

Fungarium phylogenomics reveals high species diversity in graminicolous downy mildews and upholds the distinction between *Peronosclerospora philippinensis* and *P. sacchari*

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Abstract: Graminicolous downy mildew (GDM) diseases threaten staple food crops such as corn, millets, sorghum, and sugarcane, making accurate pathogen identification critical for global food security and quarantine enforcement. However, identification is hindered by inconclusive morphological characters and unreliable host associations. Progress using molecular data is also challenging; as obligate pathogens, reference materials are often limited to historical fungarium specimens, which contain highly degraded pathogen DNA inextricably embedded within host tissue. To overcome these challenges, we used an oomycete-specific target enrichment protocol to generate high-throughput DNA sequence data for 429 orthologs for 57 fungarium specimens, including 17 types, to construct a phylogeny from 62 ortholog loci. The inclusion of type materials upheld the critical distinction between the high-risk plant pathogens *Peronosclerospora philippinensis* and *P. sacchari* and validated most historical species concepts. However, the data also revealed widespread taxonomic confusion, showing that nearly half of the examined specimens were misidentified. Critically, in three instances, presumed isolates were not conspecific with their nomenclatural types. Based on these results, we reduce *Sclerospora northii* to a synonym of *P. philippinensis*, propose the new combination *Sclerophthora isilematis*, and identify 12 distinct lineages that may represent undescribed species. This study demonstrates that GDM diversity is substantially greater than previously recognized and empirically confirms that anchoring molecular data to nomenclatural types is a critical prerequisite for building a stable taxonomy and developing accurate diagnostic tools for this group.

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INTRODUCTION

Graminicolous downy mildews (GDMs) are diseases caused by obligate plant pathogens that infect important agronomic crops of corn, millets, sorghum, sugarcane, and other *Poaceae* hosts (cereals and grasses) (Kenneth 1981, Spencer & Dick 2002). The GDM pathogens are members of the *Peronosporaceae* (*Oomycota*, *Peronosporales*) and belong to eight different genera, including three genera responsible for economically important crop diseases in many parts of the world: *Peronosclerospora*, *Sclerophthora*, and *Sclerospora* (Crouch *et al.* 2022). In tropical and sub-tropical regions of the world, yield losses of 40–100 % due to GDM diseases have been reported from staple food crops such as corn, pearl millet, and sorghum (Frederiksen & Renfro 1977, Payak 1975, Senanarong 1975).

Phytosanitary and quarantine measures have been established in many parts of the world to prevent the introduction of foreign GDM pathogens that pose a high risk to the health of commodity agricultural crops such as corn

and sugarcane. For example, the United States (USA) regulates two GDM pathogens—*Peronosclerospora philippinensis* and *Sclerophthora zeae* (formerly *Sclerophthora rayssiae* var. *zeae*)—as Select Agents (<https://www.selectagents.gov/sat/list.htm>) due to the severe threat that they pose to agriculture. *Peronosclerospora philippinensis*, the cause of Philippine downy mildew disease of corn (*Zea mays*) and other cereal crops is subject to phytosanitary regulations as an invasive pest in China, Mexico, Morocco and in regions of Europe (Crouch *et al.* 2022). *Sclerophthora macrospora* and *Sclerospora graminicola*, destructive pathogens of corn and pearl millet (*Pennisetum glaucum*), respectively, are recognized as plant pathogens of significant concern in some regions of the world and often handled under quarantine regulations.

Accurate pathogen identification is required for phytosanitary measures to be effective. Understanding the taxonomy of the GDM pathogens to underpin accurate identification has been an ongoing objective since the first major disease outbreaks affecting corn, sugarcane, and related

plants in the tropics in the early 20th century (Frederiksen & Renfro 1977). GDM pathogens were traditionally identified using asexual morphology and host range (Frederiksen & Renfro 1977, Crouch *et al.* 2022). This approach has led to confusion, as many of the GDM pathogens are distinguished from one another through minor morphological differences that may not provide definitive identification (Spencer & Dick 2002, Telle *et al.* 2011, Crouch *et al.* 2022). For some taxa, the diagnostic morphological structure may be absent, or in the case of the conidiophores, exist only for a few hours (Spencer & Dick 2002). This is further confounded by the fact that the morphology of *Peronosporaceae* is influenced by environmental conditions (Leu 1973, Dudka *et al.* 2007) and plant host (Runge & Thines 2011). Host association is another common method used to identify species causing GDM diseases, based on the hypothesis that the GDMs have a very narrow host range, as observed for other downy mildew pathogens (Gäumann 1918, Gäumann 1923, Gustavsson 1959). However, ongoing research of the downy mildews that parasitize *Poaceae* and other hosts is increasingly demonstrating how misleading host range is for downy mildew identification, with some hosts parasitized by several species, and some downy mildew species able to infect several hosts (*e.g.* Thines *et al.* 2008, Davis *et al.* 2020, Suharjo *et al.* 2020, Davis & Crouch 2022, Salgado-Salazar *et al.* 2023, Mu *et al.* 2024). For the GDM pathogens in particular, reliance on host identity to determine pathogen identity is unlikely to yield reliable conclusions. For example, *Sclerophthora macrospora* has been reported from 141 *Poaceae* hosts, and *Sclerospora graminicola* has been reported from 13 different host genera (Crouch *et al.* 2022). Conversely, corn has been reported as a host for 11 GDM species from three genera: *Peronosclerospora*, *Sclerophthora* and *Sclerospora* (Crouch *et al.* 2022).

Molecular sequence analysis has proven instrumental in advancing GDM taxonomy and identification, particularly for newly collected species and genera; however, its efficacy in resolving complex issues pertaining to older, established taxa remains a significant challenge (Thines & Choi 2016, Crouch *et al.* 2022). Great successes have been made for newly collected species and genera with distinct evolutionary trajectories and unique morphological characters that set them apart from taxa described from the late 19th century until early 20th century (Ryley & Langdon 2001, Telle *et al.* 2011, Shivas *et al.* 2012, Thines *et al.* 2015, Ryley *et al.* 2022). Novel organisms in genera such as *Baobabopsis*, *Eraphthora*, *Graminivora*, *Poakatesthia*, and *Viennotia* are readily distinguished based on morphology and phylogenetic relationships inferred from one or two DNA markers, especially the *cox2* and ITS (Göker *et al.* 2003, Thines *et al.* 2006, 2007, 2008, Telle & Thines 2012, Shivas *et al.* 2012). Outstanding taxonomic issues facing older taxa—especially *Peronosclerospora*, *Sclerospora* and *Sclerophthrora*—are less easily solved, even with molecular approaches. While methodological advances have enabled the molecular barcoding of some old oomycete specimens (Telle & Thines 2008), resolving outstanding taxonomic issues for older taxa, particularly *Peronosclerospora*, *Sclerospora*, and *Sclerophthrora*, often remains challenging. Indeed, the successful amplification demonstrated by Telle & Thines (2008) required significant effort, involving testing 19 different reactions per sample with 15 different Taq polymerases, and not all attempts

