Tar spot of maize in the Americas is caused by a complex of closely related Phyllachora species which vary in their host and geographic range

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Abstract

The genus Phyllachora contains numerous obligate fungal parasites that produce raised, melanized structures called stromata on their plant hosts. Most members of this genus are not of significant economic concern, with the exception of P. maydis, the causal agent of tar spot of maize (Zea mays). Tar spot of maize has emerged as a major threat to maize production throughout the Americas and continues to spread throughout North America. To date, species designations for Phyllachora have been based on host associations and morphology, and the origin and diversity of the pathogen that causes tar spot is unknown. We assessed the sequence diversity of 186 single stroma isolates collected from 16 hosts representing 15 countries by amplification of the ITS and LSU gene regions. Samples included both herbarium and contemporary strains that covered a temporal range from 1905-2019. These 186 isolates were grouped into 5 distinct species with strong bootstrap support. We found three closely related, but genetically distinct groups of Phyllachora are capable of infecting maize in the United States, we refer to these as the P. maydis species complex. Based on herbarium species, we hypothesize that these three groups in the P. maydis species complex originated from Central America, Mexico and the Caribbean. Although two of these groups were only found on maize, the third and largest group contained contemporary strains found on maize and other grass hosts, as well as herbarium specimens from maize and other grasses that include 10 species of Phyllachora. The herbarium specimens were identified based on morphology and host association, but our data indicates there may be significant synonymy in the Phyllachora genus and additional work on species delineation and host specificity should be considered.

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Abstract:

The genus *Phyllachora* contains numerous obligate fungal parasites that produce raised, melanized structures called stromata on their plant hosts. Most members of this genus are not of significant economic concern, with the exception of *P. maydis* , the causal agent of tar spot of maize (*Zea mays*). Tar spot of maize has emerged as a major threat to maize production throughout the Americas and continues to spread throughout North America. To date, species designations for*Phyllachora* have been based on host associations and morphology, and the origin and diversity of the pathogen that causes tar spot is unknown. We assessed the sequence diversity of 186 single stroma isolates collected from 16 hosts representing 15 countries by amplification of the ITS and LSU gene regions. Samples included both herbarium and contemporary strains that covered a temporal range from 1905-2019. These 186 isolates were grouped into 5 distinct species with

strong bootstrap support. We found three closely related, but genetically distinct groups of *Phyllachora* are capable of infecting maize in the United States, we refer to these as the *P. maydis* species complex. Based on herbarium species, we hypothesize that these three groups in the *P. maydis* species complex originated from Central America, Mexico and the Caribbean. Although two of these groups were only found on maize, the third and largest group contained contemporary strains found on maize and other grass hosts, as well as herbarium specimens from maize and other grasses that include 10 species of *Phyllachora* . The herbarium specimens were identified based on morphology and host association, but our data indicates there may be significant synonymy in the *Phyllachora* genus and additional work on species delineation and host specificity should be considered.

Introduction

Phyllachorales is a monophyletic order of biotrophic fungi comprised of approximately 1,226 recognized species (Maharachchikumbura et al. 2016, Mardones et al. 2017), but global estimates of species within this order approach 160,000 (Cannon 1997). The Phyllachorales largely contain plant parasitic fungi and are commonly associated with monocotyledonous plants across a range of habitats. These fungi are often referred to as "tar spot" fungi due to the production of stromata on plant hosts that resemble black flecks of tar (Fig 1) (Mardones et al. 2017).

Tar spot of maize (Figure 1 A-C), caused by the fungus *Phyllachora maydis* , emerged in the United States (U.S.) in 2015, with the disease expanding each year since the initial report and continuing to have a significant economic impact on maize across many production regions in the U.S. (Kleczewski et al. 2020a, Valle-Torres et al. 2020). Since first being identified in North America in 2015, *P. maydis* has spread rapidly throughout the U.S. and Canada (Kleczewski and Bowman 2020, Kleczewski et al. 2020), and resulted in yield losses exceeding $US 658 million in 2018 (Mueller et al. 2020). *Phyllachora maydis* caused significant losses again in 2021, appearing to be on par or greater than 2018 levels (authors personal observations). Although tar spot symptoms caused by members of the genus *Phyllachora* have been commonly observed on a number of grasses (Figure 1 D-F) and shrub species throughout North, Central and South America, historically the fungus has rarely been known to cause significant plant damage. However, tar spot has been occasionally reported to cause severe damage to maize in Mexico, Central America and several Caribbean Islands (Valle-Torres et al. 2020).

The origin of *P. maydis* within the U.S. is not currently known, although the presence of two distinct epicenters of maize tar spot in the Midwest and Southeast indicates at least two separate emergence events. While tar spot is a new disease on maize in the U.S. and Canada, it has been present in Mexico, several Caribbean islands including Puerto Rico, Cuba and the Dominican Republic as well as Central American Countries, such as Guatemala, Honduras, Nicaragua and Costa Rica for the last century but only caused limited damage. In addition, tar spot signs and symptoms caused by *Phyllachora* species are common on several native and weedy grass species in North America (Figure 1 D-F) (Orton 1944). The monographic work by Orton (1944) was completed solely by morphological identification and host affinity. Given our understanding of phenotypic plasticity of many fungi and the ability of biotrophic pathogens to infect multiple hosts (Morris and Moury 2019), it is possible that cryptic species or species complexes may be present.

