Molecular Ecology (2002) **11**, 2229–2239

Multilocus population structure of *Tapesia yallundae* in Washington State

G. W. DOUHAN,* T. L. PEEVER and T. D. MURRAY
Department of Plant Pathology, Washington State University, Pullman, WA 99164–6430, USA

Abstract

Population genetic structure of the fungal wheat pathogen *Tapesia yallunda*e in Washington State was determined using genetically characterized amplified fragment length polymorphic (AFLP) markers and mating-type (*MAT*1-1 or *MAT*1-2). Segregation and linkage relationships among 164 AFLP markers and *MAT* were analysed using 59 progeny derived from an *in vitro* cross. Alleles at 158 AFLP loci and the mating-type locus segregated in a 1:1 ratio. Ten unlinked markers were chosen to determine genetic and genotypic diversity and to test the hypothesis of random mating and population differentiation among five subpopulations of *T. yallundae* representative of the geographical distribution of wheat production in eastern Washington. Among 228 isolates collected, overall gene diversity was high (h = 0.425) and a total of 91 unique multilocus genotypes (MLG) were identified, with 32 MLG occurring at least twice. The overall population genetic structure was consistent with random mating based on the segregation of mating-type, index of association (I_A), parsimony tree length permutation test (PTLPT) and genotypic diversity analyses. However, clonal genotypes were found within each subpopulation and were also distributed among the five subpopulations. No significant differences in allele frequencies were found among the five subpopulations for all 10 loci based on contingency table analysis (G^2) and Wier & Cockerham's population differentiation statistic θ ($\theta = -0.008$, $P = 0.722$). *T. yallundae* appears to consist of a large homogeneous population throughout eastern Washington with both sexual and asexual reproduction contributing to the observed population genetic structure despite no report of sexual fruiting bodies of *T. yallundae* occurring under natural field conditions.

Keywords: AFLP, fungi, gametic disequilibrium, population genetics, recombination, *Tapesia*

Received 2 January 2002; revision received 12 July 2002; accepted 15 July 2002

Introduction

Plant pathogenic fungi are a very large and heterogenous group of organisms that are a driving force in the evolution of both natural and agricultural ecosystems (Burdon & Silk 1997). The largest and most economically important group of pathogenic fungi belongs to the Phylum Ascomycota. These fungi are distributed globally and have diverse and varying life history strategies, ranging from weakly pathogenic species to taxa that are associated obligately with their hosts. One of the most important aspects of pathogen fitness is the ability to reproduce and efficiently spread propagules to adjacent host plants. The reproductive capabilities of fungi in the Ascomycota vary from species that produce no propagules and spread by vegetative means alone to those that can produce only asexual (mitosporic) spores or can produce both sexual (meiosporic) and asexual spores (Taylor *et al*. 1999). The reproductive mode of fungal pathogens will influence the genetic structure of their populations, which can range from clonal to recombining or exhibit both population structures that can vary in both time and space for the same species (Kohn 1995; Milgroom 1996; Taylor *et al*. 1999).

Fungi that undergo a sexual cycle routinely usually exhibit greater genotypic diversity than fungi that reproduce exclusively by asexual means (Milgroom 1996). Increased genotypic diversity resulting from recombination may

Correspondence: T. D. Murray. Fax: 509-335-9581; E-mail: tim_murray@wsu.edu
*Present address: Department of Plant Pathology, University of California at Davis, CA, 95616, USA.

allow sexual populations of fungi to better respond to selective pressures such as the use of fungicides or resistant cultivars to control fungal diseases in agricultural systems (McDermott & McDonald 1993; McDonald 1997). In many species, however, sexual structures are not found commonly under natural field conditions and disease epidemics are the result of clonal spread of secondarily produced asexual spores (Kohn 1995). Thus, sexual reproduction may be occurring within and among populations but its importance may be masked by asexual reproduction. Additionally, sexual reproduction is not known to occur in approximately half of the Ascomycota but populations of some of these apparently asexual species have a recombined genetic structure consistent with sexual reproduction (Geiser *et al*. 1998; Taylor *et al*. 1999). Another important consideration when determining the spatial structure of fungal populations is the dispersal patterns of reproductive spores. For example, small clusters of clones may be found in pathogens with limited dispersal of asexual spores, whereas a single dominant clone may predominate in fungi with widely dispersed asexual spores, regardless of whether the initial infection was from an asexual or sexual spore (Kohn 1995; McDonald 1997).

Tapesia yallundae is an important fungal pathogen of winter wheat in many countries (Lucas *et al*. 2000, Wallwork & Spooner 1988) and is the causal agent of eyespot of wheat, one of the most important diseases of wheat in eastern Washington (Murray 1996). *T. yallundae* is a ascomycete fungus that has the ability to reproduce sexually if compatible mating types (*MAT1-1* and *MAT1-2*) are present and if environmental conditions are favourable, but also reproduces and spreads by asexual spores (Dyer *et al*. 1994; Lucas *et al*. 2000). Sexual fruiting structures (apothecia) of *T. yallundae* have been found in wheat producing regions in Germany, England, Belgium, Australia, New Zealand and South Africa, but have never been reported from North America (Lucas *et al*. 2000). However, abundant apothecia were recently found in an experimental field plot in Washington State where compatible mating types were used to inoculate winter wheat, demonstrating the potential for sexual reproduction to occur under natural field conditions in Washington (Douhan *et al*. 2002). Thus, both sexual and asexual reproduction may be important in shaping the genetic structure of *T. yallundae* populations.

Having a better understanding of the genetic structure of *T. yallundae* will enhance our knowledge of pathogen biology and help us understand the importance of evolutionary forces such as selection, migration, genetic drift and recombination (Milgroom 1996), which may lead ultimately to improved disease control strategies (Milgroom & Frey 1997). For example, knowledge of population structure of *T. yallundae* could have a significant impact on how pathologists and wheat breeders screen germplasm for new sources of resistance genes. If significant differences exist among subpopulations of *T. yallundae* in Washington or other wheat-producing areas of the Pacific Northwest (PNW), representative isolates from various regions would have to be included in the screening process to adequately challenge potential sources of resistance. Moreover, regional management practices could also be developed to maximize the efficacy of fungicide use in the absence of sufficient gene flow among *T. yallundae* subpopulations.

