Species and Mating-Type Distribution of *Tapesia yallundae* and *T. acuformis* and Occurrence of Apothecia in the U.S. Pacific Northwest

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ABSTRACT

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Eyespot of wheat is caused by the discomycete fungi *Tapesia yallundae* and *T. acuformis*. *T. yallundae* is considered the most important causal agent of the disease in this region but no apothecia of either species have been found in the U.S. Pacific Northwest (PNW). Two compatible isolates of *T. yallundae* from the PNW were used to inoculate a field plot in the fall of 1998 and apothecia developed in the spring and fall of 2000 on standing wheat stubble. In the spring of 2000, wheat stubble from eight naturally infected fields was examined for the presence of apothecia of *T. yallundae* and *T. acuformis*. Apothecia of *T.*

acuformis were found in two fields but no apothecia of *T. yallundae* were found. This is the first report of apothecia of the eyespot pathogens occurring in the PNW. Species and mating-type distribution of *T. yallundae* and *T. acuformis* in the PNW were determined from 817 isolates collected from diseased wheat over 3 years at spatial scales ranging from within fields to across states. In all, 460 isolates were identified as *T. yallundae* and 357 isolates were identified as *T. acuformis* with *MAT1-1/MAT1-2* ratios not significantly different from 1:1 based on χ^2 tests at most scales tested. The apparent increase in frequency of *T. acuformis* from previous surveys may indicate a shift in the predominant species causing eyespot. The occurrence of apothecia under field conditions, along with the widespread distribution of mating types of both species, suggests that sexual reproduction may be occurring in both species.

Eyespot disease of cereals, caused by the discomycete fungi *Tapesia yallundae* and *T. acuformis*, is a persistent and yield-limiting problem in many temperate climates where winter wheat is grown. Both pathogens over-summer between crops in colonized host debris and sporulate during cool and moist conditions in the fall and spring (19,31). Infection and colonization of stems occurs at or near the soil line, which weakens and reduces the number of tillers, resulting in yield loss from reduced grain set and lodging (5,31). In the United States, eyespot is a significant problem in the Pacific Northwest (PNW), where the disease can affect more than 500,000 ha and reduce yield by up to 50% in winter wheat-growing areas of eastern Washington, northeastern Oregon, and northern Idaho (5,24). Control of eyespot relies on the use of fungicides, delayed seeding in the fall, and by planting resistant cultivars (24).

T. yallundae and *T. acuformis*, respectively, were previously known as W- and R-pathotypes of the anamorphic species *Pseudocercospora herpotrichoides*, based on pathogenicity and cultural characteristics (13,16). W-type isolates are highly virulent to wheat but weakly virulent to rye and produce fast growing colonies with smooth edges on potato dextrose agar (PDA) (19, 30). R-type isolates are equally virulent on wheat and rye and form slow-growing colonies on PDA with feathery, uneven margins (19,30). Apothecia belonging to the genus *Tapesia* were discovered in Australia in 1987 on wheat residue (34). Single ascospore isolates derived from these apothecia yielded W-type isolates (34). Apothecia subsequently were found in England that produced R-type isolates, indicating that sexual reproduction

could occur within both pathotypes and establishing the anamorph-teleomorph connection (10). A two-allele heterothallic mating system has since been described for both pathotypes (9, 11,22), which are now considered separate biological species, *T. yallundae* (W-type) and *T. acuformis* (R-type), based on the failure of isolates from the two groups to intercross, despite having apparently morphologically identical apothecia (9,23). Additional sequence and molecular marker data supports the separation of pathotypes into distinct species (25,28,32).

Apothecia of *T. yallundae* have been found in wheat fields in Germany, England, Belgium, Australia, New Zealand, and South Africa (19). Apothecia of *T. acuformis* have only been reported from England (10), Germany (14), and Belgium (22), and are generally rare compared to apothecia of *T. yallundae* (10,19). In all cases, apothecia were found in agricultural fields where the stubble was left after harvest, or on diseased stubble that had been collected from the field and incubated in vitro. In England, apothecia of *T. yallundae* develop under natural field conditions when compatible isolates are used as inoculum, but apothecia of *T. acuformis* have not been found under similar experimental conditions (7). No apothecia of either species have been found in the U.S. PNW even though the disease has been studied intensively since the early 1900s (31).