were successful; for example, their oldest sample from 1878 yielded the fewest amplifications, underscoring the labor-intensive nature and inherent difficulty for older specimens. The obligate biotrophy of the GDM pathogens means that types and other important reference specimens are limited to finite quantities of non-living fungarium materials that can be 100–150-yr-old, often completely embedded within the plant host. Fungarium preservation methods are very damaging to DNA, resulting in highly degraded and fragmented DNA recalcitrant to PCR amplification (*e.g.* Osmundson *et al.* 2013). Taken together, these factors impede attempts to unravel complex taxonomic issues and connect contemporary GDM pathogens with established types.

Target enrichment approaches are increasingly used to improve the efficiency of obtaining DNA sequence data from multiple targeted genome regions suitable for phylogenetic analyses from sub-optimal samples such as fungarium specimens (*e.g.* Jones & Good 2016, Grewe *et al.* 2020, Nguyen *et al.* 2021, 2024). In this study, we generated high-throughput DNA sequence data using a target enrichment approach for 63 fungarium specimens, including 17 types, to clarify the taxonomy and identification of the GDM pathogens.

MATERIALS AND METHODS

DNA extraction and sequencing

The 63 fungarium samples used in this study, including 17 type specimens, are listed in Table 1. Small fragments of infected fungarium samples from the U.S. National Fungus Collections (BPI) were removed from the specimens using a sterilized scalpel and DNA was isolated using the QIAGEN DNeasy Plant Mini kit (QIAGEN, Germantown, Maryland, USA). For fungarium specimens from the Canadian National Mycological Fungarium (DAOM), two 2 mm biopsy punches (RBP-20 Robbins Instruments, New Jersey, USA) were used to excise infected regions of plant tissue. To mitigate the risk of contamination, particularly from contemporary samples, fungarium specimens were handled within a biosafety cabinet or laminar flow hood in laboratory facilities where living samples of the target species had never been processed. DNA was extracted using a Macherey-Nagel NucleoMag® 96 Trace kit (Macherey Nagel GmbH & Co. KG, Düren, Germany) following the user manual published on May 2012/Rev. 02 using a KingFisher Flex magnetic particle processor (Thermo Fisher Scientific Oy, Yntaa, Finland). Modifications to the protocol were as follows: prior to extraction samples were homogenized using liquid nitrogen and sterile disposable micro-centrifuge tube pestles (PES-15-B-SI Axygen, Corning, New York, USA), and DNA was suspended in 70 µL of elution buffer. Metadata for three *Peronosclerospora* genome sequence datasets used in the phylogenetic analysis are also given in Table 1. Genome sequence data was generated from two samples of *Peronosclerospora* spp. stored as frozen conidia (−20 °C) at USDA-ARS Fort Detrick, using Illumina sequencing technology following the protocols described in Fletcher *et al.* (2018); a full paper describing these genomes is forthcoming. The genome assembly of *P. sorghi* P6 (Fletcher *et al.* 2023; NCBI BioProject PRJNA845776) and

data from *Phytophthora sojae* DAOM BR 1079 (SRX9101188) generated from an oomycete-specific target enrichment approach (Nguyen *et al.* 2021) were also included in the analyses. *Phytophthora sojae* was included to outgroup root the phylogeny.

Enrichment of DNA using an oomycete-specific hybridization-based enrichment bait set of 426 single copy ortholog targets (Ultra Conserved Elements, or UCEs) and three markers commonly used as barcodes for oomycetes (*cox1*, *cox2*, and β -tubulin) followed the myBaits hybridization capture for targeted next-generation sequencing (NGS) protocol (v. 4.01; Arbor Biosciences, Ann Arbor, Michigan, USA; <https://arborbiosci.com/wp-content/uploads/2018/04/myBaits-Manual-v4.pdf>), and was sequenced on an Illumina MiSeq (Illumina, San Diego California, USA) as described previously (<https://github.com/AAFC-BICoE/snake-make-partial-genome-pipeline>; Nguyen *et al.* 2021). The bait set is 19798 probes for 429 UCEs and were designed from four lineages of oomycetes: *Aphanomyces invadens*, *Phytophthora consensus*, *Pythium consensus*, and *Saprolegnia diclina* (Nguyen *et al.* 2021 Supplemental File 1). A comparison of the bait sequences showed an average percent similarity of 97.42 % to *Albugo laibachii* (from 16496 probe hits), 93.14 % to *Phytophthora sojae* (from 18897 probe hits), and 93.19 % to *Peronosclerospora sorghi* (from 12729 probe hits), indicating a broader specificity across oomycete groups, even though downy mildew lineages were not used in the original bait set design.

All data processing steps were performed on the USDA CERES SCINet server. Raw sequence data information was processed using the bioinformatics pipeline developed to analyze target enrichment data (Nguyen *et al.* 2021). Kraken v. 2.1.2 was used to filter out any sequence that originated from plants, bacteria, or fungi (Wood *et al.* 2019). To determine if there was off-target oomycete contamination, all sequences were aligned to *Peronosclerospora sorghi* (GCA_026184515.1), *Albugo laibachii* (GCA_902706625.1), and the outgroup *Phytophthora sojae* (GCA_000149755.2) using BLASTn (Altschul *et al.* 1990). To remove potential oomycete contaminants, sequences with a top BLASTn hit to known contaminants like *Albugo laibachii* were removed, while sequences with a top hit to a representative downy mildew (*Peronosclerospora sorghi*) or the designated outgroup (*Phytophthora sojae*) were retained for their respective analyses.

