Population Diversity and Structure of *Podosphaera macularis* in the Pacific Northwestern United States and Other Populations

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ABSTRACT

Powdery mildew, caused by *Podosphaera macularis*, is one of the most important diseases of hop. The disease was first reported in the Pacific Northwestern United States, the primary hop-growing region in this country, in the mid-1990s. More recently, the disease has reemerged in newly planted hopyards of the eastern United States, as hop production has expanded to meet demands of local craft brewers. The spread of strains virulent on previously resistant cultivars, the paucity of available fungicides, and the potential introduction of the *MAT1-2* mating type to the western United States, all threaten sustainability of hop production. We sequenced the transcriptome of 104 isolates of *P. macularis* collected throughout the western United States, eastern United States, and Europe to quantify genetic diversity of pathogen populations and elucidate the possible origins of pathogen populations in the western United States. Discriminant analysis of principal components grouped isolates within three to five geographic populations, dependent on stringency of grouping criteria. Isolates from the western United States were phenotyped and categorized into one of three pathogenic races based on disease symptoms generated on differential cultivars. Western U.S. populations were clonal, irrespective of pathogenic race, and grouped with isolates originating from Europe. Isolates originating from wild hop plants in the eastern

United States were genetically differentiated from all other populations, whereas isolates from cultivated hop plants in the eastern United States mostly grouped with isolates originating from the west, consistent with origins from nursery sources. Mating types of isolates originating from cultivated western and eastern U.S. hop plants were entirely *MAT1-1*. In contrast, a 1:1 ratio of *MAT1-1* and *MAT1-2* was observed with isolates sampled from wild plants or Europe. Within the western United States a set of highly differentiated loci were identified in *P. macularis* isolates associated with virulence to the powdery mildew R-gene R6. The weight of genetic and phenotypic evidence suggests a European origin of the *P. macularis* populations in the western United States, followed by spread of the pathogen from the western United States to re-emergent production regions in the eastern United States. Furthermore, R6 compatibility appears to have been selected from an extant isolate within the western United States. Greater emphasis on sanitation measures during propagation and quarantine policies should be considered to limit further spread of novel genotypes of the pathogen, both between and within production areas.

Keywords: ecology and epidemiology, population biology

Population expansion into new areas occurs with many species and can have important ecological and economic consequences. Many agricultural pathogens are nonnative in areas where the organisms cause substantial crop damage (Pimentel et al. 2001). Understanding genetic structure of pathogen populations is important because of the impact population genetic diversity and structure can have on quarantine policies, breeding strategies, and other management considerations (Grünwald et al. 2016).

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Powdery mildew fungi are among the most devastating pathogens in modern agricultural crops (Dean et al. 2012). Population genetic studies of these organisms are limited due to the complexity of the genomes and the biotrophic nature of the organisms (Spanu et al. 2010; Wicker et al. 2013). Of the few powdery mildew fungi that have been studied, the genomes are estimated to be relatively large (120 to 180 Mb) but composed of a relatively small number of genes (e.g., 5,854 in *Blumeria graminis*) compared with other Ascomycetes (Spanu et al. 2010; Wicker et al. 2013). Population genetic studies can be further complicated because over 90% of the genome of some powdery mildew fungi are comprised of transposable and repetitive elements (Wicker et al. 2013). A variety of genotyping methods have been utilized in genetic studies for powdery mildews (Délye et al. 1997; Evans et al. 1997; Miazzi et al. 2003; Núñez et al. 2006; Pironi et al. 2015; Stummer et al. 2000). Given the complexity of the genomes of powdery mildew fungi and challenges associated with DNA extraction, identification of polymorphisms in single nucleotides or simple sequence repeats in the transcriptome tend to be the approaches used for characterizing genetic diversity in these organisms (Brewer and Milgroom 2010; Inuma et al. 2007; Komínková et al. 2016; Pedersen et al. 2002; Tollenaere et al. 2012; Wicker et al. 2013).

Hop powdery mildew (caused by *Podosphaera macularis*) is one of the oldest fungal diseases known on cultivated hop (*Humulus lupulus*), dating back to reports in England from the 1700s (Neve

1991; Royle 1978). *P. macularis* can now be found in most hop growing regions in the northern hemisphere (Mahaffee et al. 2009). In the United States, over 98% of commercial hop production occurs in the Pacific Northwestern states of Washington, Idaho, and Oregon (Barth et al. 1994). Powdery mildew has been recorded in eastern North America since at least the early 19th century (Blogett 1913). The fungus only became established in the Pacific Northwest in the mid-1990s (Ocamb et al. 1999) and powdery mildew becomes epidemic annually in this region. About 2% of the U.S. hop crop is grown in states outside of the Pacific Northwest, primarily in the Midwest and Northeast, mostly for a local but expanding brewing industry. Outside of the western United States, powdery mildew can be found on wild and feral hop plants (Claassen et al. 2017; Wolfenbarger et al. 2015), but at present the disease is observed only occasionally on cultivated hop plants (Allan-Perkins et al. 2019; Gent et al. 2015; Marks et al. 2018; Wolfenbarger et al. 2016b).

The origin of *P. macularis* introduced into the Pacific Northwestern United States is speculative, but the population is believed to be the result of a single introduction event because only the *MAT1-1* idiomorph of *P. macularis* is known to occur in this region (Wolfenbarger et al. 2015). In contrast, both mating types are broadly distributed in Europe and on wild and feral plants in eastern North America (Wolfenbarger et al. 2015), and the ascigerous stage frequently occurs in these populations (Blogett 1913; Claassen et al. 2017; Neve 1991; Royle 1978). Without coexisting compatible mating types, *P. macularis* can only perennate in association with living host tissue, most importantly crown buds that lead to the so-called “flag shoots” in the following season (Gent et al. 2008, 2018, 2019). Flag shoots are rare events and occur on less than 0.57% of plants in Washington and 0.05% of plants in Oregon (Gent et al. 2008, 2018, 2019).