Under laboratory conditions, apothecia of *T. yallundae* form readily when compatible mating types are crossed (11). Both mating types of *T. yallundae* recently have been identified from the PNW based on successful laboratory crosses, but only a limited number of isolates were used to determine mating type (6). In contrast, in vitro crosses between *T. acuformis* isolates rarely produce fertile apothecia (9,19,23), which has made studying mating-type distribution of *T. acuformis* difficult. Recently, the mating-type idiomorphs of *T. yallundae* have been sequenced and a multiplex polymerase chain reaction (PCR) assay developed to

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identify mating types of both species (8). The ability to differentiate mating type using a simple PCR test allows for the screening of a large number of isolates more effectively than performing in vitro crosses, which can take 6 to 12 months to complete, and also allows mating type of *T. acuformis* to be determined.

The objectives of this research were to determine if compatible isolates of *T. yallundae* could produce apothecia under field conditions and to estimate the frequency of occurrence of apothecia of either species by collecting and examining naturally diseased wheat stubble from winter wheat fields in the PNW. Moreover, the distribution of mating type of both species was investigated to test if the mating type ratio of *MAT1-1* to *MAT1-2* was in a 1:1 ratio for both species, as would be consistent with sexually reproducing species. *T. yallundae* has been the predominant species causing eyespot in the PNW since the disease was first described from Washington in the early 1900s (24,31). However, *T. acuformis* appears to be increasing in importance based on recent surveys (G. W. Douhan and T. D. Murray, unpublished data). This apparent increase in *T. acuformis* may indicate a shift in the predominant species causing eyespot, which also occurred in some parts of Europe in the early 1980s (13,19). Such a shift in the major eyespot pathogen could have an impact on disease control because little is known about resistance to *T. acuformis* in PNW winter wheat cultivars. Therefore, a second objective was to determine the relative abundance of each species in the PNW.

MATERIALS AND METHODS

Field inoculation. A field experiment was conducted at the Spillman Agronomy Farm, Pullman, WA, using winter wheat cvs. Madsen and Stephens and the *T. yallundae* isolates 90-83-5 (*MAT1-1*) and 97-1-8 (*MAT1-2*), which previously were shown to be fertile under laboratory conditions (6). Seed was sown (50 g per plot) on 23 September 1998 using a grain drill. Plots were planted in five rows of approximately 1.4 by 3.0 m into Thatuna silt loam (fine-silty, mixed, mesic xeric Agrialboll). Six plots of each cultivar were arranged in a completely randomized design with three treatments consisting of (i) inoculation of *T. yallundae* isolate 90-83-5 (*MAT1-1*) alone, (ii) inoculation of *T. yallundae* isolate 97-1-8 (*MAT1-2*) alone, or (iii) a 1:1 mixture of both isolates. Inoculum was produced by placing oat kernels colonized with the fungus on fiberglass screens outdoors in the early autumn for 4 to 6 weeks (3,4). Conidia were collected by rinsing the oats with water and adjusting the final concentration to 1×10^6 conidia

per ml using a hemacytometer. Inoculum was applied on 17 November at a rate of 262.5 liters/ha using a CO₂-pressurized backpack sprayer. The plots were harvested during the first week of August 1999 and the stubble was left standing. The plots were examined at the end of September and October 1999 and again in March and September 2000 for the presence of apothecia by randomly sampling straw from each treatment and observing 50 stems using a stereo microscope. When apothecia were found, single ascospore cultures were made from several apothecia by placing apothecia on the underside of a petri dish lid using petroleum jelly. The lids then were placed over 1.5% water agar (WA) (Sigma-Aldrich, St. Louis) amended with rifampicin (50 µg/ml; Sigma-Aldrich) and ascospores were allowed to discharge for 24 to 48 h at room temperature (20°C). Single germinated ascospores were transferred to PDA (Difco Laboratories, Detroit) 24 to 48 h after incubation at room temperature and incubated an additional 2 to 3 weeks at room temperature to confirm colony morphology. Apothecia also were examined microscopically to confirm their identity as *T. yallundae* as described by Wallwork and Spooner (34).

Field observations from naturally diseased wheat stems.