Because the genomes were already assembled from *Peronosclerospora* spp., the “harvest loci from genome” approach in PHYLUCE v. 1.67 was used for the genome data samples (Faircloth 2015). A fasta file of orthologs from the resultant datasets was used for phylogenetic analysis (<https://doi.org/10.15482/USDA.ADC/32177883>) with sequences individually aligned with MAFFT v. 7.402 (Katoh & Standley 2013) and trimmed using Gblocks 0.91b with default settings (Castresana 2000). Specimens with less than or equal to 100 orthologs were excluded from phylogenetic analysis with the exception of the type specimen BPI 187307 *Sclerospora northi* (n = 51) which was retained despite low data recovery due to its critical importance for resolving the status of this species name and the assembled genomes (n = 32-49). All data are available through the NCBI-GenBank Short Read Archive under accessions 58409512 to 58409575.

Incomplete data matrices were constructed using

PHYLUCE v. 1.67 (Faircloth 2015), based on the percentage of samples containing each ortholog, starting at 20 % (13 samples with same ortholog) and at every increment of 5 % until reaching 75 % (43 samples with same ortholog). For each data matrix the orthologs were concatenated into one alignment and phylogenetic analysis was conducted using the maximum likelihood approach via IQ-TREE v. 1.6.12 (Nguyen *et al.* 2015) with 1000 fast bootstraps and automatic model selection. The Bayesian Information Criterion was used to select the best model for maximum likelihood trees. It is important to note that due to the quality of DNA from historic specimens and the nature of the assembly process, some low complexity regions are present in the dataset, which may lead to artificially inflated genetic distances despite rigorous analytical methods.

RESULTS

Target enrichment and recovered sequences of targeted loci

On average 565,277 reads were sequenced per sample (range = 48213 to 5289428; median = 384976 reads). The average number of contigs generated from the four assembly methods was 93206 per sample (range = 5597 to 712519; median = 68319 contigs). The average number of orthologs recovered from the assemblies was 197.2, or 46.0 % of the total orthologs. After read decontamination, the remaining average ortholog per sample was 167.1, or 38.95 % of the total orthologs.

A summary of the target loci identified from each of the 63 fungarium specimens is listed in Table 1, and a table of each UCE identified to each specimen is in Supplemental Table S1. A total of 429 loci were targeted, comprising 426 oomycete-specific single-copy orthologs, plus three barcoding markers (*cox1*, *cox2*, and β -tubulin). Consistent with previous applications of this target bait set to degraded fungarium DNA, none of the three commonly used barcoding loci (*cox1*, *cox2*, and β -tubulin) were consistently recovered from the specimens (Nguyen *et al.* 2021). This outcome, despite the bait set’s broad oomycete specificity (as detailed in Methods) and the multi-copy nature of mitochondrial loci, primarily reflects the highly fragmented and degraded state of DNA in historical fungarium samples, where target fragments for these loci were likely too short for efficient hybridization capture, rather than a fundamental limitation of the enrichment approach itself. Seven target orthologs were not recovered (UCE 1, 59, 94, 211, 331, 341, 395). The remaining ortholog targets were recovered from 1 to 46 specimens, with each ortholog represented on average from 22.9 specimens.

Six of the 63 fungarium specimens yielded fewer than or equal to 100 orthologs and were excluded from subsequent analyses. The average number of orthologs recovered from the remaining 57 specimens were 165.9, corresponding to 38.7 % of the targeted loci. The largest number of orthologs recovered from an individual specimen was 241 (56.2 % of the targeted loci). Conversely, when evaluating the representation of individual orthologs across the dataset, no single UCE was recovered from 75 % or more of the specimens. The most frequently recovered UCE (UCE 221)

was present in 46 out of 63 samples (73.02 %), while the average ortholog was recovered only from 22.5 specimens (35.7 %) highlighting the challenge of consistent locus recovery across the dataset (Supplemental Table 1).

Phylogenetic analysis

The composition of the sequence alignments for each of the ortholog datasets was quite variable, with some of the ortholog targets only found in one or a limited number of specimens, while other targets were recovered from as many as 46 specimens. To determine the impact of missing ortholog data on downstream phylogenetic analysis, we constructed phylogenies using 12 datasets, ranging from 20 % to 75 % samples represented at 5 % increments (163 to 0 orthologs retained, respectively; <https://doi.org/10.15482/USDA.ADC/32177883>). Phylogenetic trees showed that datasets with 20–35 % samples represented (163 to 111 orthologs present) and 65–75 % samples represented (2 to 0 orthologs present) did not recover the genus-level separation of *Peronosclerospora* from *Sclerospora* which is well-established based on a preponderance of evidence derived from morphological characters and molecular data (Shaw 1978, Thines et al. 2008, Telle et al. 2011). In contrast, the tree topologies with 40–55 % of the sample represented (27 to 87 orthologs) provided genus-level resolution and were very similar to one another (<https://doi.org/10.15482/USDA.ADC/32177883>). This intermediate range was chosen as optimal because it provided the best balance between maximizing specimen inclusion and minimizing missing data, a necessary compromise given the degraded nature of DNA from historical fungarium samples. We therefore proceeded with phylogenetic analysis of the dataset with 45 % of the sample represented (62 orthologs retained).

The concatenated alignment of the 62 ortholog loci contained 3728 columns, 3230 distinct patterns, 1924 parsimony-informative sites, 856 singleton sites, and 947 constant sites (<https://doi.org/10.15482/USDA.ADC/32177883>). According to the Bayesian Information Criterion, the TIM3+F+G4 model—a transition model with unequal base pair frequency and a discrete Gamma model (Yang 1994)—was selected as the model best fitting the dataset.

Phylogenetic topology

Reading the tree topology (Fig. 1) from bottom to top, *Peronosclerospora* formed a well-supported monophyletic group (bootstrap = 100) that separated it from other GDM pathogens. Within the genus, three major subdivisions were observed. The first observed *Peronosclerospora* subdivision (bootstrap = 100, labeled as “A” on Fig. 1) corresponded with *P. sorghi*, comprising 16 specimens. The next major subdivision within the genus *Peronosclerospora* (bootstrap = 99.8; labeled “B” on Fig. 1) contained 12 members, including the lectotype specimens for *Peronosclerospora sacchari*, *P. philippinensis*, *Sclerospora northii*, and other specimens collected from corn and sugarcane.

