Genetic Variation of the Pathogen Causing Impatiens Downy Mildew Predating and Including Twenty-first Century Epidemics on *Impatiens walleriana*

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Abstract

Impatiens downy mildew (IDM) of cultivated *Impatiens walleriana* has had a significant economic impact on the ornamental horticulture industry in the United States and globally. Although recent IDM outbreaks started in 2003, downy mildews on noncultivated *Impatiens* species have been documented since the 1880s. To understand the relationship between the pathogen causing recent epidemics and the pathogen historically present in the United States, this work characterized genetic variation among a collection of 1,000 samples on 18 plant hosts. Samples included collections during recent IDM epidemics and historical herbarium specimens. Ten major genotypes were identified from cloned rDNA amplicon sequencing and

endpoint SNP genotyping. Three genotypes accounted for >95% of the samples, with only one of these three genotypes found on samples predating recent IDM outbreaks. Based on phylogenetic analysis integrating data from three markers and the presence of individual genotypes on multiple *Impatiens* species, there was some evidence of pathogen-specific infection of *I. noli-tangere*, but the distinction between genotypes infecting *I. walleriana* and *I. balsamina* was not upheld. Overall, this work provides evidence that the majority of rDNA genotypes recovered from recent IDM epidemics are different from historical U.S. genotypes, and that these genotypes can infect *Impatiens* spp. other than *I. walleriana*.

During the past decade, downy mildew diseases caused by oomycete pathogens in the Peronosporaceae have emerged as some of the most destructive diseases of specialty crops in the United States and worldwide (Thines and Choi 2016). Notable examples of specialty crops recently affected by downy mildew diseases include hyssop (*Agastache foeniculum*), basil (*Ocimum basilicum*), coleus (*Plectranthus scutellaroides*), cucumber (*Cucumis sativus*), rudbeckia (*Rudbeckia fulgida*), and garden impatiens (*Impatiens walleriana*) (Cohen et al. 2015; Rivera et al. 2016a,b,c; Thines and Choi 2016; Thines et al. 2009). Among

nonedible specialty crops, no plant has been impacted more by downy mildew than *I. walleriana*, a floriferous, shade-tolerant flowering annual used extensively in display gardens and landscape beds, container gardens and hanging baskets. Since the early twenty-first century, destructive outbreaks of impatiens downy mildew (IDM) have been documented from *I. walleriana* in the U.K., continental Europe, Australia, Japan, Korea, and Taiwan (Bulajić et al. 2011; Choi et al. 2009; Cunningham et al. 2008; Kirschner 2013; Lane et al. 2005; Petróczy et al. 2012; Satou et al. 2013; Shen et al. 2013; Toppe et al. 2010; Vajna 2011). In the United States, sporadic outbreaks of IDM were first reported from *I. walleriana* in commercial greenhouses in Tennessee and California in 2004 (Wegulo et al. 2004), North Carolina in 2007 (C. Warfield, *personal communication*) and from New York landscapes in 2009 (M. Daughtrey, *personal communication*). However, it was not until the 2011 and 2012 growing seasons that IDM became widespread in landscape plantings across the continental United States and the Hawaiian Islands (Baysal-Gurel et al. 2012; Conner et al. 2014; Crouch et al. 2014; McGinnis et al. 2015; Palmateer et al. 2013; Ward et al. 2013; Warfield 2012). Symptoms of downy mildew are especially severe on *I. walleriana* and include leaf yellowing, flower drop, complete defoliation, stem rot, and eventual plant death (Hansen et al. 2013; Shen et al. 2013). Losses due to IDM can be staggering, with 80 to 100% disease incidence widely reported (Baysal-Gurel et al. 2012; Bulajić et al. 2011; Choi et al. 2009; Cunningham et al. 2008; Kirschner 2013; Lane et al. 2005; Palmateer et al. 2013; Petróczy et al. 2012; Shen et al. 2013; Vajna 2011; Wegulo et al. 2004). Since the onset of IDM affecting *I. walleriana* in the United States, conservative estimates indicate that the value of this important ornamental bedding crop has decreased by \$72 million annually, or approximately 40% of the annual wholesale value (USDA-National Agricultural Statistics Service Census of Agriculture 1998 and 2014 reports, <https://www.agcensus.usda.gov>).

Impatiens downy mildew is not a new disease. In fact, the first samples of this disease were collected in Germany in the late 1870s

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and were used to describe the causal pathogen *Plasmopara obducens* from the cotyledons of *I. noli-tangere* (Schröeter 1877). *P. obducens* was subsequently documented in the United States during the 1880s (Farr and Rossman 2018). Despite the historical presence of IDM, the twenty-first century IDM outbreaks are unique in that they mark the first time commercial cultivars of *I. walleriana* have been impacted by this disease since their introduction to the ornamental industry in the late 1960s. Prior to the twenty-first century, *P. obducens* was only known from five species of *Impatiens*: *I. balsamina*, *I. capensis*, *I. pallida*, *I. noli-tangere*, and *I. textori* (Farr and Rossman 2018). With the exception of *I. balsamina*, which has been grown for centuries as a medicinal plant, these four *Impatiens* species are rarely cultivated in the horticultural trade (Morgan 2007). Of the approximately 1,000 known species of *Impatiens*, most live in the wild, commonly as understory woodland plants (Morgan 2007; Yu et al. 2016). The effect of downy mildew on the health of *Impatiens* species other than *I. walleriana* is largely unknown. Outside of the current IDM outbreaks affecting *I. walleriana*, disease symptoms are rarely documented from *Impatiens* host plants infected with the pathogen (but see Choi et al. 2009; Tanner et al. 2008). New Guinea *impatiens* hybrids (*I. hawkeri*) are another commercially grown *Impatiens* that are generally not susceptible to the disease (Hansen et al. 2013), although infection by the pathogen can be induced (C. Warfield, *personal communication*). Even the original record of *P. obducens* made from wild-grown *I. noli-tangere* in Germany describes the infected plants in good health, with the only external signs of infection occurring as dense mycelial growth on the underside of cotyledons (Schröeter 1877). Literature dealing with IDM prior to its association with cultivated *I. walleriana* focuses primarily on the organism from a morphological and taxonomic standpoint, or as a component of higher-level taxonomic assessments, rather than as a plant pathogen (e.g., Görg et al. 2017; McTaggart et al. 2015; Thines and Choi 2016; Thines et al. 2007; Voglmayr and Thines 2007; Voglmayr et al. 2004). Very little is known about the biology of the causal pathogen of IDM.

