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Citation	Wolfenbarger, S. N., Twomey, M. C., Gadoury, D. M., Knaus, B. J., Grünwald, N. J., & Gent, D. H. (2015). Identification and distribution of mating-type idiomorphs in populations of Podosphaera macularis and development of chasmothecia of the fungus. Plant Pathology, 64(5), 1094-1102. doi:10.1111/ppa.12344
DOI	10.1111/ppa.12344
Publisher	John Wiley & Sons, Inc.
Version	Version of Record
Terms of Use	http://cdss.library.oregonstate.edu/sa-termsofuse

Plant Pathology (2015) **64**, 1094–1102 Doi: 10.1111/ppa.12344

Identification and distribution of mating-type idiomorphs in populations of *Podosphaera macularis* and development of chasmothecia of the fungus

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Podosphaera macularis, the causal agent of hop powdery mildew, is known to produce chasmothecia (formerly cleistothecia) in eastern North America and Europe. Ascocarps have not yet been reported from the Pacific Northwestern region of North America. Reasons for the apparent absence of chasmothecia in the Pacific Northwest were unknown. This study established that *P. macularis* is heterothallic and ascocarp ontogeny, maturation, dehiscence and ascospore infection proceed similarly to other powdery mildew fungi. Genome sequencing of a *MAT1-1* isolate revealed the structure of the *MAT1* locus and presence of *MAT1-1-3*, demonstrating further similarities to other powdery mildew fungi. PCR assays with primers designed from conserved domains of the *MAT1* idiomorphs were developed to characterize the frequency of idiomorphs in populations of *P. macularis*. Amongst 317 samples of *P. macularis* collected during 2012 and 2013 from the Pacific Northwest only the *MAT1-1* idiomorph was found. In contrast, among 56 samples from the eastern United States and Europe, *MAT1-1* and *MAT1-2* idiomorphs were detected at equivalent frequencies. At temperatures representative of late season conditions in the Pacific Northwest, chasmothecia formed readily when a Pacific Northwest *MAT1-1* isolate was paired with a *MAT1-2* isolate collected from outside the region. Although these findings do not encompass all climatic, geographic or temporal barriers that could inhibit the formation of chasmothecia, the current absence of the ascigerious stage of *P. macularis* in the Pacific Northwest could be explained by the absence of the *MAT1-2* mating type idiomorph.

Keywords: chasmothecia, cleistothecia, hop, *Humulus lupulus*, *Podosphaera macularis*, powdery mildew

Introduction

Sexual recombination can be important for a pathogen's ability to adapt to changing environments and hosts, and survival of unfavourable environmental conditions. In some fungi a sexual resting structure can aid survival overwinter or during the absence of a host (McDonald & Linde, 2002; Agrios, 2005). The need for survival apart from the host during the intercrop period is especially acute in the case of biotrophic pathogens, such as fungi that cause powdery mildew diseases. Where the sexuality of powdery mildew fungi has been experimentally determined, most species have been found to be heterothallic (Gadoury & Pearson, 1991; Jarvis *et al.*, 2002; Gadoury *et al.*, 2010; Spanu *et al.*, 2010; Tollenaere & Laine, 2013; Wicker *et al.*, 2013). Homothallism in powdery mildew fungi has been reported in relatively few cases (Homma, 1933; Smith, 1970), and some of these same species have also been described as heterothallic (Gadoury & Pearson, 1991). Only in *Podosphaera plantaginis* has homothallism been confirmed by mating assays and molecular evidence (Tollenaere & Laine, 2013). The apparent rarity of homothallism in the powdery mildew mating systems may be because relatively few systems have been experimentally investigated. Nonetheless, heterothallism appears to be more common among powdery mildews than homothallism.

In the case of *Podosphaera macularis*, the causal organism of powdery mildew on hop, the ascigerious stage is known to exist in Europe (Neve, 1991) and eastern North America (Blodgett, 1913), but has not yet been reported in the Pacific Northwest (Gent *et al.*, 2006). Despite the hop industry being present in the Pacific Northwestern region of North America since the early 1900s (Neve, 1991; Barth *et al.*, 1994), powdery mildew was not reported in hop yards of the Pacific Northwest until 1997 (Ocamb *et al.*, 1999).

The lack of the ascigerious stage of *P. macularis* in the Pacific Northwest limits the pathogen to overwintering vegetatively in association with crown buds of dormant

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Published online 19 February 2015

Plant Pathology

hop plants or on hop plants cultivated in greenhouses (Gent *et al.*, 2008). Understanding why chasmothecia have not been observed in this region is important because the mode of perennation has implications for disease management practices (e.g. late spring pruning), and because sexual reproduction would provide additional opportunity for survival and genetic adaptation.

Confirmation of the heterothallic nature of *P. macularis*, as well as the presence or absence of mating types in the Pacific Northwest, could be accomplished through controlled crosses of isolates. However, this is very labour-intensive and limits the number of isolates that can be investigated. With the creation of degenerate primers to identify the conserved regions of the *MAT1* locus in the Erysiphales (Brewer *et al.*, 2011), there is an opportunity for expanding the body of knowledge on mating systems in powdery mildew fungi. In most heterothallic filamentous ascomycetes, the *MAT1* locus controls mating; this locus contains one of two different sequences, *MAT1-1* or *MAT1-2*, in heterothallic fungi. Because these sequences are not obviously related by structure or common descent they are known as idiomorphs and not alleles (Debuchy & Turgeon, 2006). The *MAT1-1* idiomorph is characterized by the *MAT1-1-1* gene that encodes a protein containing an alpha 1 (α1) box, while *MAT1-2* is characterized by the *MAT1-2-1* gene that encodes a protein containing a high mobility group (HMG) domain (Turgeon & Yoder, 2000).

