Newly Emerged Populations of *Plasmopara halstedii* Infecting Rudbeckia Exhibit Unique Genotypic Profiles and Are Distinct from Sunflower-Infecting Strains

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Accepted for publication 12 March 2016.

ABSTRACT

Rivera, Y., Salgado-Salazar, C., Gulya, T. J., and Crouch, J. A. 2016. Newly emerged populations of *Plasmopara halstedii* infecting rudbeckia exhibit unique genotypic profiles and are distinct from sunflower-infecting strains. *Phytopathology* 106:752-761.

The oomycete *Plasmopara halstedii* emerged at the onset of the 21st century as a destructive new pathogen causing downy mildew disease of ornamental *Rudbeckia fulgida* (rudbeckia) in the United States. The pathogen is also a significant global problem of sunflower (*Helianthus annuus*) and is widely regarded as the cause of downy mildew affecting 35 Asteraceae genera. To determine whether rudbeckia and sunflower downy mildew are caused by the same genotypes, population genetic and phylogenetic analyses were performed. A draft genome assembly of a *P. halstedii* isolate from sunflower was generated and used to design 15 polymorphic simple sequence repeat (SSR) markers. SSRs and two

sequenced phylogenetic markers measured differentiation between 232 *P. halstedii* samples collected from 1883 to 2014. Samples clustered into two main groups, corresponding to host origin. Sunflower-derived samples separated into eight admixed subclusters, and rudbeckia-derived samples further separated into three subclusters. Pre-epidemic rudbeckia samples clustered separately from modern strains. Despite the observed genetic distinction based on host origin, *P. halstedii* from rudbeckia could infect sunflower, and exhibited the virulence phenotype of race 734. These data indicate that the newly emergent pathogen populations infecting commercial rudbeckia are a different species from sunflower-infecting strains, notwithstanding cross-infectivity, and genetically distinct from pre-epidemic populations infecting native rudbeckia hosts.

Additional keyword: microsatellites.

Downy mildew diseases affecting Asteraceae hosts are found worldwide and are responsible for major economic losses to the oilseed and ornamental industries (Constantinescu and Thines 2010; Farr and Rossman 2015; Gulya et al. 1997). *Plasmopara halstedii* was first described as *Peronospora halstedii* on *Eupatorium purpureum* (sweet-scented Joe Pye weed) and six additional Asteraceae hosts: *Ambrosia artemisiifolia*, *Bidens frondosa*, *Helianthus strumosus*, *Rudbeckia laciniata*, and *Silphium terebinthaceum* (Farlow 1883). Following these early reports, any *Plasmopara* infecting plants in the Asteraceae family was regarded as *P. halstedii*, irrespective of observed morphological variation (Leppik 1966). Today, at least 35 different plant genera within the Asteroideae and Chicorideae subfamilies are reported as hosts to *P. halstedii* (Leppik 1966; Sackston 1981). Support for *P. halstedii* as a morphologically diverse species with a wide host range has come from several studies that show susceptibility of cultivated sunflower (*H. annuus*) to strains originating from other Asteraceae host genera, including *Ambrosia*, *Dimorphotheca*, *Iva*, and *Xanthium* (Gulya 2002; Komjáti et al. 2007; Leppik 1965; Viranyi 1984; Walcz et al. 2000). However, the presumed host range of *P. halstedii sensu stricto* is an ongoing subject of discussion, with many regarding the pathogen as a species

complex rather than a single species (Choi et al. 2009; Göker et al. 2007; Schröder et al. 2011; Thines et al. 2009). Several recent studies have identified discrete *Plasmopara* species on Asteraceae hosts that are genetically and/or morphologically distinct from *P. halstedii*. These include *P. angustiterminalis* on *X. strumarium*, *P. invertifolia* on *Helichrysum bracteatum*, and an unnamed species infecting *A. artemisiifolia* (Choi et al. 2009; Duarte et al. 2014; Komjáti et al. 2007).

Beginning in 2004, the emergence of *P. halstedii* as a destructive new pathogen of the ornamental plant *R. fulgida* (rudbeckia) in the United States brought this organism to the attention of the nursery and landscape industry (Dankers et al. 2004; Hong 2006; Rivera et al. 2014). This low-maintenance perennial plant is highly valued for its extended flowering period, beautiful flowers, and the absence of any significant pest or pathogen problems. Over 3.8 million rudbeckia plants are sold annually in the United States alone, valued at \$13 million (2007 Census of Horticultural Specialties, USDA, National Agricultural Statistics Service, issued December 2010; <http://www.agcensus.usda.gov>). Rudbeckia downy mildew (RDM) disease caused by *P. halstedii* is characterized by dark purple lesions on leaves and profuse white sporangial masses on the abaxial surface (Rivera et al. 2015a). Under conducive environmental conditions, RDM can be so severe that commercial greenhouses and nurseries can lose an entire year's crop (Hong 2006). The emergence of this disease represents a substantial threat to the commercial nursery and landscape industry.

The emergence of *P. halstedii* as a problematic issue for rudbeckia represents just one of several new downy mildew disease outbreaks to affect specialty crops in the United States and worldwide during the past decade (Cohen et al. 2015; Crouch et al.

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*The e-Xtra logo stands for "electronic extra" and indicates that four supplementary figures and four supplementary tables are published online.

<http://dx.doi.org/10.1094/PHYTO-12-15-0335-R>

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2013; Daughtrey et al. 2006; Rivera et al. 2015a; Thines et al. 2009). Destructive new downy mildews currently impede production of mainstay specialty crops such as impatiens, basil, coleus, hyssops, and cucurbits (Crouch et al. 2013; Daughtrey et al. 2006; Rivera et al. 2015b; Rivera et al. 2016; Thines et al. 2009). However, 10 to 15 years previous, damage to these plants due to downy mildews was virtually unknown, or as in the case of cucurbits, well controlled through resistant cultivars (Cohen et al. 2015). Also in common with other emergent downy mildews of specialty crops, the factors underlying the emergence of RDM remain a mystery. As seen in other newly emergent downy mildew pathosystems (e.g., impatiens and cucurbits), there are several pre-epidemic records of *P. halstedii* inhabiting native *Rudbeckia* spp. in the United States, going back to at least 1883 (Farr and Rossman 2015). It is unknown how the current disease outbreaks on *R. fulgida* are related to the earlier reports of *P. halstedii* on wild perennial *Rudbeckia* species. There is no evidence to indicate that the epidemic strains of *P. halstedii* are able to infect any species other than cultivated rudbeckia, and there is some anecdotal information suggesting the contrary. In the nursery industry, at least 25 species of *Rudbeckia* are commonly grown, including wild native species such as *R. subtomentosa* and *R. hirta*. However, to our knowledge, there are no contemporary reports of RDM from any other species of *Rudbeckia* other than *R. fulgida*. Only *R. fulgida* has been affected in nurseries where RDM has been reported, even where other species (e.g., *R. hirta*) are grown in adjacent areas (Hong 2006; Rivera et al. 2015a).