Species definitions within the Phyllachorales have historically been based largely on morphological characteristics and assumption of high host specificity, due to their presumed biotrophic nature. However, there are examples in the genus where this assumption of host specificity does not hold true (Cannon 1991, Cannon 1997). Furthermore, species designations based on host specificity are highly dependent on accurate identification of the host species, which may be difficult or impossible in some instances. For example, *P. graminis* (Pers.) Fuckel is considered a "dustbin" species where many specimens of isolates infecting grasses are deposited with the host not often identified to species (Parbery 1967). Furthermore, factors such as nutrients available to the fungus, temperature, light quality, light cycles, substrate type, host, and epigenetic factors may also result in alterations in fungal morphology that may result in inaccurate species designations (Slepecky and Starmer 2009, Stockinger et al. 2009, Money 2013, Francisco et al. 2019). Thus, our current understanding of the genetic diversity, host range and species delimitation within the genus*Phyllachora* is

relatively limited and requires reevaluation.

The recent emergence of *P. maydis* in the U.S. and Canada may also be associated with the ability of the fungus to better persist and spread than previously thought. Once established, the fungus can survive at least one winter at subzero temperatures on corn residue as ascospores within stromata, which are believed to be the main inoculum source the following season (Kleczewski et al. 2019, Groves et al. 2020). Under periods of moderate temperatures and wet weather it is believed that ascospores are dispersed by wind and rain-splash where they land on the foliage, stalks, and husks of corn. After spore germination and infection of the host, the fungus remains dormant for at least 2 weeks after which stromata, and associated spermatia and ascospores, are produced. Data from Central America indicated a relatively steep dispersal curve of *P. maydis* ascospores from a source (Hock et al. 1995). However, the rapid spread of this fungus throughout the Midwest, coupled with observations of "top down" infestations in fields with no history of disease and observations of infestations of isolated plots located 1,200 m from potential inoculum sources, indicate that the pathogen can travel much further across local/regional topographies than estimated previously (Kleczewski et al. 2020).

Based on this information, the emergence of *P. maydis* on corn in the U.S. and Canada could have been the result of many factors including the introduction of the fungus on infected plant material, natural northern dispersal through wind, establishment in the U.S. favored by climate change, changes in hybrid genetics, a host-jump from a grass species, or a combination of any of these four. In this study we use DNA sequence data to understand the genetic diversity of *P. maydis*populations in contemporary maize production regions in the U.S., and compare this to historical specimens of *P. maydis* from herbarium samples from Mexico, Central and South America, the Caribbean, and contemporary and herbarium species of *Phyllachora* species associated with grass hosts in the U.S. The goal of this study is to understand the genetic diversity and population dynamics within the*P. maydis* population infecting corn in North America. Differences in genetic backgrounds may imply dissimilar biology, and potentially interactions with the host. Furthermore, understanding the overall phylogenetic diversity and the potential host and geographic range of*Phyllachora* populations associated with maize and other grasses in the Americas will help to infer the potential evolutionary origins and speciation patterns in this genus.

MATERIAL AND METHODS

2.1 Sample Collection.

Samples of maize and wild grasses with characteristic stromata of*Phyllachora spp* . (Figure 1 A-F)) were collected from across North America and Mexico in 2018 and 2019 (Table 1). Field specimens of infested maize and other grasses were collected by numerous individuals from the agricultural community as described in Kleczewski et al (2020). Samples were pressed, dried at room temperature, and stored at 20°C in manilla envelopes until processed. Herbarium specimens were obtained from the U.S. National Fungus Collection (BPI, Beltsville, Maryland) and the University of Illinois Herbarium (Urbana, Illinois), which included specimens on maize and other grasses from additional hosts, countries, and years (Table 1). A total of 186 samples from 16 hosts, and 15 countries collected from 1905-2019 were included in the analyses.

2.2 DNA extraction, PCR amplification and sequencing of stroma from leaf tissue.

The DNA of individual stroma not surrounded by a necrotic halo were extracted using the X-Tract-N-AMP kit following manufacturer protocols (Sigma). The complete internal transcribed spacer region of ribosomal DNA (ITS1-5.8S-ITS2) with primers ITS1f and ITS4 (White et al. 1990). Stroma without necrotic halos were selected to reduce the potential for contamination by saprophytic fungi that may be present on necrotic tissue within these lesions.

The ITS gene region was amplified from DNA extracted from each stroma using the primer pair ITS1f and ITS4 (White 1990, Bruns and Gardes 1993) with 35 cycles of the following: 95°C 5 min, 94°C 30s 52°C 30s, 72°C 1 min followed by 72°C for 8 min and a final hold at 4°C in a Thermo Fisher SimpliAmp thermocycler (Thermo Fisher Scientific, Waltham, WA). Individual PCR products from corresponding DNA

extractions were loaded into 2% agarose gels and separated via electrophoresis for 40 minutes at 110V. All gels contained a *P. maydis* positive control, a*Fusarium graminearum* positive control, and a negative buffer control for quality assurance. Bands on gels were visualized using an Axygen gel imaging station (Axygen, Inc, Union City, CA). Stroma of*Phyllachora spp.* can be colonized by or associated with several other fungal species (Hock et al. 1992, Hock et al. 1995, McCoy et al. 2019). Consequently, samples returning a single band between 300-500 bp were considered free of additional fungal contaminants and used in subsequent analyses.

