

**CHARACTERIZATION OF THE NATURAL ENEMIES OF RUST FUNGI  
(PUCCINIALES)**

by

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*This work is dedicated to all students from developing countries who are passionate about science. Never give up pursuing your dreams.*

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## ABSTRACT

Rust fungi (Pucciniales) are plant pathogens that cause diseases on economically important crops worldwide and threaten native plants with extinction. Fungicides and disease-resistant plant varieties are the two primary options to control rust diseases. However, more aggressive rust races are emerging, and the current control methods are not as efficient at managing disease as they have been in the past. Thus, we must find other alternatives to keep rust fungi at low population levels to prevent high-yield losses and preserve our natural ecosystems. Biological control (BC) is one promising alternative to the current control methods. BC uses natural enemies (NEs), such as insects and microorganisms, to control pests and plant diseases. However, because the NEs of rust fungi are scarcely documented and not well characterized, using BC for rust diseases is not feasible with the current state of knowledge. Characterization studies of NEs of rust fungi are essential to select or discard potential biological control agents. How do NEs infect or feed the rust? Are NEs specific to rust species or genera? How diverse are they? and how adapted are they to different geographic regions? These are a few of the unresolved questions needed to answer to tap the potential of NEs in applied research for the biological control of rust diseases. This dissertation thesis had three aims: 1) to catalog the NEs of rust fungi from the Arthur Fungarium (PUR) rust collections emphasizing specimens from the Americas and providing barcoding sequences, photographs, and morphological descriptions. This was accomplished by thoroughly screening ca. 50% of the accepted rust genera collected over 200 years from across the globe and from 166 host plant families; 2) to describe new fungal species associated with rust fungi; and 3) to characterize the two most common NEs of rust fungi in aspects related to species diversity, host preference, geographic distribution, and antagonistic interactions. Of the 5,618 randomly screened rust specimens, we found 543 specimens with fungal NEs or hyperparasites. These belonged to the fungal genera *Helicobasidium*, *Ramularia*, *Sphaerellopsis*, *Simplicillium*, and *Trochila*. In addition to fungal antagonists, we found larvae of the gall midge *Mycodiplosis*, whose larval stage feeds rust spores, present in 287 specimens. Among these NEs, the fungal genus *Sphaerellopsis* and the fly larvae *Mycodiplosis* were the most commonly associated with rust fungi samples. Therefore, we focused on members of these two genera to determine their host-specificity, geographic distribution and describe early antagonistic interaction events. Our results suggest that members of *Sphaerellopsis* and *Mycodiplosis* do not prefer specific rust species or genera. Thus, further



studies on non-target species are needed to determine how broad their host range is. In addition, we found that both NEs have a cosmopolitan distribution. However, the fly larvae of *Mycodiplosis* show distribution patterns, suggesting that some of these species are regionally restricted, especially in the Americas. This result is an exciting finding for conservation biological control where native NEs could be stimulated to increase their populations and control targeted pests. Thus, *Mycodiplosis* larvae may be a good candidate for local use to control rust diseases without introducing non-native invasive species to new environments. Lastly, we describe the antagonistic interaction events between *Sphaerellopsis macroconidialis* and the urediniospores of southern corn rust, *Puccinia polysora*. Although *S. macroconidialis* was reported as a NE of rust fungi, the nature of the interaction had not been previously described. Herein, we observed how the hyphae of *S. macroconidialis* grew along the urediniospores germinative tube and tightly coiled around this structure. We also observed a turgor loss of the germinative tube a few days after interacting with the NE. Overall, these results contribute significantly to the characterization of the NEs of rust fungi for further studies in biological control and to develop sustainable agronomical practices in controlling rust diseases.

# CHAPTER 1. RUST FUNGI: ECONOMIC IMPORTANCE, CONTROL METHODS, AND NATURAL ENEMIES

## 1.1 Rust diseases

### 1.1.1 Impact on economically important crops

Rust fungi are plant pathogens that cause diseases of numerous economically important crops and threaten food security worldwide. For example, *Puccinia sorghi*, the causal agent of common corn rust (CCR), and *Puccinia polysora*, the causal agent of southern corn rust (SCR), are found worldwide and have caused severe epidemics on maize crops in the past and are likely to do so in the future. Between 1978 and 1979, CCR caused up to 50% yield losses in susceptible corn plants in the USA (Groth et al., 1983). In the 1950s, SCR caused up to 84% yield losses in the Philippines (Reyes, 1953) and 50% in West Africa (Rhind et al., 1952), and in 1998, it caused up to 53% yield losses in China (Y. Y. Liu & Wang, 1999). Outbreaks caused by SCR have also recently occurred in the USA and Canada, with estimated yield losses of 298 million bushels between 2016 and 2019 (Mueller et al., 2020). *Puccinia graminis* f. sp. *tritici*, the causal agent of wheat stem rust, *Puccinia recondita* f. sp. *tritici*, the causal agent of wheat leaf rust, and *Puccinia striiformis* f. sp. *tritici*, the causal agent of stripe rust of wheat, are also serious threats to global food security. New virulent strains of these diseases infect all cultivated wheat varieties, and less than 5–10% of all available varieties in germplasm stocks have demonstrated rust resistance (Lidwell-Durnin & Laphorn, 2020).

Not only do rust fungi infect cereal crops, but they also infect shrubs and perennial woody crops. For instance, *Hemileia vastatrix*, the causal agent of coffee leaf rust, is found in all coffee-producing countries (Arneson, 2000; Kolmer et al., 2009; Ristaino et al., 2021) and causes up to 40% annual yield losses in Latin-American countries (Cressey, 2013; Cristancho et al., 2012; Kumar et al., 2016; McCook, 2006). In addition, species of *Melampsora* cause diseases in woody plants, primarily willows and poplars planted across the globe. In Europe, *Melampsora* has caused up to 40% yield losses of willows used for biomass energy production (Pei & McCracken, 2005).

According to the United Nations, the world population will be 9.7 billion by 2050 (United Nations & United Nations, Department of Economic and Social Affairs, 2019), requiring an increase in food production of roughly 70% (FAO, 2009). It is also predicted that by 2050 the severity and incidence of rust diseases will increase substantially based on climate projection models (Lidwell-Durnin & Laphorn, 2020; Ramirez-Cabral et al., 2017; Torres Castillo et al., 2020). Unfortunately, new resistant rust strains are emerging, and the current strategies for rust disease management are not as effective as they once were because of the extensive use of monocultures and overuse of fungicides (Ellis et al., 2014; Godoy et al., 2016; Kikuhara et al., 2019; Langenbach et al., 2016; Lidwell-Durnin & Laphorn, 2020; Van De Wouw et al., 2010).

### ***1.1.2 Impact on natural ecosystems***

Rust fungi can cause devastating damage to natural ecosystems by threatening native plants with extinction. *Austropuccinia psidii*, the causal agent of myrtle rust, infects more than 522 plant species in the Myrtaceae family across 67 genera (Fernandez Winzer et al., 2018; Roux et al., 2016; Soewarto et al., 2018, 2019). This family is widely distributed and is common in biodiversity hotspots worldwide (Lucas & Bünger, 2015). At least 27 countries on four continents have already reported myrtle rust disease (Simpson et al., 2006; Soewarto et al., 2019). Because the Myrtaceae dominates the natural ecosystems of Australia, myrtle rust has rapidly invaded several regions of this continent in the last ten years (Berthon et al., 2019). The South and Western regions are the only ones where *A. psidii* is not yet present. It is predicted that almost 57% of plant species in Australian rainforests will be exposed to myrtle rust, and 16 tree species are projected to become extinct if current trends continue (Fensham & Radford-Smith, 2021). Eucalyptus and tea trees, both of which provide multiple environmental and socio-economic benefits (Birhanu & Kumsa, 2018; Mukhopadhyay & Mondal, 2017), are Myrtaceae species potentially affected by this rust disease (Berthon et al., 2019). Myrtle rust also threatens the extinction of endemic plant species to islands in the Pacific region, such as *Eugenia koolauensis* in Hawaii and *Eugenia gacognei* in New Caledonia (Fensham et al., 2020). Because of their dependence on Myrtaceae, many bird, mammal, and insect species will also be at risk if these plant species become extinct (Lughadha & Proenca, 1996). Although Australia is particularly vulnerable, the biodiversity of other continents is also threatened by myrtle rust if we do not find appropriate measures to control rust diseases in natural ecosystems.

### ***1.1.3 Biology of Rust Fungi***

Rust fungi are complex plant pathogens. They are a monophyletic group in the order Pucciniales (Basidiomycota), with approximately 8,000 described species, making them the largest known fungal plant pathogen group (Aime et al., 2017). All known rust fungi are obligate biotrophs. They reproduce and complete their life cycles only within the living tissues of their host plants and cannot grow on artificial culture media (Arthur et al., 1929), which complicates manipulation of them in the laboratory. Rusts are host-specific, and each rust species infects only a limited range of host plants (Aime et al., 2017; Arthur et al., 1929). These pathogens have specialized structures such as appressoria and haustoria to penetrate and obtain nutrients from living host cells (Kolmer et al., 2009).

Rust fungi have complex and variable life cycles. They can pass through up to five spore stages, known as spermatia, aecia, uredinia, telia, and basidia, in structures called sori (singular: sorus), except for basidia, which are produced by the germination of teliospores. Rust fungi can be categorized according to these spore stages (Arthur, 1934; Cummins & Hirastuka, 2003; Shattock & Preece, 2000; Tranzschel, 1904). Species developing five spore stages, such as wheat stem rust, have a macrocyclic life cycle. Those lacking the uredinial stage, such as cedar-apple rust, have a demicyclic life cycle. Those that lack aecia, uredinia, and in some cases, spermatia, such as chrysanthemum white rust, have a microcyclic life cycle. Rust fungi can require only one host plant or more than one taxonomically unrelated host plants to complete their life cycles. Rusts such as sunflower rust that can complete their life cycle on a single host plant are termed autoecious. Those requiring two unrelated host plants to complete their life cycle, such as wheat stem rust, are heteroecious. There are many rust species whose complete life cycle and any alternate hosts are unknown. Some of those rusts are economically important species, such as *Phakopsora pachyrhizi*, the causal agent of soybean rust, and *Hemileia vastatrix*, the causal agent of coffee leaf rust. This variation of spore stages and the ability to infect unrelated hosts at different stages makes it challenging to generalize rust fungal biology and systematically find strategies to control rust diseases.

#### ***1.1.4 Rust Fungi, dispersion, and infection***

Rust fungi can quickly spread and travel across continents (Hovmøller et al., 2008). Rust spores are dispersed by wind currents and are deposited by rainfall onto the host plant surface (Kolmer et al., 2009). Uredinia are considered the most dangerous spore stage of disease infection and dispersion because urediniospores are the only spores that can re-infect the same host continuously, as long as environmental conditions are favorable (Aime et al., 2017; Arthur et al., 1929). If a new rust strain emerges from a single urediniospore, massive amounts of genetically identical urediniospores will be produced in the same crop season and disperse quickly, increasing the risk of new epidemics (Kolmer et al., 2009).

Rust disease symptoms caused by urediniospores can appear on susceptible host plants in about a week if environmental conditions are favorable (Rowell, 1984). Upon arrival on the host plant surface, the urediniospore produces a germ tube. This tube elongates until reaching a stoma of the host plant. Since rust fungi are host-specific, stoma detection is unique to the germ tube of each rust species. Once the germ tube is in contact with the stoma, it stops elongating, and an appressorium is formed over the stoma (Voegelé & Mendgen, 2003). The appressorium pushes to penetrate the plant tissue and forms a hypha. A substomatal vesicle is formed from this hypha from which an infection hypha differentiates. Then, a haustorial cell forms a penetration peg that secretes enzymes to degrade and penetrate the plant cell wall and absorb nutrients (Lorrain et al., 2019; Voegelé et al., 2009; Voegelé & Mendgen, 2003). Sporogenous cells are later formed to break the surface of the epidermis. Urediniospores are formed from these cells, and the cycle continues. Although rust fungal structures infect the host tissues of susceptible plants and suppress plant immunity, they do not typically kill their hosts because rust fungi still need living tissue to reproduce and survive.

#### ***1.1.5 Virulence factors, genomes, and transcriptomes of rust fungi***

Rust fungi secrete proteins into their host cells to infect them, uptake nutrients, and manipulate their host's metabolism (Voegelé & Mendgen, 2003). These secretory proteins, also known as effector proteins, are usually critical to facilitating infection in the susceptible host. However, little is known about rust fungi effector proteins and their functions, primarily due to the lack of

available rust genomes and transcriptomes. Of the 8,000 currently identified rust species, the genomes of only 26 have been sequenced and assembled, and only eight are annotated (NCBI, n.d.). Assembly and annotation of rust fungal genomes are complicated when using short-read sequencing technologies because of the high proportion of repetitive elements, ranging from 18% to 75% (Aime et al., 2017; Duplessis et al., 2011; Lorrain et al., 2019; Nemri et al., 2014; Tavares et al., 2014). Unfortunately, most of the reported rust genomes have been sequenced using short reads (NCBI, n.d.), which explains the slow pace of detection and characterization of avirulent genes. However, with the development of long-read sequencing, we can expect an improvement in the quality of rust genomes assembly and, thus, detection and characterization of many new candidate avirulent genes.

Transcriptomic analyses have helped narrow the rust candidate effectors involved in the infection of economically important plants (Duplessis et al., 2012, 2014). Transcriptomes of the urediniospores are the most commonly sequenced among the five spore stages due to the importance of these spores in developing diseases on economically important crops and likely because it is easy to collect enough spores from them. Some candidate effectors have already been described in some rust species, such as *Melampsora larici-populina*, *Melampsora lini*, *Uromyces fabae*, *Phakopsora pachyrhizi*, and *Puccinia striiformis* f. sp. *tritici*, (Cantu et al., 2013; Duplessis et al., 2012; Hacquard et al., 2012; Kunjeti et al., 2016; Lorrain et al., 2019). Host-induced gene silencing (HIGS) and fluorescence tags are two approaches used to characterize the function of those candidate effectors. HIGS is an RNA interference process in which double-stranded RNA can be expressed in a plant to silence a pathogen gene (Yin & Hulbert, 2018). Fluorescent tags are fluorescent proteins that can attach chemically to effector proteins of interest to detect their location in the host cell and observe the protein functions and mechanism to infect the host. However, the lack of transcriptomic sequencing of other rust spore stages infecting alternate hosts continues to restrict advances in detecting essential effectors for infection of economically important plants (Lorrain et al., 2019). In addition, it is still unknown whether effectors act alone or together with other effectors to infect host cells. Thus, it is important to sequence the transcriptomes of more rust spore stages on several rust species and to determine the synergistic effects of several effector proteins for each rust species.

### ***1.1.6 Rust disease management***

Historically, there have been several strategies to control rust diseases, but some have become outdated and are no longer effective. In the early 20<sup>th</sup> century, the first strategy to control rust diseases was to abandon infested acres and to move crop production to areas where rust was not present (Gianessi & Reigner, 2006). This strategy did not last long because rust spores dispersed widely through wind currents, and adjacent farmlands were rapidly infested. The second strategy involved the removal of nearby alternate plant hosts of the rust to prevent the completion of its life cycle and thus reduce the pathogen's pressure on susceptible crops. This strategy was successfully executed in 1918 in North America to protect wheat crops by eradicating barberry plants, the alternate plant host of the rust (Roelfs, 1982). Although it was more effective than the abandonment strategy, eradicating the alternate host was expensive and did not eliminate the threat of rust infections. For example, rust infected-wheat crops from Mexico and Texas were able to disperse to the northern regions of North America (Stakman, 1957). Thus, this strategy was also discarded. With advances in understanding the biology of rust fungi and the advent of the Green Revolution, other strategies emerged to control rust diseases. These included genetic rust resistance and chemical control. Genetic rust resistance and chemical control are usually combined to control rust infections on economically important crops. However, the constant use of these two strategies exerts strong selective pressures on the rust. Thus, even when most rust spore populations are eliminated, the few that survive resist both chemical control and genetic resistance and can then trigger new outbreaks and epidemics.

#### ***1.1.6.1. Genetic resistance to control rust diseases***

Genetic resistance is known to be the most effective strategy to control rust diseases in susceptible crops. Employment of this strategy prevents the overuse of fungicides and reduces production costs and negative environmental impacts. There are currently three ways to confer genetic rust resistance on cultivars: race-specific, non-race-specific, and resistance gene pyramids (Mundt, 2018; Periyannan et al., 2017). Cultivars that are race-specific resistant possess resistance genes against specific races of a rust species. This resistance is an example of the classical gene-for-gene model, in which a specific host-resistance (R) gene interacts with a pathogenic avirulent (Avr) gene (Flor, 1971). Although it is known that cultivars with R genes possess high levels of resistance

to rust diseases, the intensive monoculture use has led to the selection pressure of several rust species to overcome resistance within a few years. Thus, cultivars that possess single R genes are not durable enough to confer rust resistance.