In the present study, we address the question of whether modern RDM epidemics on cultivated rudbeckia are caused by the same *P. halstedii* genotype(s) as those associated with wild, North American native rudbeckia species that were collected prior to the epidemics. At the same time, we set out to determine the relationship between rudbeckia-infecting *P. halstedii* genotypes and those responsible for sunflower downy mildew disease. In both cases, the goal is to determine whether either of these resident *P. halstedii* genotypes could have served as the source population(s) for the recent RDM outbreaks. To address these questions, we employ a combined population genetic and phylogenetic approach to evaluate a worldwide collection of *P. halstedii* infecting species of sunflower and rudbeckia, from both modern and historical sources. Because few molecular resources are available to support these investigations at the population level, we generated a draft genome sequence from *P. halstedii*, and used it as the basis for development of a set of simple sequence repeat (SSR) markers. Together with phylogenetic reconstructions, these analyses allow us to determine the genetic variability of *P. halstedii* from two economically important host plants, and clarify the relationship between pre- and postepidemic pathogen populations on rudbeckia and sunflower.

MATERIALS AND METHODS

***Plasmopara halstedii* samples and DNA extraction.** A collection of 232 *P. halstedii* from nine countries was analyzed (Table 1; Supplementary Table S1). We refer to *P. halstedii* samples collected between 1883 and 1999 as “historical” or pre-epidemic, while those collected between 2000 and 2014 were considered “modern”, or postepidemic. These broad designations are based on the timing of disease outbreaks: (i) the emergence of new virulent *P. halstedii* races that overcame important sources of disease resistance in sunflower starting around 2000 (Gulya et al. 2010; Tourvieille et al. 2000); and (ii) RDM outbreaks on rudbeckia that began in 2004. Historical samples from *H. annuus* were collected between 1984 and 1999. These samples were maintained as sporangial collections stored under liquid nitrogen at the USDA-ARS facility in Fargo, ND as described (Gulya et al. 1993). Historical samples of *P. halstedii* on the native plant species *H. strumosus*, *R. triloba*, *R. laciniata*, and *Rudbeckia* sp. were obtained from the U.S. National Fungus Collection in Beltsville, MD (herbarium BPI). The

BPI samples were originally collected between 1883 and 1936, and were maintained in acid-free paper envelopes at room temperature (24°C) as dried, pressed plant material colonized by *P. halstedii*. Ninety-four modern samples were collected between 2000 and 2014 from *R. fulgida* ‘Goldsturm’ ($n = 59$), *R. fulgida* var. *speciosa* ($n = 1$), and *H. annuus* ($n = 24$) from nurseries and landscapes across the United States and one from France (sample SDM128).

Dried sporangial masses were removed from infected leaf tissue by vacuum or by scraping the leaf surface with a sterile needle. Approximately 100 mg of sporangial tissue was homogenized using 100 mg of 0.5 mm glass beads (Scientific Industries Inc., Bohemia, NY) in a FastPrep-24 homogenizer (MP Biomedicals, Solon, OH). Total genomic DNA was extracted using a modified OmniPrep DNA Extraction protocol (G-Biosciences, St. Louis, MO). Briefly, larger quantities of lysis buffer (600 µl), DNA stripping solution (60 µl), precipitation solution (150 µl), and 70% ethanol (1 ml) were used. For samples with few sporangia, approximately 0.5 cm sections of visibly infected leaf tissue were ground using a mortar and pestle with liquid nitrogen, and genomic DNA was extracted following the DNeasy Plant Mini Kit protocol (Qiagen, Germantown, MD).

Genome sequencing and de novo assembly. Whole genome sequencing was performed from *P. halstedii* sample SDM74, collected in 2009 from *H. annuus* in Bottineau, ND. This sample was selected for whole genome sequencing based on the economic importance of the physiological race it represents. Sample SDM74 is classified as a member of physiological race 734, one of the first ‘hot’ races virulent against the *Pl₆* and *Pl₇* resistance genes in commercial sunflowers (Gulya et al. 2010). Sequencing libraries were created from genomic DNA using Illumina’s Nextera XT and Nextera Mate-Pair kits and sequenced using Illumina’s MiSeq sequencing-by-synthesis technology with 300-bp paired-end reads (Illumina, Inc., San Diego, CA). Fastq read files were imported into CLC Genomics Workbench v.7.5.1 (CLC Bio, Boston, MA) where Illumina adapters and indices, low quality reads and ambiguous bases were removed. De novo assembly of trimmed/filtered reads was performed using an empirically determined k-mer size of 40, bubble size of 100, with contigs ≥ 500 bp retained.

Additional Illumina DNA sequence reads were generated from two *P. halstedii* samples from sunflower and four samples from rudbeckia (*R. fulgida* ‘Goldsturm’) to provide a shallow depth of coverage suitable for comparison with the SMD74 reference assembly (Supplementary Table S2). Sequencing libraries for these six samples were constructed using Nextera XT, TruSeq, and TruSeq Nano kits and sequenced on an Illumina MiSeq to generate 300-bp paired-end reads.