The recent emergence of downy mildew as a destructive disease of cultivated *I. walleriana* raises a basic question: is the causal agent of the twenty-first century IDM outbreaks fundamentally the same organism that was documented from wild-grown species of *Impatiens* throughout most of the nineteenth and twentieth centuries? A recent phylogenetic study described the downy mildew pathogens on *I. walleriana* and *I. balsamina* as two novel, host-specific species of *Plasmopara*, distinct from *P. obducens* on *I. noli-tangere*, namely *P. destructor* and *P. velutina*, respectively (Görg et al. 2017). This description of novel host-specific downy mildew species on *Impatiens* is in general agreement with the high levels of host specialization

documented among oomycetes that cause downy mildew (Choi and Thines 2015; Choi et al. 2015; Göker et al. 2007; Rivera et al. 2016c; Rouxel et al. 2013, 2014; Runge et al. 2011; Thines and Choi 2016). However, the recent taxonomic proposal for *Plasmopara* associated with *Impatiens* used a small number of samples ($n = 12$), did not include the *P. obducens* type materials, and did not use any IDM samples collected prior to 2004 (Görg et al. 2017). Significant differences in morphological characteristics (i.e., sporangia, sporangiophores, ultimate branchlets) were found among the *Plasmopara* specimens from three *Impatiens* species (Görg et al. 2017). However, morphological characteristics of downy mildews may be influenced by infection of different plant hosts (Runge and Thines 2011), and the observed differences may simply be due to the isolation of the *Plasmopara* from the different *Impatiens* species, or environment-induced plasticity, especially given the wide overlapping range of measurements reported. In addition, although three markers were sequenced, the sole nuclear marker showed only two variant sites and the two mitochondrial markers overlapped within the same gene, meaning that the analysis was not a reflection of genetic variation across multiple loci. Considering these factors, it is still unclear if the pathogen responsible for recent outbreaks of IDM is genetically divergent from those identified as *P. obducens* and those historically present on *Impatiens*.

In this study, we broadly address the identity of the causal agent of IDM affecting cultivated *I. walleriana* in the twenty-first century in the United States using genotypic and phylogenetic methods. By including disease samples from historical herbarium specimens and modern samples, it was possible to test for potential genetic differentiation of the causal pathogen of the twenty-first century IDM outbreaks. In addition, by analyzing samples from multiple species of *Impatiens*, this work tested the hypothesis that genetic divergence among downy mildews on these plants is associated with host specificity. Overall, this work provides the first survey of genetic variation of the causal pathogen of IDM across populations in the United States and provides a foundation for future research into the evolution of the pathogen and control of the disease.

Materials and Methods

Sample collections. A total of 1,000 downy mildew samples were included in this study. An overview of the samples is provided in Table 1, and detailed listings are provided in Supplementary Tables S1 and S2. The collection included 984 samples of *Plasmopara obducens sensu lato* (i.e., not discriminating the species associated with *I. balsamina* and *I. walleriana* recently described by Görg et al. 2017). To simplify discussion, these *P. obducens s.l.* samples will be hereafter referred to as *P. obducens*. Sixteen samples from three closely related

Table 1. Summary of SNP and rDNA genotypes among 1,000 downy mildew samples taken from *Impatiens* and other hosts. Unless otherwise noted, all samples are *Plasmopara obducens sensu lato*.

Sample type	Locations	Origin	Total samples	Collection date(s)	rDNA/SNP genotype(s)	Host substrate(s)
U.S. samples, fresh collections (post-epidemics)	161	30 states	895	2011–2014	G1, G2, G3, G4, G7, G8, G9, G10, C	<i>Impatiens arguta</i> , <i>I. auricoma</i> , <i>I. balsamina</i> , <i>I. sp.</i> ‘Blue Angel’, <i>I. capensis</i> , <i>I. flanaganiae</i> , <i>I. hochstetteri</i> , <i>I. omeiana</i> , <i>I. walleriana</i>
U.S. samples, herbarium specimens (first U.S. specimen on <i>I. walleriana</i>)	1	Tennessee	2	2004	G5, G6	<i>I. walleriana</i>
U.S. samples, historical herbarium specimens (pre-epidemics)	54	18 states	55	1881–1942	G3, G4, B, C	<i>I. biflora</i> , <i>I. fulva</i> , <i>I. pallida</i> , <i>Impatiens sp.</i>
Non-U.S. samples, fresh collections (post-epidemics)	18	8 countries	18	2007–2013	G1, G2, G3	<i>I. walleriana</i>
Non-U.S. samples, herbarium specimens (pre- and post-epidemics)	17	7 countries	17	1877–2005	G3	<i>I. aurea</i> , <i>I. noli-tangere</i> , <i>I. textori</i> , <i>Impatiens sp.</i>
Outgroup samples (<i>Bremiella megasperma</i> , <i>Plasmopara constantinescui</i> , <i>Peronospora alsinearum</i>)	16	U.S. and Canada	16	1880s–1943	1, 3, 7, 8, B, C, H, I, K	<i>I. biflora</i> , <i>I. fulva</i> , <i>I. pallida</i> , <i>Viola tenella</i> , <i>V. tricolor</i> var. <i>arvensis</i>