Cultivars that are non-race-specific resistant possess resistance genes against all or most of the races of a rust species. This resistance generally involves only a partial phenotypic resistance, resulting in "slow rusting." For example, in wheat, non-race-specific resistance appears at later stages of development, referred to as adult plant resistance (APR). In contrast to R genes, APR genes are usually highly durable, so their resistance remains effective over long periods (Ellis et al., 2014). Unfortunately, cultivars with APR genes cannot entirely prevent high yield losses if a severe rust epidemic occurs. Lastly, resistance gene pyramids refer to multiple genes (either R, APR, or APR+R) acting together to provide synergistic effects on the host to increase rust resistance's durability (Mundt, 2018). Although this strategy seems to be the best to decrease yield losses and confer rust resistance at different developmental stages of the plant, applying resistance gene pyramids in classical cross-breeding is challenging because all the desirable genes may not be inheritable. Thus, gene editing would be the most addressed approach to obtaining plant cultivars with multiple resistant genes. However, over 20 countries do not support this application and thus have banned GMOs (*Countries That Ban GMOs*, 2022). Although genetic resistance seems to be the best strategy to control rust diseases nowadays, there is still missing information on identifying and characterizing R and APR genes. The function of resistance genes is mainly unknown when these genes interact with each other in a single cultivar (Mundt, 2018).

#### *1.1.6.2 Fungicides to control rust diseases*

Fungicides are the most common option to control rust fungi when rust-resistant cultivars are unavailable. These chemicals can inhibit or eradicate the growth of a fungal pathogen and its spores (Reis & Carmona, 2013). Fungicides can be classified based on their mobility, mode of action, and time of application (Hewitt, 1998). The most common rust fungicides are systemic. They penetrate plant surfaces and move upward through the xylem vessels. Rust fungicides are generally curative because they are applied after the infection but before symptoms appear on the leaf surface (Carmona et al., 2020). With respect to their mode of action, rust fungicides can be demethylation inhibitors (DMIs), quinone outside inhibitors (QoIs), or succinate dehydrogenase inhibitors



(SDHIs) (Carmona et al., 2020; Cools et al., 2013). DMIs inhibit sterol biosynthesis, an essential component of the fungal cell membrane, QoIs inhibit mitochondrial respiration in complex III, and SDHIs inhibit fungal respiration in the mitochondrial complex II. Like pyramiding of genetic resistance loci, the most common strategy to control rust diseases by fungicides is applying a mixture of QoIs and DMIs or QoIs and SDHIs to field crops. Mixtures of fungicides are currently used because the application of one type of inhibitor alone is usually not efficient and triggers the development of new rust strains resistant to fungicides (Oliver, 2014). However, using fungicides with combined modes of action can lead to stronger selective pressure on the pathogen that can select for more virulent rust strains and new, rapidly spreading epidemics.

#### *1.1.6.3 Impacts of genetic resistance and fungicides on rust disease control*

Although genetic resistance and fungicides are the most common options to control rust diseases, several problems are associated with either or both of these strategies. Genetic resistance is challenging because it can take decades to obtain rust-resistant cultivars, and even if new cultivars are developed, this does not necessarily provide complete crop protection against rust (Periyannan et al., 2017). Genetic resistance has also led plant breeders to keep only rust-resistant genes and the most desirable commercial traits on the cultivars. The restriction on desirable traits has also eliminated indirect plant defense traits, which help protect plants against other diseases and pests (Stenberg, 2017). Thus, even with the development of desirable rust-resistant cultivars, the elimination of intrinsic plant defenses forces the application of fungicides to control other diseases and pests.

Furthermore, the intensive use of the same resistant cultivar in monocrops has resulted in new rust outbreaks due to the strong selective pressures on the pathogen. These selective pressures eliminate susceptible rust strains but lead to the emergence of resistant and more aggressive races (Almeida et al., 2021; Omara et al., 2021; Twizeyimana & Hartman, 2012). Because monocultures lack genetic variation, if a single rust spore mutates, this one can quickly infect, reproduce, and cause epidemics. Thus, genetic resistance alone cannot control rust diseases on any crop across the globe, especially when there is only one rust-resistance gene in a cultivar.

The overuse of fungicides has triggered fungicide resistance in some rust strains of several crops. For example, *Gymnosporangium asiaticum*, the causal agent of Japanese pear rust, was not sensitive to the DMI fungicides Fenarimol and Difenconazole when it was applied to Japanese pear crops in Fukuoka Prefecture (Kikuhara et al., 2019). In South America, the Asian soybean rust *Phakopsora pachyrhizi* also evolved resistance to three fungicides with different modes of action, QoI, SDHI, and DMIs (Klosowski et al., 2015; Simões et al., 2018). Resistance to these fungicides by *P. pachyrhizi* is primarily due to the overuse of fungicides on soybean crops. Although there are no reports of cereal rusts conferring resistance to any fungicide so far, Cook et al. (2021) prompted to screen 363 wheat stem rust isolates from several wheat-growing countries for mutations associated with fungicide resistance. Their results showed a high proportion of isolates from China and New Zealand carrying a DMI resistance gene and another group of isolates carrying an SDHI resistance gene, which is homologous to that reported in Asian soybean rust. The reports of fungicide resistance by Japanese pear rust, Asian soybean rust, and wheat stem rust highlight the need to improve and find alternatives for rust disease management.

The use of fungicides on crops can also cause negative impacts on the environment and human health. For example, H. Wang et al. (2021) reported chronic pollution in aquatic systems caused by the strobilurin fungicide (QoI), widely used in Asia to control soybean rust. This type of fungicide can cause fish to become susceptible to viral diseases (H. Wang et al., 2021). The fungicide Headline®, with the active ingredient Pyraclostrobin (QoI), can cause more than 50% mortality of the great plains toad *Anaxyrus cognatus* juveniles at recommended application rates on corn and 100% mortality of tadpoles at one-tenth the label rate for corn (Belden et al., 2010). Although toxicology studies on the overuse of fungicides in human health are scarce, recent studies in Brazil now suggest a positive correlation between human exposure to pesticides with colon cancer mortality (Martin et al., 2018). These toxic effects on humans and wildlife raise essential concerns about the long-term impacts of fungicide use on public health and wildlife conservation (Nicolopoulou-Stamati et al., 2016). Thus, the overuse of fungicides in the agriculture sector must decrease. We must be able to find alternatives to control rust diseases that do not cause ecological imbalances.

In addition to the risks to human health and aquatic wildlife, the overuse of fungicides can also harm natural enemies of rust fungi (NEs). NEs generally refer to insects or microorganisms, such as bacteria and fungi, that naturally parasitize or compete with pathogens or pests in natural ecosystems (Hajek & Eilenberg, 2018). Fungicide application can alter the population of NEs, resulting in reductions in biodiversity of surrounding natural ecosystems, triggering new outbreaks in natural ecosystems, and causing vast ecological imbalances. Unfortunately, since there is little information regarding species diversity and biogeography of NEs of rust fungi across the globe, the overuse of fungicides in field crops may negatively affect the population of these organisms or, even worst, threaten extinction. Therefore, the overuse of fungicides should also be reduced to preserve NEs' diversity and study those organisms as alternatives to control rust diseases.

#### *1.1.6.4 Integrated Pest Management strategy (IPM) to control rust diseases*

Integrated pest management (IPM) is another strategy to control plant diseases and pests by integrating several preventive and curative methods to minimize chemical control and maintain ecological balance (Karlsson Green et al., 2020; Stenberg, 2017). IPM is now considered the new paradigm for crop protection due to the trend of pests resisting and surviving chemical control. Unlike chemical control, IPM does not eliminate pathogens and pests from crops but keeps them at low population levels, so they do not cause significant yield losses in crop production (Karlsson Green et al., 2020). Although IPM is more commonly used in the European Union countries, this strategy is still not widely used in other regions, including developing countries, due to a lack of knowledge, pesticide industry interference, and account for traditional pest and disease management strategies (Parsa et al., 2014). The current use of IPM has been misinterpreted as combining several curative and preventive methods with the primary goal of getting resistance cultivars to diseases and pests (Stenberg, 2017). IPM, on the contrary, aims to integrate several methods to get synergistic effects for crop protection without focusing on a single strategy (Stenberg, 2017). Unfortunately, because of the IPM misconception, this method has not yet been employed in the protection of most crops worldwide, and thus, genetic resistance and chemical control are still broadly used.

The classic IPM is usually illustrated as a pyramid with several tiers. A base tier involves preventive methods such as using disease or pest-resistant varieties, eliminating weeds and other

plants that may be an alternate host of pests and diseases, and using clean equipment to prevent the dispersion of pests. The second tier involves cultural control, such as eliminating leaf piles where the pest or pathogen may hide and overwinter. Physical or mechanical control is the third tier in the classic IPM strategy. This tier provides a physical barrier to prevent pests or diseases from infesting the crop, including mowing, hoeing, and tilling, but also using traps and the push-pull strategy. Physical and mechanical control works best when pests are visible and have easy access. The fourth tier involves biological control, whose primary purpose is reducing the population of the pest or disease to minimize significant yield losses. Finally, the last tier, and the one that should be used only when strictly necessary, is chemical control, a method that intends to eradicate or kill the disease or pest. Although IPM has been accepted and incorporated in several countries, primarily in the European Union, most crops still depend on chemical control (Hokkanen, 2015). This is because IPM is still far from being efficiently studied, and the holistic science of IPM has not arrived yet (Stenberg, 2017).

Fortunately, a modern IPM framework has been proposed in recent years to face our current global necessities in crop protection (Stenberg, 2017). Although this framework is illustrated as the traditional IPM pyramid with several tiers, a new circular tier is proposed containing five essential elements that are classified as the basis of this modern IPM framework. These elements include intrinsic heritable plant resistance, plant vaccination, inter and intra-specific botanical diversity, biorational synthetic volatiles, and biological control. Two more tiers are part of this pyramid, a base tier, which involves cultural, mechanical, physical, optic, and auditive control, and a top tier, which involves chemical control only used when strictly necessary if the other elements fail to control plant diseases and pests.

Despite the advantages that IPM offers for crop protection, the holistic science of IPM is not yet well developed. According to Stenberg (2017), we are far from understanding and developing programs to apply IPM to different crops. Many farmers worldwide continue to use individual strategies to control plant diseases, such as chemical control. There is a broad perception that the IPM strategy is less efficient and, thus, a fear of high yield losses in crop production. Integrating the five elements mentioned above and filling knowledge for IPM on plant diseases will prevent a

strong selective pressure for pests and diseases to confer resistance. Therefore, it is essential to prioritize studies on the above elements as interdisciplinary research for crop protection.

IPM of rust diseases has been mainly used as a combination of preventive methods prioritizing the use of rust-resistant cultivars. Preventive methods include planting early maturing cultivars, destroying alternate hosts of the rust if the host is known, and avoiding dense plantations to allow air circulation and light penetration into the crop (UMass, n.d.). Chemical control is also used as a curative method when no rust-resistant varieties are available or the disease has arrived early during the growing season. Nevertheless, as mentioned above, the IPM strategy requires more than a combination of preventive and curative methods. Instead, IPM aims to integrate several methods to act synergistically.

There are several reasons why IPM of rust diseases is a challenge. Most of the economically important crops affected by rust diseases are usually planted as monocrops without any botanical diversity, which is essential for IPM strategy. The lack of diversity makes the main crop wholly exposed to the same pathogens and pests, facilitating an easy overgrowth of the pest population and, thus, a significant challenge to control. In addition, because all the rust is deposited on resistant cultivars, these cultivars have likely lost their natural indirect defenses and plant vaccination to naturally combat rust diseases. If intraspecific botanical diversity were present on those crops, there would be variation in resistance genes, so not all plants would be susceptible to the disease. Interspecific botanical diversity would also benefit the main crop from associational resistance, thanks to other plant species. Furthermore, biological control is typically not used because living agents are traditionally known to attack the disease slower than the development of the disease by itself to infect and disperse in the crop. Lastly, although volatile emissions of the host plant seem to play an important role in interactions with the rust and natural enemies (Eberl et al., 2018), studies are scarce. Therefore, even though some preventive and curative methods are used in IPM for rust diseases, we are still missing studies that integrate those methods with the new elements proposed in the modern IPM framework to control rust diseases better.

## 1.2 Biological control

### 1.2.1 Definition

Biological control, or biocontrol, is currently defined as using living agents to combat pathogens, pests, and weeds for human benefit (Stenberg et al., 2021). Those living agents can use several interaction mechanisms to affect the target pathogen, pest, or weed negatively. One of these mechanisms, and perhaps the most commonly known to control plant diseases, is a direct attack on the pathogen, known as hyperparasitism, which consists of the penetration or enfolding of host structures, systemic infection, death, and ingestion of the host (Benítez et al., 2004; Weindling, 1932). Other biocontrol mechanisms, such as antibiosis, competition, and plant-induced systemic resistance (ISR), are also known for plant disease control. Antibiosis refers to the production of antimicrobial metabolites by natural enemies to degrade pathogens' cell walls (Köhl et al., 2019; Whipps & Gerhardson, 2007). Competition refers to the living agent rapidly consuming the same nutrient resources as the pathogen and colonizing space before the pathogen arrives at the plant tissue. Lastly, plant-induced systemic resistance (ISR) refers to enhancing plant defense against a broad range of pathogens by prior infection of selected beneficial microbes (Pieterse et al., 2014; Rahman et al., 2018). Other mechanisms may exist, but they are currently unknown (Stenberg et al., 2021). Regardless of the mechanism that microbial biological control agents used to combat plant pathogens, studies on their interaction are crucial to successfully controlling plant diseases and understanding the risk of using these living agents for the environment and humans (Köhl et al., 2019).

### 1.2.2 Categories of biological control

Four categories classify various approaches to biological control (Stenberg et al., 2021). When a biological control agent (BCA) is native to an ecosystem and interacts with pests or pathogens independently of any human intervention, this biological control class is categorized as *natural biological control*. However, if the BCA is native but actively stimulated by human intervention, this class is categorized as *conservation biological control*. On the contrary, if the BCA is exotic in the ecosystem and applied as an additional organism to become permanently established, this class is categorized as *classical biological control*. Lastly, if the exotic living agent is only intended to be temporarily established, this class is categorized as *augmentative biological control*.

(Stenberg et al., 2021). Nevertheless, van Lenteren (2012) posits that indigenous living agents are used in *augmentative biological* control due to the concern about the importation and release of exotic living agents to other countries.

Augmentative and classical biological control are the two most commonly used categories to control diseases, pests, and weeds. Classical biological control has been used since the second half of the nineteenth century (DeBach, 1964; Eilenberg et al., 2001; Stenberg et al., 2021) with thousands of studies and applications on pests and weeds mainly (Cock et al., 2010; van Lenteren, 2012; Winston et al., 2014). Because these two categories are known to import living agents to a local environment, they are subject to several legislations and regulations in each country. These regulations involve mainly environmental risks, including new problematic pests and a threat to biodiversity conservation (Barratt et al., 2018; Ehlers et al., 2020; EU, 2011; FAO, 2017). Thus, although augmentative and classical biological control offer an alternative to chemical control, they are still delicate methods that require intense research programs, from the biology and ecology of the living agent to the environmental risks for human health and ecological balance.

### ***1.2.3 Selection for successful biocontrol agents***

It is essential to highlight that not all native natural enemies (NEs) that combat diseases or pests have great potential as biological control agents. In fact, of hundred species of NEs combating a specific pathogen or pest, only a few are potential candidates as biological control agents (van Lenteren et al., 2019). Proceedings to select, commercialize, and implement appropriate biological control agents (as listed in table 1.1) can take about ten years, similar to developing synthetic pesticides (Fravel, 2005; van Lenteren et al., 2019). In addition, because this method is included in the IPM strategies, integrating living agents with other control strategies can also affect their efficiency (Stenberg, 2017). Thus, even though biological control is considered an eco-friendly alternative for pest control, it must still be treated cautiously to avoid the release and dispersion of new pests, as occurred in the past (Simberloff & Stiling, 1996).