SSR identification and primer design. The genome assembly of *P. halstedii* SDM74 was used for genome-wide identification of SSR motifs (MISA) (Thiel et al. 2003), primer design (Primer3; Untergasser et al. 2012) and a reciprocal BLAST search to identify multiple priming sites within the genome (Altschul et al. 1990). The analysis was completed using the automated pipeline implemented in PrimerPro v1.0 (<http://webdocs.cs.ualberta.ca/~yifeng/primerpro/>). Search parameters were performed as previously described (Salgado-Salazar et al. 2015). Using CLC Genomics Workbench, SSR motifs and Illumina sequence reads from the four *P. halstedii* samples (SDM74, RDM1, RDM4, RDM5) were mapped to the SDM74 reference assembly. Candidate SSR markers were preferentially selected by identifying SSR regions represented by sequence reads in at least one *P. halstedii* samples from each host. Because genome sequencing

TABLE 1. Summary of *Plasmopara halstedii* samples genotyped using simple sequence repeat markers designed in this study

Host	Historical (1883–1999)	Modern (2000–2014)	Unknown (data not available)	Total
<i>Helianthus</i> spp.	115	24	28	167
<i>Rudbeckia</i> spp.	5	60	–	65
Total				232

was performed directly from environmental samples, the contigs selected for SSR primer design were queried against the NCBI GenBank nucleotide database to ensure that they originated from *P. halstedii*.

De novo gene predictions on the regions containing target SSR motifs were conducted using the AUGUSTUS web interface (<http://bioinf.uni-greifswald.de/webaugustus/prediction/create>) with gene model parameters trained for *Saccharomyces cerevisiae*. BLASTp and BLASTn searches of the NCBI GenBank nonredundant database were used to provide putative identifications of the predicted proteins and noncoding regions, respectively.

SSR genotyping. Amplifications were carried out following the approach of Schuelke (2000). PCR reactions were performed in a 10 µl volume containing 2× Mango Mix, 12.5 mM of MgCl₂ (Bioline Inc., Tauton, MA), 2.5 µM forward primer with a 5' M13 tail, 5 µM reverse primer, and 5 µM M13 primer labeled with one of the following fluorophores: 6FAM (Integrated DNA Technologies, Coralville, IA), VIC, NED or PET (Applied Biosystems, Foster City, CA). Each reaction contained 1.5 µl of genomic DNA (final concentration: 10 to 100 ng/µl). Reactions were carried out in a thermal cycler (C100 Thermal Cycler, Bio-Rad Laboratories, Hercules, CA or PTC-100 Gradient Cycler, MJ Research, Watertown, MA) under the following conditions: initial denaturation at 94°C for 5 min; 30 cycles at 94°C for 30 s, 57°C annealing temperature for 45 s, and 72°C for 45 s; a labeling step of 8 cycles at 94°C for 30 s, 53°C for 45 s, and 72°C for 45 s; and a final extension step at 72°C for 10 min. All PCR reactions included a minimum of two negative controls consisting of water or DNA extracted from healthy sunflower leaf tissue. A subset of the PCR amplifications including negative controls were visually confirmed on a 2% agarose gel. Samples with no PCR amplification for a given marker were repeated once. PCR amplicons were prepared for fragment analysis by mixing 1 µl of undiluted PCR product and 9 µl of loading buffer (9 µl of HiDi formamide [Applied Biosystems] and 0.2 µl of GeneScan 500 LIZ size standard [Applied Biosystems]). SSR reactions were multiplexed in groups of four, by combining SSR markers labeled with one of each of the four fluorescent labels. Fragment analysis was performed on a 3730xl DNA Analyzer (Applied Biosystems). Results were scored using GeneMarker v2.6.3 (SoftGenetics, State College, PA). Five percent of reactions were duplicated to confirm allele calls.

Population analysis. GenAlEx v.6.5 (Peakall and Smouse 2012) was used to calculate the number of unique multilocus genotypes, total number of alleles per locus, unbiased gene diversity (*h*), and Shannon's information index. A genotype accumulation curve was calculated using the statistical language R (R Core team 2015) and the package poppr (Kamvar et al. 2014) to determine the efficiency of the SSR markers in discriminating unique multilocus genotypes in our data set. Population clusters were defined using the Bayesian clustering algorithm implemented in STRUCTURE 2.3.4 without a priori definitions (Falush et al. 2007, 2003; Hubisz et al. 2009; Pritchard et al. 2000), using a burn-in period of 500,000 and 500,000 Monte Carlo Markov Chain (MCMC) iterations for data collection and 10 independent simulations for each K (1 to 25). Admixture and correlated allele frequencies models were evaluated for every K. A second analysis in STRUCTURE was performed for each of the previously identified clusters using the same parameters and evaluating Ks from 1 to 10. The optimal K value was chosen by comparing the rate of change in the log probability of the data between K values (DeltaK) using the web-based program STRUCTURE HARVESTER (Earl and vonHoldt 2012; Evanno et al. 2005). Clusters were visualized using the default settings for CLUMPP and DISTRUCT in the beta version of the web-based pipeline Cluster Markov Packager Across K (CLUMPAK; Kopelman et al. 2015). A principal coordinate analysis (PCA) using pairwise genotypic distance between all samples was calculated with GenAlEx v.6.5 to visualize additional genetic variation patterns within and between clusters.

Multilocus sequencing and phylogenetic analysis. Seven sunflower downy mildew (SDM) and RDM samples were analyzed for phylogenetic relationships using sequence data generated from the cytochrome oxidase subunit II (*cox2*) and the rDNA internal transcribed spacer (ITS) regions. Local BLAST searches in CLC Genomics Workbench were used to identify and extract these sequences from the seven *P. halstedii* genome data sets. Four additional SDM samples were also included in the phylogenetic analysis of *cox2*. For these samples, genomic DNA was extracted from sporangial masses and infected leaf tissue as described above. PCR amplifications were performed using the primer pair Cox2-F/Cox2-RC4 (*cox2*; Choi et al. 2015; Hudspeth et al. 2000), and the primer pair ITS1-O/LR-O (ITS; Moncalvo et al. 1995; Spring et al. 2006). PCR conditions were as follows: (i) *cox2*: 95°C for 4 min followed by 36 cycles at 95°C for 40 s, 55°C for 40 s and 72°C for 60 s; 5 min at 72°C; (ii) ITS: 94°C for 3 min followed by 94°C for 30 s, 47°C for 30 s, 72°C for 1 min; 72°C for 10 min. All amplified fragments were confirmed on a 1% agarose electrophoresis gel. After removal of unused primers and nucleotides using Illustra ExoStar (GE Healthcare Life Sciences, Piscataway, NJ), PCR products were sequenced bidirectionally using BigDye Terminator v3.1 cycle sequencing chemistry (Applied Biosystems) on an ABI 3730x Genetic Analyzer (Applied Biosystems). Sequences were assembled and edited to remove low quality bases using CLC Genomics Workbench and consensus sequences were extracted for phylogenetic analysis.

