this one had a recombinant genotype in the RAPD analysis. None of the progeny had bands that were not present in the parents. For comparison, seven isolates of *M. graminicola* were included on the same polyacrylamide gel using the same AFLP enzymes and primers. There was at least one (at ~230 bp) and possibly more shared bands between *M. graminicola* and *S. passerinii*, which can be expected since these species are closely related, but bands having the same size do not necessarily have the same sequences. However, the vast majority of bands were not shared between the two species, and the AFLP patterns clearly distinguish *S. passerinii* from *M. graminicola*.

To further distinguish the *S. passerinii* progeny from *M. graminicola*, the ITS region was digested with the enzyme *Sau*3AI. All *S. passerinii* progeny showed the same pattern as both of the parental isolates, P71 and P83 (not shown). This pattern was different from the pattern of *M. graminicola* isolates IPO323, IPO94269, and T48. In addition, the ITS regions of parental isolates P71 and P83 and progeny A, E, K, and M were cloned and sequenced. The ITS sequences of all isolates were identical to one another and to archived sequences of several isolates of *S. passerinii* in a blastn search of GenBank. Isolates P71, P83, A, and B have been deposited into the culture collection of the Fungal Biodiversity Center (Centraalbureau voor Schimmelcultures) in Utrecht, the Netherlands. The one proposed progeny isolate from the cross between *S. passerinii* isolates P63 and P67 was characterized as *mat1-1*, and its ITS sequence also was identical to that of *S. passerinii*. This isolate must have been a progeny derived from isolates P63 and P67, because barley is not grown in central Indiana and *S. passerinii* has not been found on wild barley, so no source of natural inoculum exists.

3.3. Phenotyping

Plant inoculations confirmed the ability of the progeny isolates to infect barley. Inoculation of barley seedlings with spores from offspring from the cross between P71 and P83 caused the typical SSLB symptoms on barley (Fig. 2) that began as small chlorotic flecks at 10 days after inoculation. These slowly developed into larger chlorotic blotches that eventually turned necrotic at ~17 days after inoculation. These lesions contained numerous pycnidia, the asexual fructifications that produce the slender multicelled pycnidiospores typical for *S. passerinii*. In contrast, inoculations using *M. graminicola* on the barley cv. Topper 33 or *S. passerinii* on the wheat cv. Taichung 29 did not develop symptoms, even after extended incubation periods (data not shown).

4. Discussion

High genotypic diversity in natural populations, the identification of apparently intact mating-type genes, and the occurrence of both mating types within single leaves

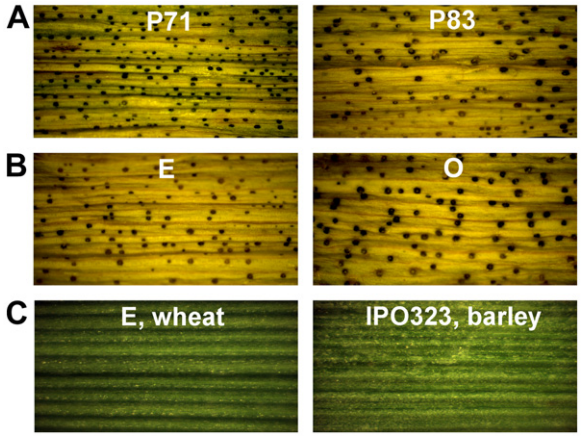

Fig. 2. Symptom development of isolates of *Septoria passerinii* and *Mycosphaerella graminicola* on the barley cultivar Topper 33 or the wheat cultivar Obelisk at 21 days post inoculation. (A) *S. passerinii* parental isolates P71 and P83 on cv. Topper 33. (B) *S. passerinii* progeny isolates E and O on cv. Topper 33. (C) Negative controls. (Left) *S. passerinii* progeny isolate E on cv. Obelisk. (Right) *M. graminicola* isolate IPO323 on cv. Topper 33.

all led to the speculation that *S. passerinii* had the capacity for sexual recombination (Goodwin et al., 2003). However, there was no concrete proof of a functional teleomorph for this fungus that was hitherto considered to be asexual (Cunfer and Ueng, 1999). Therefore, we proceeded to test the hypothesis of a functional teleomorph by crossing isolates of *S. passerinii* with opposite mating types using the *in planta* protocol developed for the closely related sexual species *M. graminicola* (Kema et al., 1996c). This led to the generation of the teleomorph for *S. passerinii* both in Europe and in the United States.