Characterizing NEs is the most critical issue for selecting a potential BCA. Correct taxonomical identification of the NE is always crucial to allow clear communication between scientists and have consistent results in the characterization of any NE (Huber et al., 2002). Introducing a poorly

identified NE can quickly introduce new pests to the environment, releasing exotic species without any beneficial purpose and leading to an ecological imbalance (De Moraes, 1987; Hoelmer & Kirk, 2005; Rosen, 1986). In addition, the mechanism by which the NE combats the pest helps us to determine how to approach the pest and when and where to release the NE to the crop (Fravel, 2005). Studies on their geographic distribution and host-specificity are also critical to understanding their behavior based on the environment where they are found and how specific they are to their target host for future risks of becoming new potential pests. Finally, whole-genome sequencing, transcriptomics, proteomics, and metabolomics analyses help us to identify which genes and proteins of the NEs are highly expressed and produced at specific interaction events while combating rust diseases.

Table 1.1 Summary of general issues for detection and commercialization of efficient BCAs

1. Knowledge of the biology of the target pathogen
2. Efficient screening for natural enemies
3. Characterization of the natural enemy
3.1 Taxonomic identification (morphology and molecular identification)
3.2 Biology and Ecology (life cycle, mechanism of action, the geographic distribution that coincides with the target pathogen, climatic adaptation, host-specificity, synchronization with the pathogen, capability to search for pest, high pathogen kill ability, good pathogen finding capacity)
3.3 Whole-genome sequencing, transcriptomics, proteomics, and metabolomics analyses
4. Mass-production
5. In-vitro essays and Greenhouse testing
6. Environmental and human health risks
7. Cost-effective mass production, shipment, and release
8. Field testing
8.1 Combined with pesticides and other chemicals
8.2 Combined with cultural and physical practices
8.3 Integrated with IPM strategies
9. Reliability of performance under practical production conditions
10. Market potential
11. Importation and registration procedures
<i>Note:</i> Adapted and modified from "Table 1. Issues related to the pre-introduction evaluation of natural enemies." Van Lenteren et al., 2019

Although the first procedure from table 1.1 does not involve the study of the pathogen by itself, understanding the biology of the target pathogen is as essential as understanding the biology of the NE. Knowledge of the target pathogen's biology facilitates the efficient screening for NEs. For example, finding where the pathogen is most vulnerable will help to understand where and when the living agent should be applied to the crop (Fravel, 2005). Similarly, the life cycle of the



pathogen, its plant host range, and genotypic diversity (Moricca & Ragazzi, 2008) help to narrow the search for the best NE candidate to control the pest or disease.

Issues four to eleven listed in table 1.1 are studied to determine the efficiency of a NE as a biological control agent. These issues aim to determine if the NE can reduce the pest or disease population, have no or few adverse effects on the crop, environment, and human health, and concerns related to production, marketing, and registration of the NE as a BCA (van Lenteren et al., 2019). Additionally, depending on the category of the biological control used, some issues have more relevance than others in selecting biological control agents. Nevertheless, according to van Lenteren et al. (2019), the issues listed in table 1.1 do not have the main objective of finding the best NE for biological control. Instead, those issues aim to exclude the NEs that are not efficient quickly, can cause adverse environmental impact, and are too expensive for mass production.

### **1.3 Natural enemies of rust fungi**

#### ***1.3.1 Overview***

A natural enemy (NE) is a native organism of a natural ecosystem that combats either pathogens, pests, or weeds without any human intervention. As mentioned above, a NE may become a great biological control agent, but not all NEs can be successfully used for biocontrol. The issues listed in Table 1.1 are needed to study in order to discard the NEs that do not have the potential as biocontrol agents. The following organisms listed in table 1.2 include all the NEs of rust fungi reported so far. Unfortunately, most of them are merely reported as present in rust fungi and others as attacking rust spores directly in pathogenicity tests. Nevertheless, most of the characterization studies and other issues listed in table 1.1 are still scarce.

Although several biocontrol agents, such as bacteria, fungi, and insects, are registered as biopesticides, none have been commercialized to target rust diseases strictly. However, due to the increase in global wheat consumption and the high risk of wheat rust diseases becoming resistant to fungicides, there has recently been a spike in the screening for natural enemies against wheat rust diseases. The diverse NEs found are first identified by morphology and molecular data and then evaluated in pathogenicity tests. Some of these natural enemies are also tested in-vitro and

greenhouse essays. Nevertheless, other aspects of the biology and ecology of these NEs are not studied yet. Detecting and commercializing efficient BCAs to combat plant disease is tedious and lengthy. However, we encourage the scientific community to continue studying those organisms that show positive results in reducing rust disease and finding a good BCA against rust fungi.

### ***1.3.2 Fungi as natural enemies of rust fungi***

Fungi are the most reported group of NEs of rusts. More than ten fungal genera from several families have been described intimately associated with rust species. In addition, several mechanisms of action have been reported to combat rusts, including a direct attack on rust spores, secretion of antibiotic metabolites reducing rust spore germination and infection, induction of systemic plant resistance, and competition for nutrients and space. Direct attack or hyperparasitism is the most commonly observed interaction between fungal NEs and rust fungi. The uredinial state is the most commonly reported as being hyperparasitized by fungal NEs either because it is the most common spore state in nature or because it is of special interest for causing epidemics and re-infect in the same season on economically important crops. Most fungal NEs belong to the phylum Ascomycota, except for *Tuberculina* (the anamorphic stage of *Helicobasidium*), which belongs to the class Pucciniomycetes. Despite the necessity to find alternatives to control rust fungi, no broad screening surveys for fungal NEs on a wide range of rust genera have been done. Thus, the diversity of fungal species that naturally parasitize rust fungi is still unclear. In addition, studies related to the host preferences, geographic distribution, and life cycle, among other characterization studies, are still scarce for most of the NEs of rust fungi.

The following fungal genera described in this literature review are the most studied NEs of rust fungi. Although additional fungal genera have been found associated with rusts, they will not be described here due to the scarce documentation to confirm their role as NEs.

#### ***1.3.2.1 The fungal genus Aphanocladium***

*Aphanocladium* W. Gams (Nectriaceae, Ascomycota) is an understudied fungal genus. Of the currently four accepted members, *Aphanocladium album* is the only species reported as a hyperparasite of rust fungi (Koç & Défago, 1983). It has also been reported as a pathogen of slime

molds (Myxomycetes) (Ing, 1974) and of the cultivated mushroom *Agaricus bisporus* (Nair et al., 1979). This species can infect several rust spore stages, including aeciospores, urediniospores, and teliospores (Koç et al., 1983; Koç & Défago, 1983). When *A. album* is found hyperparasitizing rust fungi, it looks like a cottony whitish felt mat. Pathogenicity tests of *A. album* against several rust species of *Hemileia*, *Melampsora*, *Puccinia*, *Phragmidium*, and *Uromyces* showed total colonization of rust spores (Koç & Défago, 1983). The parasitic event involved penetration of *A. album* hyphae through rust spores, either penetrating via the germ pores or directly through the spore wall. In addition, the cell rust spores' content disappeared; later, the spores collapsed at the equatorial plane. Nevertheless, besides the pathogenicity tests, no other characterization studies have been made on this hyperparasitic species against rust fungi in the last 30 years. The most recent study of *A. album* was its potential use as a bionematicide in tomato plants infected by *Meloidogyne javanica*, with favorable results against this nematode (Leoni et al., 2020).

#### 1.3.2.2 The fungal genus *Alternaria*

*Alternaria* Nees (Pleosporaceae, Ascomycota) is a ubiquitous fungal genus spread worldwide. Members of this genus are usually reported as plant pathogens and soil saprotrophs; some are allergenic (Dang et al., 2015). When reported as plant pathogens, species such as *Alternaria brassicicola* and *Alternaria solani* can cause black spot diseases on plants, particularly in the genus *Brassica*, including cabbage, broccoli, and canola (Nowicki et al., 2012). Other *Alternaria* members can produce and accumulate mycotoxins in cereals, fruits, and vegetables (Patriarca, 2016) that have also been linked to certain forms of cancer (G. Liu et al., 1991). Thus, this fungal genus is commonly considered problematic in food products as well as harmful to humans and animals.

Despite these issues, a strain of *Alternaria alternata* was recently reported as a NE of rust fungi for the first time, parasitizing rust spores of wheat stripe rust (Zheng et al., 2017). Microscopic observations showed that hyphae of *A. alternata* could penetrate and colonize urediniospores on wheat. When hyperparasitized, rust pustules were covered in a gray-colored mold. Pathogenicity tests showed a reduction in pustule density, rust spores' production, and germination rate. In addition, the strain was identified through morphology and multi-locus phylogeny. Nevertheless, despite its potential as a BCA, further characterization studies are needed to determine how

harmful the strain can be to the environment and human health, particularly in terms of host-specificity due to the other ecological strategies of this fungal genus.

#### 1.3.2.3 The fungal genus *Cladosporium*

*Cladosporium* Link (Cladosporiaceae, Ascomycota) is a fungal genus with more than 600 currently accepted species (Index Fungorum, 2022) that include several ecological strategies. Some of them are reported as endophytes (Munawer et al., 2020); others as hyperparasites (Moricca et al., 2005; Zhan et al., 2014), plant pathogens (Karimi Jashni et al., 2020), saprotrophs (Ogórek et al., 2012), as well as allergens (Grinn-Gofroń et al., 2019). *Cladosporium* species that hyperparasitize rusts are usually reported on a wide range of rust genera (Torres et al., 2017), including *Cronartium*, *Endocronartium*, *Gymnosporangium*, *Hemileia*, *Melampsora*, *Peridermium*, *Puccinia*, *Uromyces*, and *Tranzschelia* (Assante et al., 2004; Dolińska et al., 2011; Moricca et al., 2005; J. K. Sharma & Heather, 1978; Traquair et al., 1984; Yusuf et al., 2019; Zhan et al., 2014). These *Cladosporium* species usually infect several rust spore stages by direct attack, penetration, colonization, and disintegration of the rust spores' cytoplasm (Assante et al., 2004; Moricca et al., 2005; Tsuneda & Hiratsuka, 1979). Additionally, some species can secrete antimicrobial compounds to destroy rust (Nasini et al., 2004).

*Cladosporium* is currently the fungal genus most studied as a NE of rust fungi. Characterization studies such as the mode of action against rusts, production of antifungal compounds, and reduction of rust disease severity *in-vitro* and *in-planta* are the most common among *Cladosporium* species associated with rust fungi (Moricca et al., 2005). Nevertheless, there is still a gap for those hyperparasitic species in their DNA sequences. Thus, although strains of some *Cladosporium* species have demonstrated outstanding potential in controlling rust fungi, their vague molecular data hampers further studies as BCAs.

#### 1.3.2.4 The fungal genus *Helicobasidium*

*Helicobasidium* Pat., (Helicobasidiaceae, Basidiomycota) is the closest NE relative to rust fungi. Its anamorphic stage, *Tuberculina*, is usually reported as a NE of several rust species, such as *Cronartium* (Rostrup, 1890), *Gymnosporangium* (Grasso, 1954), *Puccinia*, and *Tranzschelia*

species (Bauer et al., 2004). Members of *Tuberculina* have also been reported on several rust stages, including spermogonium and aecia (Bauer et al., 2004; Goodding, 1932; Grasso, 1954; Hubert, 1932; Mielke, 1933; I. K. Sharma et al., 1977; Sundaram, 1962; Wicker & Woo, 1973). Morphological structures of *Tuberculina* can be easily recognized on rust pustules as a compact purple mass of hyphae, or sporodochia, emitting a powdery mass of conidia. Among the *Tuberculina* species reported in association with rust fungi, *Tuberculina persicina* is, perhaps, the most common species with evidence of hyperparasitic interaction. Cellular interactions between *T. persicina* and the rust *Transschelia pruni-spinosae*, for example, have shown an intimate interaction between these two species resulting in cytoplasm-cytoplasm connection through a fusion pore, suggesting a hyperparasitic event (Lutz, Bauer, Begerow, Oberwinkler, et al., 2004). Host-specificity tests have also been made for several *Tuberculina* species against different rust genera. *Tuberculina persicina* showed a broad rust host range infecting several rust genera. In contrast, *T. maxima* showed a restricted rust host range for *Gymnosporangium* and *Cronartium* and *T. sbrozzii* a host preference for *Puccinia vincae* (Lutz, Bauer, Begerow, & Oberwinkler, 2004). Unfortunately, despite the evidence of *Tuberculina* as a hyperparasite of rust fungi, not all *Tuberculina* species are likely to become potential BCAs. This is because its teleomorphic stage, *Helicobasidium*, is commonly reported as a plant pathogen causing violet root rot on several vegetables. In addition, although *T. persicina* was thought to be exclusively associated with rust fungi, a recent report from Sicily, Italy, showed that the species could also cause a plant disease on pistachio fruits (Mirabile & Torta, 2020). Thus, the broad host range of *Helicobasidium* for plants and other fungi may represent a risk for further studies in the biological control of rust diseases.

#### 1.3.2.5 The fungal genera *Akanthomyces* (*Lecanicillium*), *Simplicillium*, and *Verticillium*

*Verticillium* used to be a heterogeneous genus linked to several families of the phylum Ascomycota including Clavicipitaceae, Hypocreaceae, Nectriaceae, and Phyllachoraceae (Sung et al., 2001). This genus was classified into four sections based on morphological and cultural characteristics (Gams & van Zaayen, 1982). Later based on a combination of morphology and molecular data, some members of this section were accommodated in two new genera: *Lecanicillium* W. Gams & Zare and *Simplicillium* W. Gams & Zare (Cordycipitaceae, Ascomycota) (Gams & Zare, 2001; Zare & Gams, 2001). *Lecanicillium*, now considered a synonym of the genus *Akanthomyces*,

comprises NEs of plant pathogens, pest insects, and plant-parasitic nematodes (Kepler et al., 2017; Y.-B. Wang et al., 2020). Similarly, *Simplicillium* comprises entomopathogenic, fungicolous, and nematophagous species but also includes endophytes and parasitic species of plants and mammals.

Table 1.2 List of all reports of natural enemies of rust fungi

Natural enemy (Scientific name)	Taxonomic Classification	Rust host (Scientific name)	Research topic	Locality	References
<i>Acremonium obclavatum</i> ( <i>Simplicillium obclavatum</i> )	Incertae sedis	<i>Puccinia striiformis</i> f. sp. <i>tritici</i>	Interaction test	China	Wang et al., 2020
<i>Akanthomyces lecanii</i> ( <i>Lecanicillium lecanii</i> )	Cordycipitaceae, Ascomycota	<i>Hemileia vastatrix</i>	Suppression of disease severity	Mexico	Jackson et al., 2012
<i>Aphanocladium album</i>	Nectriaceae, Ascomycota	Several rust species	Pathogenicity tests	Switzerland	Koç et al., 1983; Koç & Défago, 1983
<i>Alternaria alternata</i>	Pleosporaceae, Ascomycota	<i>Puccinia striiformis</i> f. sp. <i>tritici</i>	A report in association with rust and pathogenicity test	China	Zheng et al., 2017
<i>Bacillus cereus</i>	Bacillaceae, Bacteria	<i>Hemileia vastatrix</i>	Suppression of disease severity	Brazil	Shiomi et al., 2006
<i>Bacillus lentimorbus</i>		<i>Puccinia striiformis</i> f. sp. <i>tritici</i>		China	Kiani et al., 2021
<i>Bacillus megaterium</i> 6A		Several rust hosts		China	Kiani et al., 2021
<i>Bacillus subtilis</i>		<i>Puccinia striiformis</i> f. sp. <i>tritici</i>		China	Li et al., 2013
<i>Bacillus subtilis</i> E1R-j		<i>Puccinia striiformis</i> f. sp. <i>tritici</i>		Denmark	Reiss & Jørgensen, 2017
<i>Bacillus subtilis</i> QST713		<i>Hemileia vastatrix</i>		Brazil	Haddad et al., 2009
<i>Bacillus sp.</i> B157					
<i>Bradybaena similaris</i>	Gastropoda, Mollusca	<i>Hemileia vastatrix</i>	Rust spore consumption	Puerto Rico	Hajian-Forooshani et al., 2020
<i>Cladosporium aecidiicola</i>	Cladosporiaceae, Ascomycota	Several rust species	Reports in association with rusts and pathogenicity test	Australia	Sharma & Heather, 1980
<i>C. cladosporioides</i>				China	Zhan et al., 2014; Zhang et al., 2022
<i>C. gallicola</i>				Canada	Tsuneda & Hiratsuka, 1979
<i>C. pseudocladosporioides</i>				Mexico	Torres et al., 2017
<i>C. uredinicola</i>				Canada	Traquair et al., 1984
<i>C. uredinophilum</i>				Paraguay	Spegazzini, 1923
<i>C. tenuissimum</i>			Interaction test and production of metabolites	Australia and European regions	Assante et al., 2004; Moricca et al., 2005; Nasini et al., 2004