Data sets for each sequence marker were aligned separately using CLC Genomics Workbench. The best fitting substitution model for each marker was determined using the software jModeltest (Darriba et al. 2012; Guindon and Gascuel 2003) and models were chosen according to the Akaike information criterion (Akaike 1974). The models TIM2+I+G and TVM+I were chosen as the best fit for the *cox2* and ITS data sets, respectively. Phylogenetic relationships were reconstructed using maximum likelihood optimality criterion implemented in the RAxML-GUI (Silvestro and Michalak 2012; Stamatakis 2006), with 1,000 nonparametric bootstrap replicates performed to assess branch support. *Peronospora belbahrii* was used as outgroup taxa to root the phylogenetic trees, consistent with the known phylogenetic relationship between the *Plasmopara* and *Peronospora* genera (Thines et al. 2008). Final trees were visualized with FigTree v1.3.1 (Rambaut 2009). DNA sequences generated in this study were deposited in GenBank (Tables 3 and 4). Voucher specimens of *P. halstedii* were submitted to herbarium BPI under accessions 909964 to 910041.

Cross-infectivity experiments. A *P. halstedii* sample from *R. fulgida* 'Goldsturm' collected from a commercial greenhouse in Maryland during 2013 (RDM5) was used to test for cross infectivity on *H. annuus* according to the protocol published by Gulya (1996). Zoosporangia rinsed from *R. fulgida* 'Goldsturm' leaves were used to inoculate 3-day-old *H. annuus* seedlings. Seedlings were immersed in a zoosporangial suspension of 20,000 sporangia/ml for 3 h, and planted in a sand-perlite mixture (3:1, vol/vol) in the greenhouse for 11 to 12 days under the following conditions: 18 to 24°C, 16 h photoperiod. Zoosporangia from the same sample were also used to inoculate a series of nine sunflower differential lines to determine the physiological race of this sample (Gulya et al. 1991).

RESULTS

Development and characteristics of new SSR markers. Five hundred fifty-nine scaffolds were assembled into a draft genome sequence for *P. halstedii* sample SDM74. Seventy percent of the Illumina sequence reads generated for RDM4 (9.4 M) and RDM5 (17.4 M) mapped to the SDM74 scaffolds, but only 54% of the Illumina reads generated for sample RDM1 (2.7 M) could be mapped to the SDM74 scaffolds. A total of 682 SSR-containing regions were identified from the 559 SDM74 scaffolds (Table 2). The predominant motifs were di-nucleotide repeats (*n* = 322),

followed by tri- and penta- nucleotide repeats ($n = 152$ and 112 , respectively). From the genome assembly and mapping file data sets, 38 SSR-containing regions were selected for primer design and evaluated for amplification and polymorphism using a set of 14 *P. halstedii* samples from two different hosts, *H. annuus* and *R. fulgida* ‘Goldsturm’. From the 38 primer pairs evaluated, seven primer pairs only amplified *P. halstedii* collected from *H. annuus*, five primer pairs were monomorphic, and seven primer pairs exhibited inconsistent amplification (data not shown). The remaining 19 polymorphic SSR primer pairs were initially used to genotype a set of 547 *P. halstedii* samples that included herbarium specimens from multiple hosts within the family *Asteraceae* (collected from 1883 to 1930s; data not shown). However, the herbarium specimens from hosts other than *Helianthus* and *Rudbeckia* did not amplify consistently and were removed from the study (data not shown), leaving 232 samples. After genotyping the remaining 232 *P. halstedii* samples, four additional SSR markers were removed from the final analysis due to inconsistent allele calls. A summary of the final 15 SSR markers selected for genotypic analyses is provided in Table 3. These markers were distributed across 15 different scaffolds of the *P. halstedii* SDM74 genome assembly, with 10 of the 15 markers predicted to fall within genic regions. Ten SSR markers exhibited high levels of similarity to hypothetical proteins of *Phytophthora parasitica* and *Phytophthora infestans*, and one marker was similar to a genomic DNA sequence from *Hyaloperonospora parasitica*. No significant hits were obtained from BLASTn searches performed using the remaining four SSR markers in nongenic regions (Table 3).

PCR reactions using the 15 SSR markers yielded amplicons ranging in size from 133 to 301 bp, with 4 to 12 alleles per locus. Unbiased gene diversity ranged from 0.34 to 0.68 and the Shannon’s information index ranged from 0.61 to 1.77. The genotype accumulation curve showed that nine loci were sufficient to discriminate 90% of the multilocus genotypes in our data set (Supplementary Fig. S1). Failed PCR amplifications were more common within the RDM samples than SDM. The highest percentages of missing data resulted from markers 2118 (17%), 354 (17%) and 4499 (16%). Marker 2118 failed to amplify all SDM and RDM herbarium samples equally, while marker 4499 failed to amplify a large percentage of RDM samples (38%). Missing data for all other loci ranged between 3 and 14%.

Population genetic structure. From 232 *P. halstedii* samples, 231 exhibited unique multilocus genotypes. Only two samples collected from the same location shared a unique multilocus genotype at 14 loci (RDM30 and RDM33). The genotypic diversity in our data set was also reflected by a large number of private alleles (i.e., alleles unique to a population). When the data set was subdivided by plant host genus, 14 alleles at eight loci were unique to the RDM samples (allele frequencies range from 0.008 to 0.67), while 34 alleles at 13 loci were unique to the SDM samples (allele frequencies range from 0.003 to 0.46). Five private alleles at four loci were also observed across all herbarium samples of RDM collected from 1883 to 1936 (allele frequencies range from 0.125 to 0.8).