DNA from samples returning a single ITS band were subject to amplification of the Large Ribosomal Subunit (LSU) region using the primer pair LROR and LR5 (Dayarathne et al. 2017) using the aforementioned thermocycler conditions. All PCR products were purified using QIAquick PCR kits (Quiagen, Inc., Hilden, Germany), and the ITS and LSU amplicons for all samples were sequenced in the forward and reverse directions at the University of Illinois Core DNA Sequencing Facility (Urbana, Illinois).

2.3 Sequence alignment, phylogenetic analysis and molecular identification

Sequences generated from this study were combined with sequences obtained from GenBank. *Exserohilum turcicum* and *Cocoicola californica* were selected as the outgroups. Sequence data was aligned and concatenated using MAFFT v.7 (mafft.cbrc.jp/alignment/server/index/html) using the G-INS-I model and manually inspected. The best fit partitioning schemes were determined using PartitionFinder (Lanfear et al. 2017) and used to build the phylogenies. Both single gene and concatenated gene sets were analyzed using a maximum likelihood (ML) analysis. The ML phylogenies were generated by RaxML under GTR model with gamma distributed rate heterogeneity with 1000 bootstrap replicates. Resulting trees were visualized with iTOL (Interactive Tree of Life) v.6 (*https://itol.embl.de/*) or MEGA. Sequences generated in this study were deposited in GenBank (Table 1)

RESULTS

3.1 DNA extraction and PCR amplification from herbarium and contemporary samples

A total of 186 samples from twelve states in the U.S. (n=130), four states in Mexico (n=13), three Central American countries (n=13), four South American countries (n=6), four Caribbean Islands (n=16), Germany (n=3), India (n=3) and The Philippines (n=2) were sequenced and analyzed as noted above. There were varying levels of success for the amplification of each genetic locus among the samples. This was particularly the case for many of the herbarium samples, some of which were more than 100 years old. The ITS region was the most successfully amplified and sequenced, with 168 sequences generated. Whereas 91 sequences were generated for the LSU locus (Table 1).

3.2 Phylogenetic diversity of *Phyllachora* isolates infecting maize and grasses

Based on both the ITS + LSU (Figure 2) and ITS (Figure S1) phylogenies, we observed five genetically distinct groups that represent individual species of *Phyllachora* with strong bootstrap support (>70%). The results suggest that tar spot on maize in the U.S. is caused by three closely related species of *Phyllachora*(Figure 2). In all, four species were found on maize but only*Phyllachora sp. 1* , *P. sp.* 2, and *P. sp .3* were recovered from contemporary maize in the U.S., while*P. sp. 4* was recovered from herbarium samples collected in Guatemala and Venezuela (Table 1).

Samples of *Phyllachora sp. 3* represent the broadest geographic and host range and was also the most frequently recovered species associated with *Phyllachora* sp. stroma on maize from both herbarium and contemporary specimens representing a span of time from 1905-2019 (Table 1). Samples of *P. sp. 3* on maize were reported and recovered from herbarium samples throughout the Americas including Bolivia, Colombia, Costa Rica, Cuba, Dominican Republic, Guatemala, Mexico, Nicaragua, Puerto Rico, and Trinidad and Tobago prior to the first report of tar spot on maize in the U.S. Importantly the type specimen of *P. maydis* (BPI638553) collected in Mexico in 1977 and the *P. maydis* isolate (BPI893226) used in the first report of tar spot in the U.S. in 2015 are both part of *P. sp. 3* and isolates of this species have since been recorded in Illinois, Indiana, Iowa, Michigan, Minnesota and Wisconsin. This represents the widest geographic range

of the maize infecting *Phyllachora* species in the U.S. among the samples included in this study. However, isolates of *P. sp. 3* were also recovered from another 10 host species including monocots and dicots, with a global distribution including 12 countries across South, Central and North America and the Caribbean, as well as Germany, India and the Philippines (Table 1; Figure 3). The herbarium samples associated with each of the 10 host species represented morphologically recognized species of *Phyllachora* including*P. graminis* , *P. heraclei, P. junci, P. chaetochloae, P. diplocarpa, P. epicampis, P. euphorbiaceae, P. rottboelliae, P. sylvatica, and P. vulgata* .

The other two contemporary maize-infecting species, *P. sp. 1* and *P. sp. 2* , have a more limited observed host and geographic range. Both species were only recovered on maize.*Phyllachora sp. 1* was only recovered from contemporary maize samples from Indiana and Ohio, whereas *P. sp. 2* was found on herbarium specimens from Colombia and Puerto Rico and contemporary specimens from Puerto Rico, Mexico (Guerrero, Oaxaca, Puebla, Veracruz), and the U.S. (Florida, Illinois and Michigan).

The other species recovered from maize was *Phyllachora sp. 4*.However, samples only included herbarium specimens from Guatemala and Venezuela and did not include any contemporary maize specimens. Interestingly, *P. sp. 4* was commonly found among grasses in the U.S. that are found in proximity to maize production fields in Illinois, South Dakota and New York (Table 1). Isolates of *P. sp. 4* were recovered from 6 grass species in 4 tribes in the U.S. representing a broad host range across a breadth of genetically diverse grass species.*Phyllachora sp. 5* was the only species not recovered from maize but was found on many of the same grass species as *P. sp. 4* , including rye, triticale and fall panicum (Table 1).