Table 1.2 continued

<i>Lecanicillium uredinophilum</i>	<b>Cordycipitaceae, Ascomycota</b>	<b><i>Pucciniastrum agrimoniae</i> and <i>Coleosporium</i> sp.</b>	<b>A report in association with rust</b>	<b>Korea</b>	<b>Park et al., 2015</b>
<i>Lymantria dispar</i>	Lepidoptera, Arthropoda	<i>Melampsora larici- populina</i>	Suppression of rust disease	Germany	Eberl et al., 2018, 2020
<i>Mycodiplosis</i> sp.	Diptera, Arthropoda	Several rust hosts	A report in association with rusts	Worldwide	Henk et al., 2011
			Host- specificity, phylogeny	Worldwide	Nelsen, 2013
<i>Mycrodochium nivale</i>	Amphiphaeriaceae, Ascomycota	<i>Puccinia striiformis</i> f. sp. <i>tritici</i>	An indirect effect of antagonistic fungus	Pacific Northwest area in North America	Littlefield, 1981
<i>Paneibacillus xylanexedens</i> 7A	Paenibacillaceae, Bacteria	<i>Puccinia striiformis</i> f. sp. <i>tritici</i>	Suppression of rust disease	China	Kiani et al., 2021
<i>Pseudomonas putida</i>	Pseudomonadaceae, Bacteria	<i>Puccinia striiformis</i> f. sp. <i>tritici</i>	Suppression of rust disease (ISR)	China	Pang et al., 2016
<i>Ramularia coleosporii</i>	Mycosphaerellaceae, Ascomycota	Several rust hosts	Reports in association with rust	India, Korea, Thailand	Baiswar et al., 2014; Bartkowska, 2007; Sun, 2017
<i>Ramularia uredinearum</i>				Poland	
<i>Ramularia uredinis</i>				Poland	
<i>Scytalidium uredinicola</i>	Leotiomyces, Ascomycota	<i>Endocronartium harknessii</i>	A report in association with rust	Canada	Moltzan et al., 2001
<i>Serratia marcescens</i> 3A	Yersiniaceae, Bacteria	<i>Puccinia striiformis</i> f. sp. <i>tritici</i>	Suppression of rust disease	China	Kiani et al., 2021
<i>Simplicillium lanosoniveum</i>	Cordycipitaceae, Ascomycota	Several rust species	Suppression of rust disease and interaction test	Costa Rica, Netherlands, USA	Gauthier et al., 2014; Ward et al., 2011; García-Nevárez & Hidalgo-Jamison, 2019
<i>Sphaerellopsis</i> sp.	Phaeosphaeriaceae, Ascomycota	Several rust species	Taxonomic classification	European regions, South Africa, Brazil	Trakunyingcharoen et al., 2014
<i>Sphaerellopsis paraphysata</i>		<i>Puccinia substriata</i>	Metabolite production	India	Ashmitha Sri et al., 2020



Table 1.2 continued

<i>Staphylococcus agnetis</i> 15A	<b>Staphylococcaceae, Bacteria</b>	<i>Puccinia striiformis</i> <b>f. sp. tritici</b>	<b>Suppression of rust disease</b>	<b>China</b>	<b>Kiani et al., 2021</b>
<i>Trichoderma harzianum</i>	Hypocreaceae, Ascomycota	<i>Puccinia graminis</i> Pers. f. sp. <i>tritici</i> ,	Induction of systemic resistance	Sri Lanka	Abeyasinghe, 2009; El- Sharkawy et al., 2018
<i>Trichoderma viride</i>		<i>Uromyces</i> <i>appendiculatus</i>		Egypt	
<i>Trichoderma</i> spp.		<i>Phakopsora pachyrizi</i>	Antifungal secondary metabolites	Germany	El-Hasan et al., 2022
<i>Trochila urediniophila</i>	Cenangiaceae, Ascomycota	<i>Cerotelium fici</i>	New species associated with rust	Trinidad	Gómez-Zapata et al., 2021
<i>Tuberculina maxima</i>	Helicobasidiaceae, Basidiomycota	<i>Gymnosporangium</i> and <i>Cronartium</i>	Host-specificity	Canada	Lutz, Bauer, Begerow, & Oberwinkler, 2004; Lutz, Bauer, Begerow, Oberwinkler, et al., 2004
<i>Tuberculina persicina</i>		Several rust species		Germany	
<i>Tuberculina sbrozzii</i>		<i>Puccinia vincae</i>		England	
<i>Typhula ishkariensis</i> ( <i>Typhula idahoensis</i> )	Typhulaceae, Basidiomycota	<i>Puccinia striiformis</i> f. sp. <i>tritici</i>	An indirect effect of antagonistic fungus	Pacific Northwest area in North America	Littlefield, 1981

Two species of *Akhantomyces* have been reported as NEs of rust fungi. For example, *Akhantomyces lecanii*, previously known as *Lecanicillium lecanii*, is an entomopathogenic species that infects the green coffee scale *Coccus viridis* (Hemiptera, Coccidae) in coffee plantations. When the insect is at high population densities, *A. lecanii* is also found parasitizing coffee leaf rust as a yellowish-white mycelium covering the rust pustules. Although coffee leaf rust is not a direct target for *A. lecanii*, its presence can cause significant suppression of the rust (Jackson et al., 2012; Vandermeer et al., 2009). *Lecanicillium uredinophilum* is another species reported as a NE of rust fungi. The species associated with the rusts *Pucciniastrum agrimoniae* and *Coleosporium* sp. was reported in South Korea and described as a new species (Park et al., 2015). Nevertheless, no further studies have been conducted to determine the interaction between *L. uredinophilum* and its rust hosts. Additionally, although *L. uredinophilum* was initially reported on rust fungi only, the species was isolated from an insect in Yunnan, China (Wei et al., 2018).

Two species of the genus *Simplicillium* are also reported as NEs of rust fungi. *Simplicillium lanosoniveum* has been reported on *Hemileia vastatrix* (Kouvelis et al., 2008) and *Phakopsora pachyrhizi* (Gauthier et al., 2014; Ward et al., 2011); and *Simplicillium obclavatum* on *Puccinia striiformis* f. sp. *tritici* (N. Wang et al., 2020) and rust pustules on the host plant *Arachis hypogaea* (Zare & Gams, 2001). In addition, pathogenicity tests clearly show an antagonistic relationship when interacting with rust fungi. For instance, a GFP-transformed strain of *Simplicillium lanosoniveum* showed hyphae colonizing and coiling around urediniospores of *P. pachyrhizi* before infection. Later, penetration occurred through the germ pores of urediniospores, and within 24 hours, rust spores showed signs of degradation (Gauthier et al., 2014). The direct attack of *S. lanosoniveum* on *P. pachyrhizi* could suggest the great potential of this NE to control rust fungi. Nevertheless, pathogenicity tests *in planta* showed that the rust must become established on the soybean plant tissue with clear signs and symptoms of rust disease for *S. lanosoniveum* to infect the rust. Otherwise, *S. lanosoniveum* did not grow or infect rust pustules (Ward et al., 2012). *In planta* pathogenicity tests were also performed with *Simplicillium obclavatum* on wheat stripe rust. The presence of *S. obclavatum* reduced the production and germination rate of *Puccinia striiformis* f. sp. *tritici* urediniospores.

#### 1.3.2.6 The fungal genus *Ramularia*

*Ramularia* Unger (Mycosphaerellaceae, Ascomycota) is a rich-species genus with more than 1,000 currently described species (*Index Fungorum*, 2022). Most *Ramularia* species are plant pathogens causing leaf spots, necrosis, and chlorosis on several hosts, some of which are economically important crops, such as barley, sugar beet, and strawberry (Videira et al., 2016). However, other species can have a saprobic or mycoparasitic strategy. For example, *Ramularia coleosporii*, *Ramularia uredinearum*, and *Ramularia uredinis* are reported as NEs of rust fungi infecting telial and uredinial stages of several rust species (Baiswar et al., 2014; Bartkowska, 2007; Videira et al., 2016). White mycelium growing exclusively on the rust pustules is the typical sign of these *Ramularia* species. Similar to other fungal NEs, most of the publications on *Ramularia* associated with rust fungi are merely reports of their presence on rust pustules. Few of them show microscopic ultrastructural observations of hyphae of *Ramularia* interacting with rust spores. In those observations, callosities similar to appressoria are formed after hyphal adhesions to the rust spore that penetrate and destroy the rust (Bartkowska, 2007). Although *Ramularia coleosporii* was thought to be a NE of rust fungi exclusively, a recent report of this species as a plant pathogen on *Perilla frutescens* in Korea, causing leaf spots (Aktaruzzaman et al., 2021) has led to a loss of interest in further BCA assays of this species.

#### 1.3.2.7 The fungal genus *Sphaerellopsis*

*Sphaerellopsis* Cooke (Leptosphaeriaceae, Ascomycota) is the most commonly reported fungal genus associated with rust fungi, whose anamorphic state has been reported on hundreds of rust species worldwide (Kranz & Brandenburger, 1981). The fungus is usually described as clumps of spherical black pycnidia covering rust spores of uredinial sori (Eriksson, 1966; Plachecka, 2005). Although most scientific publications on *Sphaerellopsis* posit it as a hyperparasite of rust fungi, its relationship with rust fungi is still poorly understood. While there is some evidence of direct interaction between the type species *Sphaerellopsis filum* and several rust species, the interaction is not consistent between *S. filum* strains. For instance, some researchers argue that *S. filum* can colonize rust spores by penetrating nonspecialized hyphae and disrupting cytoplasm (Carling, D.E. Brown, M.F. Millikan, 1976; Plachecka, 2005; Sappin-Trouffy, 1896; Whelan et al., 1997). However, other studies report no evident cytoplasmic disruptions of rust spores when *S. filum* is

present (D'Oliveira, 1941; Hulea, 1939). In-vitro assays demonstrated fast growth of the mycelium and conidioma of *S. filum* when cultured with intact or ruptured rust spores (Rambo & Bean, 1970). However, changes in fungal growth rate do not necessarily demonstrate that *S. filum* can infect rust fungi. Similarly, lab and field experiments have shown a significant reduction in rust infection when *S. filum* is present (Black, 2012; Gordon & Pfender, 2012; X. Yuan & Han, 2000), but these conclusions are contradicted by other studies (Z. W. Yuan et al., 1999). In recent years, thorough phylogenetic analyses, including several isolates named *Sphaerellopsis*, have turned out to be unrelated and belong to other genera (Trakunyingcharoen et al., 2014). Thus, the previous interaction tests of *S. filum* with rust fungi are questionable, as the *Sphaerellopsis* specimens used in those studies may no longer be placed in the genus. Four of the current seven accepted *Sphaerellopsis* species are still reported as hyperparasites of rust fungi: *Sphaerellopsis anomala*, *Sphaerellopsis filum*, *Sphaerellopsis macroconidialis*, and *Sphaerellopsis paraphysata* (Nag Raj, 1993; Trakunyingcharoen et al., 2014); and two are saprobic: *Sphaerellopsis artemisiae* and *Sphaerellopsis isthmospora* (Doilom et al., 2021; Phookamsak et al., 2019). *Sphaerellopsis hakeae* is reported in association with rust pustules and plant tissue (Crous et al., 2016), although it is unclear if the association with the host plant is parasitic or saprobic. While significant improvements have been made to this fungal genus' taxonomy (Trakunyingcharoen et al., 2014), other mycoparasitic characterization issues are not yet documented. Therefore, it is still inconclusive to suggest that *Sphaerellopsis* species associated with rust fungi have great potential as BCAs.

#### 1.3.2.8 The fungal genus *Trichoderma*

*Trichoderma* Pers. (Hypocreaceae, Ascomycota) is the fungal genus most studied as a NE of plant pathogens. Members of *Trichoderma* are known to have several mechanisms of action for controlling plant diseases, including antibiosis, competition, hyperparasitism, and induction of systemic plant resistance (ISR) (J.-Z. Sun et al., 2019; Zin & Badaluddin, 2020). Nevertheless, the main mechanisms of action of the current *Trichoderma* species as NEs of rust fungi are reported only as ISR and antibiosis. For example, the strain RU01 of *Trichoderma harzianum* studied against the bean rust *Uromyces appendiculatus* reduced the bean rust incidence by inducing plant systemic resistance under greenhouse conditions (Abeyasinghe, 2009). Furthermore, a combination of different species, such as *Trichoderma harzianum* and *Trichoderma viride*, applied as spore

suspension on wheat stem rust under greenhouse conditions, reduced significantly rust spore germination and disease severity attributed to secreted metabolites (El-Sharkawy et al., 2018). Similarly, secondary metabolites from several *Trichoderma* strains applied against the soybean rust *Phakopsora pachyrhizi* have demonstrated significant inhibition of urediniospore germination in detached leaves and whole plant experiments (El-Hasan et al., 2022). Although antifungal compounds are promising bioproducts to control rust diseases, the production of these compounds may vary when used on different rust host species and under different environmental conditions (Vinale et al., 2009).

#### *1.3.2.9 The fungal genus Trochila*

One member of the genus *Trochila* Fr. (Cenangiaceae, Ascomycota) was recently discovered in association with rust fungi. *Trochila urediniophila* was found on uredinia of *Cerotelium fici* on the underside of fig leaves in Trinidad and Tobago (Gómez-Zapata et al., 2021). The specimen was collected by chance in 1912 and deposited at the Arthur Fungarium (PUR) as a plant specimen hosting the rust species *Cerotelium fici* only. Fortunately, this new species was found due to recent surveys of NEs of rust fungi at PUR. The Morphological structures of *T. urediniophila* were easily distinguished from the rust as gregarious brown apothecia protruding from uredinial pustules. Additionally, apothecia were never observed in contact with plant tissue. Although no further characterization studies were possible on this specimen due to its nonviable structures, the species is suspected to be a NE of rust fungi.

#### *1.3.3 Arthropods and Gastropods as natural enemies of rust fungi*

Insects from two distinct classes have been reported so far, consuming directly and indirectly spores of rust fungi. The first insect is the fly larva *Mycodiplosis* which belongs to the class Diptera and is commonly known as a gall midge (Gagné & Jaschhof, 2021). Larvae of this fly genus are usually found feeding on the spores of several rust species, and powdery mildews (Holz, 1970). However, of the 49 currently accepted species, nine are also reported on other groups of fungi and consuming plant tissue (Gagné & Jaschhof, 2021). These larvae are tiny, with a body length between 1 to 3 mm in size (Holz, 1970), challenging field observations with the naked eye. Adults are similar in size to the larval stage, and because of their short life, between two to three days

(Kaushal et al., 2001), their primary purpose is to mate and lay eggs on the host plant. Although most of the literature on *Mycodiplosis* is related to reports of new species and associations with rust fungi (Henk et al., 2011; Jiao et al., 2019; Kolesik, 2019; Kolesik et al., 2022; Sicoli et al., 2017), characterization studies of this NE in terms of biology and ecology are scarce (Nelsen, 2013; Santiago-Elena et al., 2020). The second insect associated with rust fungi is the caterpillar *Lymantria dispar* which belongs to the class Lepidoptera. In contrast to *Mycodiplosis*, this insect does not feed on rust spores directly but shows preferential grazing on plants infected by rust fungi (Eberl et al., 2018, 2020). In addition, since *Lymantria dispar* is a forest pest that defoliates large areas of broadleaf forests (Wilson, 2016), its potential as a BCA is unlikely.

Gastropods are unusual NEs of rust fungi. Only one species has been reported consuming rust spores directly. This species is the land snail *Bradybaena similaris*, reported for the first time feeding on the rust spores of coffee leaf rust *Hemileia vastatrix* in Puerto Rico (Hajian-Forooshani et al., 2020). Despite this remarkable discovery, the snail has a generalist behavior, feeding on the mycoparasitic species *Lecanicillium lecanii* and consuming plant material. Since *Bradybaena similaris* is a widely distributed invasive snail with a generalist consumption behavior, its use as a potential biological control agent is questionable due to the environmental risk of becoming a more potent invasive pest on crops and natural ecosystems. However, no further studies on this species have been done. Similarly, two unidentified gastropods have been found with their guts full of rust spores of CLR. However, neither identification nor characterization studies of these gastropods have been made (Hajian-Forooshani et al., 2020). Indirect rust spore consumption by other gastropods is also recently reported. For example, the omnivorous slugs *Arion ater*, *Arion hortensis*, and *Arion fasciatus* have shown preferential grazing on plants infected by several rust species (Ramsell & Paul, 1990), but then again, no further studies on the characterization of these species as NEs have been accomplished. Whether these gastropods have potential as BCAs of rust diseases or not, records of these NEs highlight the scarcity of knowledge that exists regarding the interaction of other organisms with rust fungi.