When all 232 *P. halstedii* samples were analyzed using STRUCTURE, the deltaK was maximized when $K = 2$ (DeltaK = 4,280.8; Supplementary Fig. S2). These two sample clusters divided the 232 *P. halstedii* samples entirely according to host plant origin, with one cluster exclusive to *Helianthus* spp. (designated cluster A) and one cluster exclusive to *Rudbeckia* spp. (designated cluster B; Fig. 1A), with almost no admixture. Clustering by plant host was also observed in the PCA (Fig. 2), where 30% of the variance was explained by the first two coordinates. As with the STRUCTURE analysis, sample characteristics such as year of collection, country of origin, or physiological race did not explain secondary clustering of samples within the PCA. Interestingly, historical pre-epidemic samples collected from *Rudbeckia* spp. between 1883 and 1936 loosely clustered

between the two main plant host clusters for *P. halstedii* (Fig. 2). To explore if this was an artifact of the missing data found in the historical RDM samples, we calculated a PCA using a reduced number of loci. After discarding loci with over 60% missing data (loci 147, 354, 1213, 2010, 2118, and 5056) in historical RDM samples, the PCA analysis yielded similar results as observed in the complete PCA (data not shown).

STRUCTURE analysis performed using only SDM samples in cluster A identified eight clusters (designated subclusters A1 to A8; $K = 8$, DeltaK = 11.5; Supplementary Fig. S3; Supplementary Table S3). None of the eight SDM subclusters A1 to A8 corresponded with country of origin, year of collection, or physiological races (Fig. 1B). All SDM subclusters included samples collected over a time period of at least 11 years (A3), and up to 102 years (A7). The sole SDM herbarium specimen—collected in the year 1911 from *H. strumosus* (BPI 796453)—was placed in cluster A7, along with samples collected between 1990 and 2013. Physiological race groups were distributed across different SDM subclusters. For example, 15 samples of *P. halstedii* race 700 clustered in six different SDM subclusters (A1, A2, A4, A5, A7, and A8), while the 33 samples of race 730 were found in all eight SDM subclusters. Admixture between SDM subclusters occurred throughout the sample (Fig. 1B).

Within *P. halstedii* cluster B from *Rudbeckia* spp., STRUCTURE analysis subdivided samples into three main subclusters ($K = 3$, DeltaK = 82.0; Supplementary Fig. S4; Supplementary Table S4). Subcluster B1 contained the five historical *P. halstedii* samples collected on native *Rudbeckia* hosts from 1883 to 1936 (Fig. 1C). There was no evidence of admixture between historical subcluster B1 and the modern RDM samples in subclusters B2 and B3. Subcluster B2 consisted of 20 modern RDM samples collected from two commercial nurseries in Maryland, and landscape samples from Connecticut, Tennessee, Virginia, Ohio and Maryland during 2013 and 2014. All but two samples in subcluster B2—from a single nursery in Maryland—were collected during 2013. Subcluster B3 contained 40 RDM samples collected during 2013 and 2014 from three commercial nurseries in Maryland and from a nursery-grown sample of *R. fulgida* var. *speciosa* from Indiana. Samples collected from one commercial nursery in 2013 originated from three different supplier shipments during, April, July and August (Fig. 1C). Clustering analysis in STRUCTURE showed that *P. halstedii* genotypes from the April shipment belonged entirely to subcluster B2. In contrast, RDM samples collected from the July cluster belonged to both B2 and B3 subclusters, while the August RDM sample group from the same nursery belonged almost entirely to subcluster B3 (except one sample). Ninety-three percent of the samples collected in 2014 belonged to subcluster B3, and originated from two local commercial nurseries (Fig. 1C).

Phylogenetic analysis. Eleven *cox2* and seven ITS rDNA sequences were obtained from RDM and SDM samples (Table 4). As observed from the SSR data sets, phylogenetic analyses of these data sets revealed a separation of the SDM and RDM *P. halstedii* samples into two distinct clades that corresponded with plant host genus. Although the distinction between clades of *P. halstedii* from

TABLE 2. Summary statistics from *Plasmopara halstedii* sample SDM74 draft genome assembly

Statistics	
Draft genome statistics	
Draft genome assembly size	70 Mb
Number of scaffolds ≥ 500 bp	8,385
N50	25,074
Average scaffold length	8,352
Average coverage	192x
Simple sequence repeat (SSR) statistics	
Number of scaffolds examined	8,385
Number of scaffolds with SSRs	559
Number of identified SSRs	682

SDM and RDM was also observed in *cox2* and ITS rDNA phylogenetic trees, only the RDM samples showed high bootstrap values within both phylogenies (99%; Fig. 3). Samples from SDM clustered together with high bootstrap support only in the ITS phylogeny (97%; Fig. 3B). Despite the low branch support, phylogenetic analysis of *cox2* further divided SDM samples into two distinct clades (Fig. 3A).

Cross-infectivity between different host genera. Signs and symptoms characteristic of downy mildew disease, using sporangia originally collected from *R. fulgida* 'Goldsturm' (sample RDM5), were observed on sunflower seedlings 12 days after inoculation. Profuse sporulation was observed on cotyledons and the first pair of true leaves in 98 to 100% of all inoculated seedlings. Evaluation of this same sample on nine sunflower differential lines identified it as a member of *P. halstedii* race 734.

DISCUSSION

In this study, we developed new molecular resources and used them to determine whether recent outbreaks of downy mildew on

rudbeckia in the United States are caused by the same *P. halstedii* genotypes responsible for pre-epidemic disease outbreaks on native *Rudbeckia* sp., or disease outbreaks on cultivated sunflower. Through the analysis of 60 modern RDM samples and five historical RDM herbarium specimens using SSRs and two phylogenetic sequence markers, we observed unique genotypes that served to delineate pre- and postepidemic pathogen populations from each other, and from SDM samples. In particular, these data show that *P. halstedii* causing the modern RDM epidemics on cultivated *R. fulgida* are genetically different from the *P. halstedii* samples associated with historical disease outbreaks on native *Rudbeckia* spp. Modern RDM samples characterized in this study exhibit high genetic diversity and a lack of genetic structure, even when one considers the small geographical region and two year time frame used for sample collection. For example, the *R. fulgida* plants from which RDM samples were collected in 2013 at one nursery were received from wholesale suppliers at three different times: April, July and August. Interestingly, genetic changes in the pathogen populations can be observed between the shipments received in April (belonging exclusively to subcluster B2) and August (subcluster B3), with a genetically mixed

TABLE 3. Characteristics of the 15 polymorphic simple sequence repeat markers developed for *Plasmopara halstedii* in this study^a