A thorough evaluation of the mating system and characterization of ascocarp initiation and development has not been conducted for *P. macularis*, leading to speculation on the absence of the ascigerious stage of *P. macularis* in the Pacific Northwest. Royle (1976) stated that *P. macularis* was heterothallic, and it has been assumed that the lack of the ascigerious stage is due to absence of one of the mating types needed for sexual reproduction. Other possibilities that could explain the absence of chasmothecia are various climatic, geographic or temporal barriers that prevent sexual reproduction. The objective of this study was to clarify why the sexual stage of the pathogen is absent from the Pacific Northwest. This was accomplished by characterizing the mating type idiomorphs, developing PCR assays for rapid determination of mating type, and conducting a survey of mating type idiomorphs among populations in the Pacific Northwest and within populations in eastern North America and Europe where chasmothecia are known to occur. Physical pairings of the fungus were also conducted under three different temperature regimes to determine whether incubation temperature affects the initiation of chasmothecia, as had been reported for the closely related pathogen *Podosphaera aphanis* (Asalf *et al.*, 2013). A brief preliminary account of this research has been published (Wolfenbarger *et al.*, 2012).

Materials and methods

Plant materials

Hop plants of the powdery mildew-susceptible cultivar Symphony were propagated from softwood cuttings and maintained in a greenhouse with sulphur burned to ensure plants were free of powdery mildew. The greenhouse was maintained at 20–25°C with a 14 h photoperiod. Plants were grown in Sunshine Mix #1 (SunGro Horticulture) for approximately 21 days and were watered regularly, receiving Sunshine Technigro 16-17-17 Plus fertilizer with micronutrients (Sun Gro Horticulture) at each irrigation to promote succulent and vigorous growth. Young, unfurled leaves from the top node were detached, surface disinfected with 70% ethanol, rinsed for 30 s with water, and dried. Disinfected leaves were placed in double Petri dishes (Pearson & Gadoury, 1987) with water in the lower Petri dish, and were inoculated with isolates. Isolates were transferred to new leaves every 2–3 weeks for their maintenance.

Discovery of the *MAT1* locus in *P. macularis*

Degenerate primers designed by Brewer *et al.* (2011) were used to amplify the conserved region of the *MAT1-2* idiomorph of *P. macularis* using DNA obtained from a field population of the pathogen from the Czech Republic. The PCR reaction was carried out in a total volume of 12·5 μL. Reaction components and cycling parameters were as described by Brewer *et al.* (2011) for degenerate primers except reaction components and volume were halved. Ten microlitres of the PCR product were visualized by electrophoresis through a 1% (w/v) agarose Tris-acetate-EDTA gel. The *c.* 230 bp PCR product was purified from the gel (UltraClean GelSpin DNA Extraction kit; MO BIO Laboratories) and cloned using a TOPO TA cloning kit (Invitrogen). The cloned PCR products were sequenced in both directions at the Oregon State University Center for Genome Research and Biocomputing. Sequence data was deposited in the National Center for Biotechnology Information GenBank database (accession KJ741396).

The degenerate primers designed for the conserved region of *MAT1-1* did not amplify the *MAT1-1* idiomorph in *P. macularis* despite multiple attempts using DNA originating from the Pacific Northwest and from the Czech Republic. Therefore, a region of the *MAT1-1* of *P. macularis* was amplified using primers Pxα-10f and Pxα-156r, designed for the closely related species *P. aphanis* (Asalf *et al.*, 2013). The PCR reaction was carried out as stated above. Cycling conditions were an initial denaturation at 95°C for 2 min; 35 cycles with a denaturation step at 95°C for 30 s, annealing set as a gradient from 48 to 58°C for 30 s, extension at 72°C for 30 s; and a final extension at 72°C for 5 min. The *c.* 170 bp PCR product was gel purified, cloned and sequenced as described previously. Sequence data for the full length of *MAT1-1* was obtained and deposited in GenBank, as described below.

Development of PCR to determine mating type of *P. macularis*

Specific primers incorporating single nucleotide polymorphisms from the closely related organisms *P. xanthii* and *P. aphanis* were designed from the conserved region of *MAT1-2* of *P. macularis*. The primer sequences were M2F_2_TA (5′-GTCTTAGCAATAATCAAATATG-3′) and M2R_2_Edeg (5′-TTCCGTGGTTTGTAACGGTAA-3′). Because of the limited size of the conserved region for *MAT1-1*, the primers from Asalf *et al.* (2013) were used to identify this mating type in the present study. The PCR assays for both primer sets were carried out identically in a total volume of 25 μL in separate tubes. Reaction components comprised 10 μL Mango Taq (Bioline), 0·5 μL 50% acetonitrile in water, 0·75 μL of each 10 μM primers, and

1 μL DNA template. Cycling conditions were an initial denaturation at 95°C for 2 min; 35 cycles with a denaturation step at 95°C for 1 min, annealing at 52°C for 1 min, and extension at 72°C for 30 s; and a final extension at 72°C for 10 min. Primer sets were not multiplexed due to the PCR products being of similar size.

Verification of mating types determined by primers for *P. macularis*

The mating types of clonal isolates of *P. macularis* were tested with the above primers and, subsequently, mating experiments were performed to verify mating types. Test isolates originating from Europe were selected for mating type loci *MAT1-2* or *MAT1-1*. Clonal isolates from the Pacific Northwest, 15 in total, were paired with each of the test isolates on detached leaves for approximately 21 days at 13°C with a 14 h photoperiod. Isolates were also inoculated individually to check whether the fungus was capable of self-mating. The presence or absence of chasmothecia was confirmed 21 days after inoculation with the aid of a stereomicroscope at ×10 to ×30 magnification and the isolates were assigned to a mating type.