#### ***1.3.4. Bacteria as natural enemies of rust fungi***

Several bacterial strains have been found as antagonists of rust fungi by screening for endophytes in vulnerable and resistant plant hosts of rust diseases (Haddad et al., 2009; Kiani et al., 2021; Li

et al., 2013; Pang et al., 2016; Shiomi et al., 2006). For example, *Bacillus lentimorbus* and *Bacillus cereus* were found on leaves of *Coffea arabica* and *Coffea robusta* in Brazil. These bacteria inhibited urediniospores germination of *H. vastatrix* with values above 50% (Shiomi et al., 2006). Another study from organic coffee plantations in Brazil selected seven bacterial strains to apply in combination with copper hydroxide, calcium silicate, and water on coffee plants to measure the suppression of rust severity and incidence. Lower suppression of rust disease was detected in applications of *Bacillus* sp. B157 combined with copper hydroxide (Haddad et al., 2009). Similarly, five bacterial strains were found from screening endophytic bacteria on stripe rust-resistant wheat Pakistani cultivars, including *Serratia marcescens* 3A, *Bacillus megaterium* 6A, *Paneibacillus xylanexedens* 7A, *Bacillus subtilis* 11A, and *Staphylococcus agentis* 15A. They all showed significant inhibition of stripe wheat rust by triggering the treated seedlings' defense mechanism to high production of antioxidant enzymes and high expression of pathogenesis-related (PR) protein genes (Kiani et al., 2021).

Other bacterial strains with antagonistic backgrounds on several plant pathogens have also been studied against several rust diseases. For example, *Bacillus subtilis* was used to evaluate the reduction of disease severity with chemical fungicide products to control the soybean rust *Phakopsora pachyrhizi* in Brazil (Dorighello et al., 2020). Results showed that *B. subtilis* and fungicides applied in sequence reduced the progress of the disease by between 53 and 69%. Antibiotic culture filtrates of *Bacillus* and *Streptomyces* spp. were also applied to snap beans inoculated with the rust *U. appendiculatus* in a greenhouse to assess antifungal and translaminal activity within the plant (Wagacha et al., 2007). Results showed significant suppression of rust disease up to 10 days after application of antibiotics, thus suggesting that these bacteria produce antibiotic compounds with a systemic activity that can persist within the plant for over a week. Although *Bacillus* seems to be the most common bacteria with antagonistic activity against rust fungi, gram-negative bacteria are also reported as NEs of rusts. For example, the strain JD204 of *Pseudomonas putida* significantly reduced stripe wheat rust *Puccinia striiformis* f.sp. *tritici* and enhanced yield production, inducing plant resistance (Pang et al., 2016). Similarly, *P. putida* strain BK8661 suppressed symptoms of leaf wheat rust *Puccinia recondita* f. sp. *tritici* (Flaishman et al., 1996).

Despite the great potential of bacterial strains to reduce rust diseases, it is still unknown whether those strains combat rust fungi through the production of secondary metabolites, the induction of systemic plant resistance, or a combination of both mechanisms. Thus, further studies are still necessary to characterize those bacterial strains and detect their potential as BCAs.

#### **1.4 Concluding Remarks**

In the new crop protection era, it is crucial to start reconsidering other strategies to control rust diseases besides chemical control and plant genetic resistance. The new IPM framework offers several methods to control plant diseases that, if integrated, would be possible to keep rust inoculum at low population levels without using products with high environmental risks. As biological control is one of the IPM strategies to control pests and diseases, this strategy opens a window to discover NEs with new antifungal compounds, bioproducts, and action mechanisms that have remained hidden or understudied to combat rust diseases. Nevertheless, it is important to mention that although reports of an organism associated with rust pustules and pathogenicity tests are essential for further studies in biocontrol, these are just the first steps in characterizing NEs. If no additional studies on those NEs are done, those reports and pathogenicity tests will never be taken advantage of to control rust diseases. Thus, despite the long process of discovering NEs, characterizing them, and commercializing them, their usage as biocontrol agents against rust fungi is worth it for crop protection and environmental health in the long run.

#### **1.5 Thesis statement and objectives**

Due to the scarce knowledge of natural enemies of rust fungi, in this dissertation, I attempt to utilize multiple approaches, including morphology, DNA sequencing and phylogenetics, in-vitro interaction essays, rust inoculations in greenhouse, and data analyses, to characterize natural enemies of rust fungi. Three main objectives of this dissertation are as follows:

1. To catalog natural enemies of rust fungi from biological collections at the Arthur Fungarium, providing barcoding sequences, photographs, and morphological descriptions of these organisms, by thoroughly screening ca. 50% of the accepted rust genera collected over 200 years from across the globe and from 166 host plant families. (Chapter 2).



2. To describe new fungal species associated with rust fungi. (Chapter 3).
3. To characterize the two most common natural enemies of rust fungi in aspects related to species diversity, host preference, geographic distribution, and pathogenicity interactions. (Chapters 4-5).

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## **CHAPTER 2. THE FIRST CATALOG OF NATURAL ENEMIES OF RUST FUNGI FROM RUST COLLECTIONS AT THE ARTHUR FUNGARIUM**

### **2.1 Introduction**

Rust fungi (Pucciniales, Basidiomycota) comprise the largest group of plant pathogens with *ca.* 170 genera (Berndt & Aime, n.d.) and more than 7,000 species (Aime & McTaggart, 2020). They are obligate biotrophs and highly specialized pathogens that cannot be cultured in-vitro, which restricts many studies of their biology (Lorrain et al., 2019). Historically, these plant pathogens have been found worldwide and cause considerable economic losses in several crops, forest products, and natural ecosystems (Fensham & Radford-Smith, 2021; Kolmer et al., 2009; Moricca et al., 2005). Rust fungi are typically controlled using rust-resistant varieties and fungicides. Nevertheless, monocultures with identical rust-resistant plants and overuse of fungicides application adversely affect the environment and induce the emergence of new races of rust (Carmona et al., 2020; Singh et al., 2011), leading to outbreaks and causing yield losses. Thus, other alternatives need to be explored to manage rust diseases.

Biological control is considered an alternative method to combat diseases and pests on several crops worldwide. This method uses beneficial organisms which target the pathogen or pest and keep it at low population levels, thus reducing yield losses without causing adverse effects on the environment (Stenberg et al., 2021). However, the use of commercial biological control agents to combat rust diseases is negligible. One of the reasons is the limited information on the species diversity of beneficial organisms that target rust fungi in nature. Without this information, we cannot develop further studies to discover their potential as biological control agents, such as their mechanism of action against rust or testing their performance in field trials to reduce disease severity (Table 1.1). Thus, since we know little about the diversity of those beneficial organisms globally, advances in the biological control of rust fungi are still slow.

The first step to detecting and commercializing biological control agents of any disease is to screen for those beneficial organisms that target the disease in nature (*aka* natural enemies). Field sites are likely the most common places to screen and collect those natural enemies. However, preserved

specimens from biological collections, such as fungaria, may also provide a diverse source of natural enemies of rust fungi that are incidentally collected with the rust specimen. For instance, The Arthur Fungarium (PUR), housed at Purdue University, is one of the world's largest collections of rust fungi. It holds approximately 110,000 specimens of 5,000 species collected across a broad geographic distribution and timeline, and it is one of the most diverse collections, with 132 rust genera in 14 families in the world (Purdue Herbaria, 2022), with especially rich holdings (ca. 50%) of specimens from the Americas. Therefore, it would not be surprising to find natural enemies collected incidentally on preserved rust specimens at PUR.

Thus far, Asia and Europe have the highest records of natural enemies of rust fungi globally, with 30 reports in total. In addition, less than 15 have been recorded in the Americas (see Table 1.2). The lack of documentation of these natural enemies, especially in the tropics, is consistent with much of the undocumented fungal biodiversity (Hawksworth & Lücking, 2017). Thus, this study aims to screen rust fungus collections at PUR for the presence of natural enemies to provide a more global catalog of these, with emphasis on the less studied Americas.

## **2.2 Objective**

To catalog natural enemies of rust fungi from biological collections of the Arthur Fungarium with an emphasis on the Americas, providing a host rust list, host plant families, country of origin of these natural enemies, DNA barcoding sequences, photographs, and morphological descriptions, by thoroughly screening at least 50% of the accepted rust genera on rust specimens collected over 200 years from across the globe.

## **2.3 Materials and Methods**

### ***2.3.1 Collected samples at the Arthur Fungarium***

Rust specimens at PUR are stored in folders sorted by rust species in host plant families and geographic regions. When collecting natural enemies at PUR, we randomly screened rust specimens by selecting the top, middle, and bottom specimens from the Americas in each rust species folder. When collecting natural enemies from other geographic regions, we randomly selected one rust specimen per folder. Although we screened rust specimens collected across the

globe, the Americas was our preferred geographic region. When looking for fungal natural enemies, we screened every rust-infected leaf of each rust specimen under a stereoscope Olympus Model SZ2-ILST by looking for visible signs of mycelia or fruiting bodies developed only on the rust pustules. If we observed the same symptoms in plant tissue, these specimens were not recorded as natural enemies and thus not considered in our results. We removed one infected rust pustule with a razor blade for each fungal natural enemy detected per rust specimen. Only one razor blade was used per rust specimen to prevent cross-contamination. Then, each infected pustule was placed in a microcentrifuge tube labeled with the PUR barcode of the rust specimen and a serial number.

When looking for insects as natural enemies, we focused on collecting fungivorous larvae, with an average size of 2 mm, either on the rust pustule or dispersed on the plant tissue. Because larvae are often dislodged from the rust sori during sample pressing and processing, we collected any larvae that were present in the herbarium packet. Four fungivorous larvae were removed at the most from each rust specimen with a fine tweezer and placed individually in microcentrifuge tubes. Before collecting the larvae of another rust specimen, we cleaned the tweezers with 70% ethanol to prevent cross-contamination. Each tube was labeled with the PUR barcode of the rust specimen and a serial number.

Once we found a natural enemy, we manually recorded the following information for each rust specimen: the PUR barcode, year of collection, rust species name, host plant, country of origin, and rust spore stage. Finally, we took photographs of each natural enemy collected with an Olympus SC30 camera and image software Olympus cellSens entry version 1.14 under a stereoscope Olympus Model SZ2-ILST at PUR.

### ***2.3.2 Collected fresh samples***

We supplemented the PUR materials with live collections from several regions, including Hawaii, Indiana, and Puerto Rico in the United States, Italy, and Peru (Table 2.1). Fungal natural enemies were isolated from these collections by cutting a piece of the plant tissue containing both the rust pustule and the fungal natural enemy with a razor blade under a stereoscope. Then, the plant tissue was sterilized with 1/10 chlorine dilution bleach for one minute and washed three times with sterile water. The piece of plant tissue was inoculated onto Petri Dishes containing Potato Dextrose Agar

(PDA) and 50 mg/mL Chloramphenicol. Then, Petri Dishes were shipped to Aime Lab at Purdue University for pure isolation. Once the Petri Dishes arrived at Aime lab, we subcultured them and made pure isolations on PDA and 2% malt extract agar (MEA) with 50 mg/mL Chloramphenicol. For species identification, we extracted their DNA and stored them long-term in PDA slants at 4 °C and 15% glycerol at -80 °C. For fresh insects as natural enemies, we collected only larvae feeding directly on rust pustules. Each fungivorous larva was removed from the pustule with a fine tweezer and placed individually in microcentrifuge tubes. Tweezers were cleaned with 70% ethanol between samples to prevent contamination of rust specimens. We labeled every natural enemy found with a serial number. Finally, we pressed, dried, and deposited the collected rust specimens at PUR.

Table 2.1 Field sites screened for natural enemies of rust fungi

Site	System	Year of collection
National Forest El Yunque, Puerto Rico, USA	Natural	2018
Celery Bog, Indiana, USA	Natural	2018
Beck Agricultural Center, Indiana, USA	Wheat crop	2021
Southwest Purdue Agricultural Center, Indiana, USA	Maize crop	2022
Coffee farms, Hawaii, USA	Coffee crop	2022
Calabria, Italy	Natural	2018
Peru	Natural	2019

### 2.3.3 *Species identification of natural enemies of rust fungi*

Natural enemies of rust fungi were identified based on morphological characters and DNA sequences. We used original descriptions of fungal species associated with rust fungi as references for morphological comparison (Crous et al., 2016; Doilom et al., 2021; Khodaparast & Braun, 2005; Lutz et al., 2004a; Nonaka et al., 2013; Trakunyingcharoen et al., 2014; Videira et al., 2016; Wei et al., 2019). We also amplified the internal transcribed spacer (ITS) region to identify fungal species. In contrast, we could not use morphological traits to identify fungivorous larvae. Species identification of fungivorous insects is traditionally based on morphological traits of the adult male (Gagné & Jaschhof, 2021). Since the adult does not feed on rust fungi, this stage is usually absent on rust specimens. Thus, the only possibility to identify fungivorous larvae is through DNA

sequences. Below we describe the methodology used for species identification of fungi and larvae based on DNA sequences.

#### *2.3.3.1 DNA isolation and PCR amplifications of fungal natural enemies from preserved specimens at PUR*

The genomic DNA of each fungal natural enemy was extracted using the EZNA HP Fungal DNA kit (Omega Bio-Tek, Norcross, Georgia), following the manufacturer's instructions and modifying only the incubation time in the third step. Instead of 30 min, we incubated the samples overnight to ensure complete lysis of cells in the suspension. Leaving the specimen for a long time helped to make DNA accessible easily. Due to the typical high DNA degradation of preserved specimens, we designed the set of primers EudITS2F (5' AAC TTT CAA CAA CGG ATC TCT TGG T 3')/EudITS4R (5' ATG CTT AAG TTC AGC GGG TA 3') and EudITS2F/EuSP\_ITS\_R2 (5' ATG TGC YRM GMT YCA GGC 3') to amplify the 5.8S rDNA and ITS2 region only. All 25- $\mu$ l PCR reactions were conducted on a Mastercycler ep gradient Thermal Cycler (Eppendorf model #5341, Hauppauge, New York) that consisted of 12.5  $\mu$ l of 2 $\times$  MyTaq Mix (Bioline, Swedesboro, New Jersey), 1.25  $\mu$ l of each 10  $\mu$ M primer, and 10  $\mu$ l of either 1/10 or 2/10 diluted DNA extract. The 5.8S and ITS2 regions were amplified under the following conditions: initial denaturation at 94 °C for 5 min; followed by 45 cycles of denaturation at 94 °C for 30 sec, annealing at 51.8 °C for 45 sec, and elongation at 72 °C for 45 sec; and final extension at 72 °C for 7 min.

#### *2.3.3.2 DNA isolation and PCR amplifications of fungal natural enemies from fresh collections*

The genomic DNA of fresh fungal collections was extracted using the DNA extraction—Promega Wizard Purification Kit: for cultures and mushrooms, with Aime's lab modifications as follows:

1. Collect fresh mycelia in 1.5 mL autoclaved microtubes.
2. Add 600  $\mu$ L of Nuclei lysis solution and grind tissue with a sterile blue pestle and vortex.
3. Incubate at 65 °C for 30 min and occasional vortexing to keep contents in solution.
4. Centrifuge for 3 min at top speed.
5. Carefully pipette off approximately 500  $\mu$ L of supernatant and use it for the next step in a new tube.
6. Add 200  $\mu$ L of Protein Precipitation Solution to the supernatant. Then, shake or vortex for 20 sec. Put in 4°C for 10 min.



7. Centrifuge from 3 to 6 min at top speed. Pipette supernatant to a new tube. If the sample is cloudy, centrifuge again until the sample is not cloudy. Otherwise, proteins are still present.
8. Add 600 uL room temperature Isopropanol (100%) which precipitates DNA. Invert to mix.
9. Centrifuge for 1 min at top speed. Carefully decant without losing the pellet.
10. Add 600 uL of 70% room temperature ethanol. Invert to mix. Then, centrifuge for 1 min at top speed.
11. Remove ethanol with a pipette without disturbing the pellet. Carefully invert the tube on a clean wipe and leave it drying overnight.
12. Resuspend in 30 uL of rehydration solution or TE buffer. Then store in a -20 °C freezer.