Little is known about the population diversity of *P. macularis* in the Pacific Northwest or elsewhere. In general, newly introduced pathogen populations are typically less genetically diverse than the source pathogen populations because of smaller founder population sizes, resulting in a genetic bottleneck (Nei et al. 1975; Goodwin et al. 1994). A founder effect may persist in the newly introduced population from the source population, especially in clonal organisms as exemplified in several cases (Brewer and Milgroom 2010; Drenth et al. 1993; Goss et al. 2009; Steele et al. 2001; Wellings and McIntosh 1990). However, this pattern does not hold true in cases where multiple divergent lineages from separate source populations inhabit a new area (Dutech et al. 2010; Petit et al. 2003). In the case of *P. macularis* in the Pacific Northwest, the low frequency of overwintering in infected buds presents a potentially severe bottleneck to overwintering populations. This recurrent bottleneck event reduces census size of the population and could reduce the effective population size, or alternatively lead to intra-annual variation in population structure because of annual founder effects and random genetic drift.

Pathogenic diversity of *P. macularis* is well characterized in certain populations (Gent et al. 2017; Seigner et al. 2001; Wolfenbarger et al. 2016a). Isolates often vary in their virulence on a set of differential cultivars possessing seven major known R-genes, wild hop plants with uncharacterized resistance (Seigner et al. 2006), and even cultivars with partial resistance (Gent et al. 2017). In the primary hop production regions in the United States, the population consists predominately of three pathogenic races. These races are (i) isolates known to occur before 2012 with race Vb,V3,V5; (ii) isolates that have become epidemic since 2012 that are virulent on plants that possess R6 (race Vb,V3,V4,V5,V6); and (iii) those adapted to the partial resistance in cultivar Cascade (race Vb,V3,V5; dubbed Cascade-adapted) (Wolfenbarger et al. 2016a; Gent et al. 2017).

Important questions about the population genetic diversity and structure are unresolved in *P. macularis*. It is unknown if the population in the Pacific Northwestern United States is structured

by state or pathogenic race, if virulent races were selected from extant isolates or introduced, how the population in the United States is related to other populations in the world, and what is the origin of *P. macularis* that is now endemic in the Pacific Northwest. These questions motivated two overarching objectives of this study. We sought to determine if the population of the fungus is structured among different geographic regions and pathogen races. Secondly, we sought to characterize the relatedness of the populations in the Pacific Northwest to those in eastern North America and Europe to infer the possible origin of *P. macularis* in the primary hop production regions of the United States.

MATERIALS AND METHODS

Plant materials. Hop plants were propagated from softwood cuttings and grown in a greenhouse free of powdery mildew. The greenhouse was maintained at 20 to 25°C with a 14-h photoperiod. Plants were grown in Sunshine Mix #1 (Sun Gro Horticulture, Hubbard, OR) for approximately 14 to 21 days and were watered daily, receiving Sunshine Technigro 16-17-17 or 20-20-20 Plus fertilizer with micronutrients (Sun Gro Horticulture) at each irrigation.

Isolates of *P. macularis*. Isolates of *P. macularis* were maintained on detached hop leaves of the powdery-mildew-susceptible cultivar Symphony using standard methods for this organism (Wolfenbarger et al. 2016a), and transferred every 2 to 3 weeks. A total of 104 isolates of *P. macularis* were obtained from a variety of populations, as detailed in Table 1. These isolates were derived from the Pacific Northwestern United States (49 isolates), east of the Pacific Northwestern United States (25 isolates), England (13 isolates), and continental Europe (17).

As noted previously, three races of *P. macularis* are known to be prevalent in hopyards in the Pacific Northwest. Efforts were made to collect isolates representing each of these races from both Oregon and Washington. Powdery mildew isolates were confirmed to possess V6-virulence if (i) the isolate was obtained from a cultivar possessing the resistance gene R6 or (ii) isolates were able to grow on the R6 differential cultivar Nugget as described by Wolfenbarger et al. (2016a). Isolates with V6-virulence were assumed to be race Vb,V3,V4,V5,V6 because this virulence is invariably associated with this race in the United States (Wolfenbarger et al. 2016a). Isolates that failed to cause disease on the cultivar Nugget were inoculated to cultivar Cascade to determine Cascade adaptation (Gent et al. 2017). As noted previously, Cascade is only partially resistant to powdery mildew and only Cascade-adapted isolates cause appreciable disease on this cultivar under field conditions in the Pacific Northwestern United States. Determination of Cascade-adaptation by *P. macularis* in a given isolate requires measurement and comparison of aggressiveness on this cultivar in comparison with other isolates with known phenotypes. In this study, Cascade-adaptation was delineated based on a quantitative difference in the number of colonies produced on detached leaves of Cascade compared with a reference isolate that was not aggressive on Cascade (Gent et al. 2017; Wolfenbarger et al. 2016b). We considered isolates that caused greater than 20 colonies per leaf to be Cascade-adapted, which unambiguously dichotomized isolates into the two phenotypes. Isolates from the Pacific Northwest that did not display differential aggressiveness on Cascade and could not infect Nugget were by default categorized as matching the virulence of the pre-2012 race, Vb,V3,V5 (Gent et al. 2017; Wolfenbarger et al. 2016a).

Mating type determination. *P. macularis* is heterothallic and mating type can be assayed indirectly by detection of the *MAT1-1* and *MAT1-2* idiomorphs (Wolfenbarger et al. 2015). When mating type of the isolates was not known from previous studies (Wolfenbarger et al. 2015, 2016a), PCR assays were conducted as described by Wolfenbarger et al. (2015) to determine whether isolates were *MAT1-1* or *MAT1-2*.

RNA extraction. The first node of completely unfurled leaves of the powdery-mildew-susceptible cultivar Symphony were detached and inoculated with conidia with the aid of an insect pinning needle and stereomicroscope. Leaves were incubated at 18°C with a 14-h light regime for 14 to 17 days. Acetate film (35 µm) was cut into approximately 2.5 cm² pieces, similar to methods used by Cadle-Davidson et al. (2009). Depending on the size of the diseased leaf area, multiple pieces of the acetate film were attached to the leaf using HPLC-grade acetone applied with an eye dropper until just before runoff. Once the acetate film was dry, the film was peeled off and placed in a mortar with liquid nitrogen. Six to nine heavily diseased leaves inoculated with the same isolate

were peeled, pooled, and ground in liquid nitrogen using a mortar and pestle. One milliliter of TRIzol was added to the mortar once a powder formed and, when thawed, transferred to a 2-ml microcentrifuge tube. An additional 0.5 ml of TRIzol “wash” was added to the mortar and transferred to the same 2-ml microcentrifuge tube. RNA extraction was completed with an Ambion PureLink RNA mini kit (Thermo Fisher Scientific, Waltham, MA), following the protocol for using the TRIzol reagent with a DNase I treatment after RNA purification.