species, *P. constantinescui*, *Bremiella megasperma*, and *Peronospora alsinearum*, were included for outgroup comparisons. Samples originated from two primary sources: (i) fresh collections of detached leaves from diseased plant material; and (ii) herbarium specimens from the U.S. National Fungus Collections (BPI; USDA-ARS, Beltsville, MD). Fresh collections were made in the United States from 895 foliar samples of *Impatiens* spp. exhibiting signs of *P. obducens* or symptoms of IDM, representing 161 different geographic locations. With the exception of 47 samples, all fresh collections were made from naturally infected plants. The remaining 47 freshly collected samples, provided by Margery Daugherty (Cornell University), correspond to eight species of *Impatiens* plants (*I. arguta*, *I. auricoma*, *I. balsamina*, *I. capensis*, *I. flanaganiae*, *I. hochstetteri*, *I. omeiana*, and *I. sp.* 'Blue Angel') inoculated with spores originally collected from infected *I. walleriana* plants. To test for the presence of different *P. obducens* genotypes on individual plants within sites, multiple leaves/plants were processed independently when adequate material was available. Fifty-five U.S. herbarium samples, including *P. obducens* infecting species of noncultivated *Impatiens* and two samples from the first outbreak on *I. walleriana* in 2004, were included to compare pre-epidemic or historical samples to those collected after the first major U.S. IDM outbreak in 2004. A total of 35 *P. obducens* samples collected from outside the United States were also included in the study, comprising 18 fresh samples of IDM on *I. walleriana* and 17 samples from herbarium specimens of IDM on *Impatiens* spp. other than *I. walleriana* (Table 1).

All freshly collected samples were exposed to ambient temperatures during the collection process, which included time spent with collectors, time spent in transit to our lab either by hand delivery or via commercial shipping providers, and time spent in storage in our lab in a double-walled metal herbarium cabinet at room temperature (ca. 25°C) prior to DNA extraction (one day to one month). Long-term conditions experienced by the herbarium specimens are unknowable and may have included any number of variables (Verkley et al. 2015), but at the time of sampling, the specimens were stored within acid-free paper envelopes in double-walled metal herbarium cabinets at ambient temperature, approximately 16–18°C.

Extraction of DNA from IDM samples. Extraction of total DNA for the majority of leaf samples was performed by excising and chopping ca. 10 mm² leaf sections colonized by *P. obducens* using a sterile razor blade. For samples collected from Michigan, sporangia and sporangiospores stored in microcentrifuge tubes were provided courtesy of Mary Hausbeck (Michigan State University), from material originally harvested from the surface of infected *I. walleriana* leaf tissue. Samples were homogenized in 2.0 ml microcentrifuge tubes using 0.5 mm sterile glass beads (Cell Disruption Media; Scientific Industries, Inc., Bohemia, NY) in a FastPrep 24 instrument (MP Biomedicals, Santa Ana, CA) for two cycles of 20 s in the presence of 600 µl Lysis Buffer AP1 (QIAGEN, Inc., Gaithersburg, MD). DNA was extracted in a QIAcube (QIAGEN) instrument using the DNeasy Plant Mini Kit (QIAGEN) according to manufacturer's instructions and then quantified using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Inc., Wilmington, DE).

Ribosomal DNA PCR amplification, cloning, and sequencing.

PCR amplification of an approximate 1,100 bp segment of the partial 18S, ITS1, 5.8S, and partial ITS2 region of the rDNA was performed using forward primer DC6 (Bonants et al. 1997) and a new reverse primer designed for this study, Plob3 (5'-TTAGAAGACCAAGCAACTCG-3'). PCR amplifications were performed in a 25 µl total volume reaction containing 2.5 µl 10× PCR buffer (New England Biolabs, Ipswich, MA), with MgCl₂ at a final concentration of 1.5 mM, 0.2 mM dNTPs, 0.2 µM forward and reverse primers, 1.25 U standard *Taq* DNA Polymerase (New England Biolabs), and 2.5 µl of 1/10× concentration of the genomic DNA stock. Thermal cycling was performed by 5 min denaturation at 95°C; followed by 40 cycles of 45 s at 95°C, 30 s at 55°C, 30 s at 72°C. A final extension was performed for 10 min at 72°C.

Amplicons from DC6/Plob3 amplification were sequenced to a minimum of 2 to 5× coverage using Sanger technology, either directly from amplicons or from plasmid preps after cloning of the amplicon. Direct sequences were primed for cycle sequencing using the DC6/Plob3 PCR primers and three custom designed internal sequencing primers Plasmor2, PlasmorF1, and PlasmorR1 (5'-TGATGTACGGACACTGAT-3', 5'-CTTCCACGTGAACCGTTTCA-3', and 5'-CCTTGTTACGACTTCACCTTC-3', respectively). Cloned templates were generated by ligating DC6/Plob3 amplicon into the pGEM-T Easy vector (Promega, Madison, WI). The resultant plasmids were transformed into α-Select Gold Efficiency competent cells (BioLine, Taunton, MA), purified with the NucleoSpin Plasmid purification kit (Macherey-Nagel, Duren, Germany), and sequenced from the SP6 and T7 primer sites located on the vector and the three internal sequencing primers. Sequencing was performed using the BigDye Terminator 3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA) on an Applied Biosystems 3130xl Genetic Analyzer (Applied Biosystems). Sequences were assembled using Sequencher 4.1 software (Gene Codes Corp., Madison, WI). Unique sequences were identified using DNACollapser (<http://users-birc.au.dk/biopv/php/fabox/dnacollapser.php#>) to generate a reduced dataset, which was aligned using the ClustalX algorithm implemented in CLC Main Workbench v.6.8.1, with ends of the alignment trimmed so that all sequences were equal length. The alignment was used to construct a median joining network in SplitsTree v.4.12.3 (Huson and Bryant 2006).