Even though we have generated and characterized sexual progeny from two crosses of *S. passerinii* isolates, we cannot formally describe the sexual stage as required by the International Code of Botanical Nomenclature due to the lack of well-preserved teleomorph material. The identification of the sexual structure has been hampered by the necessity to place inoculated plants outside for approximately two months. Due in part to this, the vast majority of the ascospores discharged from the inoculated barley leaves did not originate from crosses of *S. passerinii* isolates but instead were contaminants from fungi in the environment. Likewise, the vast majority of sexual structures observed on leaves were not produced by crosses of *S. passerinii* isolates but rather by naturally occurring contaminant species. This complicated the localization of the very few ascomata generated by the teleomorph of *S. passerinii*. Furthermore, our observations suggest that the inconspicuous, thin-walled, medium-brown ascomata degenerate quickly once ascospores are discharged, which could explain our difficulty in locating ripe ascomata on leaf sections known to harbor the teleomorph. Three species of *Mycosphaerella* have been described on *Hordeum* (barley), two on *Secale* (rye), and three on *Triticum* (wheat) (Corlett, 1991), but the dimensions of their ascospores as well as their associated anamorphs indicate that they are distinct from the *Mycosphaerella* teleomorph of *S. passerinii*.

It is noteworthy that the success rate of crosses and the number of ascospores obtained from successful crosses are much lower for *S. passerinii* than for *M. graminicola*. Two explanations for the observed sporadic recombination are that either the sexual cycle is much less active in *S. passerinii* on barley than in *M. graminicola* on wheat, or that conditions of the crossing procedure for *M. graminicola* on wheat need to be adapted to meet the environmental requirements for formation of the teleomorph of *S. passerinii* on barley. Thus far, we do not have an indication of what these environmental requirements are, especially since ascospores were harvested from the two successful crosses during cold and wet conditions in Europe (November 2002) and during warm and dry conditions in the United States (May 2005). Other crossing procedures have been attempted for both *S. passerinii* and *M. graminicola*, including leaving the inoculated plants in the greenhouse instead of placing them outside, following the *in vitro* crossing method used for *Mycosphaerella citri* (Mondal et al., 2004), and others. However, only the protocol developed by Kema et al. (1996c) resulted in ascospore production in both species.

The need to place inoculated plants outside to complete the sexual cycle makes them vulnerable to infection by environmental inoculum of *S. passerinii* and other fungi. Contamination by unrelated fungi can be identified and eliminated easily, as described above. However, we also must be certain that environmental inoculum of *S. passerinii* can be identified and excluded. The possibility of contamination by environmental inoculum in Indiana is essentially zero. Barley is not grown commercially in Indiana so there is no nearby source of inoculum. The only wild barley that occurs commonly is *Hordeum jubatum*, and speckled leaf blotch has never been reported on this host in Indiana. Furthermore, an isolate from *H. jubatum* in Minnesota had a different-sized amplicon with the mating-type PCR assay and a different ITS sequence compared to typical *S. passerinii*, so was considered to represent a new, unnamed species of *Septoria* (Goodwin and Zismann, 2001). Thus, there is essentially a zero probability that the progeny isolate in Indiana could have arisen from contamination by environmental inoculum of *S. passerinii*.

It also is extremely unlikely, if not impossible, that we have isolated ascospores from environmental inoculum of *S. passerinii* in the Netherlands. Despite the fact that *S. passerinii* is endemic in the Netherlands, it is not a major pathogen of barley. Moreover, the size of the barley crop in the Netherlands is very small (~50,000 ha) and concentrated at least 150 km from the experimental site. This reduces the chance for splash-borne inoculum to zero, as conidia (pycnidiospores) of the closely related (Goodwin and Zismann, 2001) *S. tritici* are dispersed only over very short distances (on the order of meters) (Bannon and Cooke, 1998; Shaw, 1999) with half distances of about 10 cm (Shaw, 1999). Dispersal ranges of conidia of

S. passerinii have not been estimated but presumably will be similar to those for *S. tritici*. Furthermore, none of the negative controls (those inoculated with isolates of the same mating type) discharged ascospores that could be tied to *S. passerinii*, and all of the segregating markers from the AFLP and RAPD analyses came from the two inoculated isolates with no evidence of migrant alleles. An abundance of genetic data in *M. graminicola* using the same mating protocol also showed no evidence of migrant alleles (Kema et al., 1996c, 2000). We therefore conclude that there is essentially no chance that any of the progeny isolates in Indiana or the Netherlands arose from environmental inoculum of *S. passerinii*.