DNA-based marker technologies have had a significant impact on the study of population genetics of many organisms because it is now relatively easy to collect data from many loci (markers) and use these markers to test various hypotheses. However, assumptions about molecular markers such as Mendelian segregation and linkage that are critical for properly analysing multilocus population structure often are not tested, leading to erroneous conclusions (Brown 1996). Population differentiation analysis will appear more statistically powerful than it really is if the markers are linked and gametic disequilibrium can be influenced by factors other than sexual or clonal reproduction, such as hypervariable loci, physical linkage, epigenetic effects, rapid population expansion or selection among loci (Brown *et al*. 1980; Slatkin 1994; Taylor *et al*. 1999). Therefore, the objectives of this research were to first develop a set of genetically characterized molecular markers for *T. yallundae* and then to use the markers to determine genetic and genotypic diversity and to test the hypothesis of random mating and population differentiation among *T. yallundae* subpopulations in eastern Washington.

Materials and methods

Sampling

Isolates of *T. yallundae* were sampled from five wheat fields in 1998, 1999 and 2000 in eastern Washington State, USA (Fig. 1). Two different strategies were used to sample isolates in 1998–99 and 2000. In 1998–99, a single handful of diseased plants (~20–30 stems) were collected every 3 m along two parallel transects separated by 100–150 m. A total of 60 (2×30) and 100 (2×50) samples were obtained in 1998 and 1999, respectively. The sampling strategy was changed in 2000 after preliminary genetic analysis of the 1998 and 1999 subpopulations to test the effect of a finer-scale sampling strategy on estimation of population structure. In 2000, diseased plants (~10–20 stems) were collected every metre 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. For the purposes of this study, a sample of isolates from a single wheat field was considered a subpopulation of *T. yallundae*.

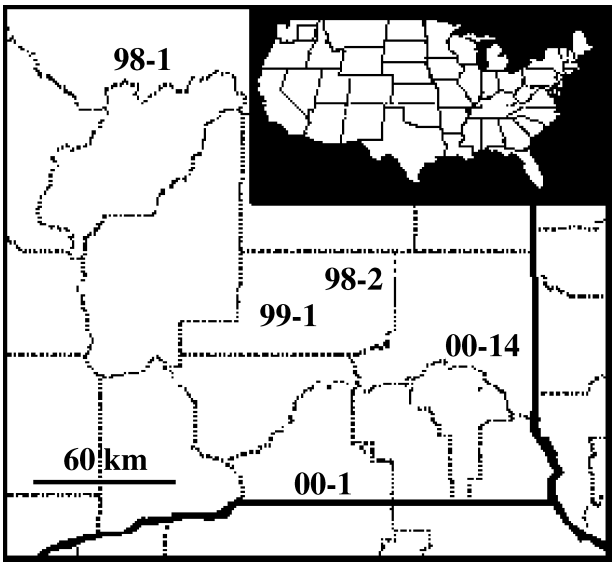

Fig. 1 Five populations of *Tapesia yallundae* collected in the winter wheat-growing region of eastern Washington between 1998 and 2000. Sample sizes were 39, 28, 63, 46, and 52 isolates for populations 98–1, 98–2, 99–1, 00–1 and 00–14, respectively. The first two numbers in the code correspond to the year the isolates were collected, whereas the second number designates individual wheat fields.

Isolation, culturing and DNA extraction

T. yallundae was isolated by removing individual lesions from diseased stems with a razor blade. Two to five diseased stem pieces (1–3 cm) per sample were surface disinfected for 1–3 min in 1% NaOCl, placed on 1.5% water agar (WA) containing rifampicin (50 μg/mL, Sigma, USA), and incubated at 14–16 °C under near UV light for 1–2 weeks. One randomly chosen isolate per sample was then transferred to 0.2X potato dextrose agar (PDA) (Difco, Detroit, MI, USA) containing rifampicin (50 μg/mL) and incubated for an additional 1–2 weeks at room temperature before a final transfer to PDA. Single spore cultures were made from each isolate in order to obtain genetically uniform individuals and were incubated at room temperature for 2–3 weeks before the surface mycelium was removed and lyophilized. Lyophilized mycelium was ground and DNA was extracted using the method of Lee & Taylor (1990) with 100 mM Tris (pH 8.0) used instead of 50 mM Tris (pH 7.2) in the lysis buffer and without the addition of 2-mercaptoethanol.

AFLP marker development

An *in vitro* cross between the Washington isolate 90-47-1 (*MAT1-1*) (Murray 1996) and the European isolate 433–22 (*MAT1-2*) (Dyer *et al*. 1993) was made following the protocol of Dyer *et al*. (1993). Fifty-nine single ascospore progeny were used to study inheritance and linkage among AFLP markers and the mating type locus. The parental isolates were chosen based on preliminary experiments in order to maximize the number of AFLP loci segregating in the cross.

The method of Vos *et al*. (1995) was used to develop AFLP markers with the exception that the restriction enzyme *Tru*9I (Boehringer Mannheim GmbH, Germany) was used instead of *Mse*I (*Tru*9I is an isoschizomer of *Mse*I). A total of 12 primer combinations with the addition of two selective nucleotides at the 3′ end were used to analyse the progeny isolates (Table 1). Two of the 12 primer

Table 1 AFLP primer sets and the number and segregation of polymorphic markers among single ascospore progeny from a cross between isolates 90-47-1 (*MAT1-1*) and 22–433 (*MAT1-2*) of *Tapesia yallundae*

	Primer combination*				
Primer set	*Eco*RI adapter +	Tru 91 adapter +	Polymorphic markers	No. of markers segregating	1:1†
A	CT	GA	20	20	
B	AT	CT	15	14	
C	AA	GA	18	16	
D	CT	CG	16	16	
E	AC	CG	15	14	
F	AG	CA	13	13	
G	CT	CT	9	9	
H	GT	GA	14	14	
I	GG	CG	12	11	
J	GC	CC	4	4	
K	GT	CA	14	13	
L	GA	CC	14	13	
	Total		164	157	

*Each primer combination consisted of nucleotide sequences homologous to the adapter sequences plus the addition of two selective nucleotides at the 3′ ends (Vos *et al*. 1995).
†Goodness-of-fit to a 1:1 ratio was tested using χ^2 tests among 50–59 single ascospore progeny at $P = 0.05$.

combinations were used subsequently on the field populations of *T. yallundae*. Each 20 μL reaction contained 1 × PCR buffer (Life Technologies, Grand Island, NY, USA), 2.5 mM $MgCl_2$, 2.5 mM each dNTP (Life Technologies), 3.75 μM of each primer, 0.5 U of Taq polymerase (Life Technologies) and 10 μL of template. Thermocycling conditions consisted of an initial hold at 72 °C and 94 °C for 1 and 4 min, respectively, followed by 44 cycles with an annealing temperature of 65 °C for 30 s and an extension temperature of 72 °C for 1 min with the annealing temperature reduced by 1 °C for the first nine cycles. All amplifications were performed in a PE-9700 thermocycler (Perkin Elmer Corp., Norwalk, CT, USA).