Endpoint SNP genotyping. To develop custom TaqMan 5'-nuclease endpoint assays to genotype *P. obducens* samples, four single nucleotide polymorphisms (SNPs) were identified from the rDNA nucleotide sequence alignment (Table 2). The assays were developed by first identifying seven SNPs across the rDNA sequence alignment that were diagnostic for G1, G2, or G3 rDNA groups (identified from cloned rDNA amplicon sequences), and then selecting the minimal number of SNPs that provided the unique genotype sufficient to characterize the rDNA group. At each of the SNP sites, only two alleles were observed from the sequence dataset. Primers and minor groove binding probe pairs were selected using the Custom TaqMan Assay Design Tool (Life Technologies, Carlsbad, CA), with the hydrolysis probe pairs labeled with either 5'-FAM or 5'-VIC fluorophores. All

Table 2. Endpoint SNP genotyping assay primers and probes

Assay name	SNP ^a	Amplicon size (bp)	Forward (F) and reverse (R) primers (5' to 3')	Reporter 1 probe sequence ^b	Reporter 2 probe sequence ^c
IDM134	A/G	67	F-TGCGTTCAACGAGTATACAACTT R-CTAGCACGATGCGCATTCAC	AAGATTACCCAGATCTATCG	ATTACCCAGACCTATCG
IDM498	A/G	101	F-CGTGAACCGTTTCAACCAAATAGT R-CCACTTCACTATGATAGGACTCGTCTA	CAGCTGCTAACTTTAA	AGCTGCTGACTTTAA
IDM604	A/T	104	F-GGACATTATAGTCCGAGCTAGTAGTA R-GCTGAAAGTTGCTATCTAGTTAAAAGCA	TCCCCACAGTATAATCAGTA	TCCCCACAGTATATTCAGTA
IDM1020	A/T	80	F-CGGCGACCAGTTTGTGTTGC R-GCGCATTGTTAGCCGAACTAAC	ACCGCAAATAAAATAC	ACCGCAAATTAATAC

^a Nucleotides present at SNP identified based on sequencing cloned rDNA amplicons from *Plasmopara obducens*.

^b Reporter probe labeled at the 5' end with 6-carboxyfluorescein (FAM) and labeled at the 3' end with a nonfluorescent quencher (NFQ). Diagnostic single nucleotide polymorphisms (SNPs) within probe sequences are identified in bold. Note all probe sequences, except assay IDM498, are listed in reverse orientation (i.e., 3' to 5') relative to the primers.

^c Reporter 2 probes were labeled at the 5' end with the VIC fluorophore and labeled at the 3' end with NFQ. Diagnostic SNPs within probe sequences are identified in bold. Note all probe sequences, except assay IDM498, are listed in reverse orientation (i.e., 3' to 5') relative to the primers.

probes were labeled with a 3' nonfluorescent quencher attached to a minor groove-binding moiety. Polymerase chain reaction amplifications were performed in white half-skirted 96-well LightCycler 480 Multiwell plates sealed with clear adhesive film (Roche Diagnostics, Indianapolis, IN) on the Roche LightCycler II 480 instrument (Roche Diagnostics). Negative controls (nuclease-free water) and positive controls (samples of known genotype, as determined through Sanger sequencing) were included on all plates. All samples were analyzed at least twice, and two or more biological replicates were included from 64 of the sample sites. Thermal cycling was performed in total volume of 10 µl containing 5 µl of 2× LightCycler 480 Probes Master Mix (Roche Diagnostics), 0.5 µl of 20X SNP genotyping assay, 3.25 µl of PCR grade water and 1.25 µl of template DNA. Cycling was performed as follows: preincubation at 50°C for 2 min, followed by initial denaturation for 15 s at 95°C, with 35 subsequent cycles of amplification (denaturation at 95°C for 15 s, annealing/extension at 61°C for 20 s, and extension at 72°C for 1 s). Fluorescence signals were collected during the annealing step using the dual color hydrolysis probe detection format in the LightCycler 480 SW1.5 software. Data were analyzed using the endpoint genotypic analysis algorithm, with alleles called relative to the sequenced positive control samples. Positive assay results for allelic discrimination of the samples were determined when the endpoint scattergrams showed clearly separated clusters that were distinct from negative controls. Endpoint SNP genotypes were analyzed by scoring each of the four markers as homozygous (1, 1 or 2, 2), or heterozygous (1, 2) and combining the data into genotypes using GenAIEx v. 6.501 (Peakall and Smouse 2012). A genotype network was constructed from the four rDNA SNPs using the median joining option in Network software v.4.612 (www.fluxus-engineering.com; Bandelt et al. 1999).

Phylogenetic analysis. Nine downy mildew samples from *I. waleriana*, *I. balsamina*, and *I. noli-tangere* were selected for phylogenetic analysis. Sequence data were generated from the three molecular marker regions used by Görg et al. (2017), namely *cox2*, *cox2-1* spacer, and ITS. The ITS region was amplified using the DC6 and Plob3 primers from all twenty-first century samples (collected after 2004). Three samples from historical (collected prior to 1950) herbarium specimens failed to produce the approximate 1,000 bp amplicon using the DC6/Plob3 primers, which was a consistent result given known levels of degradation experienced by DNA in old samples (Verkley et al. 2015). Instead, the three known variant sites within the ITS region sequenced by Görg et al. (2017) were amplified using primers from the IDM498 and IDM604 genotyping assays, each producing approximately 100 bp fragments (Table 2). Two markers were amplified from the mtDNA for all nine samples. First, an approximate 300 bp portion of the mitochondrial cytochrome oxidase subunit 2 (*cox2*) gene was amplified using the CoxFn (Hudspeth et al. 2000) and CoxRc4 (Choi et al. 2015) primers. Second, a region downstream of *cox2* (*cox2-1* spacer) was amplified using forward (5'-GGTCAATGTAGTGAGATCTGTG-3') and reverse (5'-GTTGAGAAAAGCCAACGAGTTGAC-3') primers, which were modified from those used in Görg et al. (2017) to account for poor amplification of the herbarium specimens.