Straw from eight wheat fields in Washington previously identified as having a high incidence of eyespot during the fall of 1999 was sampled on 29 March and 5 May 2000. Approximately 75 to 150 stems were randomly collected within a 25-m² area from each field. Diseased straw was collected from fields that had intact standing stubble as well as from fields where the stubble had been tilled. From 50 to 75 stems with intact lower leaf sheaths were examined for the presence of apothecia using a stereo microscope. When apothecia were found, several were examined in greater detail using a compound microscope to observe morphological characters, and single ascospore cultures were made as described above or by dilution plating crushed apothecia onto WA amended with rifampicin after microscopic examination.

Sampling of field isolates for species and mating-type distribution. A hierarchical sampling strategy was used to collect isolates of *T. yallundae* and *T. acuformis*. Five wheat fields in Washington were intensively sampled and smaller samples were collected from 55 wheat fields from eastern Washington, northern Oregon, and northern Idaho (Fig. 1; Table 1). All isolates were collected from diseased stems from March through July 1998, 1999, and 2000. Two different sampling strategies were used for the intensively sampled fields. In 1998 and 1999, diseased plants (≈20 to 30 stems) were collected every 3 m along two parallel transects separated by 100 to 150 m for a total of 60 (2 × 30) and 100 (2 × 50) samples, respectively. In 2000, the sampling strategy was modified and diseased plants (≈10 to 20 stems) were collected every meter along two transects separated by 3 m for a total of 20 (2 × 10) samples. In 1998 and 1999, an attempt was made to recover a single isolate per sample, whereas in 2000, an attempt was made to recover up to three isolates per sample. It was possible to collect samples along transects because all fields had relatively uniform disease across a large area. In contrast, individual plants were pulled randomly from the less intensively sampled fields to look for disease symptoms because aboveground symptoms were not apparent at the time of collection. Each sample was collected at least 5 to 10 m apart and was represented by 1 to 15 diseased stems per field with a maximum of 10 samples per field. An attempt was made to recover a single isolate per sample.

To isolate the pathogens, lesions were cut from diseased stems, surface disinfected for 1 to 3 min in 1% NaOCl, placed onto WA containing rifampicin (50 µg/ml), and incubated at 14 to 16°C under near UV light for 1 to 2 weeks. Hyphae growing directly from the lesions or hyphal tips from the edge of a colony growing into the agar were transferred to 0.2% PDA containing rifampicin (50 µg/ml) and incubated for an additional 1 to 2 weeks at room temperature. When isolates appearing to be *T. yallundae* and *T. acuformis* originated from the same sample, two isolates per

Fig. 1. Locations of eyespot samples from winter wheat fields in A, Washington, B, Oregon, and C, Idaho. Intensively sampled fields = ■ and less intensively sampled fields = *.

sample were made, one to tentatively represent each species. Single conidial cultures were made from the intensively sampled fields but not from the less intensively sampled fields. All isolates were incubated at room temperature on PDA for at least 2 weeks before identification was made.

DNA extraction and mating-type identification. Isolates were grown on PDA for 2 to 4 weeks at room temperature, after which surface mycelium was removed and freeze dried. DNA was extracted by the method of Lee and Taylor (17) omitting the addition of 2-mercaptoethanol and using 100 mM Tris (pH 8.0) instead of 50 mM Tris (pH 7.2) in the lysis buffer. DNA was suspended into Tris-EDTA (10 mM Tris, 0.1 mM EDTA, pH 8.0) with the addition of RNase (20 µg/ml, Sigma-Aldrich), incubated at 37°C for 2 h, diluted 1:25 in sterile H₂O, and used directly as template in PCR.

Mating type of isolates was determined using multiplex PCR as described by Dyer et al. (8). Three primers are used in the assay, one that is common to both mating types and specific primers for *MAT1-1* and *MAT1-2* sequences which are differentiated by size in agarose gels. Each 20-µl PCR reaction contained 1× PCR buffer (Life Technologies, Grand Island, NY), 2.0 mM MgCl₂, 2.5 mM each dNTP (Life Technologies), 3.75 µM of each primer, 0.5 units of *Taq* polymerase (Life Technologies), and 2 µl of diluted template DNA. Thermocycling conditions consisted of an initial hold of 94°C for 4 min followed by 30 cycles of 94°C (30 s), 55°C (30 s), and 72°C (1 min) with a final hold of 72°C for 5 min. All amplifications were performed in a PE-9700 thermocycler (Perkin Elmer Corp., Norwalk, CT). PCR products were separated in 1.5% agarose gels and stained with ethidium bromide, after which the image of the gel was captured digitally.