Following amplification, 5 μL of loading dye (98% formamide, 10 mM EDTA pH 8.0. 0.025% bromphenol blue, 0.025% xylene cyanole) was added to each reaction and the reaction mix was denatured for 4 min at 94 °C and cooled on ice. Three to 4 μL of each sample was loaded and separated on 4% acrylamide gels buffered in TBE (1.35 M Tris pH 8.0, 0.45 M boric acid, 25 mM EDTA) for approximately 2 h at 1800–1900 V using a Bio-Rad Sequi-General GT Sequencing Cell (Bio-Rad Laboratories Inc., Hercules, CA, USA). The two parental isolates were always loaded in the first two lanes adjacent to the DNA size ladders to serve as controls for the progeny and field isolates of *T. yallundae*. The gels were then silver-stained by the method of Bassam *et al*. (1991) and dried overnight before visual scoring of bands.

Mating type (*MAT*1-1 or *MAT*1-2) was identified among progeny and field isolates using mating type-specific primers in multiplex PCR reactions as described by Dyer *et al*. (2001). Each 20 μL PCR reaction contained 1 × PCR buffer (Life Technologies), 2.0 mM $MgCl_2$, 2.5 mM each dNTP (Life Technologies), 3.75 μM of each primer, 0.5 U of Taq polymerase (Life Technologies) and 0.5 μL of undiluted stock DNA. Thermocycling conditions consisted of an initial hold of 94 °C for 4 min followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min with a final hold of 72 °C for 5 min. The amplification products were separated in 1.5% agarose gels, stained with ethidium bromide, and the image was captured digitally. All amplifications were performed in a PE-9700 thermocycler (Perkin Elmer Corp.).

Segregation, linkage analysis and marker selection

Putative alleles at each polymorphic AFLP locus and mating type alleles were scored using a binary code (1, 0) corresponding to positive and null alleles for AFLP loci and *MAT*1-1 and *MAT*1-2 for mating type, respectively. For the selection of AFLP markers, only polymorphic loci that were reproducible and easily identifiable (Fig. 2) between the parents and progeny were chosen. Each putative locus was named using a letter code corresponding to the primer combination used followed by the size of the fragment. Segregation of alleles among the progeny isolates was tested for each putative AFLP locus using χ^2 analysis. Segregation ratios were tested against the null hypothesis of 1:1 segregation ($P = 0.05$), which is the ratio expected for the segregation of alleles at a single genetic locus in a haploid organism (Milgroom 1996).

Linkage analysis was performed using MAPMAKER (Macintosh version 2.0, Whitehead Institute for Biomedical Research, Cambridge Center Cambridge, MA, USA) with log likelihood (LOD) scores ranging from 4.0 to 6.0 and a recombination fraction ranging from 0.2 to 0.4. LOD scores and recombination fraction values were varied to evaluate the stability of putative linkage groups. Candidate loci for population structure studies were only considered if they segregated in a 1:1 ratio and the linkage relationships were stable at varying LOD and recombination fraction values. In addition, loci with intermediate allele frequencies in the field populations of *T. yallundae* were given preference in order to increase the statistical power of the population differentiation and multilocus gametic disequilibrium analyses (Brown 1996; Milgroom 1996). In order to test the prediction of the multilocus analysis, a preliminary analysis of the markers was tested using the progeny isolates to ensure that the markers were in random association using the methodologies outlined below for random mating tests (data not shown).

Analysis of field populations

Two types of data sets were generated for the analysis: one using all individuals within each subpopulation and another in which each multilocus genotype was represented only once in each subpopulation (i.e. cloned-corrected) (Chen & McDonald 1996). Genetic diversity and population differentiation statistics were based on allele frequencies estimated from clone-corrected data sets due to the presence of multiple clonal genotypes within each subpopulation whereas genotypic diversity was estimated from complete data sets and tests for random mating were analysed using both data sets (Milgroom 1996). Allele frequencies at each locus were estimated from each subpopulation and used to calculate standard population statistics using the software POPGENE (PC version 1.31, Molecular Biology and Biotechnology Centre, University of Alberta, Canada), FSTAT (PC version 2.9.1, Institute of Ecology, Laboratory for Zoology University of Lausanne, Switzerland), and MULTILOCUS (MAC version 1.21, Department of Biology, Imperial College at Silwood Park, UK). Genetic diversity at each locus was estimated using Nei's genetic diversity as $h = 1 - p_i^2$, where p_i is the frequency of the ith allele at each locus, and averaged over all loci (Nei 1973). Genotypic diversity was calculated as $(n/n - 1)(1 - \Sigma p_i^2)$ where p_i is the frequency of the ith genotype and n is the number of individuals sampled, which is the probability that two individuals taken at random have unique genotypes (Agapow