Within *Peronosclerospora* group B there were two well-supported sub-groups. The first subgroup (labeled “B1” on Fig. 1; bootstrap = 99.6) included three specimens,

comprising the lectotype of *P. sacchari* BPI 187331 (sugarcane host), and three specimens collected from corn. With the inclusion of the *P. sacchari* lectotype, there is no doubt that subgroup B1 corresponds to *P. sacchari*. The second subgroup in *Peronosclerospora* group B (labeled “B2” on Fig. 1; bootstrap = 100) was a sister clade to *P. sacchari* and included five individuals: the lectotype of *P. philippinensis* BPI 187314, the lectotype for *S. northii* BPI 18707, two specimens of *P. philippinensis* from corn, and one specimen labeled as *P. sacchari* from sugarcane. The third major group of *Peronosclerospora* (labeled group “C” in Fig. 1; bootstrap = 89.9) in the phylogenetic tree comprised a diverse assemblage of four named species and seven unidentified specimens. Two specimens from miscanthus grass (*Miscanthus* sp.), including the neotype for *Peronosclerospora miscanthi* BPI 187301, formed a subgroup (labeled “C1” on Fig. 1; bootstrap = 99.6), confirming their identification as *P. miscanthi*. The isotype specimens of *Peronosclerospora maydis* (BPI 89413) from corn and *P. noblei* (BPI 187306) from wild sorghum (*Sorghum leocladum*) grouped together as a subgroup within *Peronosclerospora* group C (labeled “C2” on Fig. 1; bootstrap = 93.9). A third specimen collected from miscanthus and identified as *P. miscanthi* by the original collector (BPI 187304) clustered separately from the *P. miscanthi* neotype and grouped alongside BPI 187298 collected from corn (labeled C3 on Fig. 1; bootstrap=100).

The next phylogenetically distinct subdivision (bootstrap = 100, labeled as “D” on Fig. 1), was the sister taxon provisionally identified as *Sclerospora graminicola*, comprising two specimens from wild foxtail millet (*Setaria viridis*; BPI 187210, BPI 187261). The sister group to *Peronosclerospora/Sclerospora* (bootstrap = 98.4, labeled as “E” on Fig. 1), had multiple diverse lineages composed of four named genera and at least two lineages that likely represent undescribed genera (Fig. 1 “E”). Group E was further subdivided, into two lineages labeled as E1 and E2 on Fig. 1. Subgroup E1 was the lectotype of *Graminivora graminicola* BPI 786232. Subgroup E2 was a diverse clade of four specimens from the C3 grass smooth brome (*Bromus inermis*) and one specimen from wild foxtail millet, identified by the original collectors as *Sclerophthora macrospora* and *Sclerospora graminicola*, respectively. However, E2 was only distantly related to group G (below) where the *Scl. macrospora* neotype BPI 187265 was situated, indicating that the E1 specimens are not *Scl. macrospora*.

The isotype of *V. oplismeni* BPI 784264 was sister to the clade containing group E/*Sclerospora/Peronosclerospora* (bootstrap = 87.6) and was most closely related to *G. graminicola*. The clade sister to *V. oplismeni* (labeled “G” on Fig. 1; bootstrap = 93.3) contained six specimens, including the neotype of *Scl. macrospora* (BPI 187265), the holotype of *Scl. cryophila* (DAOM 20643), lectotype and isotype of *S. farlowii* (BPI 1987077, BPI 187077, respectively), and the lectotype of *Sclerospora isilematis* (BPI 187262). Group G was characterized by two main subclades (labeled “G1”, “G2” on Fig. 1). Subgroup G1 contained the neotype for the generic type *Scl. macrospora* and is unambiguously identified as the genus *Sclerophthora*. The lectotype of *Sclerospora isilematis* BPI 187262 clustered within G1 and was the sister taxon to the *Scl. macrospora* lectotype (bootstrap = 95.8). Subclade G2 (bootstrap = 93.3), containing the lectotype

and isotype of *Sclerospora farlowii* (BPI 187077, BPI 187078, respectively), was the sister clade to G1 *Sclerophthora*. Specimen DAOM 110575, was sister to group G with low bootstrap support of 22.6. The top-most group in the tree topology comprised of a diverse lineage of five specimens identified by the original collectors as *Scl. cryophila* (labeled as “H”, bootstrap = 100).

DISCUSSION

The primary aim of this research was to develop a molecular phylogenetic hypothesis that included the nomenclatural type specimens of several GDM pathogens, including some species where reliable diagnoses are challenged by inconclusive indicators, such as variable/overlapping morphology and host association. Using an oomycete-specific target enrichment approach to generate high-throughput DNA sequence data from 62 single copy orthologs, the phylogenetic tree generated in this study supported the recognition of 13 species names. Sequence data was generated from the nomenclatural types (lectotype/ holotype/ neotype) of 11 species for the first time. From these data, original morphology-based species concepts from the late 1800s through 1948 were upheld for all but two of the 11 species, with *S. northii* identified as a later synonym of *P. philippinensis*, and *S. isilematis* included in the genus *Sclerophthora* (Fig. 1). Given the known difficulties associated with GDM identification, the correspondence between the molecular data and the original species concepts is particularly noteworthy.

The genus *Peronosclerospora*, and *P. sorghi*

The distinction of *P. sorghi* from other *Peronosclerospora* species by molecular analyses agrees with previous research based on morphology, phylogenetic analysis of the *cox2* marker, isozyme phenotypes, and SSR fragment analysis (Bonde *et al.* 1984, Micales *et al.* 1988, Thines *et al.* 2008, Crouch *et al.* 2022). The *P. sorghi* clade included the neotype BPI 187336 collected from *Sorghum bicolor* (synonym = *Sorghum vulgare*) in India in 1915. This study is the first to molecularly connect the *P. sorghi* neotype specimen to modern collections, including the invasive populations of *P. sorghi* impacting cultivated sorghum production in the Southern USA (Frederickson *et al.* 1969, Frederickson 1980).

All but one of the specimens in the *P. sorghi* clade were collected from *Sorghum* spp. BPI 1791983 was collected from corn, consistent with reports that *P. sorghi* can infect this host (Craig 1983). Within *P. sorghi*, several subclades with high bootstrap levels were identified, including clusters of specimens collected from different regions of the world and collections made many decades apart (*i.e.* USA 2005 and Zimbabwe 1932; USA 2005 and India 1912). This clustering of diverse genotypes across geographic space and time between invasive populations impacting the USA in 2005 and older populations from India and Africa suggests that multiple, diverse genotypes of *P. sorghi* may have been introduced into the USA after the pathogen first entered the country in the 1960s. Future research aimed at understanding the evolution of *P. sorghi*, and how these diverse genotypes might correspond with newly emergent phenotypic races (Isakeit & Jaster 2005, Prom *et al.* 2015)

could provide valuable empirical insight into the factors that drove the expansion and establishment of *P. sorghi* populations worldwide.