While there was limited *Phyllachora* sequence data in Genbank, we were able to include the ITS sequence of 19 isolates representing six recognized species of *Phyllachora* to determine any relationship between the isolates used in this study to those submitted previously to Genbank (Figure 3). In the case of *P. sp. 4* , two isolates referred to as *P. graminis,* one from *Hordelymus europaeus*in Germany and one of unknown origin, as well as isolates of*Phyllachora* on *Elymus kamoji* and *Roegneria sp* . from China grouped together with strong boostrap support (99%). There was also an isolate of *P. graminis* from an unknown grass in Canada that grouped together with *P. sp. 5* , and the herbarium specimen of *P. graminis* from *Agropyron repens* in Germany from this study grouped in *P. sp. 3* (Figure 3). Our results support the findings of previous observations that *P. graminis* is a poorly defined polyphyletic species, that has often been assigned to tar spot symptom on a variety of grass hosts.

Discussion

Since *P. graminis* was described by Nitschke 1870 (Fuckel 1870), over 300 species have been recorded on graminaceous hosts, and many more on non-grass hosts. However, Parbery recognized that there are fewer species associated with grasses and established that there were 95 valid graminicolous *Phyllachora* species world-wide based on morphological characteristics (Parbery 1967). In the most complete study of *Phyllachora* species in North America, Orton (1944) identified 45 morphological species from more than 100 host species (Orton 1944). While this likely represents a significant overestimation of the true number of species in North and Central America, it does demonstrate the vast number of hosts on which *Phyllachora* species have been reported. Our results based on both herbarium and contemporary samples of infected hosts indicate that there are far fewer species of*Phyllachora* in the Americas than indicated by Orton (1944) and Parbery (1967), and the species that are present have a greater host range than previously thought. The predominant species in this study,*P. sp. 3* , has a broad geographic and host range with the capacity to infect maize throughout South, Central and North America as well as seven grass species and two dicot species. This phylogenetic species also includes isolates of 11 morphologically determined species of*Phyllachora* (*P. chaetochloae, P. diplocarpa, P. epicampsis, P. euphorbiaceae, P. graminis, P. heraclei, P. junci, P. maydis, P. rottboelliae, P. sylvatica,* and *P. vulgata*) from herbarium samples collected in the Dominican Republic, Germany, India, Mexico, the Philippines, Trinidad and Tobago and the U.S., indicating global distribution of this species. This expanded host range also now complicates the taxonomic status and the name to be retained by this genetic group. An isolate of *P. graminis* collected from*Agropyron repens* from Germany was designated as the lectotype specimen for the genus (Clements and Shear 1931), and the isolate of*P. graminis* examined in

this study was also collected from *A. repens* in Germany, indicating that *P. graminis* may have precedence for the species name of *P. sp. 3* . This would have ramifications for *P. maydis* as well as several other *Phyllachora* species in *P. sp. 3* (Table 1; Figure 3) that appear to be synonyms of *P. graminis* . This is based solely on sequence data from the ITS and/or LSU region and further multi-gene phylogenetic studies of a larger representation of type material from herbaria and contemporary *Phyllachora* samples from additional hosts is needed for a thorough taxonomic assessment of this genus.

The three maize-infecting species, *P. sp. 1, P. sp. 2* and *P. sp. 3* , have overlapping geographic and host ranges, providing the opportunity for co-infection and genetic exchange. Co-infection on the same leaf tissue by *P. sp. 3* and *P. sp. 1* was observed on four occasions with herbarium samples (BPI893232_1 and BPI893232_2 , BPI893231_1 and BPI893231_2, BPI893226_1 and BPI893226_2, BPI893230_1 and BPI893230_2) from 3 counties in Indiana. A recent fungal community analysis of tar spot lesions on maize found a similar trend with two distinct OTUs occurring on 21 of 22 maize leaf samples from Michigan (McCoy et al. 2019). A similar phenomenon has also been observed in *Albugo candida* , another biotrophic pathogen with a broad host range (McMullan et al. 2015). Races of *A. candida* were not able to infect a host on their own but were able to co-infect with a race-specific isolate that suppressed host immunity in that host. The offspring of any genetic introgression or recombination resulted in a race with an expanded host range able to infect both plants infected by the parental strains of *A. candida* . A whole genome comparison of these *A. candida* races found a mosaic-like genome structure with large portions conserved between races, as well as regions with only 89% sequence similarity. This scenario may explain the wide host range and variation in morphology between hosts in *Phyllachora* species. Sexual reproduction in *P. maydis* followed by discharge of infective ascospores commonly occurs on corn leaves annually in maize producing regions of the U.S. (Kleczewski et al. 2019, Groves et al. 2020b). The presence of multiple maize infecting species in the midwestern US, and even on a single infected leaf, combined with frequent sexual recombination, ascospore release and infection, could result in novel populations and/or species of *Phyllachora* that are more virulent on maize or that have an expanded host range. This may also explain why *P. sp. 3* has such a broad host range whereas *P. sp. 1* and *P. sp. 2* were only found on maize. Individual populations may gain the ability to infect a new host but are still able to sexually recombine with the rest of the population on the original host species. Given the geographic overlap of many grass species in Central, South and North America, small populations of *P. sp. 3* may have adapted to infect a novel grass species, while maintaining the ability to recombine with the larger *P. sp. 3* complex, resulting in the expansion of the host range without specialization and speciation.