Identification of field isolates. Isolates were grown on PDA for 2 to 4 weeks at room temperature before identification was determined. *T. yallundae* isolates usually produce fast-growing, smoky gray colonies with smooth edges, whereas isolates of *T. acuformis* grow at about half the rate of *T. yallundae* and produce compact gray to cream-colored colonies with feathery to coarsely uneven margins (30). In instances when isolates produced intermediate cultural characteristics and positive identification could not be determined visually, isolate identification was determined using species-specific PCR primers designed from the internal transcribed spacer (ITS) region of the rDNA (2). Amplification cocktail mixture for species identification using species-specific primers was the same as above. Thermocycling conditions consisted of an initial hold of 94°C for 4 min followed by 30 cycles of 94°C (20 s) to 72°C (30 s) with a final hold of 72°C for 5 min. All amplifications were performed in a PE-9700 thermocycler and the PCR products were separated in 1.5% agarose gels and stained with ethidium bromide, after which the image of the gel was captured digitally.

Data analysis of field isolates. Isolates were pooled in a hierarchical fashion to investigate species and mating-type distribution by field, county, state, and the entire PNW. Isolates from the intensively sampled fields were used to represent individual fields in Washington. Isolates from the less intensively sampled fields were pooled for county comparison, and counties were pooled for state comparisons. The entire data set was pooled to represent the PNW winter wheat-growing area. The number of isolates and mating type of *T. yallundae* and *T. acuformis* at all levels were determined and χ^2 tests were used to test for deviations from the expected 1:1 ratio of *MAT1-1*/*MAT1-2* under the null hypothesis of sexual reproduction at the 95% confidence level. The χ^2 tests were not performed on sample sizes of less than 10 due to lack of statistical power (12).

RESULTS

Development of apothecia in inoculated field plots. Apothecia were first observed on wheat stubble 25 March 2000. In all, 51

and 94% of stems examined from cvs. Madsen and Stephens, respectively, had apothecia in the treatment where both mating types were used as inoculum. Only one apothecium was found on a stem of Stephens from a treatment where a single mating type was used as inoculum. The disease was more severe on Stephens compared to Madsen based on a large percentage of stems that had lodged in the Stephens plots. However, eyespot lesions were

TABLE 1. Isolates of *Tapesia yallundae* and *T. acuformis* from wheat fields in Washington (WA), Oregon (OR), and Idaho (ID) collected March 1998 to May 2000

State	County	Field no. ^a	Date collected ^b	N ^c
WA	Adams	98-2*	4/26/98	76
WA	Adams	99-1*	4/29/99	123
WA	Adams	99-3	3/31/99	18
WA	Adams	99-7	3/31/99	3
WA	Columbia	99-21	4/23/99	4
WA	Garfield	99-35	4/23/99	9
WA	Grant	99-10	4/16/99	15
WA	Grant	99-14	4/16/99	4
WA	Grant	99-16	4/16/99	13
WA	Grant	99-19	4/16/99	8
WA	Grant	99-39	4/16/99	3
WA	Lincoln	99-11	4/16/99	12
WA	Lincoln	99-12	4/16/99	9
WA	Lincoln	99-15	4/16/99	12
WA	Lincoln	99-17	4/16/99	11
WA	Lincoln	99-28	4/13/99	5
WA	Lincoln	99-37	4/16/99	1
WA	Okanogan	98-1*	4/25/98	81
WA	Walla Walla	00-1*	5/3/00	55
WA	Walla Walla	99-5	3/31/99	1
WA	Walla Walla	99-6	3/31/99	5
WA	Walla Walla	99-29	4/23/99	9
WA	Walla Walla	99-31	4/23/99	8
WA	Walla Walla	99-33	4/23/99	8
WA	Walla Walla	99-36	4/23/99	5
WA	Whitman	00-14*	6/15/00	82
WA	Whitman	99-2	4/26/99	10
WA	Whitman	99-4	3/31/99	9
WA	Whitman	99-8	4/13/99	3
WA	Whitman	99-9	4/28/99	10
WA	Whitman	99-18	4/16/99	11
WA	Whitman	99-20	4/16/99	6
WA	Whitman	99-22	4/16/99	4
WA	Whitman	99-23	4/23/99	16
WA	Whitman	99-24	4/16/99	13
WA	Whitman	99-38	4/16/99	3
WA	Whitman	99-40	4/23/99	12
WA	Whitman	99-41	4/23/99	13
WA	Whitman	99-42	4/23/99	5
WA	Whitman	99-43	4/23/99	5
WA	Whitman	99-44	4/23/99	1
WA	Whitman	00-10	5/22/00	10
WA	Whitman	00-11	5/22/00	7
WA	Whitman	00-12	5/22/00	3
WA	Whitman	00-13	5/22/00	4
OR	Gilliam	00-4	5/23/00	8
OR	Gilliam	00-5	5/23/00	4
OR	Sherman	00-2	5/23/00	7
OR	Sherman	00-3	5/23/00	6
OR	Umatilla	00-13	4/23/99	7
OR	Umatilla	00-25	4/23/99	10
OR	Umatilla	00-26	4/23/99	11
OR	Umatilla	00-27	4/23/99	10
OR	Umatilla	00-30	4/23/99	1
OR	Umatilla	00-32	4/23/99	9
OR	Umatilla	00-34	4/23/99	3
ID	Idaho	00-8	5/23/00	7
ID	Lewis	00-7	5/23/00	3
ID	Lewis	00-9	5/23/00	10
ID	Nez Perce	00-6	5/23/00	7
Total	...	...	...	828