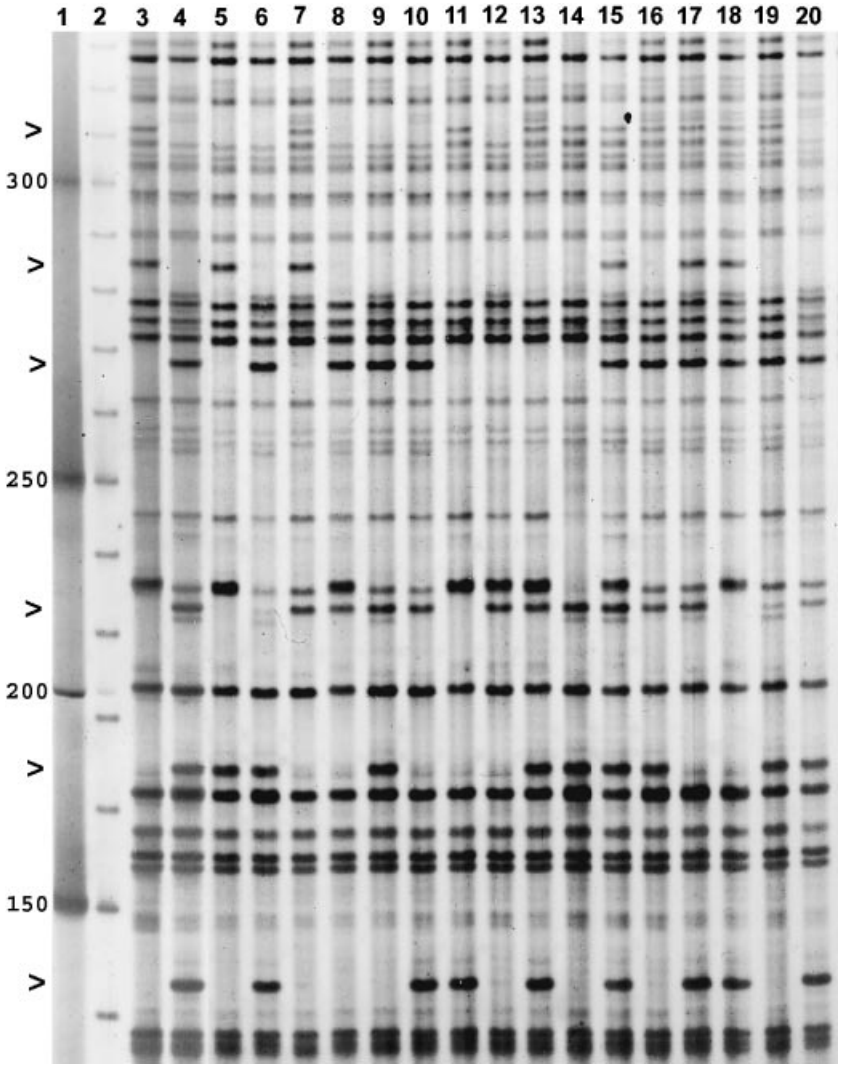

Fig. 2 Partial AFLP fingerprints using primer combination K (Tru + CA/Eco + GT) of *Tapesia yallundae* parental isolates 90–47–1 (*MAT1-1*) and 22–433 (*MAT1-2*) and progeny isolates. Parental isolates 90–47–1 and 22–433 are in lanes 3 and 4, respectively, followed by progeny isolates in lanes 5–20. Segregating polymorphic markers can easily be seen between the parental and progeny isolates (>). A 50 bp and 10 bp ladder (Invitrogen, Carlbad, CA) are in lanes 1 and 2, respectively.

& Burt 2001). This value is 0 if every individual is the same, and 1 if every individual is different. Isolates with missing data were not included in genotypic diversity estimates.

Population differentiation was tested among subpopulations by comparing allele frequencies using the log-likelihood statistic G^2 (Brown 1996) and Weir & Cockerham's θ (Weir & Cockerham 1984). The estimated G^2 and θ-values were tested under the null hypothesis of nondifferentiation among populations ($\theta = 0$, $G^2 = 0$) using nonparametric statistical methods. The observed values of G^2 and θ were compared to values estimated for datasets in which alleles were resampled without replacement 10 000 times using the programs FSTAT and MULTILOCUS, respectively.

The hypothesis of random mating was tested using four approaches. First, a χ^2 test was used to test the segregation of mating type, which should be 1:1 for a randomly mating haploid fungus (Milgroom 1996). Second, random association among loci was tested using the index of association (I_A) statistic as implemented in the program MULTILOCUS. I_A has an expected value of zero if there is no association of alleles at unlinked loci as expected in a randomly mating population. The significance of I_A was tested by randomization (1000 times) procedures by comparing the observed value of I_A to that expected under the null hypothesis of complete panmixis (Burt *et al*. 1996; Agapow & Burt 2001). Third, genotypic diversity was estimated as $G = 1/\Sigma p_i^2$, where p_i is the observed frequency of the *i*th genotype and compared to genotypic diversity expected under the null hypothesis of random mating (Hoffman 1986; Stoddart & Taylor 1988; Milgroom 1996). The significance was tested using randomization procedures (1000 times) as described above using a computer program provided by Michael Milgroom, Department of Plant Pathology, Cornell University, NY, USA. Fourth, the parsimony tree length permutation test (PTLPT) was used to determine reproductive mode (Burt *et al*. 1996). PTLPT was analysed using PAUP (version 4.0b10) (Swofford 1998) and is a phylogenetic approach based on the concept that a clonal population

Table 2 Allele frequencies, population differentiation, and genetic diversity among five subpopulations of *Tapesia yallundae* in Washington

	Population allele frequency							
Locus	98–1	98–2	99–1	00–1	00–14	Overall	G^2*	θ†
A68	0.519	0.364	0.514	0.462	0.529	0.478	1.74	–0.023
A82	0.259	0.409	0.432	0.538	0.286	0.385	5.70	0.015
A90	0.111	0.136	0.243	0.154	0.19	0.167	2.30	–0.017
A213	0.259	0.409	0.459	0.423	0.429	0.396	3.03	–0.011
A298	0.667	0.727	0.892	0.885	0.762	0.787	7.05	0.028
B83	0.185	0.364	0.378	0.500	0.333	0.352	6.20	0.018
B132	0.185	0.227	0.351	0.308	0.286	0.271	2.62	–0.014
B222	0.519	0.500	0.432	0.500	0.571	0.504	1.14	–0.028
B306	0.704	0.773	0.811	0.731	0.905	0.785	3.71	–0.006
MAT	0.407	0.381	0.417	0.346	0.500	0.410	1.18	–0.029
Overall	—	—	—	—	—		38.67	–0.008
h‡	0.391	0.420	0.426	0.422	0.408	0.425	—	—

*Log-likelihood statistic (Brown 1996). No significant differences in allele frequencies were found under the null hypothesis of $G^2 = 0$ ($0.149 < P < 0.882$). The observed values of G^2 were compared to values estimated for data sets in which alleles were resampled without replacement 10 000 times.

†Population differentiation estimator of Weir & Cockerham (1984). No significant overall population differentiation among the five subpopulations was found under the null hypothesis of $\theta = 0$ ($\theta = -0.008$, $P = 0.722$) determined using randomization procedures as described above.

‡Nei's genetic diversity (Nei 1973).

should evolve like a parsimonious tree with little homoplasy, whereas recombining populations will have little phylogenetic single due to the reshuffling of alleles during meiosis (Burt *et al*. 1996; Taylor *et al*. 1999). The significance of PTLPT was determined using randomization procedures (1000 times) as described above. Isolates with missing data were not included in tests for random mating.