Reference assembly. Sequencing was performed using paired-end 150 bp Illumina HiSeq 3000 technology at the Oregon State University Center for Genome Research and Biocomputing. These

TABLE 1. Isolates of *Podosphaera macularis* used in studies and associated meta-data

Isolate	Year	Region ^a	Subregion ^b	Source ^c	Race ^d	Mating type ^e	Batch ^f
HPM-198/BU10	1999	Continental Europe	Germany	Commercial yard	–	<i>MAT1-2</i>	1
HPM-199/Hu9	1999	Continental Europe	Germany	Breeding yard	–	<i>MAT1-1</i>	1
HPM-200/E8	2000	England	England	Commercial yard	–	<i>MAT1-2</i>	1,2,3,4,5
HPM-201/Hopfen 1	1999	Continental Europe	Germany	Commercial yard	–	<i>MAT1-1</i>	1
HPM-202/2003/198/021	2008	Continental Europe	Germany	Commercial yard	–	<i>MAT1-2</i>	1
HPM-203/F10	1999	Continental Europe	France	Commercial yard	–	<i>MAT1-1</i>	1
HPM-204/E10	2004	England	England	Commercial yard	–	<i>MAT1-1</i>	1
HPM-205/HA1	1999	Continental Europe	Germany	Commercial yard	–	<i>MAT1-1</i>	1
HPM-303	2012	Eastern U.S.A.	NY	Wild plant	–	<i>MAT1-2</i>	1
HPM-412	2012	Eastern U.S.A.	MD	Wild plant	–	<i>MAT1-2</i>	1
HPM-439	2012	Continental Europe	Slovenia	Hallertau Magnum	–	<i>MAT1-2</i>	1
HPM-527	2013	Eastern U.S.A.	MD	Wild plant	–	<i>MAT1-2</i>	1,2,3,4
HPM-593	2013	PNW	WA	Chinook	Vb,V3,V4,V5,V6	<i>MAT1-1</i>	4
HPM-609	2013	PNW	OR	Nugget	Vb,V3,V4,V5,V6	<i>MAT1-1</i>	1
HPM-643	2013	Continental Europe	Slovenia	Dana	–	<i>MAT1-1</i>	1
HPM-644	2013	Continental Europe	Slovenia	Dana	–	<i>MAT1-2</i>	2
HPM-646	2013	Continental Europe	Slovenia	Celeia	–	<i>MAT1-2</i>	1
HPM-648	2013	Continental Europe	Slovenia	Dana	–	<i>MAT1-2</i>	2
HPM-663	2014	PNW	OR	Hallertau Magnum	Vb,V3,V5	<i>MAT1-1</i>	1,2,3
HPM-693	2014	PNW	WA	Cascade	Cascade-adapted	<i>MAT1-1</i>	1,2,3
HPM-697	2014	PNW	WA	Cascade	Cascade-adapted	<i>MAT1-1</i>	1
HPM-865	2014	Continental Europe	Slovenia	Dana	–	<i>MAT1-2</i>	3
HPM-866	2014	England	England	First Gold	–	<i>MAT1-1</i>	2
HPM-867B	2014	England	England	First Gold	–	<i>MAT1-1</i>	2,3
HPM-868	2014	England	England	First Gold	–	<i>MAT1-2</i>	3
HPM-870	2014	England	England	First Gold	–	<i>MAT1-1</i>	1
HPM-951	2015	Continental Europe	Germany	Wild plant	–	<i>MAT1-1</i>	2
HPM-952	2015	Continental Europe	Slovenia	Bobek	–	<i>MAT1-2</i>	2
HPM-953	2015	Continental Europe	Slovenia	Celeia	–	<i>MAT1-2</i>	3
HPM-956	2016	PNW	OR	Cascade	Cascade-adapted	<i>MAT1-1</i>	2
HPM-967	2016	PNW	OR	Cascade	Cascade-adapted	<i>MAT1-1</i>	2
HPM-974	2016	PNW	OR	Cascade	Cascade-adapted	<i>MAT1-1</i>	2
HPM-990	2016	Eastern U.S.A.	NY	CTZ	–	<i>MAT1-1</i>	2
HPM-1023	2016	Eastern U.S.A.	CO	Chinook	–	<i>MAT1-1</i>	3
HPM-1028	2016	Continental Europe	Slovenia	Celeia	–	<i>MAT1-1</i>	4
HPM-1029	2016	Continental Europe	Slovenia	Celeia	–	<i>MAT1-1/2</i>	2
HPM-1030	2016	PNW	WA	Cascade	Cascade-adapted	<i>MAT1-1</i>	3
HPM-1040	2016	PNW	WA	Cascade	Cascade-adapted	<i>MAT1-1</i>	3
HPM-1050	2016	PNW	OR	Cascade	Cascade-adapted	<i>MAT1-1</i>	2
HPM-1051	2016	PNW	OR	Cascade	Cascade-adapted	<i>MAT1-1</i>	3
HPM-1052	2016	PNW	WA	CTZ	Vb,V3,V5	<i>MAT1-1</i>	2
HPM-1054	2016	PNW	WA	Chinook	Vb,V3,V5	<i>MAT1-1</i>	2
HPM-1055	2016	PNW	WA	Chinook	Vb,V3,V5	<i>MAT1-1</i>	4
HPM-1056	2016	PNW	WA	CTZ	Vb,V3,V5	<i>MAT1-1</i>	2
HPM-1057	2016	PNW	WA	CTZ	Vb,V3,V5	<i>MAT1-1</i>	3
HPM-1058	2016	PNW	WA	Cascade	Cascade-adapted	<i>MAT1-1</i>	3
HPM-1059	2016	PNW	WA	Cascade	Cascade-adapted	<i>MAT1-1</i>	3
HPM-1063	2016	PNW	WA	Cascade	Cascade-adapted	<i>MAT1-1</i>	3

(Continued on next page)

^a Geographic region of origin of the isolate. PNW is Pacific Northwestern United States. Eastern United States refers to any location east of the Pacific Northwest states of Idaho, Oregon, and Washington.