Specificity and sensitivity of primer sets

The specificity of the *MAT1-1* and *MAT1-2* primer sets was assessed on a range of other powdery mildew fungi found on grape (*Vitis vinifera*), cucumber (*Cucumis sativus*), zucchini (*Cucurbita pepo*), maple (*Acer* spp.), strawberry (*Fragaria* spp.), prostrate knotweed (*Polygonum* spp.), plantain weed (*Plantago* spp.), rose (*Rosa* spp.), Oregon grape (*Mahonia aquifolium*), heavenly bamboo (*Nandina domestica*), azalea (*Rhododendron* spp.), grass species (*Poaceae* spp.), cherry (*Prunus* spp.), lilac (*Syringa* spp.), blackberry (*Rubus* spp.), buckeye (*Aesculus* spp.) and sycamore (*Platanus occidentalis*). Also tested were organisms commonly found in association with powdery mildew fungi, namely *Lecanicillium attenuatum* and *Pseudozyma flocculosa*, and organisms found in association with hop, *Cladosporium herbarum*, *Fusarium* spp., *Epicoccum nigrum*, an *Alternaria* spp. and *Pseudoperonospora humuli*, as well as hop itself.

The reliability of the extraction procedure and PCR assay to detect varying quantities of *P. macularis* was established by collecting conidia and mycelia on pieces of cellophane tape of varying lengths; these were the standard 2·5 cm, and ¾, ½, ¼ and $^1/_8$ of the standard length. This was repeated independently nine times. DNA was extracted from powdery mildew isolates using a Chelex extraction procedure (Brewer & Milgroom, 2010) and amplified by PCR according to the conditions described above. The proportion of successful PCR assays was determined.

Determination of the full length sequence of the *MAT1-1* idiomorph

The *MAT1-1* idiomorph of an isolate of *P. macularis* originating from Oregon was sequenced using paired-end 100 bp Illumina HiSeq 2000 technology at the Oregon State University Center for Genomics Research and Biocomputing. *De novo* assembly was achieved using VELVET v. 1.2.09 (Zerbino & Birney, 2008) with a minimum contig length of 200 bp required for retention. Multiple initial assemblies were performed using 4–60 million reads and a k-mer length ranging from 31 to 61 bp. After all initial assemblies had been completed the contigs from each assembly were clustered into groups of homologous sequences using VMATCH v. 2.0 (Abouelhoda *et al.*, 2004). The longest sequence was retained from each cluster. These sequences, along with the sequences that did not cluster with others, were concatenated for the final assembly. *MAT1-1* and related genes were isolated from assembled contigs using GENEIOUS v. 6.0.5 (Biomatters) using gene information of other powdery mildew fungi available in GenBank.

A PCR assay was designed to amplify the region between *SLA2* and *MAT1-1*. Primers used were PM_SLA2-mat1F2 (5′-TGTAGCTATCTAGATTCGTCCAT-3′) and PM_SLA2-mat1R2 (5′-TGAAAGGATCTCGGTTCCAC-3′). The PCR reaction components and cycling conditions were the same as stated above for the mating type PCR assay, with the exception of the annealing temperature being set at 60°C. The *c.* 700 bp PCR product was gel purified, and sequenced in both directions by the Center for Genomics Research and Biocomputing.

Mating type survey

A stratified sampling method was used to collect samples of powdery mildew from the Pacific Northwest for determination of mating type. Sampling occurred three times during both 2012 and 2013: early season (early June 2012, early May 2013), mid-season (mid-July 2012, mid-June 2013), and late season (early September 2012, mid-July 2013). At least three hop yards were sampled from each of Oregon and Washington at each time point. Powdery mildew samples were also collected from Idaho during the late season in 2012, but were not available from Idaho during the other surveys because powdery mildew was not found. When possible, different cultivars of hop were selected to represent the diversity of cultivars grown in the Pacific Northwest. Powdery mildew samples from the following cultivars were tested over the course of the survey: Apollo, Bravo, Cascade, Centennial, Chelan, Chinook, Cluster, Columbus/Tomahawk, El Dorado, Galena, Glacier, Magnum, Meridian, Millennium, Mount Hood, Nugget, Santiam, Sterling, Summit, Super Galena, Symphony, Tettnanger, Warrior, Willamette, Zeus and various unnamed breeding lines. Four to five samples from separate leaves and/or cones were collected from each field during each sampling period. Samples were intentionally not reduced to clonal isolates. In addition, a total of 56 samples of hop leaves with powdery mildew or clonal isolates of *P. macularis* from known sexual reproducing populations were obtained from cooperating scientists in the eastern US and Europe. When samples were received in a viable condition, *P. macularis* was cultured and maintained without single conidial chain transfers on detached hop leaves before DNA extraction. Otherwise, 2-mm disks of infected tissue were extracted using a PowerSoil DNA isolation kit (MO BIO Laboratories) according to manufacturer's instructions. Extracted DNA was used in PCR assays as described above for detection of *MAT1-1* and *MAT1-2*. Negative and positive controls were included in every PCR assay using DNA extracted from confirmed *MAT1-1* and *MAT1-2* isolates. In 2012 the ITS region was also amplified using ITS primers ITS1 and ITS4 (White *et al.*, 1990) as a further control to ensure amplification of the template DNA was possible. To further guarantee that the products of the PCR reactions were obtained from *P. macularis*, every PCR product or a subsample of PCR products was confirmed by bidirectional sequencing in 2012 and 2013, respectively. Sequences were aligned to each other and to *P. aphanis* and *P. xanthii* using BIOEDIT (Hall, 1999) to verify that SNPs associated with other powdery mildew fungi were not present.