Recently, many presumably asexual fungi have been found to be sexual, such as: *Colletotrichum acutatum* (teleomorph *Glomerella acutata*), a pathogen of flowering plants (Guerber and Correll, 2001); *Phaeoacremonium aleophilum* (teleomorph *Togninia minima*), associated with Petri disease in grapevines (Mostert et al., 2006); and *Beauveria bassiana* (teleomorph *Cordyceps bassiana*), a widely used biological control agent against insects (Huang et al., 2002). Similarly, the identification of mating-type genes in *S. passerinii* has led to the current discovery of a cryptically active sexual cycle. However, mating-type genes have been identified in many other fungal species in which a sexual cycle has not yet been confirmed. One such example is the barley pathogen *Rhynchosporium secalis*. After a phylogenetic analysis showed that this pathogen probably has a teleomorph in the genus *Tapesia* (Goodwin, 2002), two groups cloned its mating-type genes using degenerate primers designed from sequences of *T. yallundae* and *Pyrenopeziza brassicae* (Foster and Fitt, 2003; Linde et al., 2003). Screening of natural populations of *R. secalis* revealed high genetic diversity and a 1:1 ratio for *mat1-1*: *mat1-2* in most populations sampled (Linde et al., 2003). Another example is *Fusarium oxysporum*, a well-studied plant pathogen with a wide host range (Armstrong and Armstrong, 1981). Mating-type genes from *F. oxysporum* have been cloned by Arie et al. (2000). However, attempts to cross isolates of *F. oxysporum* with opposite mating types have not yielded sexual spores (S. Ware, unpublished), nor have these spores been found in nature, although high genotypic diversity in natural populations of *F. oxysporum* also suggests the possibility of a sexual cycle (Baayen et al., 2000; Bao et al., 2002). More recently, Paoletti et al. (2005) found evidence for sexuality in the opportunistic human pathogen *Aspergillus fumigatus*.

Almost certainly, many presumably asexual fungi are sexually recombining (see review by Taylor et al., 1999, for a parallel opinion with expanded arguments). In addition to the examples given already, a brief review of findings for the human pathogen *Cryptococcus neoformans* represents an excellent example of why the reproductive capabilities of fungi should not be underestimated. The anamorph *C. neoformans* was first described by Busse (1894) and was presumed to be asexual until the discovery of a bipolar heterothallic mating system in 1976, which led to the naming of the teleomorph *Filobasidiella neoformans* (Kwon-Chung, 1976). Twenty years later, monokaryotic fruiting between isolates with the same mating type was reported in *C. neoformans*, but this type of reproduction was considered to be strictly mitotic and asexual based on descriptions in other fungi (Wickes et al., 1996). However, in 2005 this monokaryotic fruiting was proven to be a second sexual form of mating for this pathogen (Lin et al., 2005). Thus, major ideas on mating for *C. neoformans* have changed three times since the description of the anamorph, and even a completely new type of sexual reproduction in fungi has been discovered. Therefore, the possibility and even probability of sexual recombination for presumably asexual fungi cannot be excluded, as has been demonstrated in our study.

The discovery of a functional sexual cycle for *S. passerinii* has potentially important consequences for future study of this pathogen as well as for resistance breeding efforts in the host. In a comparison between *S. passerinii* and *M. graminicola*, the time lapse between the description of the anamorph and the discovery of the corresponding teleomorph is similar (123 and 130 years, respectively). *S. tritici*, the anamorph of *M. graminicola*, was first reported in 1842. The teleomorph was discovered in 1894, but it was not linked to *S. tritici* until 1972 (Sanderson, 1972). Once this link was made, the emphasis of research efforts extended from epidemiological studies (Royle and Shaw, 1986; Shaw and Royle, 1993) to studies on population genetics (McDonald et al., 1995, 1999) and host-pathogen interactions (Kema et al., 1996a,b, 2000). The development of fungal genetics in *M. graminicola* (Kema et al., 1996c) had an important impact on the identification of resistance genes in wheat (Brading et al., 2002). To date, at least 12 resistance genes have been identified that are currently being used in practical breeding programs (Chartrain et al., 2005). In this study, we have identified the existence of the sexual stage of *S. passerinii* and report a crossing protocol that potentially can, with some adaptation, be used to generate a mapping population of *S. passerinii* progeny to study the genetics of avirulence on barley. We hypothesize that this will substantially benefit resistance breeding in barley to this economically important pathogen.