Peronosclerospora philippinensis and *P. sacchari*

Our analysis provides the first molecular connection between the *P. sacchari* lectotype specimen to modern collections. Specimen BPI 187313, collected at the same time and country as the lectotype for *P. philippinensis* (1919, Philippines), and annotated by Weston as *P. philippinensis*, was recently listed as an isotype of *P. philippinensis* (Crouch *et al.* 2022) based on the identification/collection by Weston from the same general locale/date when *P. philippinensis* was described (Los Banos, Philippines; Weston 1920). However, based on the results of the current phylogenetic analysis, BPI 187313 is identified as *P. sacchari*.

This is first time molecular data has been used to connect the lectotype specimens and species concepts of *P. philippinensis* and *S. northii*. The clustering of these five specimens in *Peronosclerospora* subgroup B2 supported three important conclusions. First, based on the inclusion of the BPI 187314 lectotype specimen, subgroup B2 was unambiguously identified as *P. philippinensis*. Although subgroup B2 also included the lectotype specimen for *S. northii*, *P. philippinensis* was described in 1920, giving it priority over *S. northii*, which was not published until 1924 (Weston 1920, 1924). Second, the host origin of the five B2 subgroup members was consistent with the published host range of *P. philippinensis* as a parasite of both corn and *Saccharum* species, confirming that host range is of limited value for diagnosing the identity of this pathogen. And third, with the lectotypes of *P. philippinensis* and *P. sacchari* clustering in two separate clades, both taxa were upheld as distinct species, and are not conspecific as some authors have suggested based on morphology, shared host range, and isozyme phenotypes (Weston 1920, Bonde *et al.* 1984, Micales *et al.* 1988).

In addition to subclades B1 and B2, *Peronospora* group B included two specimens of *Peronosclerospora* (BPI 789414, BPI 1789417) that were not members of *P. sacchari* or *P. philippinensis*. Both specimens were collected from corn in the early 20th century, and identified by their collectors as *P. maydis*, the destructive pathogen responsible for Java downy mildew of corn. Based on their position in the phylogeny, BPI 789414 and BPI 1789417 were part of a larger clade that included *P. sacchari* (bootstrap = 78.5), suggesting that the specimens might be a part of that species. Since neither of these two specimens grouped with *P. maydis* isotype specimen BPI 789413 (part of *Peronosclerospora* group C, described below), and the affinity with *P. sacchari* is not well supported, these specimens are conservatively identified as *Peronosclerospora* sp.

Peronosclerospora group C: A diverse species assemblage

The phylogenetic results for Group C reveal a complex mosaic of both confirmed species and widespread historical misidentifications, highlighting it as a hotspot of taxonomic confusion. Similar reports of high species

diversity in the *Peronosclerospora* have been made from Australian grasses (Ryley et al. 2022). Two specimens from miscanthus grass (*Miscanthus* sp.), including the neotype for *Peronosclerospora miscanthi* BPI 187301 (which is sequenced here for the first time), formed a subgroup, confirming their identification as *P. miscanthi*. The long branch lengths within subgroup C1 reflect a high level of diversity and suggest that these two specimens might represent divergent taxa, but the limited sampling precludes inference on this topic. The isotype specimens of *Peronosclerospora maydis* (BPI 89413) from corn and *P. noblei* (BPI 187306) from wild sorghum (*Sorghum leocladum*) grouped together, which agrees with evolutionary relationships inferred using the *cox2* (Suharjo et al. 2020). Long branch lengths separating the *P. maydis* and *P. noblei* specimens supported their distinction as separate species. Notably, the *P. maydis* isotype was not grouped with any of the eight other specimens identified by the original collectors as *P. maydis*. Instead, specimens BPI 187263, BPI 197264, BPI 789414, BPI 789415, BPI 789417, BPI 789418, BPI 789422, BPI 789983 were identified from the phylogenetic analysis as *P. sacchari*, *P. philippinensis*, *P. sorghi*, or *Peronosclerospora* spp.

Two type specimens of *Peronosclerospora spontanea*—lectotype BPI 187043 and isotype BPI 187073—clustered separately. These results indicate that BPI 187333, despite its origination from the same collection as BPI 187043, is not a specimen of *P. spontanea*, and is identified here as an unknown *Peronosclerospora* sp. A third specimen collected from miscanthus and identified as *P. miscanthi* by the original collector (BPI 187304) clustered separately from the *P. miscanthi* neotype and grouped alongside BPI 187298 collected from corn. Based on the phylogenetic analysis, both specimens in subgroup C3 correspond to a yet-to-be identified species of *Peronosclerospora*.

While this study has clarified the phylogenetic placement of several *Peronosclerospora* type specimens, several unidentified *Peronosclerospora* sp. clades remain, highlighting the ongoing taxonomic complexity within the genus. For instance, the importance of *P. neglecta* underscores a known challenge, as this species has historically been confused with *P. maydis* and may represent some of the currently unassigned specimens. A comprehensive resolution of *P. neglecta* 's phylogenetic position, and its relationship to the unidentified *Peronosclerospora* lineages observed here, represents a critical next step for future research.

Sclerospora graminicola*: Sister group to *Peronosclerospora

The identification of *Sclerospora graminicola* is considered provisional because the type specimen of *S. graminicola* (collected from *S. viridis*) was not a part of this study. Most taxonomic works point to *S. graminicola* as a species complex in need of revision (Thines & Choi 2016, Petrželová et al. 2017, Crouch et al. 2022). The shared host range, geographic origin (western Europe), morphological characters and time of collection held in common with the *S. graminicola* type (1870s; Fungi Europaei Exsiccati #2498, Mycotheca Veneta, Sistens Fungos Venetos Exsiccatos #496) suggests these specimens are authentic specimens of *S. graminicola* but this

cannot be concluded with certainty without comparison to the type specimen. Sister relationship to *Peronosclerospora* supports the identification of this clade as a *bona fide* *Sclerospora* (Muis et al. 2023).

***Graminivora*, *Sclerophthora*, *Viennotia* and undefined taxa**

The lineages basal to the core *Peronosclerospora*/*Sclerospora* clade reveal a complex interplay of established genera and several previously unrecognized, distinct evolutionary lines, particularly surrounding the historically problematic genus *Sclerophthora*. The lectotype of *Graminivora graminicola* BPI 786232, sequenced here for the first time (labeled E1 on Fig. 1), was sister to the isotype of *Viennotia oplismeni* (BPI 784264) which is consistent with phylogenetic relationships inferred from rDNA sequence data from non-type materials (Thines et al. 2006).