Results

Progeny analysis and marker selection

One hundred and sixty-four polymorphic AFLP markers were scored using 12 primer combinations, with four to 20 markers scored per primer combination (Table 1). Addition of the mating-type locus resulted in a total of 165 markers scored. One hundred and fifty-eight markers of 165 (96%) segregated in a 1:1 ratio among the ascospore progeny (Table 1). Ten markers were chosen to be used in the analysis of field populations of *T. yallundae* which segregated in a 1:1 ratio and were unlinked at several LOD scores and recombination fractions (data not shown). Ten markers were found to be sufficient for population structure analyses based on preliminary analysis of field population data by computing genotypic diversity and plotting it against the number of loci, from 1 to 10 using randomization procedures in MULTILOCUS. It was determined that only seven to eight markers were needed to account for 95–99% of the observed variation (data not shown).

Genetic and genotypic diversity

All loci were polymorphic in all subpopulations with gene diversities ranging from 0.391 to 0.426 (Table 2). Many more additional polymorphic markers were found within the field isolates that were either absent or monomorphic in the parental isolates, but no data were collected on these polymorphisms because the genetics of these additional markers could not be determined. A total of 91 unique multilocus genotypes (MLG) were identified among all subpopulations, with 32 MLG occurring at least twice among the five subpopulations (Table 3). Within each subpopulation, the number of unique MLG ranged from 16 to 36, with genotypic diversities ranging from 0.889 to 0.960 (Table 3). Clonal genotypes were distributed within and among the five subpopulations of *T. yallundae*. Three clonal genotypes were found in four of the subpopulations, five clonal genotypes were found in three of the subpopulations, 16 clonal genotypes were found in two subpopulations and eight clonal genotypes were found at least twice within the same subpopulation (Table 3). The most common clonal genotype was isolated 24 times, accounting for 10.9% of the entire collection of isolates, but most clonal genotypes accounted for less than 2.0% of the entire collection of isolates (Table 3).

Population subdivision

No significant differences ($0.149 < P < 0.882$) in allele

Table 3 Genotypic diversity and the occurrence of multilocus genotypes (MLG) detected at least twice among five subpopulations and the entire sampled population of *Tapesia yallundae* in Washington

	Population					
MLG	98–1	98–2	99–1	00–1	00–14	Total
1000100110	4	2	8	4	—	18
1110100111	4	2	7	—	2	15
1000101011	5	4	5	—	10	24
0101110010	2	1	3	—	—	6
0000000111	1	1	—	—	1	3
0001001011	1	1	—	1	—	3
1101100100	1	—	—	1	1	3
0001111010	—	1	4	—	5	10
1000000010	1	1	—	—	—	2
1000101010	1	—	1	—	—	2
1110000111	1	—	1	—	—	2
0001101011	1	—	1	—	—	2
0000100110	1	—	2	—	—	3
1000110000	2	—	—	1	—	3
0100110100	1	—	—	1	—	2
0000100100	1	—	—	7	—	8
1001100111	1	—	—	—	1	2
0000000011	1	—	—	—	1	2
0101111110	—	1	1	—	—	2
0001100010	—	1	—	1	—	2
1101100111	—	1	—	1	—	2
0000111010	—	—	1	1	—	2
1111100010	—	—	1	1	—	2
0000110110	—	—	1	—	6	7
0110000110	—	2	—	—	—	2
1101110110	—	—	4	—	—	4
0000110000	—	—	—	5	—	5
0100111110	—	—	—	3	—	3
0000110001	—	—	—	4	—	4
1001101111	—	—	—	—	3	3
1001110010	—	—	—	—	10	10
0000000110	—	—	—	—	2	2
N *	39	27	62	45	47	220
No. genotypes†	27	21	36	26	16	91
D‡	0.968	0.974	0.960	0.953	0.889	0.978

*Total number of isolates included in the analyses for each (sub)population.
†Number of unique genotypes in each (sub)population.
‡Genotypic diversity calculated as $(n/n–1)(1–\Sigma p_i^2)$ where p_i is the frequency of the ith genotype and n is the number of individuals sampled, which is the probability that two individuals taken at random have unique genotypes (Agapow & Burt 2001).

frequencies were found among the five subpopulations for all 10 loci by contingency table analysis using the G^2 statistic. Similarly, no significant overall population differentiation was found among the five subpopulations based on Wier & Cockerham's population differentiation statistic θ (θ = –0.008, P = 0.722) (Table 2).

Random mating

Most tests were not consistent with random mating when all isolates were included in the analyses except for the segregation of mating type. Subpopulations 98–1, 98–2, 99–1 and 00–14 were not significantly different from a 1:1 segregation, whereas subpopulation 00–1 and the combined analysis of all isolates were significantly different from 1:1 (Table 4). The only other test that was consistent with random mating was I_A (P = 0.085) for subpopulation 98–1. In contrast, most tests were consistent with random mating when clonal genotypes were removed from the data sets. Random mating was rejected only in subpopulation 99–1 based on I_A (P = 0.020), but all other tests were consistent with random mating for that subpopulation (Table 4).

Discussion

Apothecia of *T. yallundae* have not been found under natural field conditions in North America but we detected a history of recombination among the genetic loci analysed, suggesting that sexual reproduction occurs in this fungus. Clone-corrected data sets were used to make inferences on random mating of *T. yallundae* to minimize the effects of linkage due to resampling the same genotypes (Chen & McDonald 1996). A criticism of this approach is that the power to reject the null hypothesis can also decline dramatically when sample sizes are clone-corrected due to the reduction in sample size (Milgroom 1996). To test this, we pooled and clone-corrected the data (N = 91) from all five subpopulations, because there was no evidence of population differentiation, and random mating could still not be rejected based on I_A, PTLPT and genotypic diversity analyses with this large sample size. Mating-type was also in a 1:1 ratio and apothecia have recently developed under field conditions when compatible isolates were artificially inoculated onto wheat in Washington State (Douhan *et al*. 2002). Therefore, sexual reproduction is probably a regular component of the life cycle of this pathogen, but apothecia have not yet been found under natural field conditions.