PCR amplifications were performed using 25 µl reactions containing 12.5 µl MangoMix (Bioline, Taunton, MA, USA), 2 µl of each primer (10 µM), 1 µl MgCl₂ (50 mM), 5.5 µl PCR-grade water, and 2 µl template DNA. Thermal cycling was performed by 5 min denaturation at 95°C, followed by 30 cycles of 1 min at 95°C, 1 min at annealing temperature, 1 min at 72°C. A final extension was performed for 10 min at 72°C. All reactions used an annealing temperature of 55°C except the *cox2-1* spacer, which used an annealing temperature of 52°C. *Cox2* and *cox2-1* spacer amplicons were directly sequenced using the forward and reverse primers used in the initial PCR reaction. To account for heterozygosity in the region, the ITS amplicons were cloned using the TOPO TA cloning kit with the pCR 2.1-TOPO vector (Life Technologies, Carlsbad, CA) and One Shot TOP10 Chemically Competent cells following manufacturer's instructions. Cloned ITS amplicons were sequenced from plasmid DNA using the M13-F and M13-R primers. Sequencing was performed using the BigDye Terminator 3.1 cycle sequencing

kit (Applied Biosystems, Foster City, CA) as described in previous sections.

Chromatograms were processed and sequences were assembled into contigs using Geneious v10.2.3 (Kearse et al. 2012). Sequence data from 11 additional downy mildew samples on *Impatiens* spp. covering the same three molecular markers were obtained from NCBI GenBank (Görg et al. 2017; accessions MF372718 to MF372750) and from the reference genome of *Plasmopara viticola* isolate INRA-PV221 (assembly ASM169559v1). For each marker, sequences were aligned using MUSCLE (Edgar 2004) and viewed in AliView v1.18.1 (Larsson 2014) to evaluate alignment quality. Internal gaps were left in the alignment and treated as missing data in downstream analysis. Alignments from the three markers were concatenated using FaBox (Villesen 2007; <http://users-birc.au.dk/biopv/php/fabox/>).

A maximum likelihood phylogenetic tree was constructed from the concatenated alignment using RAxML v8.0.0 (Stamatakis 2014). Different substitution models were evaluated using jModelTest 2 (Darriba et al. 2012) and selected based on Akaike information criterion values and the ability to use a given model in RAxML. Tree construction used a general time reversal model with gamma distributed rate variation among sites and estimate of invariable sites (GTRGAMMAI in RAxML) and 100 bootstrap replications. Trees were visualized using the program FigTree v1.4.3 (<http://tree.bio.ed.ac.uk/software/figtree/>) and the R package APE (Paradis et al. 2004; R Development Core Team 2015).

Data and resource curation. Fifty-four herbarium voucher specimens corresponding to new *P. obducens* samples collected in this study were deposited in the U.S. National Fungus Collections (BPI). DNA sequences from cloned amplicons generated from the IDM498 and IDM604 assays from the three historical herbarium samples were not deposited in NCBI GenBank as they do not meet the minimum length requirements of 200 nucleotides; these sequences are provided in the Supplementary Fig. S1. All other nucleotide sequences have been deposited in GenBank (accession numbers MG805315 to MG805338).

Results

Ribosomal DNA sequencing of *P. obducens* from fresh collections on *impatiens*. Thirty-seven samples of *P. obducens* collected between 2007 and 2013 were assessed by Sanger sequencing of the DC6/Plob3 rDNA PCR amplicons, which corresponded to a portion of the partial 18S, ITS1, 5.8S, and ITS2 regions of the nuclear rDNA. PCR amplification yielded products of approximately 1,100 bp when visualized on agarose gels (data not shown). Sanger sequencing reactions primed directly from the DC6/Plob3 rDNA amplicons produced chromatograms with mixed peaks at some nucleotide sites (data not shown). These mixed peaks might have been due to allelism and/or the presence of more than a single individual genotype in the sample. Therefore, all subsequent nucleotide sequencing data from nuclear loci reported in this study were generated from cloned amplicons.

A total of 150 DC6/Plob rDNA clones were sequenced from the 37 *P. obducens* samples, as summarized in Supplementary Table S3. BLASTn searches against NCBI GenBank databases using the most common rDNA haplotype (H30 haplotype, *n* = 54) as the query showed that the sequence shared 99 to 100% identity with specimens of *P. obducens* (DQ665666, EF553506 and KF366378 to KF366380; Crouch et al. 2014; Voglmayr and Constantinescu 2008), and 95% identity with a voucher specimen of *P. nivea* (EF553507; Voglmayr and Constantinescu 2008). Relatively high levels of nucleotide identity (88 to 92%) were also observed between the *P. obducens* rDNA amplicon sequences and other oomycete genera that cause downy mildew diseases, including *Bremia lactucae* (e.g., DQ235794), *Hyaloperonospora parasitica* (e.g., AY211008), *Novotlenovaea scorzonerae* (KT249142), *Peronospora belbahii* (KJ960193), and *Pe. pulveracea* (FJ384778; Choi and Thines 2015; Choi et al. 2003; Petželová et al. 2015; Warfield et al. 2009).