The specimens in subgroup E2 and *Scl. macrospora* are not derived from the same ancestor, with E2 more closely related to *Graminivora*. This indicates that E2 specimens are not members of a monophyletic *Sclerophthora*. The divergence between the E2 specimens and *Sclerophthora* is not unexpected, as many authors have considered *Scl. macrospora* a species complex (Telle et al. 2011, Telle & Thines 2012, Thines et al. 2015). Within subgroup E2, three clades were recovered (bootstrap = 79.5 to 99.8) with long branches, consistent with previous reports of high phylogenetic diversity among *Scl. macrospora* collections (Telle & Thines 2012).

The isotype of *V. oplismeni* BPI 784264 was sister to the clade containing group E/*Sclerospora*/*Peronosclerospora*, and was most closely related to *G. graminicola*, in agreement with molecular phylogenies generated from rDNA, *cox2*, and β -tubulin sequence data (Göker et al. 2007).

Group G was characterized by two main subclades (labeled "G1", "G2" on Fig. 1). Given the inclusion of the neotype for the generic type *Scl. macrospora*, subgroup G1 is unambiguously identified as the genus *Sclerophthora*. The lectotype of *Sclerospora iseilematis* BPI 187262 clustered within *Sclerophthora*. The original generic identification of *Sclerospora iseilematis* was based on oogonial structures, in the absence of diagnostic asexual characters, and is considered not reliable (Crouch et al. 2022).

Subclade G2, containing the lectotype and isotype of *Sclerospora farlowii* (BPI 187077, BPI 187078, respectively), was the sister clade to G1 *Sclerophthora*. Given the close relationship between the G1 and G2 subclades, we cannot rule out the possibility that *S. farlowii* should be included in the genus *Sclerophthora*. In an abstract, Kenneth (1979) proposed the inclusion of this taxon in *Sclerophthora*, citing host range and sporangial characters without providing details. No subsequent taxonomic treatments followed, so the report cannot be confirmed. However, based on the distant relationship between the *S. farlowii* lectotype to specimens of *S. graminicola*, and the phylogenetic affinity of the *S. farlowii* lectotype with *Sclerophthora*, a reasonable conclusion is that *S. farlowii* is not a member of the genus *Sclerospora*. Further research of this species is warranted, with particular attention needed on resolving the boundaries of *Sclerophthora* to determine if *S. farlowii* should be included in that genus.

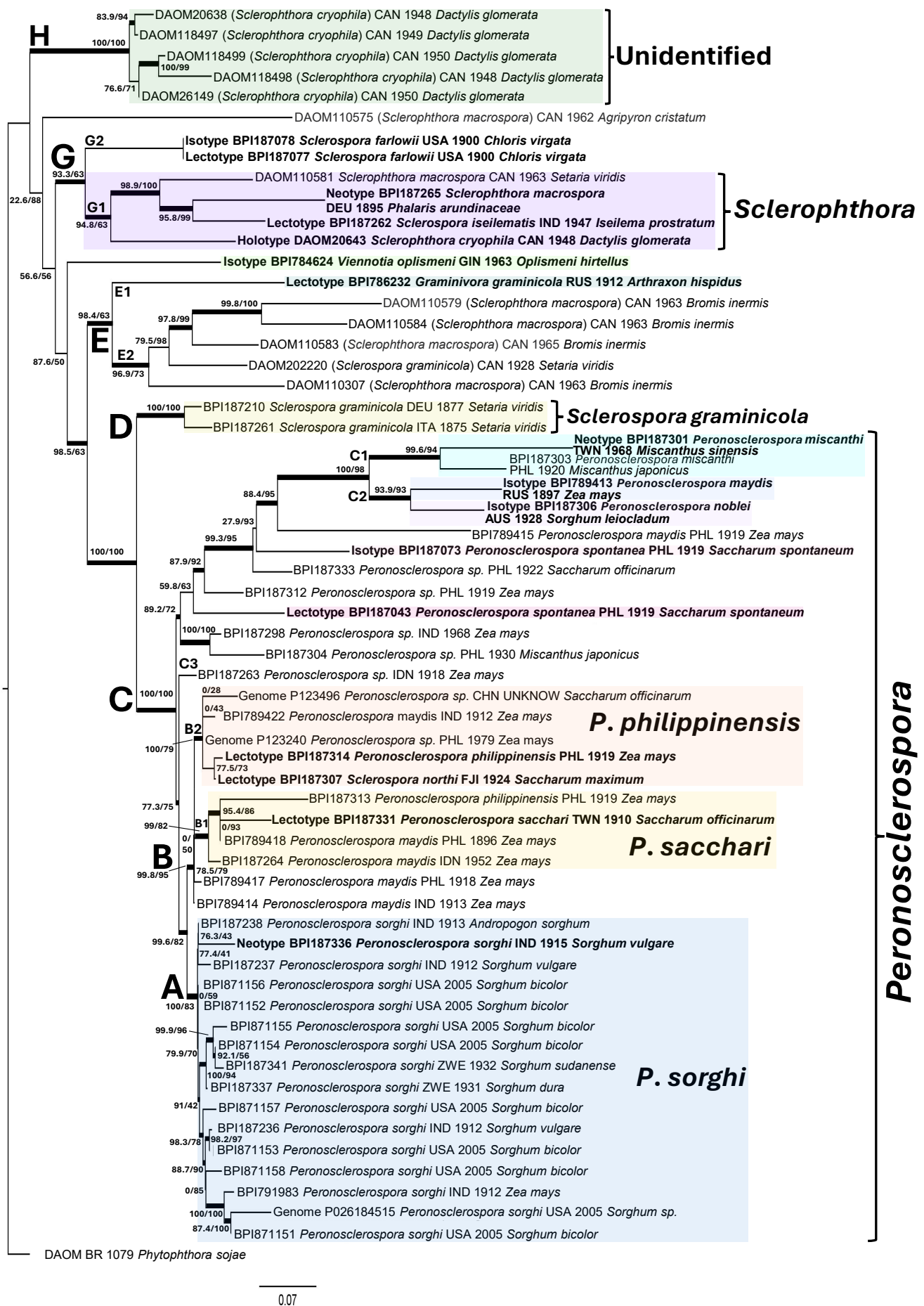


Fig. 1. Maximum likelihood phylogenetic tree computed from 62 filtered, trimmed, and concatenated ortholog sequences. The tree was outgroup rooted to *Phytophthora sojae*. Bootstraps are displayed on the branches as approximate likelihood ratio test Shimodaira–Hasegawa/ultrafast bootstrap values.