Taylor *et al*. (1999) suggest that truly asexual taxa are extremely rare, with most putative asexual taxa showing a signature of recombination within their population genetic structures. For *T. yallundae*, it is not surprising that we detected a genetic structure consistent with recombination in Washington State as apothecia have been found in every other wheat-producing area where the disease occurs (Lucas *et al*. 2000), both mating types are widely distributed, and the environmental conditions in Washington appear to be conducive for sexual reproduction to occur (Douhan *et al*. 2002). However, an important caveat to these analyses is that the statistical tests used in this and other studies appear to be very sensitive and statistical power of these tests are not known. For example, I_A measures the association

Table 4 Segregation of mating type and tests for random mating among five subpopulations and the entire sampled population of *Tapesia yallundae* in Washington

		Mating type segregation‡			Index of association§		Genotypic diversity¶			PTLPT**	
Population	N†	*MAT*1-1:*MAT*1-2	χ^2	*P*	I_A	*P*	Gobs	Gexp	*P*	L	*P*
98–1	39	18:21	0.231	0.631	0.121	0.085	17.48	30.65	< 0.001	16	< 0.001
98–2	27	12:15	0.333	0.564	0.209	0.019	16.20	24.83	0.002	33	< 0.001
99–1	62	25:37	2.32	0.127	0.456	< 0.001	18.13	48.17	< 0.001	48	< 0.001
00–1	45	12:33	9.80	0.002	0.479	< 0.001	14.57	35.59	< 0.001	37	< 0.001
00–14	47	21:26	0.532	0.466	0.520	< 0.001	7.70	34.35	< 0.001	24	< 0.001
Total	220	88:132	8.80	0.003	0.199	< 0.001	28.09	121.93	0.003	103	< 0.001
98–1cc*	27	11:16	0.926	0.336	–0.010	0.507	27.0	23.62	1.00	36	0.226
98–2cc	21	8:13	1.19	0.275	0.113	0.149	21.0	19.69	1.00	34	0.343
99–1cc	36	15:21	1.00	0.317	0.141	0.020	36.0	32.43	1.00	50	0.191
00–1cc	26	9:17	2.46	0.117	0.083	0.165	26.0	24.02	1.00	38	0.145
00–14cc	16	9:7	0.250	0.617	–0.021	0.554	16.0	14.96	1.00	25	0.187
Totalcc	91	37:54	3.18	0.075	0.041	0.110	91.0	71.17	1.00	102	0.241

*Clone-corrected (cc); one isolate representing each unique multilocus genotype.
†Total number of isolates used in the analysis.
‡Segregation ratio of mating type determined by multiplex PCR (Dyer *et al*. 2001). χ^2 value based on a 1:1 ratio with 1 degree of freedom and the probability (*P*) of a greater χ^2 value under the null hypothesis of 1:1 segregation.
§Index of association (I_A) statistic (Brown *et al*. 1980). The significance of I_A was tested with randomization (1000 times) procedures by comparing the observed value of I_A to that expected under the null hypothesis of complete panmixis (Burt *et al*. 1996).
¶Observed (Gob) and expected (Gexp) genotypic diversity (Stoddart & Taylor 1988). The significance of the test was determined with randomization (1000 times) procedures by comparing the observed genotypic diversity to that expected under the null hypothesis of random mating (Hoffman 1986; Stoddart & Taylor 1988; Milgroom 1996).
**Parsimony tree length permutation test (PTLPT). The significance of the test was determined with randomization (1000 times) procedures by comparing the observed tree length (L) to tree lengths of 1000 artificially recombined data sets (Burt *et al*. 1996).

among alleles in a population and it takes only a few individuals reproducing sexually per generation to create enough recombination to ~~reject~~ the null hypothesis (Burt *et al*. 1996). This makes it impossible to determine how often sexual reproduction is occurring or if the underlying recombining genetic structure is due to present, past or parasexual recombination events (Geiser *et al*. 1998; Taylor *et al*. 1999). Inconsistencies between tests can also occur, such as in the analysis of subpopulation 99–1 where we rejected random mating based on I_A, but all other tests were consistent with recombination (Table 4). Similar, but opposite, results have been found for *Coccidiodes immitis*, where association between loci was found based on PTLPT, but random mating was not rejected based on I_A (Burt *et al*. 1996). Such discrepancies with these analyses warrant theoretical investigations to understand better how parameters such as the marker system used, number of loci, number of individuals, linkage and allele frequency distribution effect the outcome of these tests, especially if inferences about sexual reproduction are going to be made without additional evidence.

T. yallundae, like many fungal plant pathogens, has an 'epidemic' population structure consistent with the definition of Maynard Smith *et al*. (1993), in which populations undergo sexual reproduction followed by clonal spread via asexual propagates (Boeger *et al*. 1993; McDermott & McDonald 1993; Milgroom 1996). Similar results have been found for the rice-blast pathogen *Magnaporthe grisea* (Kumar *et al*. 1999). Kumar *et al*. (1999) failed to reject random mating in populations of *M. grisea* collected in the Himalayan region of India. *M. grisea* is thought to reproduce asexually in nature and Kumar *et al*. (1999) hypothesized that sexual reproduction may occur in this region because it is the proposed centre of origin for the pathogen. Additional evidence supporting this was the frequent recovery of sexually fertile hermaphroditic isolates. Moreover, they found that over 3 years of sampling that one population shifted from putatively recombining and genetically diverse to almost completely dominated by a single clonal lineage, demonstrating the possible effects of clonal selection (Kumar *et al*. 1999).

No single *T. yallundae* genotype predominated in this study and the repeated isolation of certain genotypes within subpopulations was probably a function of the sampling scale. For example, more clonal genotypes were found on average in subpopulations 00–1 and 00–14 (sampled in 2000) when the sampling scale was reduced from that used in 1998 and 1999. The repeated sampling of clonal genotypes in these subpopulations was also reflected in the lower genotypic diversity estimates (Table 3). Finding more clonal

genotypes when finer-scale sampling was employed was not unexpected, because asexual spores of *T. yallundae* move only short distances and are dispersed by rain (Sprague & Fellows 1934; Bruehl *et al*. 1968). The importance of localized genotypic dispersal is also evident because there is a significant correlation between pairwise θ-values between subpopulations and geographical distance (data not shown), even though the overall allele frequencies among the subpopulations are not significant. These results confirm the importance of asexual reproduction in the eyespot disease cycle, but also raise some questions about dispersal of asexual propagules. For example, clonal genotypes were found separated by as little as 1 m to as much as 200 km and some clonal genotypes were found in four of five fields sampled over 3 years. Asexual spores (conidia) of *T. yallundae* are hyaline, long and slender, and do not appear morphologically adapted for long-distance dispersal. However, *T. yallundae* also produces melanized pseudoparenchymatous stromata within lesions that could potentially serve as a survival structure, and which might also be dispersed long distances via wind during and following harvest.