Seventy-two unique rDNA haplotypes were identified from the 150 *P. obducens* clone sequences. Despite the fact that 50% of the

clones possessed unique haplotypes, nucleotide diversity was low among the *P. obducens* samples. With only one exception, pairwise comparisons between the 72 *P. obducens* rDNA haplotypes showed a shared nucleotide identity of 98.8–99.9%. The sole exception, haplotype H56, corresponding to *P. obducens* sample H12_194P1 clone 1, shared only 93.4–94.1% nucleotide identity with the other *P. obducens* haplotypes. For the 26 *P. obducens* samples where two or more clones were sequenced, there was variation between cloned sequences. For example, sample CTIDM3, from which nine clones were sequenced, produced eight haplotypes (H19, H48 to H54) that differed from one another by 2 to 10 bp ($<0.01\%$ overall difference). From sample FL1, 27 clones produced just three haplotypes (H7, H30, H55) that differed by 3 to 11 bp ($\leq 0.01\%$ overall difference).

Cluster analysis of the *P. obducens* rDNA amplicon sequence dataset showed that the 72 unique haplotypes formed three main groups, which were designated G1, G2, and G3 rDNA groups (Fig. 1). Sequences in the G1 rDNA group were derived from 15 clones with 14 unique haplotypes. The G1 group clones originated from three locations: two in Costa Rica and one in the United States (Southern California). Sequences in the G2 rDNA group were derived from 104 clones and included 34 unique haplotypes. The G2 clones originated from 28 locations across the United States and one Australian location. Sequences in the G3 rDNA group were derived from 22 clones and included 15 unique haplotypes. The G3 clones originated from five locations in the northeastern United States. There were also nine haplotypes that fell at intermediate positions between rDNA groups G1, G2, and G3 (Fig. 1).

Not all of the DC6/Plob rDNA haplotypes from within individual locations fell within the same rDNA group. Nine locations yielded haplotypes that fell within the G2 group and at intermediate positions between the three main groups in the median joining network. Three locations yielded haplotypes that grouped with the G2 rDNA group and the G3 rDNA group (Fig. 1).

Endpoint SNP genotyping. To analyze rDNA profiles from 1,000 downy mildew samples, a set of custom TaqMan SNP genotype assays were developed to detect polymorphisms across the sequenced rDNA ITS region described above. The four SNPs selected for use in the endpoint SNP genotype assays are referred to as assays IDM134, IDM498, IDM604, and IDM1020 hereafter based on their position

Fig. 1. Median joining network constructed from 150 rDNA amplicon sequence data representing 72 unique haplotypes of *Plasmopara obducens sensu lato*. Each tip label represents a unique rDNA haplotype. The G1, G2, and G3 clusters represent rDNA groups and correspond to the G1, G2, and G3 rDNA genotypes.

or earlier) (G3 and G4, Fig. 2). Approximately 40% of the G3 rDNA genotypes were from historical *P. obducens* samples. One of the five samples with the G4 rDNA genotype was also observed from a historical sample. Overall, the presence of only two genotypes in

historical samples highlighted the increase in the number of *P. obducens* rDNA genotypes recovered from modern samples.

The different genotypes also displayed little evidence of differentiation based on host origin. Aside from the recovery of the nine

Table 3. Endpoint rDNA SNP genotypes generated at four nucleotide sites within the nuclear ribosomal DNA from *Plasmopara obducens* and related downy mildew species in *Plasmopara* and *Bremiella*

Species ^a	Samples ^b	SNP genotype ^c	rDNA genotype ^d	IDM134 ^e	IDM498 ^e	IDM604 ^e	IDM1020 ^e	Het. Pos. ^f
<i>P. obducens</i>	741	F	G2	AG	GG	AT	AT	3
<i>P. obducens</i>	148	A	G3	GG	GG	AA	TT	0
<i>P. obducens</i>	69	G	G7	AG	GG	AT	AA	2
<i>P. obducens</i>	9	E	G1	GG	AA	AA	AT	1
<i>P. obducens</i>	5	D	G4	GG	GG	AA	AT	1
<i>P. obducens</i>	2	J	G9	AA	GG	TT	AA	0
<i>P. obducens</i>	1	4	G10	GG	AA	AA	AA	0
<i>P. obducens</i>	1	2	G5	GG	AG	AA	AT	2
<i>P. obducens</i>	1	6	G6	AG	AG	AT	AT	4
<i>P. obducens</i>	1	5	G8	AG	GG	AT	TT	2
<i>Peronospora alsinearum</i> (n=1)	7	C		GG	GG	AT	AT	2
<i>P. constantinescui</i> (n=2)								
<i>P. obducens</i> (n=4)								
<i>P. constantinescui</i> (n=2)	3	B		GG	GG	TT	AT	1
<i>P. obducens</i> (n=1)								
<i>B. megasperma</i>	3	K		AA	GG	AT	AA	1
<i>B. megasperma</i>	2	I		AA	GG	AT	AT	2
<i>B. megasperma</i> (n=1)	2	H		AA	GG	TT	AT	1
<i>P. obducens</i> (n=1)								
<i>P. constantinescui</i>	2	3		GG	GG	AT	TT	1
<i>P. constantinescui</i>	1	1		GG	AA	AT	TT	1
<i>B. megasperma</i>	1	7		AA	GG	AA	AT	1
<i>B. megasperma</i>	1	8		AA	GG	AA	TT	0

^a Taxonomic classification of original sample.

^b Number of samples out of 1,000 total classified to the genotype in SNP genotype column.

^c Genotype label output from the GenAlEx genotyping analysis corresponding to the four SNPs in columns labeled with e.

^d Reclassification of SNP genotypes to correspond to the G1, G2, and G3, rDNA haplotypes. G4 to G10 do not correspond to rDNA groups, but represent SNP genotypes for samples originally classified as *P. obducens*.