^b Abbreviations are United States Postal Codes.

^c Cultivar from which the isolate was collected is given if known. Otherwise, the source is indicated generally as from a breeding yard, commercial yard, or wild (or feral) plant.

^d Pathogen race was determined for isolates derived from the Pacific Northwestern United States as described in the text.

^e Mating type was obtained from previous studies or determined by PCR using primers specific to each idiomorph (Wolfenbarger et al. 2015).

^f Batch refers to the group (1 to 5) from which RNA was extracted, libraries constructed, and sequenced. Isolates with multiple numbers were considered technical replicates to determine variability in variant calls throughout the genotyping process.

RNAs were extracted independently and included in each sequencing run as technical replicates to understand genotyping errors and other sources of error throughout the process (annotated in Table 1 as batches). We refer to these samples as technical replicates. A de novo transcriptome was assembled using RNA sequence data from isolate HPM-663. We selected this isolate as a reference because it represented the pre-2012 race of the fungus and was sequenced deeply in one of the batches. Reads from this isolate were mapped to the hop reference genome based on cultivar Shinsu Wase (Natsume et al. 2015; accessed from <http://hopbase.cgrb.oregonstate.edu>) using bwa 0.7.10 (Li 2013; Li and Durbin 2009, 2010) to reduce hop-related transcripts that may have been sequenced. Reads that did not map to the hop genome were assembled using SPAdes 3.8.0 (Bankevich et al. 2012). The

resulting assembly was filtered to only contain contigs that were at least 1 kbp in length.

Population level variant calling. Reads were mapped to the reference using bwa 0.7.10 as described above. Duplicate reads were marked using Picard tools 2.5.0 (<https://broadinstitute.github.io/picard/>). Mate pair information, MD tags, and sorting were performed using SAMtools 1.3.1 (Li et al. 2009). Variants, both single nucleotide polymorphisms (SNPs) and short indels as described in Danecek et al. (2011), were called from the resulting bam files using the GATK 3.5 by first creating genomic variant call format (gVCF) files and calling variants over all gVCF files with the HaplotypeCaller (DePristo et al. 2011; McKenna et al. 2010; Van der Auwera et al. 2013). The variants that resulted from these steps we refer to as raw variants.

TABLE 1. (Continued from previous page)