Speciation has likely occurred in instances where geographic isolation of a new host prevented further introgression with the original population. As maize is commonly grown from Argentina to Canada, it represents a common host for which distinct *Phyllachora* populations may infect and recombine resulting in potentially new and more virulent populations that are still part of the same species. It is unclear if geographic or genetic barriers lead to speciation between the closely related *P. sp. 1, P. sp. 2* and *P. sp. 3* , but the significant overlap in host and geography would indicate a genetic barrier. While *P. sp. 1* and *P. sp. 2* were only recovered from maize, our sampling scheme was strongly biased towards maize. It is possible that *P. sp. 1* and *P. sp. 2* are present on other grass and non-grass hosts in Central and North America and were not sampled in this study. These non-sampled hosts, if only infected by one of the *Phyllachora* species, may represent the isolation that led to adaptation and speciation.

For now the name *Phyllachora maydis* will be retained by *P. sp. 3* as the *P. maydis* type material (BPI638553) clustered with this group. However, the presence of three maize-infecting species, the lack of type material of *P. graminis* , and the potential taxonomic synonymy with *P. graminis* and several other *Phyllachora* species makes it difficult to determine which of the maize infecting species will retain the name *P. maydis* . Therefore, we recommend referring to *P. sp. 1, P. sp. 2* and *P. sp. 3* as the *Phyllachora maydis* species complex until further morphological and multi-gene phylogenetic studies can properly delineate these species.

In this work, we conducted the most comprehensive assessment of *Phyllachora maydis* reported to date and provided evidence that our understanding of this species and genera is limited and requires significant

attention. The reasons for the emergence of tar spot, caused by three different species of *Phyllachora* that have been present in Central America, Mexico and the Caribbean for over 75 years is still unclear. Several scenarios may explain the recent emergence and severity of tar spot caused by *Phyllachora spp* . in the upper Midwest of the U.S. While *P. sp. 2* and *P. sp. 3* have been present in both Mexico and Puerto Rico for the last century, it is possible that when the fungus was able to be dispersed via wind and rain to the U.S. it could not overwinter in colder climates and the disease could not get established. In fact, according to the herbarium specimens, *P. sp. 3* has been present in the U.S. since the 1940s in California and Arizona on native grasses but not maize. However, recent studies have demonstrated that *Phyllachora* spp. can overwinter in Illinois (Kleczewski et al. 2019). Shorter and warmer winters due to climate change could be playing a role in the ability of*Phyllachora* spp. to survive further north in the U.S. Changes in climate patterns during the growing season may also have an impact on this disease as increased temperature and precipitation may promote epidemics of this disease. Finally, a change in maize genetics may also play a role in the increased severity of tar spot. Since maize breeding programs were not selecting for resistance to tar spot, any partial resistance that may have been present in U.S. germplasm may have been lost through genetic drift. The loss of this resistance may not have been noticed until *Phyllachora* spp. arrived in the primary maize growing region of the U.S. The disease remains of minor importance in Mexico and Central American maize production, as resistance to this disease would be selected for in breeding programs. The most likely scenario for the emergence of tar spot in the U.S includes a combination of these factors: 1) introduction of multiple species of*Phyllachora* from Mexico, Puerto Rico or other Central American countries through movement of infected plant tissue or possible long-distance movement via wind, rain, hurricane/tropical storm system, etc., 2) change in climate in the Midwestern maize growing region more hospitable to the growth, reproduction and survival of *Phyllachora spp.* ; and 3) lack of resistance in maize germplasm grown in the Midwestern U.S.

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Table 1. The sample ID, genetic cluster, geographic and host origin, year collected and source of the 186 *Phyllachora* specimens used in this study.

SampleID	**Genetic Cluster[a]**	**Species[b]**	**State**	**Country**	**Host**
BPI893226_2	1	*Phyllachora maydis*	Indiana	USA	*Zea mays*
BPI893227_2	1	*P. maydis*	Indiana	USA	*Zea mays*
BPI893229_2	1	*P. maydis*	Indiana	USA	*Zea mays*
BPI893230_2	1	*P. maydis*	Indiana	USA	*Zea mays*
BPI893231_2	1	*P. maydis*	Indiana	USA	*Zea mays*
BPI893232_2	1	*P. maydis*	Indiana	USA	*Zea mays*
C18001-2	1	*P. maydis*	Indiana	USA	*Zea mays*
C18001-3	1	*P. maydis*	Indiana	USA	*Zea mays*
C18003-1	1	*P. maydis*	Indiana	USA	*Zea mays*
C18003-2	1	*P. maydis*	Indiana	USA	*Zea mays*
C18003-3	1	*P. maydis*	Indiana	USA	*Zea mays*
C18009-1	1	*P. maydis*	Indiana	USA	*Zea mays*
C18009-2	1	*P. maydis*	Indiana	USA	*Zea mays*
C18011-3	1	*P. maydis*	Indiana	USA	*Zea mays*
C18024-1	1	*P. maydis*	Indiana	USA	*Zea mays*
C18024-2	1	*P. maydis*	Indiana	USA	*Zea mays*
C18024-3	1	*P. maydis*	Indiana	USA	*Zea mays*
C18161-1	1	*P. maydis*	Ohio	USA	*Zea mays*
C18161-2	1	*P. maydis*	Ohio	USA	*Zea mays*
C18161-3	1	*P. maydis*	Ohio	USA	*Zea mays*
C18162-1	1	*P. maydis*	Ohio	USA	*Zea mays*
C18162-3	1	*P. maydis*	Ohio	USA	*Zea mays*
C18164-1	1	*P. maydis*	Ohio	USA	*Zea mays*
C18164-2	1	*P. maydis*	Ohio	USA	*Zea mays*
C18164-3	1	*P. maydis*	Indiana	USA	*Zea mays*
C19043-1	1	*P. maydis*	Indiana	USA	*Zea mays*
C19043-3	1	*P. maydis*	Indiana	USA	*Zea mays*
BPI638548_1	2	*P. maydis*	Cundinamarca	Colombia	*Zea mays*
BPI638554_1	2	*P. maydis*	Añasco	Puerto Rico	*Zea mays*
BPI638578_1	2	*P. maydis*	Vega Baja	Puerto Rico	*Zea mays*
BPI910562_1	2	*P. maydis*	Michigan	USA	*Zea mays*
C18026-3	2	*P. maydis*	Puebla	Mexico	*Zea mays*
C18030-1	2	*P. maydis*	Guerrero	Mexico	*Zea mays*
C18030-2	2	*P. maydis*	Guerrero	Mexico	*Zea mays*
C18030-3	2	*P. maydis*	Guerrero	Mexico	*Zea mays*
C18031-1	2	*P. maydis*	Veracruz	Mexico	*Zea mays*
C18031-2	2	*P. maydis*	Veracruz	Mexico	*Zea mays*
C18031-3	2	*P. maydis*	Veracruz	Mexico	*Zea mays*
C18033-2	2	*P. maydis*	Oaxaca	Mexico	*Zea mays*
C18033-3	2	*P. maydis*	Oaxaca	Mexico	*Zea mays*
C18038-3	2	*P. maydis*	Guerrero	Mexico	*Zea mays*
C18040-1	2	*P. maydis*	Florida	USA	*Zea mays*
C18040-2	2	*P. maydis*	Florida	USA	*Zea mays*