^e One of four targeted SNPs, with letters indicating homozygosity (e.g., GG) or heterozygosity (e.g., AG) corresponding to the genotype in the column labeled with c.

^f The total number of the four SNPs that display heterozygosity.

Fig. 2. Median joining network constructed using SNP genotypes from 1,000 downy mildew samples generated from four individual SNP genotyping assays. Network topology reflects output from the program Network, while size of individual nodes are rescaled for readability.

Phylogenetic analysis. A phylogenetic analysis was performed using sequence data from the ITS, *cox2*, and *cox2*-1 spacer regions of the nuclear and mitochondrial genomes of nine *P. obducens*

One sample from *I. noli-tangere* (BPI871273), collected from South Korea in 2005, did not fall within any of the four clades (Fig. 3). None of the individual gene trees constructed from the ITS, *cox2*, and *cox2-1* datasets could independently resolve all three species described by Görg et al. (2017) into well supported phylogenetic clades (Supplementary Fig. S2).

Discussion

The primary question of this research was to determine if the downy mildew pathogen causing the recent twenty-first century IDM epidemics on *I. walleriana* is the same organism that was described from historical herbarium records from multiple noncultivated species of *Impatiens* (e.g., *I. biflora*, *I. palida*, *I. fulva*, *I. noli-tangere*). As modern IDM samples were infected with all 10 of the *P. obducens* rDNA genotypes and historical IDM samples were infected with only two of these 10 major rDNA genotypes (G3 and G4), the increased presence of the most dominant and widespread IDM rDNA genotypes (e.g., the heterozygous G2 and G7) after 2004 may represent the primary source of genetic variants of the pathogen responsible for twenty-first century IDM epidemics. The homozygous G3 rDNA genotypes found on the majority of the historical samples worldwide were also routinely recovered in modern samples of the disease affecting *I. walleriana* collected in the northeastern region of the United States. These findings indicate that even though certain rDNA genotypes are only found in samples from the recent epidemic affecting *I. walleriana*, downy mildew pathogens historically documented on *Impatiens* spp. in the United States and globally still live in the environment alongside the more contemporary genotypes.

The evidence that modern *P. obducens* infecting *I. walleriana* is genetically different from historical herbarium specimens of *P. obducens* shares similarities with other downy mildews in the Peronosporaceae and has implications for managing these pathogens. Within the genus *Plasmopara*, modern *P. halstedii* infecting *Rudbeckia* spp. are genetically differentiated from historical herbarium specimens of *P. halstedii* infecting this same host species (Rivera et al. 2016b). Similarly, the re-emergence of cucurbit downy mildew in the United States caused by *Pseudoperonospora cubensis* is likely due to the displacement of historical pathogen genotypes by emerging genotypes not previously found in the United States (Thomas et al. 2017). Overall, the significant shifts in pathogen genotypes associated with the emergence or re-emergence of downy mildews on known and novel hosts highlights the importance for continued monitoring of genetic variation among these pathogens. Further integration of those data with research on the life-cycle of these pathogens will refine our understanding of disease epidemiology and contribute to the development of robust disease management strategies.

The secondary question of this work was to evaluate evidence for host specificity among downy mildews infecting *Impatiens* species. Host specificity among pathogens causing downy mildews on other plant host species is documented from several studies based on phylogenetic affinities (Choi and Thines 2015; Choi et al. 2015; Göker et al. 2007; Rivera et al. 2016b; Rouxel et al. 2013, 2014; Runge et al. 2011; Thines and Choi 2016) and was recently used as a basis for the description of two new species of *Plasmopara* as specific to *I. balsamina* and *I. walleriana*, distinct from the species originally described from *I. noli-tangere* (Görg et al. 2017). Based on the recovery of all three of the most common rDNA genotypes from IDM samples collected from *I. balsamina*, *I. capensis*, and *I. walleriana*, we found little evidence for host specificity on those species in the present study. The only exception was the G1 rDNA genotype, which was only identified from *I. walleriana*. However, the small number of samples ($n = 9$) encompassed by the G1 rDNA genotype limits our ability to confidently assert its host specific infection of *I. walleriana*.

The recovery of the same *P. obducens* genotypes from multiple *Impatiens* species and different pathogen genotypes from the same host species highlights a lack of host specificity. Although downy mildews are often regarded as highly host-specific pathogens, this specificity is not always found at the host species level. For example, grape downy mildew caused by *Plasmopara viticola* can be found on

different species of cultivated and native *Vitis* (Farr and Rossman 2018). In addition, isolates from different phylogenetic clades within *P. viticola* show variation in the number of different *Vitis* spp. they infect, and representatives from different phylogenetic clades can be recovered from the same host species (Rouxel et al. 2013, 2014). Considering these observations, inferring host specificity of poorly understood downy mildews like *P. obducens* should be done with caution and only when an appropriate sampling of the pathogen has been performed that includes observations over multiple years and from multiple geographic locations.

A supplementary analysis was added to this work to determine how conclusions drawn from rDNA genotyping data compare with recent phylogenetic studies that suggested high levels of host specialization among different phylogenetic clades in *P. obducens*. Sequencing of rDNA and two molecular markers from the mtDNA *cox2* region provided evidence that only the infection of *I. noli-tangere* species appears to be limited to an individual phylogenetic clade of *Plasmopara*, which includes only isolates from the G3 rDNA SNP genotype (Fig. 3, Görg et al. 2017). As an individual sample from this host did not fall within the primary clade, future phylogenetic studies would need to include a greater number of samples from *I. noli-tangere*. Infection of the remaining *Impatiens* species (*I. balsamina* and *I. walleriana*) is not limited to any phylogenetic group of *Plasmopara*. In particular, we found that samples with similar genotypes as the recently described species *P. destructor* are present on both *I. walleriana* and *I. balsamina*. *Plasmopara* genotypes originally harvested from *I. walleriana* plants were capable of causing infection on plants from other species of *Impatiens*, including *I. arguta*, *I. auricoma*, *I. capensis*, *I. flanaganiae*, and *I. omeiana*. In addition, we identified a new sister clade (G1 rDNA genotypes) to *P. destructor* that is also capable of infecting *I. walleriana*. Taken together, these results demonstrate the species described as *P. destructor* is not limited to *I. walleriana* and that a second phylogenetic clade of downy mildew pathogens is also found on this host. These results further demonstrate a lack of host specificity of isolates from modern collections of IDM on *I. walleriana* and *I. balsamina*, and conflict with the conclusions of Görg et al. (2017), who used this trait as one of three criteria (the other two being morphological and phylogenetic differences) to describe these as two novel species of downy mildews.