^a * = intensively sampled fields.

^b Month/day/year.

^c Total number of *T. yallundae* and *T. acuformis* isolates collected per field.

readily observed on Madsen stems but no disease ratings were made on either cultivar.

Developmental stages of apothecia ranged from immature initials to fully exposed cups on the lower leaf sheaths below the first node of each cultivar. The number of mature and developing apothecia on each stem ranged from a few to several hundred, and more apothecia generally were found on cvs. Stephens than Madsen. Mature apothecia of *T. yallundae* also were found on 25 September 2000 on 10% of stems examined from Stephens. Apothecia were not abundant compared with the observations made the previous spring. Only one stem had a cluster of apothecia (≈ 10) and the remaining four stems had one or two. No apothecia were found on stems of Madsen. The identification of apothecia belonging to the genus *Tapesia* was made by morphological observations and the final identification as *T. yallundae* was confirmed by cultural characteristics of progeny isolated from apothecia and by PCR as described above.

Development of apothecia under natural field conditions. One to six individual apothecia per stem were found on 2 and 4% of the sampled wheat stems collected from two fields, respectively, in the vicinity of Walla Walla, WA. Most of the apothecia were fully open, mature, and had already discharged many ascospores based on the number of empty asci and condition of the apothecia. Attempts were made to produce single ascospore cultures from all apothecia found; however, only three apothecia yielded viable cultures. The identification of apothecia belonging to the genus *Tapesia* was made by morphological observations and the final

identification as *T. acuformis* was confirmed by cultural characteristics of progeny isolated from apothecia and by PCR as described above.

Identification and species distribution of field isolates. Problems with PCR amplification of either the mating-type locus or the ITS region were encountered with 11 isolates; therefore, data is presented for only 817 isolates (Table 1). Most isolates (789) were easily identified as *T. yallundae* or *T. acuformis* based on cultural characteristics on PDA. Only 28 isolates produced intermediate cultural characteristics and the majority of these isolates were identified as *T. acuformis* by PCR.

In all, 460 isolates were identified as *T. yallundae* and 357 as *T. acuformis*, with *T. yallundae* more commonly isolated at all scales sampled (Tables 2 and 3). *T. yallundae* and *T. acuformis* were isolated from all fields sampled, except for Columbia and Garfield Counties, WA, where *T. yallundae* was not isolated, and Gilliam and Sherman Counties, OR, where *T. acuformis* was not isolated (Table 3). Even though both species were widely distributed, a greater proportion of *T. yallundae* isolates were collected from southeastern Washington and northeastern Oregon compared with *T. acuformis*.