C18040-3	2	*P. maydis*	Florida	USA	*Zea mays*
C18069-1	2	*P. maydis*	Illinois	USA	*Zea mays*
C18069-2	2	*P. maydis*	Illinois	USA	*Zea mays*
C18069-3	2	*P. maydis*	Illinois	USA	*Zea mays*
C19001-1	2	*P. maydis*	Florida	USA	*Zea mays*
C19001-2	2	*P. maydis*	Florida	USA	*Zea mays*
C19001-3	2	*P. maydis*	Florida	USA	*Zea mays*
92794-1	3	*P. chaetochloae*	Santiago	Dominican Republic	*Setaria sp.*
92794-2	3	*P. chaetochloae*	Santiago	Dominican Republic	*Setaria sp.*
92794-3	3	*P. chaetochloae*	Santiago	Dominican Republic	*Setaria sp.*
92812-1	3	*P. diplocarpa*	California	USA	*Distichilis spicata*
92821-1	3	*P. epicampsis*	Arizona	USA	*Muhlenbergia emersley*
92821-2	3	*P. epicampsis*	Arizona	USA	*Muhlenbergia emersley*
92821-3	3	*P. epicampsis*	Arizona	USA	*Muhlenbergia emersley*
92825-1	3	*P. euphorbiaceae*	Mumbai	India	*Euphorbia sp.*
92825-2	3	*P. euphorbiaceae*	Mumbai	India	*Euphorbia sp.*
92825-3	3	*P. euphorbiaceae*	Mumbai	India	*Euphorbia sp.*
92845-3	3	*P. graminis*	Mittelfranken	Germany	*Agropyron repens*
92922-3	3	*P. heraclei*	Hessen	Germany	*Heracleum spondylium*
92925-2	3	*P. junci*	Holstein	Germany	*Juncus effusus*
92938-1	3	*P. maydis*	Arecibo	Puerto Rico	*Zea mays*
92938-2	3	*P. maydis*	Arecibo	Puerto Rico	*Zea mays*
92938-3	3	*P. maydis*	Arecibo	Puerto Rico	*Zea mays*
92940-2	3	*P. maydis*	Arecibo	Puerto Rico	*Zea mays*
92940-3	3	*P. maydis*	Arecibo	Puerto Rico	*Zea mays*
93013-1	3	*P. rottboelliae*	Luzon	Philippines	*Rottboellia*
93013-2	3	*P. rottboelliae*	Luzon	Philippines	*Rottboellia*
93064-1	3	*P. sylvatica*	California	USA	*Festuca idahoensis*
93064-2	3	*P. sylvatica*	California	USA	*Festuca idahoensis*
93126-2	3	*P. vulgata*	Arizona	USA	*Muhlenbergia glauca*
93126-3	3	*P. vulgata*	Arizona	USA	*Muhlenbergia glauca*
BPI638546_1	3	*P. maydis*	Maracas Valley	Trinidad and Tobago	*Zea mays*
BPI638553_1	3	*P. maydis*		Mexico	*Zea mays*
BPI638556_1	3	*P. maydis*	Valle del cauca	Colombia	*Zea mays*
BPI638558_1	3	*P. maydis*	Antigua	Guatemala	*Zea mays*
BPI638559_1	3	*P. maydis*	Matagalpa	Nicaragua	*Zea mays*
BPI638561_1	3	*P. maydis*	Veracruz	Mexico	*Zea mays*
BPI638564_1	3	*P. maydis*		Mexico	*Zea mays*
BPI638567_1	3	*P. maydis*	Havana	Cuba	*Zea mays*
BPI638568_1	3	*P. maydis*	Alajuela	Costa Rica	*Zea mays*
BPI638570_1	3	*P. maydis*	Vega Baja	Puerto Rico	*Zea mays*
BPI638571_1	3	*P. maydis*	Turrialba	Costa Rica	*Zea mays*
BPI638572_1	3	*P. maydis*	Chimaltenanco	Guatemala	*Zea mays*
BPI638574_1	3	*P. maydis*	Arecibo	Puerto Rico	*Zea mays*
BPI638575_1	3	*P. maydis*	Chimaltenanco	Guatemala	*Zea mays*
BPI638577_1	3	*P. maydis*	Nor Yungas	Bolivia	*Zea mays*
BPI638579_1	3	*P. maydis*		Guatemala	*Zea mays*
BPI638580_1	3	*P. maydis*	Santander	Colombia	*Zea mays*
BPI638581_1	3	*P. maydis*	Vega Baja	Puerto Rico	*Zea mays*
BPI638582_1	3	*P. maydis*	Guatemala	Guatemala	*Zea mays*
BPI638584_1	3	*P. maydis*	Antigua	Guatemala	*Zea mays*