Locus	Repeat motif	Primer sequence (5'→3')	Allele size range	Number of alleles	Gene diversity ^b	I ^c	Predicted function ^d	GenBank accession
147	(TTC) ₅	F: TCAAGACCGCCCATAGTCTC R: GGGTTCGTTGATCGAGTTA	134–152	7	0.68	1.31	Hypothetical protein F444_17999 [<i>Phytophthora parasitica</i> P1976]	KT891414
250	(GAT) ₅	F: AGGAACCTTAACGACGAGCGA R: TGTATCTTTTCGAGCCGAGG	133–172	7	0.49	1.00	Predicted intergenic region. No significant BLASTn hit	KT891415
282	(GGT) ₅	F: GCAGTTGCACGACATCACTT R: AGCGAACTACGAGGAGGTCA	135–144	4	0.50	0.72	Conserved hypothetical protein (PITG_15922) [<i>Phytophthora infestans</i> T30-4]	KT891421
354	(CTACTGCTG) ₆	F: CGTGGTACTCGCTCTGTTTG R: TGGATGCATTCTGACCAAG	262–301	12	0.77	1.70	TKL protein kinase [<i>Phytophthora parasitica</i> P1569]	KT891418
919	(GGAC) ₅	F: AACCGACATTTTAAGCGCAC R: TAGCACGGTCGGAACCTTACC	149–161	5	0.50	0.78	Predicted intergenic region. No significant BLASTn hit	KT891419
1213	(TCAATA) ₆	F: GCCCACTGCCAATTAGAACA R: GACCCATGGTTCGACATTTT	192–225	10	0.78	1.77	Predicted intergenic region. BLASTn: DNA sequence clone HpEmoy2-12D7 [<i>Hyaloperonospora parasitica</i>]	KT891423
1338	(TTC) ₅	F: TCGCTTGAATCACTCTCCCT R: CTCGACTGAAGTGGAGGAGC	137–164	8	0.50	0.77	Aspartyl protease family A22B, putative [<i>Phytophthora infestans</i> T30-4]	KT891422
1813	(CAA) ₉	F: TAGTGCCTGCCACTTCACAG R: GCATTTGTTGCATTGTGCT	158–191	7	0.51	0.97	Hypothetical protein F443_17385 [<i>Phytophthora parasitica</i> P1569]	KT891416
1886	(TGAG) ₆	F: CCGATGACCATCTGTACGTG R: AGCGACAGGGAATGAGAGA	177–193	5	0.68	1.27	Predicted intergenic region. No significant BLASTn hit	KT891417
2010	(TCA) ₅	F: GGCTCCAAGAGTTTCAGTTGC R: TCTGGGCTGATAATGAAGGG	257–269	4	0.34	0.61	Dentin sialophosphoprotein-like protein [<i>Phytophthora infestans</i> T30-4]	KT891424
2118	(TTC) ₇	F: CTCCATCGTAGCATTTCCGT R: AACATCTCTCCAACGCGACT	269–281	4	0.54	0.85	Transcriptional repressor TUP1-like protein (PITG-11423) mRNA [<i>Phytophthora infestans</i> T30-4]	KT891413
3593	(ATG) ₅ ctgatgat(GAA) ₆	F: GCTTGGTGGAGATTGTTGGT R: TTGCTTCTTGGTGCTTTGTG	226–265	6	0.43	0.76	Dentin sialophosphoprotein-like protein [<i>Phytophthora infestans</i> T30-4]	KT891420
4367	(TCA) ₅	F: GCCTCTAGCTCGACTTCGTC R: CATCGGGCAAAAATCTCTCT	135–159	9	0.60	1.34	Hypothetical protein F442_04312 [<i>Phytophthora parasitica</i> P10297]	KT891425
4499	(TCCG) ₅	F: TAGCACGGTCGGAACCTTACC R: AACCGACATTTTAAGCGCAC	138–165	9	0.56	1.01	Predicted intergenic region. No significant BLASTn hit	KT891426
5056	(TGT) ₇	F: AGGGGCAAAAGTTTTTGGTT R: AGCTACAGCCAAAGACCGAA	209–230	6	0.45	0.89	AP-4 complex subunit epsilon, putative [<i>Phytophthora infestans</i> T30-4]	KT891412

^a Fluorophores were added to forward primers as described in Schuelke 2000.

^b Gene diversity (unbiased heterozygosity $[2n/2n - 1(1 - \sum p_i^2)]$) was calculated using GenAlEx v.6.5 (Peakall and Smouse 2012).

^c I = Shannon's information index, $-1 \times \sum (p_i \times \ln(p_i))$, was calculated using GenAlEx v.6.5 (Peakall and Smouse 2012).

^d Predicted genic regions are based on results from AUGUSTUS web interface and BLAST queries of the NCBI nonredundant database.

sample group collected in July (subclusters B2 and B3; Fig. 1C). The predominant genetic subcluster (B3) identified at the end of the 2013 growing season at this nursery was also the predominant subcluster sampled in 2014 at all sites. Although this pattern may have been influenced by the localized nature of our sampling, our data highlight the striking presence of multiple genetic groups within a single growing season and within a single commercial operation. Oomycete plant pathogens are known to evolve rapidly and overcome strategies for disease management in a very short time (Ahmed et al. 2012; Callaghan and Guest 2015; Gulya et al. 2013). The results of our study support the existence of high levels of genetic diversity in *P. halstedii* infecting *Rudbeckia* spp., and the potential for rapid evolution of its populations.

Analysis of 232 modern and historical samples identified the existence of two well-defined clusters using SSR markers and molecular phylogenetic trees. The differentiation between RDM and SDM samples is consistent with a species-level diversification, based on the reciprocal monophyly in phylogenetic analyses and multilocus genotype clustering. The two main clusters corresponded, unequivocally, to the two plant host genera examined in our study, regardless of the type of marker employed. The SSR markers developed in our study revealed a high level of genetic diversity (up to 12 alleles/locus) and discriminative power between samples of these two putative *Plasmopara* species. Furthermore, the lack of transferability of these SSR markers among *P. halstedii* isolates collected from different Asteraceae hosts indicates that these markers are highly specific to the two host isolate groups. This observation suggests the existence of multiple divergent species associated with other Asteraceae hosts, such as *Bidens* spp., *Eupatorium* spp. and *Erigeron* spp. Our finding is in agreement with the narrow species concept proposed for *P. halstedii* by Novotél'nova (reviewed by Choi et al. 2009) based on morphometric characters, and with more recent molecular work (Komjáti et al. 2007; Spring et al. 2006, 2003; Voglmayr et al. 2004). Molecular and morphological work by Choi et al. (2009) revealed that the downy mildew on *Ambrosia artemisiifolia* (common ragweed) is also caused by a *Plasmopara* sp. different from the previously reported *P. halstedii* and *P. angustiterminalis*. Similarly, *P. sphagneticolae* (on *Sphagneticola*; McTaggart et al. 2015) and *P. majewskii* (on *Arctotis*