Mating-type distribution of field isolates. Mating type was readily identified in *T. yallundae* and *T. acuformis* following amplification of an 812-bp *MAT1-1*-specific product or of a 418-bp *MAT1-2*-specific product (Fig. 2). Mating-type ratio of *MAT1-1*/*MAT1-2* for the entire PNW collection of *T. yallundae* was 216:244, which did not deviate significantly from a 1:1 ratio,

TABLE 2. Species and mating-type distribution of *Tapesia yallundae* and *T. acuformis* within individual fields sampled in Washington

Field	<i>T. yallundae</i>			<i>T. acuformis</i>		
	Number sampled	<i>MAT1-1</i> / <i>MAT1-2</i> ^a	χ^2 ^b	Number sampled	<i>MAT1-1</i> / <i>MAT1-2</i> ^a	χ^2 ^b
98-1	43	21:22	0.02	36	12:24	5.16*
98-2	27	12:15	0.34	49	22:27	0.52
99-1	66	26:40	2.96	57	26:31	0.44
00-1	46	12:34	10.5*	8	7:1	...
00-14	53	22:31	1.86	28	16:12	0.58
Total	235	93:142	10.2*	178	83:95	0.08

^a Mating type determined by multiplex polymerase chain reaction (8).

^b χ^2 value based on a 1:1 ratio with 1 degree of freedom; * indicates $P < 0.05$.

TABLE 3. Species and mating-type distribution of *Tapesia yallundae* and *T. acuformis* within counties, states, and the entire inland Pacific Northwest (PNW) region of Washington (WA), Oregon (OR), and Idaho (ID)

State, county	<i>T. yallundae</i>			<i>T. acuformis</i>		
	Number sampled	<i>MAT1-1</i> / <i>MAT1-2</i> ^a	χ^2 ^b	Number sampled	<i>MAT1-1</i> / <i>MAT1-2</i> ^a	χ^2 ^b
WA						
Adams	12	4:8	1.34	9	4:5	...
Columbia	...	...	...	3	3:0	...
Garfield	...	...	...	9	4:5	...
Grant	24	7:17	4.16*	19	10:9	0.06
Lincoln	18	10:8	0.06	31	10:21	3.90*
Walla Walla	25	17:8	2.46	9	3:6	...
Whitman	64	40:24	4	79	35:44	1.5
Subtotal	143	78:65	1.08	159	69:90	3.18
OR						
Gilliam	12	8:4	1.34	...	...	...
Sherman	13	4:9	1.92	...	...	...
Umatilla	42	25	1.14	8	1:7	...
Subtotal	67	37:30	0.66	8	1:7	...
ID						
Idaho	5	2:3	...	2	1:1	...
Lewis	4	2:2	...	9	5:4	...
Nez Perce	6	4:2	...	1	0:1	...
Subtotal	15	8:7	0.06	12	6:6	0
Total	225	123:102	1.96	179	76:103	4.08*
PNW total ^c	460	216:244	1.70	357	159:198	4.26*

^a Mating type determined by multiplex polymerase chain reaction (8).

^b χ^2 value based on a 1:1 ratio with 1 degree of freedom, * indicates $P < 0.05$.

^c Entire PNW total including results from Table 2.

whereas the *MAT1-1*/*MAT1-2* ratio for *T. acutiformis* was 159:198, which did deviate significantly from a 1:1 ratio (Table 3). With the exception of *T. yallundae* population 00-1 and *T. acutiformis* population 98-1, the ratio of mating types for field populations of *T. yallundae* and *T. acutiformis* in Washington did not deviate significantly from 1:1 (Table 2). Only two county comparisons, one each for *T. yallundae* and *T. acutiformis*, deviated significantly from a 1:1 ratio (Table 3). Both mating types of each species were widely distributed across all sampling scales studied in the U.S. PNW. Sample sizes for county and state comparisons in Oregon and Idaho were small compared with most of the Washington samples, which prevented statistically meaningful χ^2 tests for some samples; however, 1:1 ratios still were apparent in most comparisons for *T. yallundae* and *T. acutiformis* (Table 3).

DISCUSSION

This is the first report of apothecia of *T. yallundae* and *T. acutiformis* occurring under field conditions in the U.S. PNW. Apothecia of *T. acutiformis* were found under natural field conditions, whereas apothecia of *T. yallundae* were found in a field plot inoculated with isolates of compatible mating type. The discovery of apothecia of *T. acutiformis* under natural field conditions was unexpected because apothecia of *T. acutiformis* are rare compared with *T. yallundae* in other parts of the world (19). The role of apothecia of either pathogen in the eyespot disease cycle in the PNW is unknown. The formation of apothecia could serve as a source of primary inoculum, long-distance dispersal of inoculum, and in generating increased genotypic variation through recombination (21).