Effects of temperature on initiation of chasmothecia

To determine if temperatures typical of the growing season for hop in the Pacific Northwest might suppress initiation of chasmothecia as reported by Asalf *et al.* (2013), compatible isolates were paired on mildew-free leaves as described previously. The inoculated leaves were incubated in a diurnal temperature regime in which hourly temperatures ranged from 11 to 27·9°C. Values for the hourly temperatures were derived from the 30-year average daily temperatures for Prosser, WA, USA for the date 1 September as described by De *et al.* (1978). Weather data was retrieved from the Western Region Climate Center (Prosser, WA, USA). A second group of paired isolates was also incubated on detached leaves at a constant temperature of 13°C, while a third group was incubated at ambient room temperature, which ranged from 16 to 26°C over the course of the experiment. Treatments were replicated on nine leaves, and the entire experiment was conducted three times. At 28 days after inoculation, leaves were examined for the presence of chasmothecia within powdery mildew colonies. Differences among the proportion of colonies forming chasmothecia at different temperature regimes were analysed in a generalized linear mixed model with the response distribution specified as the beta distribution and a logit link function using the GLIMMIX procedure in SAS v. 9.4 (SAS Institute).

Maturation process of chasmothecia and viability of ascospores

Leaves bearing chasmothecia in various maturation stages were gathered from feral hop plants near Sharpsburg, Maryland in October of 2012. Disks approximately 2 mm in diameter with chasmothecia present were cut from the leaves. Disks for scanning electron microscopy were fixed in a solution of 3% glutaraldehyde in water overnight. Samples were then dehydrated in a five-step ethanol series (starting at 10% ethanol and ending at 100% ethanol), critical-point dried, mounted and sputter coated with gold before examination. Samples intended for light microscopy were fixed in formalin/acetic acid/alcohol (10% formalin, 5% glacial acetic acid, 50% ethanol (95% concentration); Johansen, 1940) and dehydrated in an ethanol series, infiltrated with paraffin, and sectioned at 10–15 μm. The sections were adhered to glass microscope slides, dewaxed in Histoclear, and stained with 1% safranin in 50% ethanol, followed by counterstaining in 0·1% Fast Green in clove oil (Johansen, 1940). Samples were examined under a light microscope at ×10 to ×40 magnification.

To confirm viability of ascospores, chasmothecia were mounted on glass microscope slides in 0·5% (w/v) fluorescein diacetate (FDA). The wall of the chasmothecium was ruptured by applying gentle pressure to the glass coverslip using fine forceps whilst the ascocarp was observed at ×100 magnification. The ascus contents were then allowed to absorb the FDA stain for 5 min before viewing and photomicrography at ×200 magnification under both bright-field and fluorescence illumination (325–500 nm excitation filter and transmission filter >530 nm). Chasmothecia prepared as above were also stained with Sudan Black B and examined at ×200 magnification under bright-field illumination to assess the lipid content of the ascus and ascospores (Gadoury *et al.*, 2010), as reabsorbtion and metabolism of lipids has been correlated with maturation of ascocarps in *P. aphanis* (Gadoury *et al.*, 2010) and *Erysiphe necator* (Gadoury & Pearson, 1990).

To further demonstrate the viability and infectivity of ascospores, leaves bearing chasmothecia were collected in September 2013 from a feral hop plant in Seneca Castle, NY, placed in envelopes made from fibreglass window screen, and allowed to overwinter on the ground in Geneva, NY. At monthly intervals from January to March and at 2-week intervals thereafter, 2-cm disks bearing at least 20 ascocarps were cut from the leaves, attached to a 9 cm disk of filter paper wetted with distilled water, and suspended above mildew-free detached hop leaves of cultivar Symphony for 24 h at 20–22°C. After exposure to chasmothecia, detached leaves were enclosed in 9-cm polystyrene Petri dishes and incubated at 20–22°C for 14 days. Leaves were then examined at ×20 magnification for the presence of mildew colonies.

Results

Verification of primers for *P. macularis*

PCR products of the expected α1 box and HMG domains were obtained with the *MAT1-1* and *MAT1-2* test isolates originating from Europe. Physical pairings conducted with a putative *MAT1-1* genotype isolate of *P. macularis* from the Pacific Northwest and isolates identified as *MAT1-1* and *MAT1-2* genotypes by PCR confirmed the genotypes (Table 1). Chasmothecia developed only when isolates of opposite mating types were paired and were not produced when two isolates of the same mating type from the Pacific Northwest were paired. Using the test isolates, 15 additional pairings were conducted with clonal isolates from the Pacific Northwest: all were positive for *MAT1-1* as determined by PCR.

Specificity and sensitivity of primer sets

The *MAT1-1* primers were able to amplify DNA from the powdery mildew fungi from strawberry, blackberry and rose. The *MAT1-2* primers amplified DNA derived from the powdery mildew fungus growing on cherry. The PCR assays did not amplify from DNA of the other organisms tested. Both the *MAT1-1* and *MAT1-2* primer sets strongly amplified a DNA product in every experiment when using only $^1/_8$ of the quantity of material normally used in the extraction procedure.