For species identification of fresh fungal cultures, we used the primers ITS1F (Gardes & Bruns, 1993) and ITS4 (White et al., 1990) to amplify the internal transcribed spacer 1 region (ITS1), the 5.8S rDNA, and the internal transcribed spacer 2 region of the ribosomal DNA (ITS2). All 25- $\mu$ l PCR reactions were conducted on a Mastercycler ep gradient Thermal Cycler (Eppendorf model #5341, Hauppauge, New York) that consisted of 12.5  $\mu$ l of 2 $\times$  MyTaq Mix (Bioline, Swedesboro, New Jersey), 1.25  $\mu$ l of each 10  $\mu$ M primer, and 10  $\mu$ l of 1/10 diluted DNA extract. The ITS locus was amplified under the following conditions: initial denaturation at 95 °C for 5 min; followed by 40 cycles of denaturation at 95 °C for 30 sec, annealing at 45 °C for 45 sec, and elongation at 72 °C for 45 sec; and final extension at 72 °C for 7 min.

#### *2.3.3.3 DNA isolation and PCR amplifications of fungivorous larvae associated with rust fungi*

The genomic DNA from each larva was extracted using InstaGene Matrix catalog #732-6030: DNA preparation from whole blood with several modifications as follows: skip steps 1 through 4 from the manufacturer's protocol. Add a single larva into a microtube. Then, add 50 uL of InstaGene Matrix to the tube containing the larva. Macerate the larva using autoclaved pestles or 200 uL micropipette tips. After that, place the microtube at 56 °C for 30 minutes. Mix the tube to maintain the matrix in suspension and vortex at high speed for 10 seconds. Then, place the tube in a 100 °C heat block for 8 minutes and vortex it at high speed for 10 seconds. Spin the tube at 15,000 rpm for 3 minutes and store it at -20 °C for PCR reaction.

### ***2.3.4 Electrophoresis and sequencing***

We ran the PCR products of fungal and insect natural enemies in 1% agarose and stained with GelRed (RGB4102, Phoenix Research Products) for 35 min at 110 V in a Bio-Rad electrophoresis tank to visualize PCR products. PCR products of samples that showed bands were sent to Genewiz (South Plain- field, New Jersey) for purification and subsequent sequencing in both directions. Raw sequence reads were edited manually and assembled using Sequencher version 5.2.3 (Gene Codes Co., Ann Arbor, Michigan).

### ***2.3.5 Sequence alignment and phylogenetic trees***

The edited sequences were blasted against the NCBI GenBank nucleotide database (Altschul et al., 1990) to search for the closest relative species. We downloaded the CO1 and 16S sequences from peer-reviewed articles that included morphology descriptions and phylogenetic analyses of fungivorous larvae of rust fungi (Table 2.2). Similarly, we downloaded the ITS sequences from fungal species that shared the highest percentage of identity with our specimens and their types species when available (Table 2.2). The DNA sequences were aligned using MUSCLE version 3.7 (Edgar, 2004) in MEGA7 (Kumar et al., 2016). Then, the aligned sequences were trimmed using trimAl version 1.2 (Capella-Gutiérrez et al., 2009) with a minimum percentage of positions to conserve [0-100]: 50, and gap threshold, the fraction of positions without gaps in a column [0-1]: 0.6. We performed maximum likelihood (ML) inference using IQ-TREE (Minh et al., 2020) under partitioned models for larval sequences (Chernomor et al., 2016) and selected the best nucleotide substitution model under Akaike's information criterion corrected for small sample size (AICc) using ModelFinder (Kalyaanamoorthy et al., 2017). An ultrafast bootstrap analysis was implemented with 1,000 replicates (Hoang et al., 2018). Finally, phylogenetic reconstructions with bootstrap values (BS) were visualized in FigTree version 1.4.3 (<http://tree.bio.ed.ac.uk/software/figtree/>).

Table 2.2 Reference sequences used in phylogenetic analyses

NCBI Reference	Species Name	Gene region	Reference
NR171717	<i>Sphaerellopsis filum</i>	ITS	Trakunyingcharoen et al., 2014
AY607011	<i>Sphaerellopsis filum</i>	ITS	Liesebach & Zaspel, 2004
AY607012	<i>Sphaerellopsis filum</i>	ITS	Liesebach & Zaspel, 2004
AY607013	<i>Sphaerellopsis filum</i>	ITS	Liesebach & Zaspel, 2004
AY607014	<i>Sphaerellopsis</i> sp.	ITS	Liesebach & Zaspel, 2004
AY607015	<i>Sphaerellopsis</i> sp.	ITS	Liesebach & Zaspel, 2004
AY587134	<i>Sphaerellopsis</i> sp.	ITS	Nischwitz et al., 2005
KP170661	<i>Sphaerellopsis paraphysata</i>	ITS	Trakunyingcharoen et al., 2014
KP170662	<i>Sphaerellopsis paraphysata</i>	ITS	Trakunyingcharoen et al., 2014
MT957065	<i>Sphaerellopsis artemisiae</i>	ITS	Doilom et al., 2021
MK387925	<i>Sphaerellopsis isthmospora</i>	ITS	Phookamsak et al., 2019
NR155859	<i>Sphaerellopsis hakeae</i>	ITS	Crous et al., 2016
AY607022	<i>Sphaerellopsis macroconidialis</i>	ITS	Liesebach & Zaspel, 2004
AY607023	<i>Sphaerellopsis macroconidialis</i>	ITS	Liesebach & Zaspel, 2004
KP170659	<i>Sphaerellopsis macroconidialis</i>	ITS	Trakunyingcharoen et al., 2014
KC584247	<i>Alternaria consortialis</i>	ITS	Woudenberg et al., 2013
AY292450	<i>Helicobasidium purpureum</i> I	ITS	Lutz et al., 2004
AY460132	<i>Helicobasidium purpureum</i> I	ITS	Lutz et al., 2004a
AY460137	<i>Helicobasidium purpureum</i> I	ITS	Lutz et al., 2004a
AY292451	<i>Helicobasidium purpureum</i> I	ITS	Lutz et al., 2004
AY460176	<i>Helicobasidium purpureum</i> I	ITS	Lutz et al., 2004a
AY460165	<i>Helicobasidium purpureum</i> I	ITS	Lutz et al., 2004a
AY460170	<i>Helicobasidium purpureum</i> I	ITS	Lutz et al., 2004a
AY460136	<i>Tuberculina maxima</i> I	ITS	Lutz et al., 2004a
AY460150	<i>Tuberculina maxima</i> II	ITS	Lutz et al., 2004a
AY460151	<i>Tuberculina maxima</i> II	ITS	Lutz et al., 2004a
AB043964	<i>Helicobasidium mompa</i>	ITS	Matsumoto et al., 2000
AY292429	<i>Helicobasidium mompa</i>	ITS	Lutz et al., 2004
AY292428	<i>Helicobasidium mompa</i>	ITS	Lutz et al., 2004
AY254188	<i>Helicobasidium longisporum</i> I	ITS	Lutz et al., 2004
AY292444	<i>Helicobasidium longisporum</i> I	ITS	Lutz et al., 2004
AY292443	<i>Helicobasidium longisporum</i> I	ITS	Lutz et al., 2004
AY254194	<i>Helicobasidium longisporum</i> I	ITS	Lutz et al., 2004
AY292447	<i>Helicobasidium longisporum</i> II	ITS	Lutz et al., 2004
AY292445	<i>Helicobasidium longisporum</i> II	ITS	Lutz et al., 2004
AB043967	<i>Helicobasidium longisporum</i> II	ITS	Matsumoto et al., 2000a
AY292426	<i>Helicobasidium longisporum</i> II	ITS	Lutz et al., 2004
AY292452	<i>Helicobasidium longisporum</i> II	ITS	Lutz et al., 2004
HQ012446	<i>Puccinia chunjei</i>	ITS	Liu & Hambleton, 2012
KP894239	<i>Ramularia endophylla</i>	ITS	Videira et al., 2015
NR154949	<i>Ramularia hydrangeicola</i>	ITS	Videira et al., 2016
KX287394	<i>Ramularia cynarae</i>	ITS	Videira et al., 2016
KX287462	<i>Ramularia lamii</i> var. <i>lamii</i>	ITS	Videira et al., 2016
GU214694	<i>Ramularia uredinicola</i>	ITS	Crous et al., 2009
GU939180	<i>Ramularia uredinicola</i>	ITS	Kellner et al., n.d.
KX287523	<i>Ramularia uredinicola</i>	ITS	Videira et al., 2016
KX287526	<i>Ramularia uredinicola</i>	ITS	Videira et al., 2016
KX287530	<i>Ramularia uredinicola</i>	ITS	Videira et al., 2016
KX287521	<i>Ramularia uredinicola</i>	ITS	Videira et al., 2016
KX287524	<i>Ramularia uredinicola</i>	ITS	Videira et al., 2016

Table 2.2 continued

KX287528	<i>Ramularia uredinicola</i>	ITS	Videira et al., 2016
KX287525	<i>Ramularia uredinicola</i>	ITS	Videira et al., 2016
NR175043	<i>Ramularia uredinicola</i>	ITS	Videira et al., 2016
KX287522	<i>Ramularia uredinicola</i>	ITS	Videira et al., 2016
KX287527	<i>Ramularia uredinicola</i>	ITS	Videira et al., 2016
KX287529	<i>Ramularia uredinicola</i>	ITS	Videira et al., 2016
KX287531	<i>Ramularia uredinicola</i>	ITS	Videira et al., 2016
GU214692	<i>Ramularia coleosporii</i>	ITS	Crous et al., 2009
KX287374	<i>Ramularia coleosporii</i>	ITS	Videira et al., 2016
KX287381	<i>Ramularia coleosporii</i>	ITS	Videira et al., 2016
MW470869	<i>Ramularia coleosporii</i>	ITS	Videira et al., 2016
KX287384	<i>Ramularia coleosporii</i>	ITS	Videira et al., 2016
KF924738	<i>Ramularia coleosporii</i>	ITS	Baiswar & Ngachan, n.d.
KX287362	<i>Ramularia coleosporii</i>	ITS	Videira et al., 2016
KX287363	<i>Ramularia coleosporii</i>	ITS	Videira et al., 2016
MH974744	<i>Ramularia coleosporii</i>	ITS	Aktaruzzaman, n.d.
KP878302	<i>Ramularia coleosporii</i>	ITS	Sun et al., n.d.
KF550285	<i>Ramularia coleosporii</i>	ITS	Baiswar & Ngachan, n.d.-b
MH865126	<i>Zymoseptoria halophila</i>	ITS	Vu et al., 2019
AJ292396	<i>Simplicillium lanosoniveum</i>	ITS	Zare et al., 2000
MH856100	<i>Simplicillium lanosoniveum</i>	ITS	Vu et al., 2019
KM035981	<i>Simplicillium lanosoniveum</i>	ITS	Baiswar & Ngachan, n.d.-b
KM035982	<i>Simplicillium lanosoniveum</i>	ITS	Baiswar & Ngachan, n.d.-b
MG807436	<i>Simplicillium lanosoniveum</i>	ITS	Skaptsov et al., n.d.
MT508802	<i>Simplicillium lanosoniveum</i>	ITS	Rego & Soares, n.d.
AB603992	<i>Simplicillium minatense</i>	ITS	Nonaka et al., 2013
AB604001	<i>Simplicillium subtropicum</i>	ITS	Nonaka et al., 2013
AJ292394	<i>Simplicillium obclavatum</i>	ITS	Zare et al., 2000
AB604002	<i>Simplicillium aogashimaense</i>	ITS	Nonaka et al., 2013
MF066034	<i>Simplicillium coffeanum</i>	ITS	Gomes et al., 2018
JQ410324	<i>Simplicillium chinense</i>	ITS	F. Liu & Cal, 2012
FJ515771	<i>Akanthomyces lecanii</i>	ITS	Diaz et al., 2009
AF163020	<i>Cordyceps militaris</i>	ITS	Lee et al., 2000
MK511238	<i>Mycodiplosis puccinivora</i>	CO1	Jiao et al., 2019
MN191325	<i>Mycodiplosis coniophaga</i>	CO1	Dorchin et al., 2019
MN191326	<i>Mycodiplosis sphaerothecae</i>	CO1	Dorchin et al., 2019
MN191328	<i>Obolodiplosis robiniae</i>	CO1	Dorchin et al., 2019
MN191360	<i>Youngomyia podophyllae</i>	CO1	Dorchin et al., 2019
MN201513	<i>Mycodiplosis coniophaga</i>	16S	Dorchin et al., 2019
MN201515	<i>Obolodiplosis robiniae</i>	16S	Dorchin et al., 2019

### 2.3.6 Morphological studies

We confirmed molecular identifications with morphological observations by looking at the structures of each specimen under an Olympus compound microscope BH2-RFCA. We took mycelium or fruiting bodies and mounted them in water or 2% potassium hydroxide (KOH). If the

fungus structures did not match the sequence output, we considered the sequence a contaminant and discarded it from our results.

### ***2.3.7 Data cleaning and analyses***

We digitized in an excel spreadsheet file the manually recorded data by filling each piece of information in a separate column. Once digitized, we cleaned the data by fixing incorrect entries and removing duplicates in Excel. Then, we analyzed the total number of rust specimens screened per rust genus, country, year of collection, and host plant family for data analyses. Similarly, we analyzed the same information for the fungal and insect natural enemies found in our screening survey.

## **2.4 Results**

### ***2.4.1 Rust specimens screened for the presence of natural enemies at PUR***

We randomly screened 5,618 rust specimens in 99 rust genera, which represents 58% of the accepted 170 rust genera (Berndt & Aime, n.d.) for the presence of natural enemies of rust fungi (see Supplementary data Table S1). The five rust genera whose rust specimens we screened the most were *Puccinia* (2276 specimens), *Uromyces* (681 specimens), *Melampsora* (495 specimens), *Coleosporium* (471 specimens), and *Phakopsora* (271 specimens), which covers 75% of the total specimens (Fig. 2.1). Our survey includes 166 host plant families. Asteraceae (960 specimens), Poaceae (930 specimens), Fabaceae (436 specimens), Salicaceae (373 specimens), and Rosaceae (238 specimens) are the most representative, covering 52.3% of the total screening (see Supplementary Fig. S1). Additionally, we screened rust specimens with an extended timeline from 1787 to 2019. The rust specimens we screened the most were collected between 1919 and 1924, with 1,320 samples (see Supplementary Fig. S2). Finally, our survey covered rust specimens across the globe, including 105 countries. The five countries with the rust specimens we screened the most were The United States (1288 specimens), Brazil (498 specimens), Argentina (467 specimens), Ecuador (405 specimens), and Bolivia (337 specimens) (see Supplementary Fig. S3).

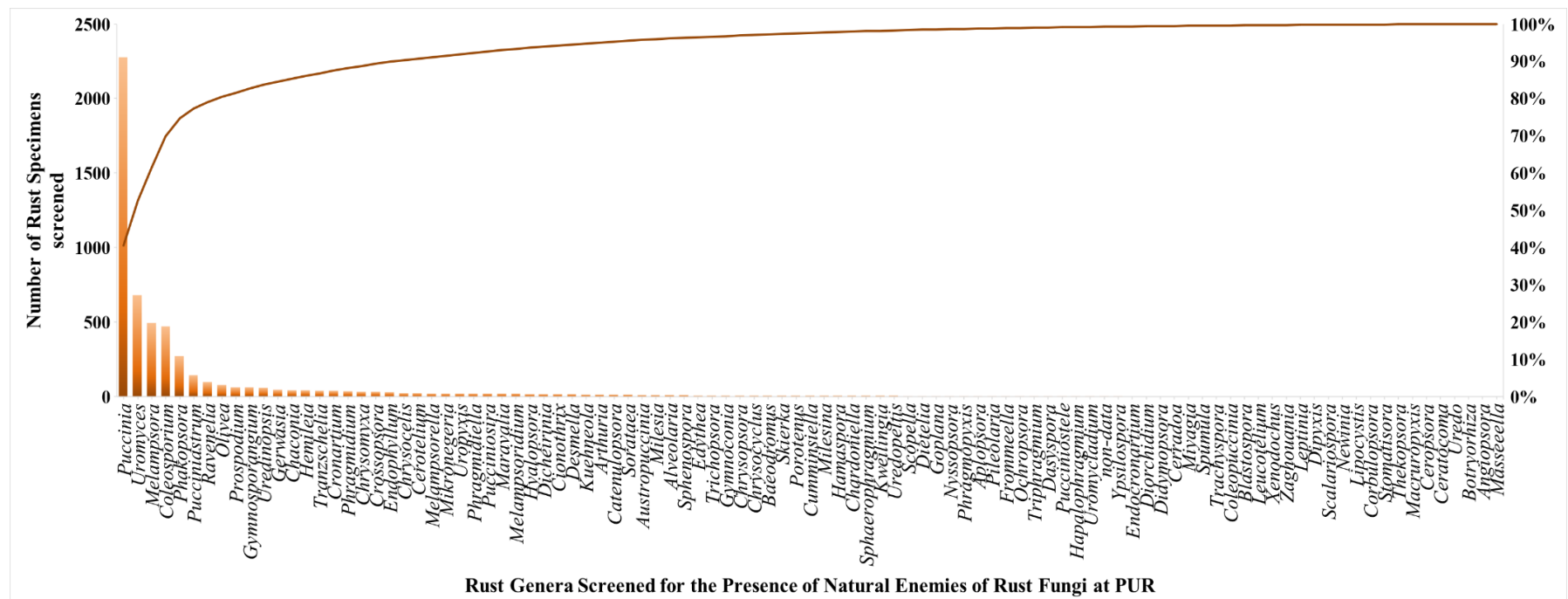


Figure 2.1 Pareto chat. Rust genera screened for the presence of natural enemies of rust fungi at PUR

#### 2.4.2 Fungal natural enemies of rust fungi

Of the 5,618 rust specimens we screened for the presence of natural enemies of rust fungi at PUR, 543 are associated with fungal natural enemies in 30 rust genera (see Table 2.3 and supplementary table S2 for more information). 12% of the *Puccinia* specimens that we screened are associated with fungal natural enemies, 15% of the *Uromyces* specimens, and 13% of the *Melampsora* specimens (Fig. 2.2). Most of these fungal natural enemies were found in the uredinial rust stage (501 specimens), followed by the telial stage (48 specimens). In addition, we found fungal natural enemies in 6% of the specimens we screened from the United States, 18% from Bolivia, 12% from Brazil, and 13% from Argentina (Fig. 2.3). Finally, most of these fungal natural enemies were found from specimens collected in 1920.