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References

Adhikari, T.B., Anderson, J.M., Goodwin, S.B., 2003. Identification and molecular mapping of a gene in wheat conferring resistance to *Mycosphaerella graminicola*. Phytopathology 93, 1158–1164.

Arie, T., Kaneko, I., Yoshida, T., Noguchi, M., Nomura, Y., Yamaguchi, I., 2000. Mating-type genes from asexual phytopathogenic ascomycetes *Fusarium oxysporum* and *Alternaria alternata*. Mol. Plant Microbe Interact. 13, 1330–1339.

Armstrong, G.M., Armstrong, J.K., 1981. Formae speciales and races of *Fusarium oxysporum* causing wilt diseases. In: Nelson, P.E., Toussoun, T.A., Cook, R.J. (Eds.), *Fusarium*: Diseases, Biology and Taxonomy. The Pennsylvania State University Press, University Park, pp. 391–399.

Baayen, R.P., O'Donnell, K., Bonants, P.J.M., Cigelnik, E., Kroon, L.P.N.M., Roebroeck, E.J.A., Waalwijk, C., 2000. Gene geneologies and AFLP analyses in the *Fusarium oxysporum* complex identify monophyletic and nonmonophyletic formae speciales causing wilt and rot disease. Phytopathology 90, 891–900.

Bannon, F.J., Cooke, B.M., 1998. Studies on dispersal of *Septoria tritici* pycnidiospores in wheat-clover intercrops. Plant Pathol. 47, 49–56.

Banttari, E.E., Anderson, W.H., Rasmusson, D.C., 1975. Helminthosporium head blight resistance in six-row spring barleys. Plant Dis. Rep. 59, 274–277.

Bao, J.R., Fravel, D.R., O'Neill, N.R., Lazarovits, G., van Berkum, P., 2002. Genetic analysis of pathogenic and nonpathogenic *Fusarium oxysporum* from tomato plants. Can. J. Botany 80, 271–279.

Brading, P.A., Verstappen, E.C.P., Kema, G.H.J., Brown, J.K.M., 2002. A gene-for-gene relationship between wheat and *Mycosphaerella graminicola*, the septoria tritici blotch pathogen. Phytopathology 92, 439–445.

Buchannon, K.W., 1961. Inheritance of reaction to *Septoria passerinii* Sacc., and *Pyrenophora teres* (Died.) Drechsl., and a row number, in barley. Ph.D. thesis, University of Saskatchewan, Canada.

Busse, O., 1894. Über parasitäre zelleinschlüsse und ihre züchtung. Zentbl. Bakteriol. 16, 175–180.

Chartrain, L., Joaquim, P., Berry, S.T., Arraiano, L.S., Azanza, F., Brown, J.K.M., 2005. Genetics of resistance to septoria tritici blotch in the Portuguese wheat breeding line TE 9111. Theoret. Appl. Genet. 110, 1138–1144.

Corlett, M., 1991. An annotated list of the published names in *Mycosphaerella* and *Sphaerella*. Mycol. Mem. 18, 1–328.

Cunfer, B.M., Ueng, P.P., 1999. Taxonomy and identification of *Septoria* and *Stagonospora* species on small grain cereals. Annu. Rev. Phytopathol. 37, 267–284.

Flier, W.G., Grunwald, N.J., Kroon, L.P.N.M., Sturbaum, A.K., van den Bosch, T.B.M., Garay-Serrano, E., Lozoya-Saldana, H., Fry, W.E., Turkensteen, L.J., 2003. The population structure of *Phytophthora infestans* from the Toluca Valley of Central Mexico suggests genetic differentiation between populations from cultivated potato and wild *Solanum* spp. Phytopathology 93, 382–390.

Foster, S.J., Fitt, B.D.L., 2003. Isolation and characterisation of the mating-type (MAT) locus from *Rhynchosporium secalis*. Curr. Genet. 44, 277–286.

Goodwin, S.B., 2002. The barley scald pathogen *Rhynchosporium secalis* is closely related to the discomycetes *Tapesia* and *Pyrenopeziza*. Mycol. Res. 106, 645–654.

Goodwin, S.B., Dunkle, L.D., Zismann, V.L., 2001. Phylogenetic analysis of *Cercospora* and *Mycosphaerella* based on the internal transcribed spacer region of ribosomal DNA. Phytopathology 91, 648–658.