Isolate	Year	Region ^a	Subregion ^b	Source ^c	Race ^d	Mating type ^e	Batch ^f
HPM-1064	2016	PNW	WA	Cluster	Vb,V3,V5	<i>MAT1-1</i>	5
HPM-1065	2016	PNW	WA	Cluster	Vb,V3,V4,V5,V6	<i>MAT1-1</i>	3
HPM-1066	2016	PNW	WA	Simcoe	Vb,V3,V5	<i>MAT1-1</i>	3
HPM-1068	2016	PNW	WA	Nugget	Vb,V3,V4,V5,V6	<i>MAT1-1</i>	2
HPM-1070	2016	PNW	WA	Apollo	Vb,V3,V4,V5,V6	<i>MAT1-1</i>	2
HPM-1071	2016	PNW	WA	Apollo	Vb,V3,V4,V5,V6	<i>MAT1-1</i>	3
HPM-1072	2016	PNW	WA	Bravo	Vb,V3,V4,V5,V6	<i>MAT1-1</i>	2
HPM-1073	2016	PNW	WA	Bravo	Vb,V3,V4,V5,V6	<i>MAT1-1</i>	3
HPM-1074	2016	PNW	WA	Bravo	Vb,V3,V4,V5,V6	<i>MAT1-1</i>	2
HPM-1075	2016	PNW	WA	Bravo	Vb,V3,V4,V5,V6	<i>MAT1-1</i>	4
HPM-1076	2016	PNW	WA	Nugget	Vb,V3,V4,V5,V6	<i>MAT1-1</i>	2
HPM-1077	2016	PNW	WA	Nugget	Vb,V3,V4,V5,V6	<i>MAT1-1</i>	3
HPM-1078	2016	PNW	OR	Cascade	Cascade-adapted	<i>MAT1-1</i>	2
HPM-1080	2016	PNW	OR	Cascade	Cascade-adapted	<i>MAT1-1</i>	2
HPM-1081	2016	PNW	OR	Cascade	Cascade-adapted	<i>MAT1-1</i>	3
HPM-1082	2016	PNW	OR	Cascade	Cascade-adapted	<i>MAT1-1</i>	2
HPM-1083	2016	PNW	OR	Cascade	Cascade-adapted	<i>MAT1-1</i>	2
HPM-1084	2016	PNW	OR	Cascade	Cascade-adapted	<i>MAT1-1</i>	4
HPM-1085	2016	Eastern U.S.A.	CO	Chinook	–	<i>MAT1-1</i>	2
HPM-1087	2016	Eastern U.S.A.	CO	Cascade	–	<i>MAT1-1</i>	3
HPM-1088	2016	Eastern U.S.A.	CO	Cascade	–	<i>MAT1-1</i>	4
HPM-1089	2016	Eastern U.S.A.	WI	Wye Challenger	–	<i>MAT1-1</i>	2
HPM-1094	2016	PNW	OR	Willamette	Vb,V3,V5	<i>MAT1-1</i>	2
HPM-1096	2016	Eastern U.S.A.	NY	Commercial yard	–	<i>MAT1-1</i>	2
HPM-1098	2016	Eastern U.S.A.	NY	CTZ	–	<i>MAT1-1</i>	3
HPM-1099	2016	Eastern U.S.A.	NY	CTZ	–	<i>MAT1-1</i>	2
HPM-1100	2016	Eastern U.S.A.	MN	Wild plant	–	<i>MAT1-1</i>	2
HPM-1101	2016	Eastern U.S.A.	MN	Wild plant	–	<i>MAT1-1</i>	2
HPM-1103	2016	Eastern U.S.A.	MN	Wild plant	–	<i>MAT1-1</i>	4
HPM-1104	2016	Eastern U.S.A.	MN	Wild plant	–	<i>MAT1-1</i>	4
HPM-1107	2016	Eastern U.S.A.	MN	Wild plant	–	<i>MAT1-2</i>	2
HPM-1112	2016	PNW	OR	Apollo	Vb,V3,V4,V5,V6	<i>MAT1-1</i>	4
HPM-1113	2016	PNW	OR	Apollo	Vb,V3,V4,V5,V6	<i>MAT1-1</i>	4
HPM-1117	2016	PNW	OR	Delta	Vb,V3,V4,V5,V6	<i>MAT1-1</i>	2
HPM-1119	2016	PNW	OR	Nugget	Vb,V3,V4,V5,V6	<i>MAT1-1</i>	3
HPM-1120	2016	PNW	OR	Nugget	Vb,V3,V4,V5,V6	<i>MAT1-1</i>	4
HPM-1122	2016	PNW	OR	Nugget	Vb,V3,V4,V5,V6	<i>MAT1-1</i>	3
HPM-1123	2016	PNW	OR	Nugget	Vb,V3,V4,V5,V6	<i>MAT1-1</i>	4
HPM-1124	2016	PNW	WA	Ekuantot	Vb,V3,V5	<i>MAT1-1</i>	4
HPM-1126	2016	PNW	WA	Cascade	Cascade-adapted	<i>MAT1-1</i>	2
HPM-1137	2016	Eastern U.S.A.	MD	Wild plant	–	<i>MAT1-1/2</i>	3
HPM-1146	2016	PNW	OR	Sorachi Ace	Vb,V3,V4,V5,V6	<i>MAT1-1</i>	3
HPM-1151	2016	Eastern U.S.A.	MN	Breeding yard	–	<i>MAT1-1</i>	4
HPM-1152	2016	Eastern U.S.A.	MN	Breeding yard	–	<i>MAT1-1</i>	4
HPM-1154	2016	Eastern U.S.A.	NC	Cashmere	–	<i>MAT1-1</i>	3
HPM-1155	2016	Eastern U.S.A.	NC	Cashmere	–	<i>MAT1-1</i>	2
HPM-1169	2017	Eastern U.S.A.	MI	Cascade	–	<i>MAT1-1</i>	4
HPM-1182	2017	Eastern U.S.A.	WI	Galena	–	<i>MAT1-1</i>	4
HPM-1183	2017	Eastern U.S.A.	WI	Southern Cross	–	<i>MAT1-1</i>	4
HPM-1190	2017	England	England	Wild plant	–	<i>MAT1-2</i>	5
HPM-1193	2017	England	England	Wild plant	–	<i>MAT1-2</i>	5
HPM-1196	2017	England	England	Wild plant	–	<i>MAT1-2</i>	5
HPM-1198	2017	England	England	Wild plant	–	<i>MAT1-2</i>	5
HPM-1200	2017	England	England	Wild plant	–	<i>MAT1-1/2</i>	5
HPM-1202	2017	England	England	Breeding yard	–	<i>MAT1-1</i>	5
HPM-1203A	2017	England	England	Breeding yard	–	<i>MAT1-1/2</i>	5
<i>Humulus lupulus</i>	2017	–	–	Symphony	–	–	2,3

Quality control and filtering. Extensive quality control and filtering was necessary given that we sequenced the transcriptome of an obligate pathogen, potentially resulting in variable gene expression, contamination with host genes, and presence of PCR and sequencing error. The VCF files (Danecek et al. 2011) resulting from variant calling were read into R (R Core Team 2017) and further processed using *vcfR* (Knaus and Grünwald 2017) for quality assurance and control (Grünwald et al. 2017). Violin plots (Wickham 2016) were used to explore the distribution of allele depth (AD), depth (DP), genotype quality (GQ), and phred-scaled likelihood for isolates using the raw VCF data. Genotypes with a genotype quality less than 99 were marked as missing data. Genotypes which were sequenced at a depth of less than the 10th percentile or greater than the 90th percentile, for each isolate, were also marked as missing data. Genotypes sequenced at a depth below 4× were marked as missing data. Samples with less than 50% missing data were then retained, and variants with less than or equal to 5% missing data were retained. In order to avoid spurious allele calls that may have been the result of genotyping error, only variants where the second most abundant allele over all samples appeared at least four times were retained. In an attempt to avoid batch effects that may have occurred among the five lanes of Illumina sequencing performed during the project, the samples were grouped by lane, G'_{ST} (Hedrick 2005) was calculated, and only variants that had a G'_{ST} less than 0.2 were retained. Technical replicates were examined visually using histograms of pairwise differences among each replicate to ensure that run-to-run variation between the same isolates was less than the overall variation in the quality-filtered variants. The variants that resulted from these steps we refer to as the production data set.

Global structure of hop powdery mildew populations. In order to explore the global population structure of *P. macularis*, we used discriminant analysis of principal components (DAPC) (Jombart et al. 2010). The production VCF data were read into R and converted to a *genlight* object using *vcfR* (Knaus and Grünwald 2017). Principal components analysis was performed on the data using *ade4::dudi.pca()* (Dray et al. 2007). The first 40 axes described 88.5% of the variation in the data set and were used for subsequent analysis. The function *adegenet::find.clusters()* was used to group the isolates into groups, ranging from 1 to 10 groups (K). In order to explore variation within each value of K , the process was repeated 10 times for each value of K . The value of K that yielded the most parsimonious fit to the data was determined by minimizing the value of the Bayesian information criterion (BIC). Based on BIC, DAPC was performed for $K = 2, 3, 4$, and 5 using 40 principal components and three discriminant axes. Results were plotted using *ggplot2* (Wickham 2016).