Table 1 Presence (+) and absence (−) of chasmothecia in different pairings of *Podosphaera macularis* isolates putatively identified by PCR assays for *MAT1* locus

Powdery mildew isolate	PNW (*MAT1-1*)	Tester 1 (*MAT1-1*)	Tester 2 (*MAT1-2*)
PNW (*MAT1-1*)[a]	–	–	+
Tester 1 (*MAT1-1*)[b]	–	–	+
Tester 2 (*MAT1-2*)[c]	+	+	–

[a]PNW, Pacific Northwest isolate with putative genotype *MAT1-1*.
[b]Test isolate from Europe, with *MAT1-1* genotype.
[c]Test isolate from Europe, with *MAT1-2* genotype.

Determining the full length sequence of the *MAT1-1* idiomorph

One lane of paired-end 100 bp Illumina HiSeq 2000 sequencing resulted in 210 557 723 pairs of reads. The assembly of a subset of these reads resulted in 347 507 contigs ranging in size from 200 bp (the lower threshold) to 16 809 bp in length. The reference included a total of 126 225 625 nucleotides with an N50 of 329 bp. A single contig of approximately 2200 bp was isolated containing the full length of the *MAT1-1-1* gene (GenBank accession KJ922755) of *P. macularis* found in the *MAT1-1* idiomorph. In addition, fragments of genes associated with the *MAT1-1* idiomorph, *SLA2* (GenBank accession KJ922754), *APN2* (GenBank accession KJ830621), and *MAT1-1-3* (GenBank accession KJ830622) were found in the sequence data. These sequences were isolated on separate contigs and were unable to be joined to *MAT1-1-1*. However, similar to other powdery mildew fungi (Brewer *et al.*, 2011; Wicker *et al.*, 2013), the contig containing *MAT1-1-1* and the contig containing *SLA2* were later joined by PCR amplification across the missing region. The loci were separated by approximately 677 bp. The distance between *MAT1-1-3* or *APN2* from *MAT1-1-1* is undetermined as sequences could not be connected.

Mating type survey

In 2012 and 2013, 183 and 134 hop samples bearing colonies of *P. macularis* were collected from the Pacific Northwest, respectively (Table 2). Only the *MAT1-1* idiomorph was found. The 99% confidence interval for a binomial test of proportions was 0·98–1, providing a very strong indication that *MAT1-2* is not present in the Pacific Northwest region. The mating types of *P. macularis* isolates on 56 samples collected from populations outside the Pacific Northwest were identified. Of the 56 samples collected, 38 tested positive for *MAT1-1*, 44 tested positive for *MAT1-2* and of these, 23 samples were positive for both *MAT1-1* and *MAT1-2* (Table 3). The ratio of idiomorphs detected was consistent with both mating types being distributed in a ratio approximating 1:1 in both North America ($\chi^2 = 0{\cdot}438$, $P = 0{\cdot}508$) and Europe ($\chi^2 = 0{\cdot}04$, $P = 0{\cdot}841$), with a combined ratio also similar to 1:1 ($\chi^2 = 0{\cdot}439$, $P = 0{\cdot}508$).

Table 2 Survey for *MAT1* idiomorphs within *Podosphaera macularis* samples from the Pacific Northwest[a]

State	Total no. of samples	No. of locations[b]	*MAT1-1*	*MAT1-2*
2012				
Oregon	68	10	68	0
Washington	85	19	85	0
Idaho	30	6	30	0
2013				
Oregon	60	12	60	0
Washington	74	14	74	0
Total	317	56	317	0

[a]Samples were collected in early, mid and late season and maintained for 3–4 weeks on a powdery mildew susceptible cultivar. All isolates were screened for the presence or absence of *MAT1-1* and *MAT1-2* by PCR. The resulting ratio for *MAT1-1* to *MAT1-2* was 1:0 (chi square test for 1:1 ratio rejected at $P < 0{\cdot}0001$).

[b]The number of locations given is the number of unique locations sampled. Some sites were sampled multiple times over this study.

Table 3 Survey for mating type idiomorphs in *Podosphaera macularis* samples from sexually reproducing populations

Location[a]	Samples	Samples with chasmothecia at time of assays	*MAT1-1*	*MAT1-2*	*MAT1-1* and *MAT1-2*
Maryland	14	14	14	14	14
New York	17	8	11	14	8
Minnesota	4	0	1	3	0
Germany	4	0	0	4	0
Germany*	5	0	3	2	0
England*	2	0	1	1	0
France*	1	0	1	0	0
Slovenia	5	1	4	2	1
Czech Republic	4	NA	3	4	0
Total	56	23	38	44	23

[a]Locations with an asterisk (*) indicate clonal (single conidial chain) isolates were assayed, whereas the others are from heterogeneous inoculum sources. The resulting ratio for *MAT1-1* to *MAT1-2* was 1:1 ($\chi^2 = 0{\cdot}439$, $P = 0{\cdot}508$). Samples from the Czech Republic were received as DNA.

Effects of temperature on initiation of chasmothecia

Irrespective of temperature treatments, from 38 to 52% of the leaves inoculated with compatible isolates formed abundant chasmothecia within 28 days after inoculation (Table 4). The incidence of chasmothecium initiation did not differ significantly ($P = 0{\cdot}05$) between leaves incubated at a constant temperature of 13°C compared to the diurnal temperature cycle, and was only slightly

Table 4 Formation of chasmothecia by sexually compatible isolates of *Podosphaera macularis* in physical pairings at different temperature regimes

Temperature regime	High (°C)	Low (°C)	Colonies with chasmothecia (%)[a]
Pacific Northwest climate	27·9	11	38·4 a
Constant 13°C	13	13	44·1 ab
Room temperature	26	16	52·7 b

[a]Pairings of clonal Pacific Northwest isolates with test isolate of *MAT1-2* genotype, grown in different temperature regimes.
Means with same letters are not significantly different according to a generalized linear mixed model analysis (temperature treatment $F = 3{\cdot}81$; $P = 0{\cdot}0395$).

elevated at ambient room temperatures ranging from 16 to 26°C (Table 4).