BPI638585_1	3	*P. maydis*		Guatemala	*Zea mays*
BPI638586_1	3	*P. maydis*	Lima	Peru	*Zea mays*
BPI638587_1	3	*P. maydis*	La Vega	Dominican Republic	*Zea mays*
BPI638588_1	3	*P. maydis*		Guatemala	*Zea mays*
BPI893226_1	3	*P. maydis*	Indiana	USA	*Zea mays*
BPI893228_1	3	*P. maydis*	Indiana	USA	*Zea mays*
BPI893230_1	3	*P. maydis*	Indiana	USA	*Zea mays*
BPI893231_1	3	*P. maydis*	Indiana	USA	*Zea mays*
BPI893232_1	3	*P. maydis*	Indiana	USA	*Zea mays*
BPI893233_1	3	*P. maydis*	Illinois	USA	*Zea mays*
BPI893234_1	3	*P. maydis*	Illinois	USA	*Zea mays*
BPI910560_1	3	*P. maydis*	Wisconsin	USA	*Zea mays*
BPI910561_1	3	*P. maydis*	Iowa	USA	*Zea mays*
C18046-1	3	*P. maydis*	Illinois	USA	*Zea mays*
C18046-2	3	*P. maydis*	Illinois	USA	*Zea mays*
C18046-3	3	*P. maydis*	Illinois	USA	*Zea mays*
C18047-1	3	*P. maydis*	Illinois	USA	*Zea mays*
C18047-2	3	*P. maydis*	Illinois	USA	*Zea mays*
C18047-3	3	*P. maydis*	Illinois	USA	*Zea mays*
C18049-1	3	*P. maydis*	Illinois	USA	*Zea mays*
C18049-2	3	*P. maydis*	Illinois	USA	*Zea mays*
C18050-2	3	*P. maydis*	Wisconsin	USA	*Zea mays*
C18075-3	3	*P. maydis*	Illinois	USA	*Zea mays*
C18119-2	3	*P. maydis*	Wisconsin	USA	*Zea mays*
C18119-3	3	*P. maydis*	Wisconsin	USA	*Zea mays*
C18136-1	3	*P. maydis*	Wisconsin	USA	*Zea mays*
C18148-1	3	*P. maydis*	Iowa	USA	*Zea mays*
C18148-2	3	*P. maydis*	Iowa	USA	*Zea mays*
C18148-3	3	*P. maydis*	Iowa	USA	*Zea mays*
C18149-1	3	*P. maydis*	Iowa	USA	*Zea mays*
C18149-2	3	*P. maydis*	Iowa	USA	*Zea mays*
C18153-1	3	*P. maydis*	Wisconsin	USA	*Zea mays*
C18153-2	3	*P. maydis*	Wisconsin	USA	*Zea mays*
C19007-1	3	*P. maydis*	Iowa	USA	*Zea mays*
C19007-2	3	*P. maydis*	Iowa	USA	*Zea mays*
C19007-3	3	*P. maydis*	Iowa	USA	*Zea mays*
C19008-1	3	*P. maydis*	Illinois	USA	*Zea mays*
C19008-2	3	*P. maydis*	Illinois	USA	*Zea mays*
C19008-3	3	*P. maydis*	Illinois	USA	*Zea mays*
C19012-2	3	*P. maydis*	Minnesota	USA	*Zea mays*
C19012-3	3	*P. maydis*	Minnesota	USA	*Zea mays*
C19022-2	3	*P. maydis*	Illinois	USA	*Zea mays*
C19022-3	3	*P. maydis*	Illinois	USA	*Zea mays*
C19025-1	3	*P. maydis*	Illinois	USA	*Zea mays*
C19025-2	3	*P. maydis*	Illinois	USA	*Zea mays*
C19025-3	3	*P. maydis*	Illinois	USA	*Zea mays*
C19040-1	3	*P. maydis*	Michigan	USA	*Zea mays*
C19040-2	3	*P. maydis*	Michigan	USA	*Zea mays*
C19040-3	3	*P. maydis*	Michigan	USA	*Zea mays*
C19043-2	3	*P. maydis*	Indiana	USA	*Zea mays*
C19072-1	3	*P. maydis*	Wisconsin	USA	*Zea mays*