Aside from potential inconsistencies regarding the proposed host specificity of the new *Plasmopara* species on *I. walleriana* and *I. balsamina* described above, the conclusions regarding species-level diversification on *Impatiens* sp. based on morphological characteristics warrant additional consideration. Although significant differences were reported based on sporangia and sporangiophore morphology among the three proposed *Plasmopara* species when large numbers of characters were measured (Görg et al. 2017), these differences may simply be due to the recovery of the downy mildew pathogens from different host species, or a result of plasticity due to variable environmental conditions, especially given the wide range of measurements recorded and the extensive overlap of characteristics observed (Runge and Thines 2011). In addition, morphological characterizations did not include the type specimen of *P. obducens* described in the 1800s (Schröeter 1877), which limits the power of the measurements used to differentiate *P. obducens* from *P. destructor* and *P. velutina*. Indeed, measurements obtained from the original *P. obducens* syntype collections from *I. noli-tangere* made by Schröeter and archived at the U.S. National Fungus Collection in Beltsville, MD shows similarities in morphological characteristics across all of the proposed *Plasmopara* species on *Impatiens* (Rabenhorst Fungi Europaei #2344 exsiccata collection, data available upon request). Broadly overlapping ranges from the surveyed morphological characters effectively precludes the unambiguous differentiation of samples as distinct species (Görg et al. 2017), and would render any type of confident species-level identification within a nonmolecular setting such as a disease diagnostic clinic impossible. Considering this information, and in combination with observed inconsistencies in host association criteria, the recent taxonomic differentiation of *P. obducens* and other proposed *Plasmopara* species from *Impatiens*

may not be practically aligned with biologically significant groups. This is compounded by the fact that only the combined ITS/cox2/cox2-1 spacer phylogeny consistently provides support for differentiation of *P. obducens* s. str., *P. destructor*, and *P. velutina*. With the exception of the G1 rDNA genotypes from *I. walleriana*, individual gene trees provided only limited support for additional species-level subdivisions, although species-level hypotheses were not refuted by these analyses. Taken together, these data suggest a more conservative approach for species delimitation may be warranted for this group of organisms (Carstens et al. 2013) until a more comprehensive multilocus phylogenetic analysis can be completed.

This research also raises some important methodological considerations for studying genetic variation of downy mildews. Although allelic variation from nuclear loci is not typically addressed in molecular phylogenetic studies of the Peronosporaceae, including the recent taxonomic investigations of *Plasmopara* on *Impatiens*, our findings show that the diploid nature of these organisms needs to be explicitly addressed in order to eliminate potential sources of error. In the present study, accounting for heterozygosity within the rDNA using cloned rDNA amplicons and endpoint genotype assays showed that the rDNA region is heterozygous at several nucleotide sites in several genetic groups of *P. obducens* s.l. and three other species evaluated in this study: *B. megasperma*, *Pe. alsinearum*, and *P. constantinescui*. Of the 13 unique *P. obducens* rDNA genotypes observed from the four SNP endpoint genotype profiles, only three rDNA groups were homozygous at all four sites (G3, G9, G10; $n = 151$). The remaining 10 *P. obducens* rDNA groups, comprising 833 samples, would not have been accurately discriminated without incorporating allelic variation into the analyses, as these groups were heterozygous at least one of the four SNPs. The presence of multiple haplotypes from individual leaf samples also raises questions as to the nature of host infection by *P. obducens*. One explanation is that the samples represent a single *P. obducens* individual that displays variation across the different copies of rDNA loci in its nuclear genome. Such intragenomic variation in multiple copy rDNA loci has been observed in other eukaryotes (Bik et al. 2013; Simon and Weiss 2008). An alternative explanation is that individual plant samples are infected with more than one *P. obducens* individual. Future work will need to employ methods that provide information on the frequency of different alleles and ploidy of the pathogens to resolve these issues.

This work provides the first analysis of genetic variation among large populations of the pathogen causing downy mildew on *Impatiens* species, with particular focus on samples from recent IDM epidemics in the United States and historical downy mildew samples on noncultivated *Impatiens* species. Overall, the genetic variation of these organisms highlights the presence of genotypes commonly associated with recent epidemics, but not present in historical herbarium specimens. This observation provides evidence that the genetic variants of the pathogen in modern samples may be responsible for the twenty-first century epidemics. Despite this evidence, the rDNA SNP genotyping and complementary phylogenetic analysis performed herein are relatively low-resolution and likely fail to fully capture genetic variation across downy mildew pathogen genomes. Future research should apply higher resolution genotyping methods (e.g., simple sequence repeat markers or genotype-by-sequencing) to measure genetic variation among the organisms causing downy mildew on *impatiens*. Polymorphic simple sequence markers mined from the genome of *P. obducens* have been developed, and their application may provide the resolution required to go beyond single genetic loci to model population processes and divergence (Salgado-Salazar et al. 2015). Performing these higher resolution genotyping methods will provide further insight into the evolution of these destructive pathogens and may contribute to the development of IDM disease control strategies.

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