Epidemics of eyespot are dependent upon the amount of primary inoculum and environmental conditions, particularly temperature and moisture (19,29,31). Fall infections are generally more important in the PNW than spring infections because the environmental conditions are usually favorable for disease development, but spring infections also can result in severe eyespot (29). The primary source of inoculum for eyespot under PNW conditions has been attributed to conidia produced on infested residue during the fall and spring. However, it is now apparent that apothecia can form under PNW field conditions, making ascospores a potential source of inoculum. Apothecia first were observed in early spring on stubble in the inoculated field plot and on stubble of naturally infected plants, indicating they could be a potential source of inoculum for spring infections. However, apothecia with viable ascospores also were found the following fall in the inoculated field plot, indicating they also could be a potential source of inoculum in the fall because they developed on host residue over two growing seasons. Apothecia may have not developed in the fall of 1999, the first year after harvest, because of the unusually dry conditions. Only 0.8 mm of precipitation was recorded in September 1999 compared with 41.1 mm in September 2000 and 32.4 mm in September averaged over the proceeding 5 years, excluding 1999 (weather data from the Spillman Agronomy Farm, Pullman, WA). Therefore, in a more typical year, apothecia may develop in the fall following harvest and continue to develop into the spring, as was found in the United Kingdom, where apothecia of *T. yallundae* capable of discharging viable ascospores developed over a 6-month period following harvest in plots inoculated with compatible mating types (7).

Additional evidence supporting the potential for sexual reproduction to occur regularly in the PNW was the widespread distribution of compatible mating types of both *T. yallundae* and *T. acutiformis* in wheat fields sampled over 3 years. However, reproductive inferences drawn from mating-type data alone should be made with caution (21,27). PCR tests based on conserved mating-type regions have revealed the presence of both *MAT1-1* and *MAT1-2* isolates in field populations of supposedly asexual fungi for which no sexual stage has yet been described (1), and skewed

segregation ratios of sexually reproducing fungi may be the result of selection acting on the mating type or a closely linked locus (21,27). Additionally, deviations from the expected 1:1 ratio of mating types in sexually reproducing fungi also may be the result of repeated isolation of clonal genotypes due to prolific asexual reproduction (21), which was the case for *T. yallundae* population 00-1 based on the analysis of molecular markers (G. W. Douhan and T. D. Murray, unpublished data). This also may explain some of the deviations from the 1:1 ratio of mating type in *T. acutiformis* in this study.

The ability of *T. yallundae* and *T. acutiformis* to sexually reproduce also will depend on factors besides the presence of both mating types within a population, such as fertility between compatible isolates, the presence of suitable substrates, and proper environmental conditions. Apothecia of both species can develop under PNW field conditions and both mating types were found in approximately equal proportions at all geographic scales sampled;

Fig. 2. Multiplex polymerase chain reaction detection of mating type of *Tapesia yallundae* and *T. acutiformis* (8). Three primers were used in all reactions; one primer common to both mating type regions and a primer specific for *MAT1-1* and *MAT1-2* sequences. The *MAT1-1*-specific product is 418 bp and the *MAT1-2*-specific product is 812 bp. Lanes 2 through 7 in the upper gel are six representative *T. yallundae* isolates and lanes 2 through 7 in the lower gel are six representative *T. acutiformis* isolates. Lane 1 in both gels contains a 100-bp ladder (Life Technologies, Grand Island, NY).

therefore, fertility between compatible mating types may be the only factor limiting apothecial development. Fertility may be a population-specific phenomenon (15) and additional genes that segregate independent of mating type may be involved in ascocarp production, as has been determined in the rice pathogen *Magnaporthe grisea* (20). It is not known if such a phenomenon exists in *T. yallundae* or *T. acuformis*.

Apothecia from naturally diseased plants were found only on standing stubble and not on stubble that had been cultivated. Although this observation was made from a relatively small sample, it is consistent with previous reports (7). Apothecia were found only on host residue that had over-summered and not on living green tissues. Other cereal pathogens, such as *Mycosphaerella graminicola*, *Leptosphaeria nodorum*, and *Pyrenophora teres*, also require the presence of stubble or residue for sexual reproduction to occur under field conditions (7). An increase in apothecial development in Europe has been attributed to a set-aside policy implemented in 1988 and revised in 1992 that has resulted in large areas with standing wheat stubble (19). In the PNW, increased no-till farming for soil conservation purposes and the elimination of stubble burning for environmental reasons may give the eyespot pathogens more opportunity to reproduce sexually by providing a suitable substrate.