and *Arctotheca*; Constantinescu and Thines 2010) are newly described species, morphologically and genetically distinct from *P. halstedii* infecting different hosts within the family Asteraceae. As suggested previously by Thines (2014) and supported by our results, *P. halstedii sensu lato* is a complex of species that warrants an in-depth taxonomic revision to elucidate the limits of its host range. A taxonomic reclassification of the *Plasmopara* species causing downy mildew disease on rudbeckia and sunflower, while necessary, is outside the scope of our study, and thus needs to be comprehensively addressed in future research.

Despite the clear genetic distinction between *P. halstedii* samples originating from *Rudbeckia* spp. and *Helianthus* spp., our pathogenicity assay determined that cross infection from a *P. halstedii* isolate from rudbeckia onto sunflower seedlings is still possible under controlled conditions. Few studies have reported on the pathogenicity of *P. halstedii sensu lato* between different host genera relative to its wide host range. To our knowledge, demonstration of cross infectivity between *P. halstedii* isolates from other Asteraceae genera and sunflower is limited to just five genera: *Ambrosia*, *Dimorphotheca*, *Iva*, *Rudbeckia*, and *Xanthium* (Gulya 2002; Leppik 1965; Viranyi 1984; Walcz et al. 2000; and this study). This phenomenon, if common in nature, could provide a large suite of plants acting as reservoirs for *P. halstedii* to infect economically important crops. However, similar pathogenicity assays in the field are lacking and molecular studies consistently suggest that field isolates collected on different host genera are genetically distinct (Choi et al. 2009; Komjáti et al. 2007). In our study, the same isolate used for the cross infectivity assay (RDM5) was found to be genetically distinct from isolates on *Helianthus* sp. using SSR and phylogenetic analysis. While we did not inoculate rudbeckia with *P. halstedii* isolates from sunflower host, previous attempts indicate that systemic infection on rudbeckia seedlings is not possible (T. J. Gulya, unpublished data). Together, these data suggest that although cross-infectivity is possible under controlled conditions, movement of different *P. halstedii* genotypes between different Asteraceae hosts may be rare or nonexistent in natural populations under ordinary conditions.

The collection of *P. halstedii* isolates from cultivated sunflower included in this study encompasses 14 different physiological races

TABLE 4. Collection information on the *Plasmopara* samples used for phylogenetic analyses

Species	Host	Sample ID	ITS GenBank accession number	cox2 GenBank accession number
<i>Plasmopara halstedii</i>	<i>Helianthus annuus</i>	SDM1	–	KU232275
	<i>H. annuus</i>	SDM33	KU232270	KU232280
	<i>H. annuus</i>	SDM74	KU232269	KU232279
	<i>H. annuus</i>	SDM142	–	KU232276
	<i>H. annuus</i>	SDM146	–	KU232277
	<i>H. annuus</i>	SDM147	–	KU232278
	<i>H. annuus</i>	SDMR710	KU232274	KU232285
	<i>H. annuus</i>	–	DQ665670	–
	<i>H. annuus</i>	KUS-F 18911	–	EU743813
	<i>Rudbeckia fulgida</i> 'Goldsturm'	RDM1	KU232272	KU232282
	<i>R. fulgida</i> 'Goldsturm'	RDM4	–	KU232283
	<i>R. fulgida</i> 'Goldsturm'	RDM5	KU232273	KU232284
	<i>R. fulgida</i> 'Goldsturm'	RDM37	KU232271	KU232281
<i>P. angustiterminalis</i>	<i>Xanthium strumarium</i>	Pa676	DQ993167	–
<i>P. angustiterminalis</i>	<i>Xanthium</i> sp.	MT X03A1	–	EU826097
<i>P. baudysii</i>	<i>Berula erecta</i>	HV 571	–	EU826098
<i>P. nivea</i>	<i>Aegopodium podagraria</i>	H.V. 2191	EF553508	–
<i>P. obducens</i>	<i>Impatiens balsamina</i>	–	DQ665666	–
<i>P. obducens</i>	<i>I. noli-tangere</i>	HV306	–	DQ365757
<i>P. pimpinellae</i>	<i>Pimpinella major</i>	HV 634	–	DQ365758
<i>P. pusilla</i>	<i>Geranium sibiricum</i>	WU30280	HM004512	–
<i>P. siegesbeckiae</i>	<i>Siegesbeckia glabrescens</i>	KUS-F 21312	–	EU743814
<i>P. sphagneticolae</i>	<i>Sphagneticola trilobata</i>	BRIP 61010	–	KM085175
<i>P. viticola</i>	Unknown	–	AY742739	–
<i>P. viticola</i>	Unknown	UASWS SG1	–	EF426553
<i>Peronospora belbahrii</i> (outgroup)	<i>Plectranthus scutellarioides</i>	BPI893222	KT828759	–
<i>Peronospora belbahrii</i> (outgroup)	<i>Ocimum basilicum</i>	HV2280	–	EU863410

collected from nine countries between 1984 and 2013. This allowed us to explore the potential for intraspecific (i.e., intra- and inter-race) genetic patterns. The eight SDM subclusters identified in our study do not correspond to country of origin, year of collection, or physiological races. There is large variation in the characteristics of the samples within each subcluster. Most of the subclusters have a sample collection time range of 15 to 28 years, and all subclusters contain samples collected worldwide. SDM subcluster A7 contains the only herbarium sample from the native perennial *H. strumosus* (collected in 1911), but also includes samples collected from sunflower over a 102 year span (1990 to 2013). Only SDM subcluster A6 exclusively contains samples collected prior to 2000, however, there is no other unique pattern from race or country of origin. A lack of correspondence between known physiological races in *P. halstedii* and molecular data has also been reported using RAPD markers (Roeckel-Drevet et al. 2003) and ITS sequence data (Spring et al. 2006). Using 12 expressed sequence tag (EST)-derived markers, Delmotte et al. (2008) was only able to identify three distinct genetic groups from the 14 races present in France. Even when using single nucleotide polymorphisms identified from putative CRN cytoplasmic effectors, intraracial polymorphisms were the most commonly observed (As-sadi et al. 2011). Despite numerous efforts, no study performed to date has successfully developed molecular markers capable of replicating physiological race typing data that is generated through the labor-intensive