C19072-2	3	*P. maydis*	Wisconsin	USA	*Zea mays*
C19072-3	3	*P. maydis*	Wisconsin	USA	*Zea mays*
C19106-1	3	*P. maydis*	Iowa	USA	*Zea mays*
C19106-2	3	*P. maydis*	Iowa	USA	*Zea mays*
C19106-3	3	*P. maydis*	Iowa	USA	*Zea mays*
BPI638565	4	*P. maydis*		Venezuela	*Zea mays*
BPI638576	4	*P. maydis*	Mazatenango	Guatemala	*Zea mays*
BPI638583	4	*P. maydis*		Guatemala	*Zea mays*
NC19004-1	4	*Phyllachora sp.*	New York	USA	*Thinopyrum intermed*
NC19004-2	4	*Phyllachora sp.*	New York	USA	*Thinopyrum intermed*
NC19004-3	4	*Phyllachora sp.*	New York	USA	*Thinopyrum intermed*
NC19026-1	4	*Phyllachora sp.*	Illinois	USA	*triticale*
NC19026-2	4	*Phyllachora sp.*	Illinois	USA	*triticale*
NC19026-3	4	*Phyllachora sp.*	Illinois	USA	*triticale*
NC19029-1	4	*Phyllachora sp.*	Illinois	USA	*unknown*
NC19029-2	4	*Phyllachora sp.*	Illinois	USA	*unknown*
NC19029-3	4	*Phyllachora sp.*	Illinois	USA	*unknown*
NC19030-1	4	*Phyllachora sp.*	Illinois	USA	*unknown*
NC19030-2	4	*Phyllachora sp.*	Illinois	USA	*unknown*
NC19030-3	4	*Phyllachora sp.*	Illinois	USA	*unknown*
NC19032-1	4	*Phyllachora sp.*	Illinois	USA	*fall panicum*
NC19032-2	4	*Phyllachora sp.*	Illinois	USA	*fall panicum*
NC19032-3	4	*Phyllachora sp.*	Illinois	USA	*fall panicum*
NC19034-1	4	*Phyllachora sp.*	Illinois	USA	*fescue*
NC19034-2	4	*Phyllachora sp.*	Illinois	USA	*fescue*
NC19034-3	4	*Phyllachora sp.*	Illinois	USA	*fescue*
NC19035-1	4	*Phyllachora sp.*	Illinois	USA	*rye*
NC19035-2	4	*Phyllachora sp.*	Illinois	USA	*rye*
NC19035-3	4	*Phyllachora sp.*	Illinois	USA	*rye*
NC19111-1	4	*Phyllachora sp.*	South Dakota	USA	*brome grass*
NC19111-2	4	*Phyllachora sp.*	South Dakota	USA	*brome grass*
NC19111-3	4	*Phyllachora sp.*	South Dakota	USA	*brome grass*
NC19027-1	5	*Phyllachora sp.*	Illinois	USA	*triticale*
NC19027-3	5	*Phyllachora sp.*	Illinois	USA	*triticale*
NC19028-1	5	*Phyllachora sp.*	Illinois	USA	*unknown*
NC19028-2	5	*Phyllachora sp.*	Illinois	USA	*unknown*
NC19028-3	5	*Phyllachora sp.*	Illinois	USA	*unknown*
NC19033-2	5	*Phyllachora sp.*	Illinois	USA	*fall panicum*
NC19037-1	5	*Phyllachora sp.*	Illinois	USA	*rye*
NC19037-2	5	*Phyllachora sp.*	Illinois	USA	*rye*
NC19037-3	5	*Phyllachora sp.*	Illinois	USA	*rye*

[a] The genetic cluster was determine as a result of the phylogenetic analysis of the combined DNA sequences from the ITS and LSU regions. These are displayed in Figure 2, 3 and Suppl. Figure 1

[b] For contemporary material collected from field samples during this study specimens of *Phyllachora* from maize were assumed to be *P. maydis* and specimens from grass species were treated as unknown *Phyllachora* sp. For herbarium specimens we included the species name from the herbarium label.

Figure Legend

Figure 1. Signs and symptoms of *Phyllachora spp* . on grasses. *P. maydis* on maize at severe levels (A);

with ascospores being extruded from stroma (B) and showing characteristic tapering ends of mature stromata (C). *Phyllachora spp* . on *Elymus* in Michigan (D), Fall Ryegrass in Illinois \euro, and an unidentified grass in Indiana (F). Photo credit N. Kleczewski

Figure 2 . Maximum likelihood phylogenetic tree based on combined ITS and LSU sequence data from the stroma of 76*Phyllachora* isolates from herbarium and contemporary samples of infected maize and other grass hosts. *Exserohilum turcicum* was used as the outgroup.

Figure 3. Maximum likelihood phylogenetic tree based on ITS sequence data from geographically representative isolates of the 5 genetic groups of maize and grass infecting *Phyllachora* from this study and *Phyllachora* species available from GenBank. Specimens highlighted in blue a contemporary isolates collect after 2015 and those highlighted in orange are herbarium samples collected between 1905-1977.

Suppl. Figure 1 . Maximum likelihood phylogenetic tree based on the ITS sequence data from the stroma of 169 *Phyllachora* isolates from herbarium and contemporary samples of infected maize and other grass hosts. *Exserohilum turcicum* was used as the outgroup.

Data Accessibility Statement. All DNA sequence data generated by this project were deposited in Genbank as accessions OL314402-OL314494 and OL342781-OL342949.

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Author Contribution

NK designed experiments and oversaw data collection; ZD and KV conducted DNA extractions, sequencing and data generation, GIB and KB analyzed data and generated figures. KB, GIB and NK wrote and prepared the manuscript. All other authors oversaw sample collection and distribution. All authors assisted in editing and revising the manuscript.

Competing Interest

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