Maturation process of chasmothecia and viability of ascospores

Appendages typical of the myceloid appendages for the genus *Podosphaera* began to appear once chasmothecia had reached a diameter of approximately 15–20 μm (Fig. 1). These appendages continued to grow as the chasmothecium increased in diameter and eventually grew down and became entangled in the pannose mycelium of the mildew colony, effectively anchoring the chasmothecium to the developing mildew colony. By the time the chasmothecium was 40–50 μm in size, its wall had changed from hyaline to yellow, most probably due to the accumulation of a yellow lipid compound that was darkly stained by Sudan Black B (Fig. 2). At 80–90 μm in diameter, the chasmothecium wall was composed of thick-walled dark cells and a single ascus, containing eight ascospores, was present (Fig. 2). Ascospores fluoresced when stained with FDA (Fig. 2). As this process occurred, chasmothecia and appendages became further entangled with the mildew colony and thus host tissue due to overgrowth by the pannose mycelium (Fig. 1). After overwintering, chasmothecia often dehisced when exposed to free water, followed by rapid swelling of the ascus. The ascus was observed to rupture and forcibly discharged ascospores within 5 min of dehiscence at 22–25°C (i.e. room temperature) as long as the chasmothecium remained in contact with a film of water. Colonies of *P. macularis* formed on leaves subtending overwintered chasmothecia after approximately 14 days of incubation (Fig. 2), but not on control leaves similarly incubated without chasmothecia.

Discussion

The results indicate that the absence of the ascigerious state of *P. macularis* in the Pacific Northwest region is due to the exclusive presence of only one mating type: *MAT1-1*. Over two seasons, 317 *P. macularis* samples were tested and all were positively identified as *MAT1-1*. In contrast, in other regions where ascocarps are known to occur, *MAT1-1* and *MAT1-2* were detected at approximately equal frequencies. Isolates of *MAT1-1* collected within the Pacific Northwest region readily formed ascocarps when paired with *MAT1-2* across a range of temperatures that would occur during the growing season in the region. Some powdery mildew fungi such as *P. aphanis* require brief periods of low temperature to initiate ascocarps (Asalf *et al.*, 2013). However,

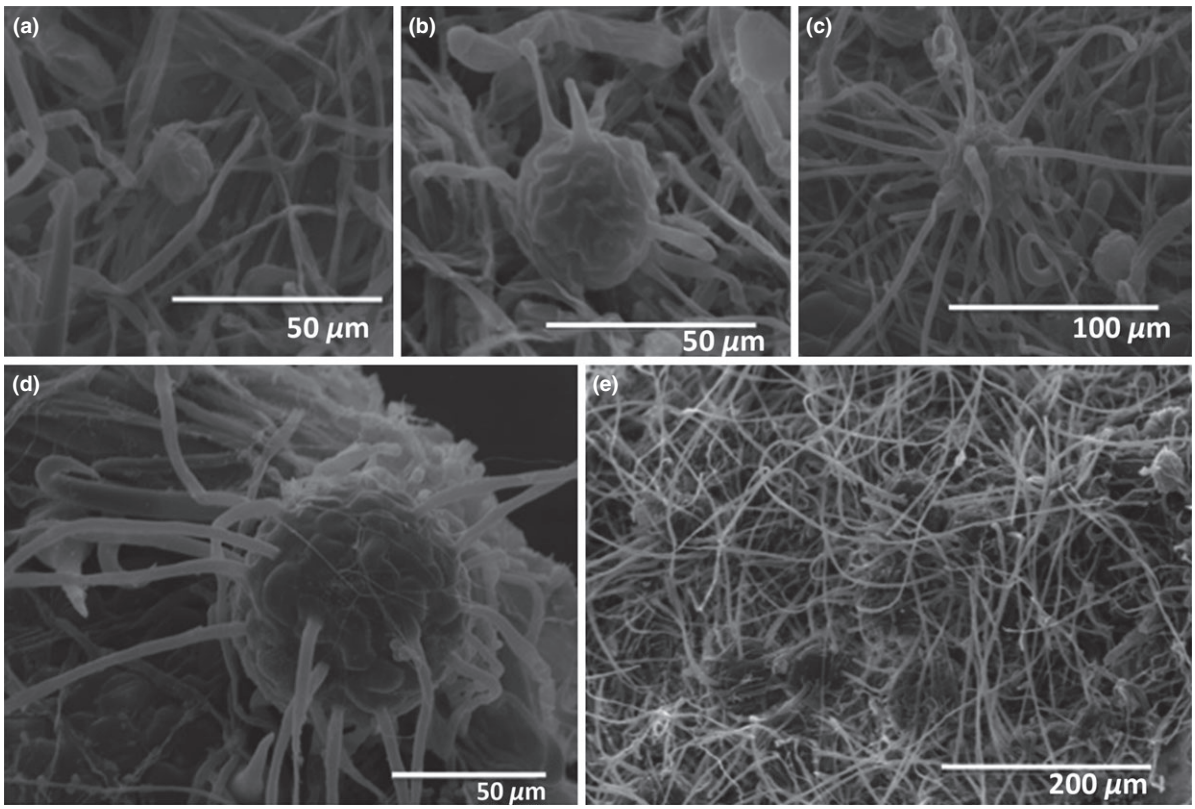

Figure 1 Scanning electron micrographs of the development of chasmothecia of *Podosphaera macularis*. (a) An initial, approximately 15 μm in diameter; (b) chasmothecium, approximately 25 μm in diameter, beginning to form appendages; (c) chasmothecium, approximately 50 μm in diameter; (d) mature chasmothecium, approximately 90 μm in diameter; (e) colony with numerous chasmothecia with appendages embedded in and intertwined with pannose mycelia, anchoring the ascocarps to the infected leaf.