phenotypic experiments with sunflower differential lines. It is known that physiological races of *P. halstedii* evolve rapidly in response to selection pressures manifested by the deployment of resistant hybrid plants (Gulya et al. 2013), and it is possible that the molecular markers that have been used to date are simply not sufficiently variable, or that they are not specifically targeted to the portions of the genomes that are affected by selective pressures. In the present study we preferentially selected loci that would produce PCR amplification across two distinct genetic groups from both sunflower and rudbeckia. Thus, it is likely that these fixed regions of the genome are more conserved across species than rapidly evolving regions associated with pathogenicity such as effector genes. For example, 10 of the SSR markers regions used in our study were recognizable in the distantly related oomycetes *Phytophthora infestans*, *Phytophthora parasitica*, and *H. arabidopsis*.

Parasexual recombination has also been previously suggested as an explanation for the appearance of new *P. halstedii* pathotypes (Delmotte et al. 2006; Spring et al. 2006), however, most investigators regard *P. halstedii* as an organism with a sexual stage in its lifecycle (reviewed in Gascuel et al. 2015). Either sexual or asexual recombination events could provide an explanation for the large number of unique multilocus genotypes in the *P. halstedii sensu lato* samples from sunflower. Recombination processes could also be responsible for the genetic clustering patterns observed in RDM from a single commercial nursery (commercial nursery 1) and

Fig. 1. Assignment of *Plasmopara halstedii* samples to clusters based on simple sequence repeat data using STRUCTURE software, as visualized by CLUMPAK. On the horizontal axis, vertical bars represent the individual *P. halstedii* samples. The vertical axis indicates the membership proportion for individual samples. **A**, Two clusters were identified from 232 *P. halstedii* samples from *Helianthus* sp. (sunflower) and *Rudbeckia* sp. (rudbeckia) corresponding with the plant host. **B**, A total of 167 *P. halstedii* samples from sunflower. Individual samples were assigned to one of eight clusters. Black dots identify modern samples collected from 2000 to 2014. **C**, A total of 65 *P. halstedii* samples from rudbeckia. Individual samples were assigned to one of three clusters.

between years (Fig. 1C). These rapid microevolutionary changes in RDM and SDM populations may pose new challenges to disease prevention, management and control.

Historical samples allow scientists to take snapshots of organismal diversity from the past and compare it with the present (Choi and Thines 2015; Rossman 1996; Verkley et al. 2015; Yoshida et al.

2013). In the current study, historical specimens collected from native *Rudbeckia* spp. possess intermediate SSR genotypes, positioned between the cluster of modern RDM samples from cultivated *R. fulgida* and the cluster populated by SDM samples. *Plasmopara halstedii* and many of its Asteraceae hosts are considered native to North America, thus, populations of *P. halstedii sensu*

Fig. 2. Principal coordinate analysis of pairwise genotypic distance between 232 *Plasmopara halstedii* historical and modern samples from *Helianthus* spp. (sunflower; SDM) and *Rudbeckia* spp. (rudbeckia; RDM) downy mildew samples. The first two principal coordinates are shown. Samples are coded by the shape of the data point (triangle = sunflower host; circle = rudbeckia host) and the color (historical samples = open triangles/circles; modern samples = full color).

Fig. 3. Maximum likelihood best trees for **A**, *cox2* and **B**, rDNA internal transcribed spacer regions. Bootstrap values are indicated at branches (1,000 replicates). Scale represents expected number of changes per site. Both trees were outgroup rooted with *Peronospora belbahrii*.

lato on multiple Asteraceae hosts have coexisted in space and time. Given this, an evolutionary pattern of cospeciation between host and pathogen would be expected. However, recent work in *Bremia* suggests that host shifts followed by radiation and speciation may better explain the diversity and evolution of downy mildew pathogens (Choi and Thines 2015). Similar conclusions were drawn from studies of the grape downy mildew pathogen *P. viticola*, where host range expansion from wild to cultivated grapes in North America led to an increase in pathogen aggressiveness (Rouxel et al. 2013). Such a scenario of host range expansion followed by diversification would also be consistent with our observations of *P. halstedii* population structure on sunflower and rudbeckia. Sympatric diversification of *P. halstedii* between the two plant genera may have been a result of an increased cultivation and hybrid selection of both sunflower and rudbeckia. This diversification could have resulted in a host range expansion and specialization of modern populations, initially resulting in host shifts from sunflower onto native *Rudbeckia* spp. populations, and more recently leading to the outbreaks of downy mildew disease on cultivated *R. fulgida*.

The rapid evolution of downy mildew pathogens coupled with the lack of accurate species classification and knowledge of host range leaves global agricultural systems at risk and challenges global agricultural trade. The examination of the genetic diversity in historical and modern samples of *P. halstedii* presented in our study emphasizes the importance of downy mildews as emergent pathogens. The results of this research may provide critical information for plant breeding and disease management programs in the ornamental horticulture and sunflower industries.

ACKNOWLEDGMENTS

We thank J. Byrne, D. Clement, T. Creswell, M. Daughtrey, S. Jensen, K. Snover-Clift, R. Leatherwood, C. Misar, R. Peterman, L. Pundt, K. Rane, M. Romberg, and A. Windham for providing fresh samples of *P. halstedii* on sunflower and rudbeckia; N. Saied, C. Misar, and M. Gilley for technical support; S. Sheffer and M. Lewis for the use of the ABI 3730xl instrument; and the U.S. National Fungus Collection for allowing access to herbarium specimens. We are particularly indebted to A. Gould for her continued partnership and support, and for her ongoing mentorship of Y. Rivera and C. Salgado-Salazar. This research was funded by a 2013 USDA-APHIS-PPQ Farm Bill grant to JAC and USDA-ARS project 8042-22000-279; Y. Rivera and C. Salgado-Salazar were supported by 2013–2015 USDA-APHIS-PPQ Farm Bill grants to J. A. Crouch. Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture. The USDA is an equal opportunity provider and employer.

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