T. yallundae and *T. acuformis* accounted for 56 and 44%, respectively, of the total eyespot pathogen isolates collected over 3 years in the PNW and represents a significant increase in the occurrence of *T. acuformis* in the PNW compared with previous studies (24). Murray (24) found that approximately 82% of 677 eyespot isolates collected in 1989–90 were *T. yallundae*. There was a similar increase in frequency of *T. acuformis* in some parts of Europe in the 1980s (13,19). It is unknown if a similar transition is occurring in the PNW or why *T. acuformis* has increased over the past 10 years, but this change could have a significant impact on the management of eyespot. Little is known about the virulence of *T. acuformis* on the winter wheat cultivars currently grown in the PNW. Fungicide application practices in the PNW have been developed specifically for *T. yallundae* and it is unclear if *T. acuformis* isolates from the PNW respond similarly to these fungicides or if a greater potential for resistance development exists in this species.

The apparent shift in eyespot populations from *T. yallundae* to *T. acuformis* in many parts of Europe has been attributed to differential resistance to fungicides and host preference (13,19,24). The two most common classes of fungicides used to control eyespot are benzimidazoles and sterol demethylation inhibitors (DMIs) (19). Resistance to DMIs in *Tapesia* spp. has not been reported from the PNW, but has been reported in Europe (18). Leroux and Gredt (18) found that *T. acuformis* is intrinsically less sensitive toward most DMIs compared with *T. yallundae*, which could have facilitated the increase of *T. acuformis* in Europe. The increase of *T. acuformis* populations in the PNW may be attributed to selection due to the use of DMIs for control of eyespot, although this seems unlikely due to the small acreage of winter wheat treated in the PNW compared with the total area surveyed where *T. acuformis* was found. It has also been suggested that the production of more winter barley in Europe may have selected for *T. acuformis* because *T. yallundae* is less virulent on barley than *T. acuformis* (13). However, *T. yallundae* is still predominant in some areas of Europe and there is no clear explanation for the distribution of these pathogens in Europe (24). In the PNW, barley is grown on less than one-quarter of the small grain acreage and it has not significantly increased over the past 10 years (35), making host preference an unlikely explanation for the increase of *T. acuformis* in the PNW.

An alternative explanation for the increase in frequency of *T. acuformis* in the PNW may be its ability to preferentially colonize winter wheat cultivars with eyespot resistance more successfully than *T. yallundae*. The resistance gene *Pch1* is the only known

source of eyespot resistance in commercially grown PNW winter wheat (24), which saves PNW farmers approximately \$4 to 6 million per year in reduced fungicide applications (T. D. Murray, unpublished data). The majority of research in screening for resistance in the PNW has used *T. yallundae* as an inoculum source and the effectiveness of *Pch1* against *T. acuformis* has not been accurately investigated. Preliminary data suggests that *T. acuformis* is more virulent than *T. yallundae* against Madsen, the most widely grown eyespot resistant winter wheat (G. W. Douhan and T. D. Murray, unpublished data). Additionally, resistance to *T. acuformis* has been found only in *Dasyphyrum villosum* (33) and the genetic control of resistance in this wheat relative differed between *T. acuformis* and *T. yallundae*. Therefore, the effectiveness of *Pch1* and newly found resistance genes should be evaluated against both species when new cultivars are developed, especially because *T. acuformis* is now common throughout the PNW.

In conclusion, *T. acuformis* has increased significantly in recent years, apothecia of both species can develop under field conditions, and both mating types of each species are widely distributed. These results suggest that *T. acuformis* is more important than previously thought and that sexual reproduction may occur in both species. Additionally, some populations of *T. yallundae* and *T. acuformis* from the intensively sampled fields in this study have a genetic structure consistent with random mating based on multi-locus analysis of amplified fragment length polymorphic and mating type markers (G. W. Douhan and T. D. Murray, unpublished data). However, multiple-year studies with more intensive sampling are needed to understand the temporal importance of the species composition in the PNW and to establish the timing and frequency of apothecial development to better understand the importance of the sexual stages in the disease cycle of the eyespot pathogens in the PNW.

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