Differentiation of populations based on group numbers of $K = 3, 4$, and 5 were confirmed by conducting analysis of molecular variance (AMOVA) (Excoffier et al. 1992) separately for each value of K . From the VCF data, a matrix of pairwise Hamming distances was created using the function *poppr::bitwise.dist()* (Kamvar et al. 2015), and AMOVA was performed using *pegas::amova()* (Paradis 2010). A total of 1,000 permutations were performed to establish significance for each AMOVA.

Loci discriminating Pacific Northwest races. Analyses were conducted to identify variant loci that were highly differentiated between the three races of the fungus present in the Pacific Northwestern United States. To do this, we examined the data from isolates derived from the Pacific Northwest. Due to the low level of genetic polymorphism observed among the races of the fungus in this region and a desire to find variants that segregated among the races, we returned to the raw variants to retain the maximum possible number of variants relevant to this population. There were 46 isolates retained, all from the Pacific Northwestern United States, after quality filtering as described above. A histogram was constructed for G'_{ST} (Hedrick 2005)

values for all variants for these isolates. Based on this distribution, we examined loci that had G'_{ST} values of >0.88 , which was near the extremes of the right-tail of the histogram. This threshold was selected arbitrarily simply to subset the data to a manageable number of loci at the extreme of the distribution that could be examined in depth. The resulting 16 variant loci that were the most highly differentiated were visualized in matrix plots of each isolate by locus.

This process was then repeated omitting isolates with V6-virulence to identify variants that differentiated the pre-2012 isolates (race Vb,V3,V5) from Cascade-adapted isolates. The reduced data set contained 28 isolates and 19 loci that exceeded the G'_{ST} threshold we imposed.

As described below, there was clear differentiation of most isolates with V6-virulence from the pre-2012 and Cascade-adapted isolates. To understand the genes potentially associated with the differentiation of the races, we conducted a BLASTX (Altschul et al. 1990) search with an e-value threshold of 0.01 in the National Center for Biotechnology Information database (nonredundant) utilizing the entire contig on which each of the 16 variant loci were identified. The taxids from the results were used in the R package *taxize* (Chamberlain and Szocs 2013) to look up the superkingdom and family for each BLASTX result. Data from the search was summarized in word clouds by family and functional annotation in GenBank. A family or functional annotation had to occur five or more times to be included in a word cloud. Word clouds were produced using the R package *ggwordcloud* (Le Pennec and Slowikowski 2019).

RESULTS

Reference assembly. The assembly using isolate HPM-663 as a reference resulted in 5,269 transcripts containing 8.49 million base pairs with an N50 of 1,626. These transcripts were slightly enriched for A/T with most transcripts being about 60% A/T. No International Union of Pure and Applied Chemistry (IUPAC) polymorphic loci or missing data were present in the final reference assembly (Supplementary Fig. S1).

Population level variant calling. Sequencing resulted in 122 isolates, including hop and repeated sequencing of the technical replicates, being genotyped and identification of 166,601 variants in the raw variant set. After quality control, 114 unique isolates of *P. macularis* (including all but one technical replicate as described below) were retained and included 243 variants for the production data set.

Quality control and filtering. Technical replicates were similar to one another but typically (and expectedly) not identical (Fig. 1A). The number of pairwise genetic differences observed among technical replicates was approximately 10 differences at the greatest density of the distribution. In contrast, the number of differences in the rest of the data set was bimodal at densities of approximately 12.5 and 35 (Fig. 1B). One technical replicate of isolate HPM-200 had a substantial number of differences (about 40) between the genotype calls in one sequencing batch and the four other replicates of this isolate in other sequencing batches. The data for the suspect technical replicate of isolate HPM-200 was determined to be of low quality and was omitted from further analyses. In contrast, most technical replicates contained around four differences out of the 243 variants, for an error rate of around 1.6%. Therefore, the genetic differences between isolates far exceeded those associated with technical error and thus represent real, biological differences.

Global population structure. K -means clustering demonstrated little if any improvement in BIC when samples were divided into more than three groups (Fig. 2A). A scatter plot of the discriminant functions indicated that even if assigning isolates to five groups, three of these groups cluster together resulting in only three well distinguished clusters (Fig. 2B). Barplots of

posterior probabilities of group membership indicated that the European and eastern U.S. population typically included the highest number of groups (Fig. 2C). At $K = 4$, continental Europe and England shared a group that was not observed elsewhere. At $K = 5$, England had a low frequency group that was not observed elsewhere. At $K = 5$, the eastern United States had a low frequency group that was not observed elsewhere. Isolates from the Pacific Northwest were all assigned to one group independent of the value of K , indicating clonality, and this group was shared among all populations. Analysis of molecular variance confirmed differentiation of the populations based on discriminant analysis of principal components, with P values = 0 in all analyses due to complete separation of isolates with either $K = 3, 4$, or 5 groups (data not presented).

In the eastern United States, membership of the isolates into genetic groups varied depending on the number of groups defined and also whether isolates originated from wild or cultivated hop plants (Fig. 3). The population originating from wild plants was unique from all other populations, independent of the number of groups defined. In contrast, on cultivated plants (at all values of K) 11 of the 15 isolates (73.3%) belonged to the genetic group that contained all isolates from the Pacific Northwestern United States. When assigned to five groups, three isolates from wild and two isolates from cultivated plants were atypical. These five isolates belonged to a lower frequency genetic group not found elsewhere. These isolates were obtained from an experimental breeding yard in Minnesota, a commercial yard in North Carolina, and wild plants in Maryland and New York (Table 1).

Mating type assays indicated further similarity between isolates originating from the Pacific Northwest and those found on cultivated plants in the eastern United States. All isolates from the western United States were mating type *MAT1-1*, as expected (Table 1). However, all 15 isolates from cultivated plants in the eastern United States also were identified as *MAT1-1* (Table 1). Although only nine isolates were assayed from wild plants, both mating type idiomorphs were detected at similar frequencies in isolates derived (four isolates with *MAT1-1*, four isolates with *MAT1-2*, and both *MAT1-1* and *MAT1-2* in one isolate). The frequency of mating types in populations from Europe also was approximately a 1:1 ratio of idiomorphs (13 isolates with *MAT1-1*,

14 isolates with *MAT1-2*, and three genotyped as both mating types) (Table 1).