Specimen DAOM 110575, identified as *Scl. macrospora* by the original collector, was sister to *Sclerophthora* group G, with no support for its inclusion in the genus.

A diverse lineage of five specimens identified by the original collectors as *Scl. cryophila* were collected by the same person, from the same locale as the *Scl. cryophila* holotype (DAOM 20643), from the same host (orchard grass; *Dactylis glomerata*), and within a few months or years of the *Scl. cryophila* holotype collection. However, the group H specimens were distantly related to *Scl. cryophila* and fell outside the boundaries of a monophyletic genus *Sclerophthora* or any known taxon.

The genus *Sclerophthora* has long been considered taxonomically challenging and elusive, a complexity that is further illuminated by the molecular data presented here. While the observed diversity might raise concerns about potential artifacts from co-infections or inflated genetic distances due to degraded DNA, particularly when working with historical specimens, our rigorous filtering protocols (as detailed in Methods) largely mitigate the risk of contamination from other oomycetes. Therefore, the pronounced diversity within *Sclerophthora* groups, such as the *S. cryophila* specimens falling outside a monophyletic *Sclerophthora* and the distant relation of E2 specimens to the *Scl. macrospora* neotype, likely reflects either the inherent quality issues of DNA from historic specimens, leading to potentially inflated genetic distances, or, significantly, it could represent genuine, substantial phylogenetic diversity within a historically understudied and complex group. This finding is consistent with previous research, as *Sclerophthora* has long been considered a species complex (Telle et al. 2011, Telle & Thines 2012, Thines et al. 2015), and our data provides further molecular evidence supporting this complexity.

Despite the inherent challenges of working with these degraded samples, the inclusion of *Sclerophthora* type specimens and related materials provides critical insights. This study presents the first molecular data for several key *Sclerophthora* type specimens, unambiguously establishing the distinction between *Sclerophthora* and *Peronosclerospora*, a fundamental aspect of GDM taxonomy. The observed phylogenetic patterns strongly suggest that *Sclerophthora* is either a robust species complex or that historical morphological identifications have led to significant misclassifications. While the *Peronosclerospora* data allowed for more definitive conclusions, the *Sclerophthora* data provides an invaluable foundation for future research. Resolving the true generic and species boundaries within *Sclerophthora* will require further dedicated investigation, ideally incorporating a broader range of authenticated specimens and potentially leveraging advanced sequencing techniques more robust to DNA degradation.

Methodological considerations

The application of the oomycete-specific target enrichment approach proved instrumental in generating molecular data from challenging historical fungarium specimens, a primary aim of this study. However, the analysis also highlighted inherent limitations and trade-offs. The bait set, designed from broad oomycete lineages (e.g., *Aphanomyces*,

Pythium, *Phytophthora*), rather than specifically from downy mildews, meant it had a broader specificity across oomycete groups. While this broad design was effective in capturing diverse oomycete DNA, it likely contributed to the observed difficulty in consistently recovering complete sequences for all expected barcode loci, including the multi-copy mitochondrial genes, as noted in the results. Specifically, the extreme fragmentation of DNA in historical fungarium samples means that even if these multi-copy loci were abundant, their individual fragments were often too short for stable binding to the capture baits. This challenge is further compounded by the low overall abundance of oomycete DNA within the host plant tissue, which reduces the probability of successful enrichment for these particular loci. This outcome, along with the presence of low complexity regions and artificially inflated genetic distances, primarily reflects the highly fragmented and degraded nature of DNA inherent in historical fungarium samples, rather than a fundamental flaw in the enrichment approach itself. Despite these challenges, rigorous blastn filtering confirmed that the overwhelmingly majority of the sequences ultimately used for phylogenetic analysis were indeed of downy mildew origin, effectively mitigating concerns about contamination from other oomycetes. This underscores that while working with such degraded material demands careful methodological consideration and transparent reporting of limitations, the UCE approach remains a powerful tool for unlocking the phylogenetic relationships of taxonomically challenging and historically significant specimens.

The generation of phylogenies from highly degraded historical DNA, as presented here, necessitated careful methodological considerations, particularly in the construction of ortholog matrices. Our analysis revealed that an intermediate range of ortholog representation (40–55 % of samples) was optimal for resolving genus-level relationships, a finding that might initially raise questions regarding data quality or contamination. However, as detailed in the Methods, extensive filtering steps were employed to mitigate off-target oomycete contamination. Instead, this optimal range reflects the inherent trade-off when working with fragmented DNA from historical specimens: balancing the inclusion of a maximal number of samples with sufficient overlapping orthologs to provide a strong phylogenetic signal. Both excessively low and high thresholds for ortholog representation led to a loss of resolution, underscoring the pragmatic approach required to extract meaningful phylogenetic information from such challenging material. This strategic construction of the data matrix, while acknowledging the limitations of working with degraded DNA, ultimately allowed for the generation of reliable phylogenetic hypotheses that align with established taxonomic knowledge. Ultimately, these findings serve as a critical reminder that for historical specimens, even presumed isotypes from the same collection event cannot be taxonomically relied upon without direct molecular comparison to the nomenclatural type.

CONCLUSIONS

This study addressed the persistent challenges in graminicolous downy mildew taxonomy by applying a target

enrichment approach to historical fungarium specimens, successfully generating the first molecular data for the nomenclatural types of 11 species. This work provides a critical foundation for developing reliable molecular diagnostic tools. By directly linking the nomenclatural type of the high-risk pathogen *P. philippinensis* with modern genome data, for example, we establish an authenticated reference for creating species-specific assays essential for quarantine and disease monitoring.

From a taxonomic perspective, the phylogenomic analysis of 62 orthologs largely validated historical, morphology-based species concepts for most taxa described between the late 1800s and 1948. This high correspondence is a significant finding, given the known diagnostic difficulties in this group. At the same time, our data provided the necessary resolution to formally reduce *Sclerospora northii* to a synonym of *P. philippinensis* and justify the transfer of *Sclerospora isilematis* to *Sclerophthora*.