No evidence of population differentiation was found among the five subpopulations analysed. All alleles were found in all subpopulations, no significant differences in allele frequency were found among any of the 10 loci, and the overall level of population differentiation was not significant ($\theta = -0.008$, $P = 0.722$). Additionally, six of the 10 loci had θ-values that were negative, which can occur because θ is an unbiased estimator of population differentiation and there is equal probability of the value being negative or positive when the true value is close to zero (Weir 1990; Weir & Cockerham 1984). From a genetic point of view, this indicates that the sampled alleles were more related among compared to within populations (Weir 1990). Lack of genetic differentiation in other fungal systems has also been found using the differentiation statistic G_{ST} (overall G_{ST} in this study = 0.025, data not shown). For example, subpopulations of *Mycosphaerella graminicola* from California and Oregon ($G_{ST} = 0.039$) (Boeger *et al*. 1993) and nine subpopulations of *Phaeosphoaeria nodorum* from Switzerland ($G_{ST} = 0.03$) (Keller *et al*. 1997) were found not to be genetically subdivided. Low levels of genetic differentiation in both studies were thought, in part, to be caused by sexual reproduction followed by migration and gene flow. In contrast, seven subpopulations of *Pythium irregulare* ($G_{ST} = 0.542$) from Australia (Harvey *et al*. 2000) and five subpopulations of *Pyrenophora teres* ($G_{ST} = 0.46$) from North America and Europe (Peever & Milgroom 1994) were found to be significantly subdivided. Evidence for random mating was also found in some subpopulations of both of these latter two pathogen species, but the genetic differentiation was attributed to founder effects and limited migration followed by random genetic drift. However, it is often difficult to compare studies due to different sampling strategies and the life history of the fungus sampled. For example, Peever & Milgroom (1994) compared subpopulations of *P. teres* between North America and Europe, which would limit the possibility of movement among subpopulations. Harvey *et al*. (2000) sampled only from Australia with subpopulations as close as 40 km, but isolated *P. irregulare* from up to three host plants within the same field and found that isolates were more genetically variable when isolated from multiple hosts than those from the same host.

The recombined population structure plus the lack of population differentiation in *T. yallundae* suggests that sexual reproduction followed by extensive migration and gene flow is occurring throughout the area sampled in this study. This hypothesis is supported by the indirect analysis of multilocus population structure and by the recent direct observation of apothecial development under PNW experimental field conditions (Douhan *et al*. 2002). However, the extent of apothecial development cannot be determined from the data nor can the amount of migration or gene flow be determined. There are several indirect approaches used to measure gene flow (McDermott & McDonald 1993), but they are all developed for populations at equilibrium (Slatkin 1985, 1987) and most fungal populations under agricultural conditions are not likely to be at evolutionary equilibrium (Peever & Milgroom 1994; Milgroom 1996). When populations are not at equilibrium, it is not possible to distinguish current gene flow from past gene flow because not enough time has elapsed for the effects of drift and migration to reach equilibrium (Slatkin 1985, 1987).

An alternative hypothesis to explain the lack of differentiation is that the PNW *T. yallundae* population was founded recently from a common source population. Under this scenario, there would be limited migration among subpopulations and founder effects within subpopulations to cause genetic differentiation within and among subpopulations due to genetic drift (Goodwin *et al*. 1993). However, a low level of genetic variation might also be predicted under this hypothesis due to potential genetic bottlenecks associated with the introduction (Nei *et al*. 1975; Goodwin *et al*. 1993). This does not appear to be the case for the *T. yallundae* subpopulations studied here. Goodwin *et al*. (1993) found similar results in subpopulations of *Rynchosporium secalis*, the causal agent of barley scald, from Europe, Australia and the United States. The higher than expected variation in *R. secalis* populations was attributed to adequate founder population sizes sufficient enough to maintain variation, which could also explain the genetic diversity found in Washington *T. yallundae* subpopulations. Additional populations of *T. yallundae* from other regions need to be analysed to test this hypothesis.

The results of this study indicate that *T. yallundae* has a population genetic structure consistent with random

mating and there is no evidence of population subdivision based on the analysis of genetically characterized molecular markers. Therefore, it appears that there is one large population of *T. yallundae* in Washington and that sexual reproduction, followed by extensive dispersal of ascospore, conidial or mycelial inoculum, is adequate to homogenize genetically the entire population. Sexual reproduction may also be important for maintaining the observed high level of genetic and genotypic variation found and in the creation and dissemination of recombined genotypes throughout the state. However, the importance of ascospore inoculum in the disease cycle of this pathogen is not known and therefore remains to be determined.

Acknowledgements

Plant Pathology New Series 0311 College of Agriculture and Home Economics Research Center, Washington State University, Pullman 99164–6430. Financial support of the Washington State Wheat Commission is gratefully acknowledged. We thank Michael Milgroom for the use of his computer program to analyse genotypic diversity and his insightful comments during the review process. We also thank Austin Burt for an early beta release of MULTILOCUS and his help with some interpretation and technical assistance with the program.

References

Agapow PM, Burt A (2001) Indices of multilocus linkage disequilibrium. *Molecular Ecology Notes*, **1**, 101–102.

Bassam BJ, Caetano-Anolles G, Gresshoff PM (1991) Fast and sensitive silver staining of DNA in polyacrylamide gels. *Annals of Biochemistry*, **196**, 80–83.

Boeger JM, Chen RS, McDonald BA (1993) Gene flow between geographic populations of *Mycosphaerella graminicola* (Anamorph *Septoria tritici*) detected with restricted fragment length polymorphism markers. *Phytopathology*, **83**, 1148–1154.

Brown JKM (1996) The choice of molecular marker methods for population genetic studies on plant pathogens. *New Phytologist*, **133**, 183–195.

Brown AHD, Feldman MW, Nevo E (1980) Multilocus structure of natural populations of *Hordeum spontaneum*. *Genetics*, **96**, 523–536.

Bruehl GW, Nelson WL, Koehler F, Vogel OA (1968) Experiments with Cercosporella foot rot (strawbreaker) disease of winter wheat. *Washington Experimental Station Bulletin*, **694**, 14pp.

Burdon JJ, Silk J (1977) Sources and patterns of diversity in plant-pathogenic fungi. *Phytopathology*, **87**, 664–669.

Burt A, Carter DA, Koenig GL, White TJ, Taylor JW (1996) Molecular markers reveal cryptic sex in the human pathogen *Coccidiodes immitis*. *Proceedings of the National Academy of Sciences of the USA*, **93**, 770–773.

Chen RS, McDonald BA (1996) Sexual reproduction plays a major role in the genetic structure of populations of the fungus *Mycosphaerella graminicola*. *Genetics*, **142**, 1119–1126.