Loci discriminating Pacific Northwest races. Histograms of G'_{ST} supported low levels of genetic differentiation among most isolates given the right-skewed distribution (Fig. 4). Despite this low level of differentiation, variants that differentiated the races were observed. A total of 16 loci were identified above the threshold G'_{ST} value in the data with all three races and 19 in the data omitting V6-virulent isolates. For the former group, matrix plots of allelic state indicated that these variants generally distinguished the V6-virulent isolates from the other two races, that these variants were nearly but not entirely diagnostic for pathogen race, and that there was a low degree of missingness among variant loci (Fig. 5). These variants were mostly SNPs, but one was a small indel (Table 2).

After omitting the V6-virulent isolates and repeating this process, the highly differentiated loci were visualized for the Cascade-adapted and the pre-2012 isolates. A matrix of allelic state by isolate for variant loci indicated much less consistent discrimination of the races as compared with the V6-virulent isolates and that these variants included a relatively high degree of missing genotypes (Fig. 6).

The BLASTX search of the variants associated with V6-virulence identified diverse genera and functional annotations of the 10 contigs on which the 16 variants were located (Fig. 7). The closest matches in GenBank were most often associated with fungi, specifically those in the phylum Ascomycota. However, certain plants were represented, including potato, pepper, and soybean, among others. According to the functional annotations in GenBank, the contigs harboring the variants were predominantly associated with RNA-dependent RNA polymerases, conserved hypothetical proteins, viral coat proteins, mitochondrial import proteins, and retrovirus-related Pol (reverse transcription) proteins.

DISCUSSION

We have established that the population of *P. macularis* in the western United States, the primary region of hop production in the country, is genetically clonal and highly similar to populations of the pathogen found in England and continental Europe. However, the population is differentiated from that found on wild and feral hop plants in eastern North America. Given the clonal structure of

Fig. 1. Genetic diversity among five isolates that served as technical replicates (A) was less than the genetic diversity in the remainder of the data (B). The mean of the distribution of the number of pairwise genetic differences observed among technical replicates was approximately 10 differences while the mean number of differences in the rest of the data set was bimodal at approximately 12.5 and 35. The four values in the technical replicates above 40 were due to differences in one technical replicate of isolate HPM-200 and four other technical replicates of this isolate. This was used to justify the removal of this isolate from other analyses.

the pathogen population in the United States it is not possible to assign probabilistic functions to the likelihood that *P. macularis* in the western United States originated in Europe. Nonetheless, the genetic structure of the population is consistent with a European origin of the western U.S. population (Fig. 2). Dissemination of *P. macularis* from Europe to the western United States in association with plant material seems plausible given the geographic isolation of the production regions.

The genetic and phenotypic relatedness of the population in the western United States and the population found on cultivated plants in eastern North America also indicates that *P. macularis* may have spread from the western United States to re-emerging production

regions in the eastern United States. With few exceptions, isolates from both populations belonged to the same genetic group and invariably were the *MAT1-1* idiomorph. As we discussed previously, *P. macularis* is broadly distributed on wild and feral hop plants in the Midwestern United States and eastern North America (Blogett 1913; Wolfenbarger et al. 2015). The genetic groups found on these plants were distinct from the populations found on cultivated plants, with the exception of isolates originating from research plots in Minnesota. Although the sample size from wild or feral plants was small, mating type idiomorphs originating from these plants were approximately a 1:1 ratio. This is consistent with the findings of Wolfenbarger et al. (2015), where 56 samples of

Fig. 2. Discriminant analysis of principal components indicating that isolates of *Podosphaera macularis* could be arranged parsimoniously into three groups. **A**, *K*-means clustering (10 replicates for each value of *K*) indicating that the data could be explained by three groups (*K* = 3) because of no appreciable decline in Bayesian information criterion (BIC) occurred when the number of groups was increased. **B**, Scatterplot of linear discriminants (LD) indicating that even when five groups were defined groups 1, 2, and 4 clustered closely, indicating that they are poorly differentiated. **C**, Barplots of the posterior probability of group membership for each individual based on *K* = 2 to 5. Note that at *K* = 2, populations of the fungus are differentiated only within and between the eastern United States. At *K* = 3 a group unique to Europe appeared. Larger values of *K* generate new groups within the geographic regions but with small numbers of isolates within some of these groups. Distribution of the genetic groups on wild versus cultivated plants in the Eastern United States is shown in detail in Figure 3.

P. macularis were examined from noncultivated hop plants. Therefore, the dominant population that occurs on wild or feral plants are distinct from the population on cultivated plants. The near absence of commercial hop production in eastern North America

prior to the emergence of powdery mildew in Washington State in 1996 (Mahaffee et al. 2003; Ocamb et al. 1999) would preclude the possibility that the powdery mildew fungus was introduced from commercial hopyards in eastern North America to the western

Fig. 3. Posterior probability of group membership with varying groups (K) for isolates of *Podosphaera macularis* originating from cultivated hop yards or wild plants in the eastern United States. Color scheme of the groups is identical to those in Figure 2.

Fig. 4. Histograms summarizing G'_{ST} measures of differentiation for each variant in the transcriptome of isolates of *Podosphaera macularis* from the Pacific Northwestern United States. Data for all three extant races of *P. macularis* are presented in **A**, (dubbed V6-virulent, pre-2012, and Cascade-adapted); **B**, only data from pre-2012 and Cascade-adapted isolates are shown. Although most variants were undifferentiated (values near 0), the red line at 0.88 indicates the threshold value of G'_{ST} used to identify variant loci discriminating the races.

United States. The most plausible explanation for the intra-continental spread of *P. macularis* in the United States is in association with planting material harboring inoculum of the pathogen originally from the western United States as new yards were established in other areas of the United States.