Goodwin, S.B., Waalwijk, C., Kema, G.H.J., Cavaletto, J.R., Zhang, G., 2003. Cloning and analysis of the mating-type idiomorphs from the barley pathogen *Septoria passerinii*. Mol. Gen. Genomics 269, 1–12.

Goodwin, S.B., Zismann, V.L., 2001. Phylogenetic analyses of the ITS region of ribosomal DNA reveal that *Septoria passerinii* from barley is closely related to the wheat pathogen *Mycosphaerella graminicola*. Mycologia 93, 934–946.

Green, G.J., Bendelow, V.M., 1961. Effect of speckled leaf blotch, *Septoria passerinii* Sacc., on the yield and malting quality of barley. Can. J. Plant Sci. 41, 431–435.

Green, G.J., Dickson, J.G., 1957. Pathological histology and varietal reactions in septoria leaf blotch of barley. Phytopathology 47, 73–79.

Guerber, J.C., Correll, J.C., 2001. Characterization of *Glomerella acutata*, the teleomorph of *Colletotrichum acutatum*. Mycologia 93, 216–229.

Helm, J.H., Cortez, M.J., Juskiw, P., Salmon, D.F., Stewart, W.M., 2001. Registration of 'Peregrine' Barley. Crop Sci. 41, 1991–1992.

Huang, B., Chun-Ru, L., Zhen-Gang, L., Mei-Zhen, F., Zeng-Zhi, L., 2002. Molecular identification of the teleomorph of *Beauveria bassiana*. Mycotaxon 81, 229–236.

Kema, G.H.J., Annone, J.G., Sayoud, R., van Silfout, C.H., van Ginkel, M., de Bree, J., 1996a. Genetic variation for virulence and resistance in the wheat-*Mycosphaerella graminicola* pathosystem. I. Interactions between pathogen isolates and host cultivars. Phytopathology 86, 200–212.

Kema, G.H.J., Sayoud, R., Annone, J.G., van Silfout, C.H., 1996b. Genetic variation for virulence and resistance in the wheat-*Mycosphaerella graminicola* pathosystem. II. Analysis of interactions between pathogen isolates and host cultivars. Phytopathology 86, 213–220.

Kema, G.H.J., Verstappen, E.C.P., Todorova, M., Waalwijk, C., 1996c. Successful crosses and molecular tetrad progeny analyses demonstrate heterothallism in *Mycosphaerella graminicola*. Curr. Genet. 30, 251–258.

Kema, G.H.J., Verstappen, E.C.P., Waalwijk, C., 2000. Avirulence in the wheat septoria tritici leaf blotch fungus *Mycosphaerella graminicola* is controlled by a single locus. Mol. Plant Microbe Interact. 13, 1375–1379.

Koble, A.F., Peterson, G.A., Timian, R.G., 1959. A method of evaluating the reaction of barley seedlings to infection with *Septoria passerinii* Sacc. Plant Dis. Rep. 43, 14–17.

Kwon-Chung, K.J., 1976. Morphogenesis of *Filobasidiella neoformans*, the sexual state of *Cryptococcus neoformans*. Mycologia 68, 821–833.

Lin, X., Hull, C.M., Heitman, J., 2005. Sexual reproduction between partners of the same mating type in *Cryptococcus neoformans*. Nature 434, 1017–1021.

Linde, C.C., Zala, M., Ceccarelli, S., McDonald, B.A., 2003. Further evidence for sexual reproduction in *Rhynchosporium secalis* based on distribution and frequency of mating-type alleles. Fungal Genet. Biol. 40, 115–125.

Mathre, D.E., 1997. Compendium of Barley Diseases, second ed. American Phytopathological Society Press, St. Paul.

McDonald, B.A., Pettway, R.E., Chen, R.S., Boeger, J.M., Marinez, J.P., 1995. The population genetics of *Septoria tritici* (teleomorph *Mycosphaerella graminicola*). Can. J. Botany 73 (Suppl. 1), S292–S301.

McDonald, B.A., Zahn, J., Yarden, O., Hogan, K., Garton, J., Pettway, R.E., 1999. The population genetics of *Mycosphaerella graminicola* and *Stagonospora nodorum*. In: Lucas, J.A., Bowyer, P., Anderson, H.M. (Eds.), *Septoria* on Cereals: A Study of Pathosystems. CABI Publishing, Wallingford, pp. 44–69.