Douhan GW, Murray TD, Dyer PS (2002) Species and mating-type distribution of *Tapesia yallundae* and *T. acuformis* and occurrence of apothecia in the U.S. Pacific Northwest. *Phytopathology*, **92**, 703–709.

Dyer PS, Bateman GL, Lucas JA, Peberdy JF (1994) Seasonal development of apothecia of the cereal pathogen *Tapesia yallundae* on straw stubble in the UK. *Annals of Applied Biology*, **125**, 489–500.

Dyer PS, Furneaux PA, Douhan GW, Murray TD (2001) A multiplex PCR test for determination of mating-type applied to the plant pathogens *Tapesia yallundae* and *Tapesia acuformis*. *Fungal Genetics and Biology*, **33**, 173–180.

Dyer PS, Rezanoor HN, Lucas JA, Peberdy JF (1993) Two-allele heterothallism in *Tapesia yallundae*, the teleomorph of the cereal eyespot pathogen *Pseudocercosporella herpotrichoides*. *Physiological and Molecular Plant Pathololgy*, **43**, 403–414.

Geiser DM, Pih JI, Taylor JW (1998) Cryptic speciation and decombination in the aflatoxin – producing fungus. *Aspergillus Flavus*.

Goodwin SB, Saghai Maroof MA, Allard RW, Webster RK (1993) Isozyme variation within and among populations of *Rhynchosporium secalis* in Europe, Australia, and the United States. *Mycological Research*, **97**, 49–58.

Harvey PR, Butterworth PJ, Hawke BG, Pankurst CE (2000) Genetic variation among populations of *Pythium irregulare* in southern Australia. *Plant Pathology*, **49**, 619–627.

Hoffman RJ (1986) Variation in contribution of asexual reproduction to the genetic structure of populations of the sea anemone *Metridium senile*. *Evolution*, **40**, 357–365.

Keller SM, Wolfe MS, McDermott JM, McDonald BA (1997) High genetic similarity among populations of *Phaeosphaeria nodorum* across wheat cultivars and regions in Switzerland. *Phytopathology*, **87**, 1134–1139.

Kohn LM (1995) The clonal dynamic in wild and agricultural plant populations. *Canadian Journal of Botany*, **73** (Suppl. 1), S1231–S1240.

Kumar J, Nelsen RJ, Zeigler RS (1999) Population structure and dynamics of *Magnaporthe grisea* in the Indian Himalayas. *Genetics*, **152**, 971–984.

Lee SB, Taylor JW (1990) Isolation of DNA from fungal mycelia and single spores. In: *PCR Protocols: a Guide to Methods and Applications* (eds Innis MA, Gelfand DH, Sninsky JJ, White TJ), pp. 282–287. Academic Press, San Diego.

Lucas JA, Dyer PS, Murray TD (2000) Pathogenicity, host-specificity, and population biology of *Tapesia* spp., causal agents of eyespot disease of cereals. *Advances in Botanical Research*, **33**, 225–258.

Maynard Smith J, Smith NH, O'Rourke M, Spratt BG (1993) How clonal are bacteria? *Proceedings of the National Academy of Sciences of the USA*, **90**, 4384–4388.

McDermott JM, McDonald BA (1993) Gene flow in plant pathosystems. *Annual Review of Phytopathology*, **31**, 353–373.

McDonald BA (1997) The population genetics of fungi: tools and techniques. *Phytopathology*, **87**, 448–453.

Milgroom MG (1996) Recombination and the multilocus structure of fungal populations. *Annual Review of Phytopathology*, **34**, 457–477.

Milgroom MG, Frey WE (1997) Contributions of population genetics to plant disease epidemiology and management. In: *Advances in Botanical Research, Vol. 24. Incorporating Advances in Plant Pathology* (eds), pp. 1–30. Academic Press, San Diego.

Murray TD (1996) Resistance to benzimidazole fungicides in the cereal eyespot pathogen. *Pseudocercosporella herpotrichoides in the Pacific Northwest 1984 to 1990*. *Plant Disease*, **80**, 19–23.

Nei M (1973) Analysis of gene diversity in subdivided populations. *Proceedings of the National Academy of Sciences of the USA*, **70**, 3321–3323.

Nei M, Maruyama T, Chakraborty R (1975) Bottleneck effect and genetic variability in populations. *Evolution*, **29**, 1–10.

Peever TL, Milgroom MG (1994) Genetic structure of *Pyrenophora teres* populations determined with random amplified polymorphic DNA markers. *Canadian Journal of Botany*, **72**, 915–923.

Slatkin M (1985) Gene flow in natural populations. *Annual Review of Ecology and Systematics*, **16**, 393–430.

Slatkin M (1987) Gene flow and geographic structure of natural populations. *Science*, **236**, 787–792.

Slatkin M (1994) Linkage disequilibrium in growing and stable populations. *Genetics*, **137**, 331–336.

Sprague R, Fellows H (1934) Cercosporella foot rot of winter cereals. *USDA Technical Bulletin*, **428**, 24pp.

Stoddart JA, Taylor JF (1988) Genotypic diversity: estimation and prediction in samples. *Genetics*, **118**, 705–711.

Swofford DL (1998) *PAUP*: Phylogenetic Analysis Using Parsimony (*and Other Methods)*, Version 4.0. Sinauer Associates, Sunderland, MA.

Taylor JW, Jacobson DJ, Fisher MC (1999) The evolution of asexual fungi: reproduction, speciation and classification. *Annual Review of Phytopathology*, **37**, 197–246.

Vos P, Hogers R, Bleeker M *et al.* (1995) AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Research*, **23**, 4407–4444.

Wallwork H, Spooner B (1988) Tapesia yallundae — *the teleomorph of* Pseudocercosporella herpotrichoides. *Transactions of the British Mycological Society*, **91**, 703–705.

Weir BS (1990) *Genetic Data Analysis*. Sinauer Associates, Sunderland, MA.

Weir BS, Cockerham CC (1984) Estimating *F*-statistics for the analysis of population structure. *Evolution*, **38**, 1358–1370.

Greg Douhan is currently a postdoctoral researcher working on the population genetics of mycorrhizal fungi in an oak–grassland ecosystem in the Sierra Nevada foothills of California. This research represents a portion of his PhD research dealing with the population biology of two fungal pathogens of winter wheat in the US Pacific Northwest. Tobin Peever's research interests involve the population genetics and evolutionary biology of plant-pathogenic fungi. Tim Murray studies the ecology and epidemiology of soilborne fungal pathogens and the genetics of disease resistance.