We did not find evidence of pronounced genetic differentiation among the three pathogenic races of *P. macularis* that occur in the western United States. This supports the hypothesis that these races were selected from an extant isolate in the western United States through a selective sweep, coincident with shifts in cultivars with different sources of resistance to powdery mildew (Gent et al. 2017; Wolfenbarger et al. 2016a). We were, however, able to identify variants associated with V6-virulence in isolates causing epidemics in the western United States since 2012. The subset of loci discriminating among the three pathogenic races in the western United States examined were nearly monomorphic for most isolates with V6-virulence. However, three isolates phenotyped as having

V6-virulence appeared to have the wild-type alleles at these loci (Fig. 5). This may be due to independent evolution of V6-virulence in multiple lineages of *P. macularis* in the western United States or, quite possibly, contamination during phenotyping. Due to the enormous task of culturing and maintaining isolates of obligate parasites, we were not able to maintain all isolates used in this study indefinitely and ceased maintenance of individual isolates after phenotypic and genotypic information was obtained. Thus, it is not possible to confirm the phenotype of the three atypical isolates. Going forward, we will continue correlating the genetic loci discriminating races with the races observed.

The variants associated with V6-virulence are not necessarily causal mutations that result in the pathogen phenotype. More likely, these variants may be chance associations or hitchhiking alleles following a selective sweep of the causal mutation(s) that conferred V6-virulence (Hartl and Clark 2007). Contigs possessing the variants were most often associated with genes annotated as

Fig. 5. Matrix plot of allelic state at various loci (rows) for isolates (columns) of *Podosphaera macularis* originating from the Pacific Northwestern United States. The pathogenic race of the isolates was determined and classified as virulent to cultivars possessing the resistance gene R6 (noted as V6), adapted to the resistance in the cultivar Cascade (noted as Cascade), or neither (noted as pre-2012). Note specifically that most, but not all, isolates possessing V6-virulence were of an allelic state that was different from the allelic state observed in the other two races. In the legend, 0 indicates the wild type allele, 1 indicates a variant allele, and NA indicates missing data.

TABLE 2. Position and allelic state of transcriptome variants that differentiate isolates of *Podosphaera macularis* from the Pacific Northwestern United States that possess virulence on the hop resistance gene R6 versus isolates that do not

Variant	Node	Position	Reference ^a	Alternative
1	NODE_1456_length_1804_cov_707.907	357	C	T
2	NODE_1456_length_1804_cov_707.907	1422	T	C
3	NODE_2251_length_1520_cov_50.9591	942	G	A
4	NODE_2407_length_1484_cov_34.2402	458	A	T
5	NODE_2816_length_1390_cov_164.476	718	C	T
6	NODE_3456_length_1258_cov_137.793	352	C	T
7	NODE_4382_length_1116_cov_19.3744	931	C	T
8	NODE_4483_length_1102_cov_179.143	791	G	A
9	NODE_4483_length_1102_cov_179.143	826	A	T
10	NODE_4483_length_1102_cov_179.143	884	C	A,G
11	NODE_4483_length_1102_cov_179.143	886	TCA	GCA,T
12	NODE_4483_length_1102_cov_179.143	901	G	A
13	NODE_4752_length_1066_cov_115.835	626	G	A
14	NODE_5050_length_1023_cov_1249.36	268	A	G
15	NODE_5050_length_1023_cov_1249.36	637	T	A
16	NODE_674_length_2273_cov_146.996	1408	T	C

^a The reference transcriptome was assembled de novo from isolate HPM-663. This isolate possess neither V6-virulence nor adaptation to the cultivar Cascade. Therefore, the reference allele corresponds to the pre-2012 race and the alternative allele is in isolates called after 2012 that possess V6-virulence.

viral-related sequences and retrotransposons such as RNA-dependent RNA polymerases, conserved hypothetical proteins, viral coat proteins, and retrovirus-related Pol (reverse transcription) proteins (Fig. 7). We did not attempt to validate the annotations in the NCBI database, and some of the annotations undoubtedly may be incorrect. The genomes of powdery mildew fungi are exceptionally complex, typically with over 60% of the genome classified as transposable elements (Jones et al. 2014; Spanu et al. 2010; Wicker et al. 2013). Transposable elements expand genome plasticity and virulence (Faino et al. 2016). In *Blumeria graminis*, candidate effector genes occur in genomic regions enriched in transposable elements (Menardo et al. 2017). Perhaps the causal mutation (or mutations) for V6-virulence in *P. macularis* is similarly located within a region enriched in transposable elements.

Independent of the mechanism conferring V6-virulence, the variants identified could be suitable targets for developing diagnostic assays for rapidly determining pathogen phenotype in the western U.S. population. Such assays based on SNPs have, for instance, been developed to determining lineages of *Phytophthora infestans* (Hansen et al. 2016). Similar assays are in development for *P. macularis* (Block et al. 2019), which could be useful for several purposes including tracking spatial and temporal spread of V6-virulent *P. macularis* from the western United States to other regions (Del Castillo Munera et al. 2016).

Given the evidence presented here for inter- and intracontinental spread of *P. macularis*, greater emphasis on sanitation measures during propagation and more rigorous quarantine policies should be considered to limit further spread of novel genotypes of the

Fig. 6. Matrix plot of allelic state at various loci (rows) for isolates (columns) of *Podosphaera macularis* from the Pacific Northwestern United States identified as having a specific adaptation to cultivar Cascade (noted as Cascade) or not (noted as pre-2012). In the legend, 0 indicates the wild type allele, 1 is a variant allele, 2 is a second alternate allele, and NA is missing data.

Fig. 7. Word clouds illustrating BLASTX matches for contigs possessing variants that were highly differentiated between isolates from the Pacific Northwestern United States that possessed V6-virulence versus those that did not. **A**, Taxonomic family of the matching organism and **B**, the functional annotation reported in the National Center for Biotechnology Information (NCBI) GenBank database are depicted. **A**, NCBI superkingdoms are indicated by color.

pathogen on hop. The most recent discovery of *P. macularis* isolates pathogenic to both hop and hemp (Weldon et al. 2020) would indicate a presently unknown potential for distribution of isolates of *P. macularis* pathogenic to hop on *Cannabis sativa*. Quarantine measures are in place in the Pacific Northwestern states to prevent or delay the introduction of the second mating type of *P. macularis* into the region (Wolfenbarger et al. 2015). However, educational and research efforts are needed in other areas of the United States, and other regions of the world, where hop and *Cannabis* plants are propagated and shipped to minimize spread of disease to new plantings and production areas.

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