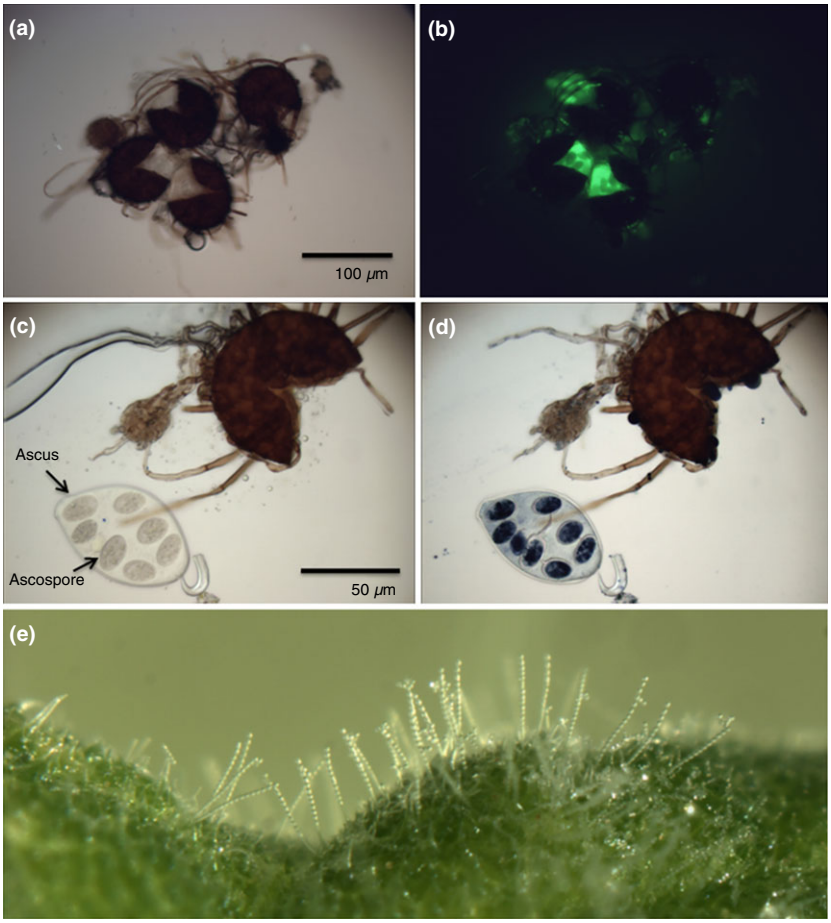

Figure 2 Ascospore viability and infectivity of *Podosphaera macularis*. (a) Crush mount of chasmothecia; (b) crush mount of chasmothecia shown in (a), stained with FDA demonstrating viability of asci and ascospores; (c) crush mount of chasmothecium with elliptically shaped ascus containing eight immature ascospores; (d) crush mount of chasmothecium shown in (c), stained with Sudan Black B showing stained lipids; (e) 2-week-old colony derived from ascospore infection.

P. macularis appears to lack a strict temperature requirement for initiation of ascocarps, readily forming chasmothecia at all temperature regimes evaluated, in addition to constant 18°C (data not shown). This supports the hypothesis that *MAT1-2* is absent in the Pacific Northwest because the temperatures found during late season appear to be conducive for ascocarp formation if the two mating types were present. Thus, three lines of evidence (assays using specific primers, crosses of isolates, and controlled environment studies) converge to support the conclusion that only *MAT1-1* is currently present in the region. This may be the result of an isolated introduction of *P. macularis* to the region during the mid-1990s, either consisting only of strains of the *MAT1-1* idiomorph or perhaps both idiomorphs, with the *MAT1-2* type strains being unsuccessful at establishing or surviving for unknown reasons.

It is often the case that establishment of a pathogen in a new region is the result of only one or a few isolates of the pathogen being introduced, resulting in a genetic founder effect (Goodwin *et al.*, 1994; Brown & Hovmoller, 2002; Sotirovski *et al.*, 2004). If this new, derived population remains isolated from the original population and has only a small subset of the genetic variation found in the larger original population, then this founder effect may persist. This is especially observed in clonal organisms such as certain populations of oomycetes and fungi. Understanding how and when sexual reproduction occurs in a population and modes of perennation for a pathogen is thought to be important in disease management (McDonald & Linde, 2002; Grünwald & Goss, 2011), particularly for foliar fungal pathogens capable of long distance dispersal (Brown & Hovmoller, 2002). Sexually reproducing powdery mildews could potentially exhibit field resistance to certain fungicide groups more quickly than non-sexual populations due to a greater frequency of perennation and increased inoculum potential, thereby increasing effective population sizes and generating greater allelic diversity at a locus under selection. Fungicides that are presently deployed in the Pacific Northwest, including both DMI and strobilurin compounds, are still effective in suppressing *P. macularis*, as field resistance has not been reported. This may be due, in part, to the severe population bottleneck that occurs each winter caused by the absence of the *MAT1-2* idiomorph of *P. macularis*, and therefore the ascigerious stage, in the Pacific Northwest. This forces the pathogen to survive the winter in association with living host tissue provided by dormant crown buds on the perennial root system (Gent *et al.*, 2008). However, perennation of the pathogen occurs on less than 0·69% of hop plants in Washington and 0·02% of plants in Oregon. This greatly

limits the potential for establishment of fungicide-resistant fungi that may be selected for during a given growing season.