Perhaps one of the most critical and unexpected findings was that isotypes of two species, *P. philippinensis* and *P. spontanea*, were not conspecific with their respective lectotypes, despite being collected by the same person at the same time and place. This highlights a significant risk in relying on isotype material, which is often more accessible for research than nomenclatural types. Our results demonstrate that a shared collection event does not guarantee genetic identity, and they underscore the vital importance of including nomenclatural type specimens in taxonomic studies to ensure the accuracy of species concepts and the diagnostic tools derived from them.

Taxonomy

Peronosclerospora philippinensis (W. Weston) C.G. Shaw, *Mycologia* **70**: 596. 1978.

Basionym: *Sclerospora philippinensis* W. Weston, *J. Agric. Res., Washington* **19**: 118. 1920.

Synonyms: *Sclerospora northii* W. Weston [as 'nothi'], *Phytopathol.* **19**: 965. 1929.

'*Sclerophthora northii*' (W. Weston) Thirum. *et al.*, *Bull. Torrey Bot. Club* **80**: 300. 1953. [nom. nud., Art. 36.1, 39.1]

'*Sclerospora maydis*' Reinking, *Philipp. J. Sci., A* **13**: 1. 1918. [nom. illegit., Art. 53.1]

Possible synonym: *Sclerospora indica* E.J. Butler, *Fungi of India* (Calcutta): **7**. 1931.

Typus: **Philippines**, Laguna Province, Los Banos, *Zea mays*, 9 Feb. 1919, W.H. Weston (**lectotype** BPI 187314).

Notes: In the most recent treatment of the graminicolous *Peronosporaceae* (Crouch *et al.* 2022), BPI 789414 was designated as an isotype of *P. philippinensis*, but molecular data refutes that designation and places BPI 789414 in *P. sacchari*. Attempts were made to generate ortholog sequence data for two additional two isotypes (BPI 187044 and BPI 187311) using the oomycete bait set (Nguyen *et al.* 2021) but were unsuccessful. Given the discrepancy between the *P. philippinensis* lectotype and presumed isotype BPI 789414, only BPI 187314 is currently accepted as a *bona fide* type. Additional molecular analysis is recommended before making a determination of whether the specimens

BPI 187044 and BPI 187311 or other isotypes are part of *P. philippinensis*.

Phylogenetic analysis of the 62 ortholog dataset leaves no doubt that *S. northii* is a member of the genus *Peronosclerospora*. *S. northii* was only reported once, at the time of its original description; no asexual morph was described (Weston 1929). Because the asexual morph is the only definitive morphological basis to distinguish members of the genus *Sclerospora* from *Peronosclerospora*, this species was not transferred to *Peronosclerospora* along with other taxa (*P. dichanthiicola*, *P. miscanthi*, *P. noblei*, *P. philippinensis*, *P. sorghi*, *P. spontanea*, *P. westonii*) by Shaw in 1978. Based on the phylogenetic tree inferred from the 62 single-copy ortholog dataset, the lectotype of *Sclerospora northii* is part of a well-supported clade of five *Peronosclerospora* specimens (bootstrap = 100) that also includes the *P. philippinensis* lectotype. There were two potential ways to deal with the inclusion of *S. northii* as a member of the genus *Peronosclerospora*. The first option would have been to establish a new nomenclatural combination, with *Peronosclerospora northii* as a singleton taxon, distinct from the nomenclatural type of *P. philippinensis*. This approach would have excluded the other three closely related specimens that reside within the same clade (BPI 1789422, P123240, P123496, Fig. 1, B2) from *P. philippinensis*. However, the observed phylogenetic relationships did not provide any support for subdividing the clade B2 taxa into separate species. On the contrary, the short within-clade branch lengths and absence of any well-supported sub-clade structure may reflect intraspecific diversity, similar to intraspecific diversity observed in *P. sorghi* (Fig. 1A). The more conservative approach applied here, and one that is supported by the phylogenetic data analysis, is to accept *S. northii* as a later synonym of *P. philippinensis*.

Using the oomycete ortholog dataset to genotype the lectotype specimens, the distinction of *P. philippinensis* from *P. sacchari* was upheld. With *P. philippinensis* subject to quarantine regulation in the USA, China, Mexico, Morocco and in regions of Europe, this finding may have important implications. For example, in the USA, *P. philippinensis* is listed as a regulated Select Agent plant pathogen as *P. philippinensis*/*P. sacchari* (www.selectagents.gov/sat/list.htm).

Peronosclerospora spontanea (W. Weston) C.G. Shaw, *Mycologia* **70**: 597. 1978.

Basionym: *Sclerospora spontanea* W. Weston, *J. Agric. Res., Washington* **20**: 678. 1921.

Typus: **Philippines**, Laguna Province, Los Banos, Luzon, on leaves and shoots of *Saccharum spontaneum* (*Panicoideae*, *Andropogoneae*), 17 Aug. 1921, W.H. Weston (**lectotype** BPI 187043).

Notes: BPI 187073 was designated as an isotype of *P. spontanea* in the most recent treatment of the graminicolous *Peronosporaceae* (Crouch *et al.* 2022), but molecular data derived from 62 ortholog sequences in the current study refutes that designation, with identification of the specimen *Peronosclerospora* sp.

Sclerophthora iseilematis (Thurum. & Naras.) J.A. Crouch, **comb. nov.** MB 863595 .

Basionym: *Sclerospora iseilematis* Thurum. & Naras., *Indian Phytopathol.* **2**: 49. 1949.

Typus: **India**, Mysore, Nandi Hills, *Iseilema prostratum* (as *Iseilema laxum*; *Panicoideae*, *Andropogoneae*), 20 Jan. 1947, M.J. Narasimhan & H.C. Govindu [**holotype** BPI 187262; **isotype** IMI 38399].

Notes: The lectotype of *Sclerospora iseilematis* (BPI 187262) clustered within the *Sclerophthora* clade in molecular phylogenetic analyses and formed a sister relationship with the *Sclerophthora macrospora* neotype. As *S. macrospora* is the type species of *Sclerophthora* and holds nomenclatural priority, transfer of *S. iseilematis* to *Sclerophthora* is warranted.

Data Availability

The sequencing data is available at NCBI Bioproject PRJNA1462011 and in the Short Read Archive <https://www.ncbi.nlm.nih.gov/sra/58409512> to <https://www.ncbi.nlm.nih.gov/sra/58409575>. The supplemental data is available at the USDA Ag Data Commons <https://doi.org/10.15482/USDA.ADC/32177883>.

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Supplementary material

Table S1. Distribution and summary statistics of each ultra conserved element (UCE) found in each specimen.