Fungal structures of those 543 fungal natural enemies of rust included black fruiting bodies, violet mycelia, pink mycelia, and white mycelia. 96% of these rust specimens contained black fruiting bodies resembling typical pycnidia of *Sphaerellopsis*, 2% contained violet mycelia resembling sporodochia of *Helicobasidium*, and less than 1% of these specimens showed other fungal structures than the previously mentioned, resembling the genera *Ramularia*, and *Trochila*. However, because most of the morphological characteristics we observed, such as fruiting bodies, hyphae, and spores, are likely similar to a broad number of genera within the phylum Ascomycota, the ITS region helped us confirm these characteristics and determine most of them at the species level. Below, we describe each fungal genus that we found in association with rust specimens screened at PUR as follows:

Table 2.3 Catalog of natural enemies of rust fungi from field sites and preserved specimens at the Arthur Fungarium. New reports are written in bold red; a dagger (†) indicates fresh specimens; NA: not available data; ND: not determined.

Species name of Natural enemy of rust	Host rust	Country	PUR barcode	Notes
<i>Cladosporium tenuissimum</i>	NA	Puerto Rico, USA	PAZ17-5(3-1)/MCA7823 †	White Mycelium. DNA sequences and macro photographs provided
<i>H. longisporum</i> Group II	<i>Puccinia arechavaletae</i>	Trinidad	<b>PURF6375</b>	
<i>H. longisporum</i> Group II	<i>Puccinia arechavaletae</i>	Trinidad	<b>PURF6376</b>	
<i>Helicobasidium</i> sp.	NA	Puerto Rico, USA	PAZ08purple/PURN23064 †	Also presence of black fruiting bodies of <i>Sphaerellopsis</i> sp.
<i>Helicobasidium</i> sp.	<i>Prospodium bicolor</i>	Trinidad	PURF11627	Purple Mycelium. Macro photograph provided only due to scarce material
<i>Helicobasidium</i> sp.	<i>Uromyces megalospermus</i>	Bolivia	PURF15153	Purple Mycelium. Macro and Microphotographs provided only due to scarce material
<i>Helicobasidium</i> sp.	<i>Uromyces colombianus</i>	Trinidad	PURF3638	Purple Mycelium. Macro photograph provided only due to scarce material
<i>Helicobasidium</i> sp.	<i>Puccinia colosseae</i>	Bolivia	PURF8203	Purple Mycelium. Macro and Microphotographs provided only due to scarce material
<i>Helicobasidium</i> sp.	<i>Prospodium appendiculatum</i>	Argentina	PURN11109	Purple Mycelium. Macro photograph provided only due to scarce material
<i>Helicobasidium</i> sp.	<i>Porotenus depallens</i>	Ecuador	PURN6633	Purple Mycelium.
<i>Helicobasidium</i> sp.	<i>Cionothrix praelonga</i>	Ecuador	PURF8737	Also presence of unknown fungivorous larvae.
<i>Helicobasidium</i> sp.	<i>Baeodromus</i> sp.	Peru	PURN4612	Also presence of unknown fungivorous larvae.
<i>Mycodiplosis</i> sp.	<i>Melampsora warburgii</i> aff.	Russia	PURN12557-1	Also presence of black fruiting bodies. They cannot be identified at species level due to scarce material
<i>Mycodiplosis</i> sp.	<i>Melampsora warburgii</i> aff.	Russia	PURN12557-2	Also presence of black fruiting bodies. They cannot be identified at species level due to scarce material
<i>Mycodiplosis</i> sp.	<i>Austropuccinia psidii</i>	Venezuela	<b>PURN15011</b>	
<i>Mycodiplosis</i> sp.	<i>Austropuccinia psidii</i>	Venezuela	<b>PURN15014</b>	
<i>Mycodiplosis</i> sp.	<i>Austropuccinia psidii</i>	Venezuela	<b>PURN15015-3</b>	
<i>Mycodiplosis</i> sp.	<i>Austropuccinia psidii</i>	Brazil	PURN7676-1	



Table 2.3 continued

<i>Mycodiplosis sp.</i>	<i>Austropuccinia sp.</i>	USA	PURN23059/ PAZ03_3L
<i>Mycodiplosis sp.</i>	<i>Coleosporium solidaginis</i>	USA	BOG03 †
<i>Mycodiplosis sp.</i>	<i>Coleosporium solidaginis</i>	USA	BOG08 †
<i>Mycodiplosis sp.</i>	<i>Coleosporium solidaginis</i>	USA	BOG09 †
<i>Mycodiplosis sp.</i>	<i>Coleosporium solidaginis</i>	USA	BOG12 †
<i>Mycodiplosis sp.</i>	<i>Coleosporium solidaginis</i>	USA	BOG16 †
<i>Mycodiplosis sp.</i>	<i>Coleosporium solidaginis</i>	USA	BOG17 †
<i>Mycodiplosis sp.</i>	<i>Coleosporium solidaginis</i>	USA	BOG21 †
<i>Mycodiplosis sp.</i>	<i>Cerotelium sabiceae</i>	Guyana	<b>ML320</b>
<i>Mycodiplosis sp.</i>	<i>Chaconia ingae</i>	Peru	<b>PURN16391</b>
<i>Mycodiplosis sp.</i>	<i>Coleosporium plumeriae</i>	Peru	<b>PURN16395</b>
<i>Mycodiplosis sp.</i>	<i>Coleosporium plumeriae</i>	Peru	<b>PURN16396</b>
<i>Mycodiplosis sp.</i>	<i>Coleosporium plumeriae</i>	Brazil	PURN9648
<i>Mycodiplosis sp.</i>	<i>Coleosporium solidaginis</i>	USA	BOG02 †
<i>Mycodiplosis sp.</i>	<i>Puccinia sp.</i>	USA	larvawheat2 †
<i>Mycodiplosis sp.</i>	<i>Coleosporium solidaginis</i>	USA	lafayette5 †
<i>Mycodiplosis sp.</i>	<i>Coleosporium solidaginis</i>	USA	PAGZ 1(3) †
<i>Mycodiplosis sp.</i>	<i>Coleosporium sp.</i>	USA	MCA7983-2
<i>Mycodiplosis sp.</i>	<i>Coleosporium sp.</i>	USA	MCA7983-3
<i>Mycodiplosis sp.</i>	<i>Coleosporium solidaginis</i>	USA	PAGZ 1(4) †
<i>Mycodiplosis sp.</i>	<i>Coleosporium solidaginis</i>	USA	West-lafayette8 †

Table 2.3 continued

<i>Mycodiplosis sp.</i>	<i>Coleosporium sp.</i>	USA	MCA7983-1
<i>Mycodiplosis sp.</i>	<i>Coleosporium sp.</i>	USA	PURN23057/P AZ01-1L
<i>Mycodiplosis sp.</i>	<i>Gymnoconia sp.</i>	USA	PURN22411/M CA3888
<i>Mycodiplosis sp.</i>	<i>Hemileia vastatrix</i>	Hawaii, Kona	B1_PV5923 †
<i>Mycodiplosis sp.</i>	<i>Hemileia vastatrix</i>	Hawaii, Kona	B2_PV5924 †
<i>Mycodiplosis sp.</i>	<i>Hemileia vastatrix</i>	Hawaii, Kona	B3_PV5925 †
<i>Mycodiplosis sp.</i>	<i>Puccinia pelargonii- zonalis</i>	Peru	<b>p.malva jorge.peru2 †</b>
<i>Mycodiplosis sp.</i>	<i>Hemileia vastatrix</i>	Hawaii, Kona	B4_PV5926 †
<i>Mycodiplosis sp.</i>	<i>Hemileia vastatrix</i>	Hawaii, Kona	K2_PV5890 †
<i>Mycodiplosis sp.</i>	<i>Hemileia vastatrix</i>	Hawaii, Kona	K3_PV5891 †
<i>Mycodiplosis sp.</i>	<i>Hemileia vastatrix</i>	Hawaii, Kona	M1midge_PV5 888 †
<i>Mycodiplosis sp.</i>	<i>Hemileia vastatrix</i>	Hawaii, Ka'u	O1_PV5927 †
<i>Mycodiplosis sp.</i>	<i>Hemileia vastatrix</i>	Hawaii, Ka'u	O2_PV5928 †
<i>Mycodiplosis sp.</i>	<i>Hemileia vastatrix</i>	Hawaii, Ka'u	O3_HCO_PV5 929 25 †
<i>Mycodiplosis sp.</i>	<i>Hemileia vastatrix</i>	Hawaii, Ka'u	O4_PV5930 †
<i>Mycodiplosis sp.</i>	<i>Hemileia vastatrix</i>	Hawaii, Ka'u	O5_PV5931 †
<i>Mycodiplosis sp.</i>	<i>Hemileia vastatrix</i>	Hawaii, Hilo	P1_PV5917 †
<i>Mycodiplosis sp.</i>	<i>Hemileia vastatrix</i>	Hawaii, Hilo	P2_PV5918 †
<i>Mycodiplosis sp.</i>	<i>Hemileia vastatrix</i>	Hawaii, Hilo	P3_PV5919 †
<i>Mycodiplosis sp.</i>	<i>Uromyces blainvilleae</i>	Peru	<b>PURF11174B</b>

Table 2.3 continued

<i>Mycodiplosis sp.</i>	<i>Hemileia vastatrix</i>	Hawaii, Hilo	P4_PV5920 †
<i>Mycodiplosis sp.</i>	<i>Hemileia vastatrix</i>	Hawaii, Hilo	P5_PV5921 †
<i>Mycodiplosis sp.</i>	<i>Hemileia vastatrix</i>	Hawaii, Hilo	P6_PV5922 †
<i>Mycodiplosis sp.</i>	<i>Hemileia vastatrix</i>	USA	PURN11725-1
<i>Mycodiplosis sp.</i>	<i>Hemileia vastatrix</i>	Bolivia	<b>PURN15328/ PURF3514</b>
<i>Mycodiplosis sp.</i>	<i>Hemileia vastatrix</i>	Peru	<b>PURN22448-4</b>
<i>Mycodiplosis sp.</i>	<i>Puccinia leonotidicola</i>	Trinidad	<b>PURF7260-2</b>
<i>Mycodiplosis sp.</i>	<i>Hemileia vastatrix</i>	Peru	<b>PURN22449</b>
<i>Mycodiplosis sp.</i>	<i>Hemileia vastatrix</i>	Peru	<b>PURN22450-1</b>
<i>Mycodiplosis sp.</i>	<i>Hemileia vastatrix</i>	Panama	<b>PURN22537-1</b>
<i>Mycodiplosis sp.</i>	<i>Hemileia vastatrix</i>	Panama	<b>PURN22625-2</b>
<i>Mycodiplosis sp.</i>	<i>Hemileia vastatrix</i>	Hawaii, Kona	W1_PV5892 †
<i>Mycodiplosis sp.</i>	<i>Puccinia sorghi</i>	USA	PURN11116-2
<i>Mycodiplosis sp.</i>	<i>Puccinia sorghi</i>	USA	PURN11116-3
<i>Mycodiplosis sp.</i>	<i>Hemileia vastatrix</i>	Hawaii, Kona	W2_PV5893 †
<i>Mycodiplosis sp.</i>	<i>Hemileia vastatrix</i>	Hawaii, Kona	W3_PV5894†
<i>Mycodiplosis sp.</i>	<i>Melampsora epiphylla</i>	Japan	PURN11585
<i>Mycodiplosis sp.</i>	<i>Melampsora occidentalis</i>	Canada	<b>PURN11705-2</b>
<i>Mycodiplosis sp.</i>	<i>Melampsora epitea</i> complex	USA	PURN16055-2
<i>Mycodiplosis sp.</i>	<i>Hemileia vastatrix</i>	USA	PURN11725-3
<i>Mycodiplosis sp.</i>	<i>Hemileia vastatrix</i>	USA	PURN11725-4
<i>Mycodiplosis sp.</i>	<i>Melampsora gelmii</i>	Italy	Italy-1L †
<i>Mycodiplosis sp.</i>	<i>Melampsora sp.</i>	Russia	PURN12029-4
<i>Mycodiplosis sp.</i>	<i>Melampsora</i> <i>humboldtiana</i>	USA	PURN12037-1
<i>Mycodiplosis sp.</i>	<i>Melampsora</i> <i>humboldtiana</i>	USA	PURN12037-2

Table 2.3 continued

<i>Mycodiplosis</i> sp.	<i>Melampsora humboldtiana</i>	USA	PURN12037-3
<i>Mycodiplosis</i> sp.	<i>Melampsora humboldtiana</i>	USA	PURN12880-1
<i>Mycodiplosis</i> sp.	<i>Melampsora humboldtiana</i>	USA	PURN12880-2
<i>Mycodiplosis</i> sp.	<i>Melampsora humboldtiana</i>	USA	PURN12880-3
<i>Mycodiplosis</i> sp.	<i>Melampsora humboldtiana</i>	Canada	<b>PURN15051</b>
<i>Mycodiplosis</i> sp.	<i>Austropuccinia psidii</i>	Venezuela	<b>PURN15011-1</b>
<i>Mycodiplosis</i> sp.	<i>Austropuccinia psidii</i>	Venezuela	<b>PURN15011-2</b>
<i>Mycodiplosis</i> sp.	<i>Melampsora larici-populina</i>	Germany	PURN5750-1
<i>Mycodiplosis</i> sp.	<i>Melampsora medusae</i>	Canada	<b>PURN11700</b>
<i>Mycodiplosis</i> sp.	<i>Melampsora occidentalis</i>	Canada	<b>PURN11705-1</b>
<i>Mycodiplosis</i> sp.	<i>Melampsora</i> sp.	USA	MT3-merie
<i>Mycodiplosis</i> sp.	<i>Melampsora</i> sp.	Russia	PURN12029-1
<i>Mycodiplosis</i> sp.	<i>Melampsora</i> sp.	Japan	PURN15091-3
<i>Mycodiplosis</i> sp.	<i>Melampsora</i> sp.	Japan	PURN15091-2
<i>Mycodiplosis</i> sp.	<i>Melampsora</i> sp.	Japan	PURN15118-1
<i>Mycodiplosis</i> sp.	<i>Melampsora</i> sp.	Japan	PURN15118-2
<i>Mycodiplosis</i> sp.	<i>Melampsora</i> sp.	Japan	PURN15118-3
<i>Mycodiplosis</i> sp.	<i>Melampsora</i> sp.	USA	PURN16030
<i>Mycodiplosis</i> sp.	<i>Melampsora</i> sp.	Germany	PURN16119-1
<i>Mycodiplosis</i> sp.	<i>Olivea tectonae</i>	Bolivia	<b>PURN15331b</b>
<i>Mycodiplosis</i> sp.	<i>Melampsora</i> sp.	Canada	<b>PURN22739</b>
<i>Mycodiplosis</i> sp.	<i>Melampsora</i> sp.	China	PURN22989
<i>Mycodiplosis</i> sp.	<i>Melampsora tremulae</i>	Japan	PURN15118
<i>Mycodiplosis</i> sp.	<i>Melampsora epitea</i> complex	USA	PURN16055-3
<i>Mycodiplosis</i> sp.	<i>Mikronegeria alba</i>	Chile	<b>PURN16373</b>
<i>Mycodiplosis</i> sp.	NA	NA	PUR68963