Because the ascigerious stage of the pathogen is absent, primary inoculum of *P. macularis* can be substantially reduced in the Pacific Northwest through spring pruning of hop plants to destroy the emerging flag shoots, either by mechanical or chemical means (Gent *et al.*, 2012). The absence of ascosporic inoculum in the Pacific Northwest greatly simplifies the management programme compared to other regions where both flag shoots and ascosporic inoculum may occur.

Recently, a new strain of *P. macularis* capable of overcoming a widely deployed resistance gene in hop, *R6*, has been observed in the Pacific Northwest (Wolfenbarger *et al.*, 2014). Sampling in the present study of *P. macularis* from cultivars possessing *R6* verified that this virulent strain is only present as the *MAT1-1* genotype and its virulence pattern is similar to extant strains in the population (D. H. Gent, unpublished data). Thus, the virulent strain may be an extant but rare *MAT1-1* strain or a spontaneous mutant of an extant *MAT1-1* strain selected by widespread deployment of the *R6* resistance gene. Alternatively, it may be a newly introduced *MAT1-1* strain of *P. macularis* from another region, but this seems unlikely given that mating type idiomorphs occur in approximately equal ratios on infected hop leaves outside the Pacific Northwest.

The maturation process of *P. macularis* appears very similar to that of *P. aphanis* (Gadoury *et al.*, 2010) and other powdery mildew fungi (Gadoury & Pearson, 1988; Jarvis *et al.*, 2002). Colonies resulting from ascosporic infection were observed in laboratory experiments. Liyanage & Royle (1976) described powdery mildew on hop early in the growing season that appeared to be the result of ascospore infection based on circumstantial evidence. However, their attempts to demonstrate ascospore infectivity experimentally failed. The present study is, to the authors' knowledge, the first documentation of ascospore viability and infectivity in *P. macularis*.

There is limited molecular information on mating type systems of most powdery mildew fungi. In an attempt to better understand the architecture of the *MAT1* locus in *P. macularis* and to create a molecular database for future studies, genomic sequencing was conducted on a *MAT1-1* isolate originating from Oregon. In other ascomycetes, there is a conserved structure of the *MAT1* locus where the mating type idiomorph and related genes are flanked by the genes *APN2* and *SLA2*. While the *MAT1-1-1* genes in *E. necator* (Brewer *et al.*, 2011) and *Blumeria graminis* f. sp. *tritici* (Wicker *et al.*, 2013) are flanked by the gene *SLA2*, *MAT1-1-1* genes in these powdery mildew fungi could not be joined by sequencing to *APN2*. Similarly, the *MAT1-1-1* gene of *P. macularis* is flanked on one side by the *SLA2* gene but could not be connected to *APN2* by sequencing, despite *APN2* being present in the genome. Idiomorphs may also contain other mating type-related genes such as *MAT1-1-3*, which is found in addition to *MAT1-1-1* in some species of ascomycetes (Debuchy & Turgeon, 2006) as well as the powdery mildew fungi *E. necator* (Brewer *et al.*, 2011), *B. graminis* f. sp. *tritici* (Wicker *et al.*, 2013) and *Podosphaera plantaginis* (Tollenaere & Laine, 2013). The *MAT1-1-3* gene was detected in the genome of *P. macularis* but sequencing was again unable to connect it to the *MAT1-1-1* gene, as has been found with other powdery mildews (Brewer *et al.*, 2011; Wicker *et al.*, 2013). The unique architecture of the *MAT1* locus in powdery mildews could be related to the high levels of transposable elements found in these genomes. For instance, in *B. graminis* f. sp. *tritici*, over 90% of the genome was classified as transposable elements (Wicker *et al.*, 2013). In *B. graminis* f. sp. *hordei*, 64% of the genome is composed of transposable elements (Spanu *et al.*, 2010).

The molecular characterization of mating type idiomorphs conducted in this research extends knowledge and genetic resources available for the genus *Podosphaera* and potentially other genera of Erysiphaceae. With the confirmation that this population is presently surviving the intercrop period asexually, this system could be a model for population genetic studies of asexually reproducing populations cycled through austere annual bottleneck events. In addition, the research presented here has provided information that has resulted in changes in quarantine regulations by the state departments of agriculture in Washington, Oregon and Idaho to reduce the likelihood of the introduction of the second mating type of *P. macularis*. It is thought that the presence of the second mating type and the ascigerious stage would substantially increase seasonal carryover of inoculum and decrease the effectiveness of current management strategies for powdery mildew (Gent *et al.*, 2012). Long term, the presence of the ascigerious stage would undoubtedly change the diversity of the population of *P. macularis* found in the Pacific Northwest.

Acknowledgements

Financial support was provided by USDA-ARS CRIS 5358-21000-046-00D, the Hop Research Council, Washington State Commission on Pesticide Registrations, and the Oregon Hop Commission. The authors thank Jason Scott and Lindsey Thiessen for their suggestions and comments that improved this paper. They also thank Marin Brewer for assistance in trouble-shooting PCR assays, Nanci Adair for her excellent technical support of this project, and Susan Haig for the construction of the Illumina library. The documentation of chasmothecia ontogeny was possible thanks to Mary-Jean Welser. The authors are also thankful to the many people who provided powdery mildew samples and DNA for this project: Stephen Massie, Josef Patzak, Sebastjan Radisek, Charlie Rowher and Elisabeth Seigner.

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