Metcalfe, D.R., Buchannon, K.W., McDonald, W.C., Reinbergs, E., 1970. Relationships between the 'Jet' and 'Milton' genes for resistance to loose smut and genes for resistance to other barley diseases. Can. J. Plant Sci. 50, 995–999.

Mondal, S.N., Howd, D.S., Brlansky, R.H., Timmer, L.W., 2004. Mating and pseudothecial development in *Mycosphaerella citri*, the cause of citrus greasy spot. Phytopathology 94, 978–982.

Mostert, L., Groenewald, J.Z., Summerbell, R.C., Gams, W., Crous, P.W., 2006. Taxonomy and pathology of *Togninia* (Diaporthales) and its *Phaeoacremonium* anamorphs. Stud. Mycol. 54, 1–115.

Paoletti, M., Rydholm, C., Schwier, E.U., Anderson, M.J., Szakacs, G., Lutzoni, F., Debeaupuis, J., Latgé, J., Denning, D.W., Dyer, P.S., 2005. Evidence for sexuality in the opportunistic fungal pathogen *Aspergillus fumigatus*. Curr. Biol. 15, 1242–1248.

Passerini, G., 1879. Funghi parmensi enumerati. Atti Soc. Crittog. Ital. Milano (Ser. 2) 2, 20–47.

Rasmusson, D.C., Rogers, W.E., 1963. Inheritance of resistance to *Septoria* in barley. Crop Sci. 3, 161–162.

Royle, D.J., Shaw, M.W., 1986. Recent advances towards reliable forecasting of *Septoria* diseases in winter wheat. In: Balkern, A.A. (Ed.), Integrated Crop Protection in Cereals. Brookfield, Rotterdam, pp. 207–215.

Sanderson, F.R., 1972. A *Mycosphaerella* species as the ascogenous state of *Septoria tritici* Rob. and Desm. N. Z. J. Bot. 10, 707–710.

Shaw, M.W., 1999. Epidemiology of *Mycosphaerella graminicola* and *Phaeosphaeria nodorum*: an overview. In: van Ginkel, M., McNab, A., Krupinsky, J. (Eds.), Septoria and Stagonospora Diseases of Cereals: A Compilation of Global Research. CIMMYT, Mexico, DF, pp. 93–97.

Shaw, M.W., Royle, D.J., 1993. Factors determining the severity of epidemics of *Mycosphaerella graminicola* (*Septoria tritici*) on winter wheat in the UK. Plant Pathol. 42, 882–899.

Steffenson, B.J., 2003. Stopping the blotch: Resistance to septoria speckled leaf blotch in barley. In: Proceedings of the 34th Barley Improvement Conference. San Francisco, pp. 16–19.

Taylor, J.W., Jacobson, D.J., Fisher, M.C., 1999. The evolution of asexual fungi: reproduction, speciation and classification. Annu. Rev. Phytopathol. 37, 197–246.

Toubia-Rahme, H., Johnston, P.A., Pickering, R.A., Steffenson, B.J., 2003. Inheritance and chromosomal location of *Septoria passerinii* resistance introgressed from *Hordeum bulbosum* into *Hordeum vulgare*. Plant Breeding 122, 405–409.

Toubia-Rahme, H., Steffenson, B.J., 1999. Sources of resistance to *Septoria passerinii* in *Hordeum vulgare* and *H. vulgare* subsp.*spontaneum*. In: van Ginkel, M., McNab, A., Krupinsky, J. (Eds.), Septoria and Stagonospora Diseases of Cereals: A Compilation of Global Research. CIMMYT, Mexico, D.F, pp. 156–158.

Toubia-Rahme, H., Steffenson, B.J., 2004. Sources of resistance to septoria speckled leaf blotch caused by *Septoria passerinii* in barley. Can. J. Plant Pathol. 26, 358–364.

Waalwijk, C., Mendes, O., Verstappen, E.C.P., de Waard, M.A., Kema, G.H.J., 2002. Isolation and characterization of the mating-type idiomorphs from the wheat septoria leaf blotch fungus *Mycosphaerella graminicola*. Fungal Genet. Biol. 35, 277–286.

Wickes, B.L., Mayorga, M.E., Edman, U., Edman, J.C., 1996. Dimorphism and haploid fruiting in *Cryptococcus neoformans*: association with the α-mating type. Proc. Natl. Acad. Sci. USA 93, 7327–7331.