Table 2.3 continued

<i>Mycodiplosis</i> sp.	<i>Olivea</i> sp.	Brazil	PUR87962
<i>Mycodiplosis</i> sp.	<i>Olivea tectonae</i>	Bolivia	<b>PURN15331</b>
<i>Mycodiplosis</i> sp.	<i>Phakopsora cherimoliae</i>	Ecuador	<b>PURF8883</b>
<i>Mycodiplosis</i> sp.	<i>Phakopsora nishidana</i>	Dominican Republic	<b>PUR44594</b>
<i>Mycodiplosis</i> sp.	<i>Puccinia pelargonii-zonalis</i>	Peru	<b>PURN16381</b>
<i>Mycodiplosis</i> sp.	<i>Puccinia sorghi</i>	USA	PURN11116-1
<i>Mycodiplosis</i> sp.	<i>Puccinia arundinariae</i>	Peru	<b>PURN16378</b>
<i>Mycodiplosis</i> sp.	<i>Puccinia caeomatiformis</i>	Ecuador	<b>PURN16</b>
<i>Mycodiplosis</i> sp.	<i>Puccinia distenta</i>	Bolivia	<b>PURF6270</b>
<i>Mycodiplosis</i> sp.	<i>Puccinia leonotidicola</i>	Trinidad	<b>PURF7260-1</b>
<i>Mycodiplosis</i> sp.	<i>Hemileia vastatrix</i>	Panama	<b>PURN22537-2</b>
<i>Mycodiplosis</i> sp.	<i>Puccinia mogiphanis</i>	Ecuador	<b>PURF5972</b>
<i>Mycodiplosis</i> sp.	<i>Puccinia mogiphanis</i>	Ecuador	<b>PURN5852</b>
<i>Mycodiplosis</i> sp.	<i>Puccinia oxalidis</i>	Venezuela	<b>PURN15018</b>
<i>Mycodiplosis</i> sp.	<i>Puccinia pelargonii-zonalis</i>	Peru	<b>p.malva jorge.peru1 †</b>
<i>Mycodiplosis</i> sp.	<i>Puccinia pelargonii-zonalis</i>	Peru	<b>PURN23083-1</b>
<i>Mycodiplosis</i> sp.	<i>Puccinia polysora</i>	USA	larvacornb1 †
<i>Mycodiplosis</i> sp.	<i>Puccinia sorghi</i>	Peru	<b>PURN23084/C JP1 †</b>
<i>Mycodiplosis</i> sp.	<i>Puccinia</i> sp.	USA	larvawheat1 †
<i>Mycodiplosis</i> sp.	<i>Puccinia sorghi</i>	Peru	<b>PURN23084-2/CJP2 †</b>
<i>Mycodiplosis</i> sp.	<i>Puccinia triumfettae</i>	Ecuador	<b>PURF6438</b>
<i>Mycodiplosis</i> sp.	<i>Puccinia tubulosa</i>	USA	PURN23060/P AZ04-5L
<i>Mycodiplosis</i> sp.	<i>Puccinia unicolor</i>	Bolivia	<b>PURF8146</b>
<i>Mycodiplosis</i> sp.	<i>Austropuccinia psidii</i>	Brazil	PURN7676-2
<i>Mycodiplosis</i> sp.	<i>Austropuccinia psidii</i>	Brazil	PURN7676-3

Table 2.3 continued

<i>Mycodiplosis sp.</i>	<i>Puccinia verbesinae-dentatae</i>	Ecuador	<b>PURF8323</b>	
<i>Mycodiplosis sp.</i>	<i>Uromyces cestri</i>	Bolivia	<b>PURF9848</b>	
<i>Mycodiplosis sp.</i>	<i>Uromyces blainvilleae</i>	Peru	<b>PURF11174</b>	
<i>Mycodiplosis sp.</i>	<i>Uromyces colombianus</i>	Trinidad	<b>PURF3638-2</b>	
<i>Mycodiplosis sp.</i>	<i>Uromyces hedysari-paniculati</i>	Trinidad	<b>PURF2926</b>	
ND	<i>Uromyces clignyi</i>	Cuba	PUR11667	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Uromyces dactylidis</i>	USA	PUR12160	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Coleosporium asterum</i>	USA	PUR1442	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Uromyces neurocarpi</i>	El Salvador	PUR15885	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia dolosa</i>	Cuba	PUR18279	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Phakopsora compressa</i>	Cuba	PUR18450	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia graminis</i>	USA	PUR19458	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Melampsora medusae</i>	USA	PUR2045	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Melampsora medusae</i>	USA	PUR2065	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Melampsora medusae</i>	USA	PUR2090	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Melampsora medusae</i>	USA	PUR2102	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Melampsora medusae</i>	USA	PUR2143	Black fruiting bodies. It cannot be identified at species level due to scarce material

Table 2.3 continued

ND	<i>Melampsora paradoxa</i>	USA	PUR2392	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia eleocharidis</i>	USA	PUR26778	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Melampsora epitea</i>	USA	PUR2916	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Melampsora epitea</i>	USA	PUR2933	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia dioicae</i>	USA	PUR29466	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Melampsora epitea</i>	Canada	PUR2956	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Melampsora epitea</i>	USA	PUR2992	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia dioicae</i>	USA	PUR30117	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Melampsora epitea</i>	USA	PUR3027	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Uromyces tranzschelii</i>	USA	PUR38665	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Uromyces holmbergii</i>	USA	PUR42945	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Phakopsora uva</i>	USA	PUR44002	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia idonea</i>	Guatemala	PUR49026	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Uromyces antiguanus</i>	Guatemala	PUR49058	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Uromyces scleriae</i>	Guatemala	PUR50437	Black fruiting bodies. It cannot be identified at species level due to scarce material

Table 2.3 continued

ND	<i>Uromyces rhynchosporae</i>	Honduras	PUR51697	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia hyptidis</i>	USA	PUR51738	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia dioicae</i>	Canada	PUR51997	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Uromyces commelinae</i>	Trinidad	PUR52266	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Uromyces setariae-italicae</i>	USA	PUR54761	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia ammophilae</i>	USA	PUR54820	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Melampsora ribesii-purpureae</i>	USA	PUR55326	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia subnitens</i>	USA	PUR55912	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia boutelouae</i>	Mexico	PUR57385	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia setariae</i>	Mexico	PUR58718	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Cronartium conigenum</i>	Mexico	PUR59405	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia anthephorae</i>	Guatemala	PUR59587	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Dasyscypha mesoamericana</i>	Mexico	PUR60025	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia versicolor</i>	Mexico	PUR60747	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia conoclinii</i>	Mexico	PUR61858	Black fruiting bodies. It cannot be identified at species level due to scarce material



Table 2.3 continued

ND	<i>Uromyces tenuistipes</i>	Mexico	PUR65124	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia obtecta</i>	USA	PUR65329	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia duthiae</i>	Mexico	PUR66096	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia arundiariae</i>	USA	PUR66097	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia galopinae</i>	Nigeria	PUR66892	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Tranzschelia fusca</i>	USA	PUR6931	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Prospodium abortivum</i>	Barbados	PUR7416	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Uromyces eragrostidis</i>	Argentina	PUR78615	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Tranzschelia arthuri</i>	Ecuador	PUR83763	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia oahuensis</i>	Brazil	PUR87966	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia bistortae</i>	USA	PUR88387	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Coleosporium asterum</i>	USA	PUR88852	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Coleosporium asterum</i>	USA	PUR88856	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Uromyces clignyi</i>	Brazil	PUR89105	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Melampsora larici-populina</i>	Argentina	PURF10348	Black fruiting bodies. It cannot be identified at species level due to scarce material

Table 2.3 continued

ND	<i>Puccinia graminis</i>	Argentina	PURF10351	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Tranzschelia discolor</i>	Argentina	PURF10356/PU R77256	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Tranzschelia discolor</i>	Argentina	PURF10358/PU R77258	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia dietelii</i>	Uganda	PURF10380	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia oxalidis</i>	Colombia	PURF10661	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Uromyces euphorbiae</i>	Colombia	PURF10662	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia graminis</i>	Colombia	PURF10668	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia brachypoi var. poaememoralis</i>	Argentina	PURF10920	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Catenulopsora praelonga</i>	Argentina	PURF10937	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia thaliae</i>	Argentina	PURF10942	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia lepturi</i>	Japan	PURF10952	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia baccharidis</i>	Argentina	PURF11244	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia pusilla</i>	China	PURF11270	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia polysora</i>	Trinidad	PURF11374	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia polysora</i>	Trinidad	PURF11376	Black fruiting bodies. It cannot be identified at species level due to scarce material

Table 2.3 continued

ND	<i>Catenulopsora praelonga</i>	Argentina	PURF11391	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia purpurea</i>	St. Vincent & the Grenadines	PURF11448	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia polysora</i>	Trinidad	PURF11595	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia cephalotes</i>	Trinidad	PURF11662	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia abnormis</i>	Argentina	PURF11695	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia solidaginis-microglossae</i>	Argentina	PURF11696	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia graminis</i>	Argentina	PURF11700	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia graminis</i>	Argentina	PURF11701	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia polygoni-amphibii</i>	Argentina	PURF11704	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Uromyces eragrostidis</i>	Argentina	PURF11715	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia cacao</i>	Indonesia	PURF11797	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia polysora</i>	Colombia	PURF11875	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia subtilipes</i>	Trinidad	PURF11895/(P URF16658)	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Melampsora euphorbiae-dulcis</i>	China	PURF11955	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia levis</i>	Japan	PURF13014	Black fruiting bodies. It cannot be identified at species level due to scarce material

Table 2.3 continued

ND	<i>Puccinia polypogonis</i>	Argentina	PURF13044	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia solidaginis-microglossae</i>	Argentina	PURF13061	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Tranzschelia discolor</i>	Ecuador	PURF1325	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Tranzschelia arthuri</i>	Ecuador	PURF1326/PU R68226	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia liberta</i>	Ecuador	PURF14603	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia sorghi</i>	Ethiopia	PURF14605	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Uromyces scleriae</i>	Trinidad	PURF14684	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia sorghi</i>	Colombia	PURF14715	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia polypogonis</i>	Argentina	PURF14840	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia liberta</i>	Trinidad	PURF14932	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Phragmidium mucronatum</i>	Germany	PURF1511	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Uromyces peglerae</i>	Philippines	PURF15111	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Phakopsora setariae</i>	Malawi	PURF15133	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia dioicae</i>	Bolivia	PURF15195	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia opuntiae</i>	Peru	PURF15395	Black fruiting bodies. It cannot be identified at species level due to scarce material

Table 2.3 continued

ND	<i>Puccinia vexans</i>	Peru	PURF15396	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia levis</i>	Venezuela	PURF15436	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia opipara</i>	Peru	PURF15448	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Kweilingia wangii</i>	Taiwan	PURF15493	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia piptochaetii</i>	Uruguay	PURF15523	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia piptochaetii</i>	Uruguay	PURF15524	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia piptochaetii</i>	Uruguay	PURF15525	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia conspersa</i>	Argentina	PURF15557	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia sp.</i>	Ecuador	PURF15576	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia polysora</i>	Venezuela	PURF15909	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Cerotelium clerodendri</i>	Ghana	PURF16253	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Uromyces coronatus</i>	Japan	PURF16265	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Uromyces eragrostidis</i>	Argentina	PURF16902	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Uromyces niteroyensis</i>	Argentina	PURF16914	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Uromyces viciae-fabae</i>	Ecuador	PURF16917	Black fruiting bodies. It cannot be identified at species level due to scarce material

Table 2.3 continued

ND	<i>Puccinia flaccida</i>	Indonesia	PURF16951	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Uromyces danthoniae</i>	New Zealand	PURF17236	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia recondita</i>	Chile	PURF17278	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia gymnopogonis</i>	British Guiana	PURF17384	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia subtilipes</i>	Trinidad	PURF17386	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia brachypodii</i>	Falkland Islands	PURF17658	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia chaetochloae</i>	Brazil	PURF17721	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia ellisiana</i>	Brazil	PURF17723	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Uromyces setariae-italicae</i>	Argentina	PURF17745	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia neorotundata</i>	Venezuela	PURF17883	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia polysora</i>	Colombia	PURF18021	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Phakopsora zeae</i>	Colombia	PURF18031	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Phakopsora zeae</i>	Colombia	PURF18135	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia polysora</i>	Colombia	PURF18139	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Uromyces setariae-italicae</i>	Argentina	PURF18304	Black fruiting bodies. It cannot be identified at species level due to scarce material

Table 2.3 continued

ND	<i>Puccinia brachypodii</i> <i>var. major</i>	Chile	PURF18480	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia magnusiana</i>	Australia	PURF18528	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia schileana</i>	Brazil	PURF18892	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Uredinopsis pteridis</i>	Brazil	PURF19234	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia brachypodii</i>	Argentina	PURF19469	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Uromyces commelinae</i>	Argentina	PURF19529	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Melampsora aecidioides</i>	Uruguay	PURF19564	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Uromyces eragrostidis</i>	Bolivia	PURF2358	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Uromyces eragrostidis</i>	Bolivia	PURF2361	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Uromyces eragrostidis</i>	Bolivia	PURF2362	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Uromyces cuspidatus</i>	Bolivia	PURF2366	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Uromyces cuspidatus</i>	Bolivia	PURF2367	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Uromyces cuspidatus</i>	Bolivia	PURF2381	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Uromyces epicampis</i>	Chile	PURF2387	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Uromyces epicampis</i>	Bolivia	PURF2389 (PUR69289)	Black fruiting bodies. It cannot be identified at species level due to scarce material

Table 2.3 continued

ND	<i>Uromyces pencanus</i>	Chile	PURF2406	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Uromyces pencanus</i>	Chile	PURF2407	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Uromyces tenuicutis</i>	Chile	PURF2416	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Uromyces tenuicutis</i>	Bolivia	PURF2417	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Uromyces tenuicutis</i>	Bolivia	PURF2418	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Uromyces tenuicutis</i>	Ecuador	PURF2420	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Uromyces tenuicutis</i>	Trinidad	PURF2427	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Uromyces niteroyensis</i>	Uruguay	PURF2436	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Phakopsora lenticularis</i>	Trinidad	PURF2452	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Uromyces setariae-italicae</i>	Peru	PURF2453	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Uromyces setariae-italicae</i>	Peru	PURF2465	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia infuscans</i>	Bolivia	PURF2488	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia infuscans</i>	Bolivia	PURF2491	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Uromyces oblectaneus</i>	Brazil	PURF2506	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Uromyces magellanicus</i>	Chile	PURF2547	Black fruiting bodies. It cannot be identified at species level due to scarce material



Table 2.3 continued

ND	<i>Uromyces junci</i>	Bolivia	PURF2573	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Uromyces silphii</i>	Chile	PURF2587	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Uromyces bunsteri</i>	Chile	PURF2687	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Uromyces iresines</i>	Ecuador	PURF2794	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Uromyces hedysari-paniculati</i>	Trinidad	PURF2925	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Uromyces hedysari-paniculati</i>	Ecuador	PURF2927	Pink Mycelium. Macrophotograph provided only due to scarce material
ND	<i>Uromyces trifolii-repentis</i>	Bolivia	PURF3131	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Uromyces trifolii-repentis</i>	Bolivia	PURF3137	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Uromyces trifolii</i>	Bolivia	PURF3167	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Uromyces euphorbiae</i>	Trinidad	PURF3340	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Uromyces triquetus</i>	Chile	PURF3421	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Uromyces myrsines</i>	Bolivia	PURF3434	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Uromyces myrsines</i>	Bolivia	PURF3437	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Uromyces asclepiadis</i>	Peru	PURF3488	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Uromyces asclepiadis</i>	Peru	PURF3489	Black fruiting bodies. It cannot be identified at species level due to scarce material

Table 2.3 continued

ND	<i>Uromyces wulfiae-stenoglossae</i>	Trinidad	PURF3629	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Uromyces bidenticola</i>	Bolivia	PURF3645	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia brachypodii</i> var. <i>poaememoralis</i>	Bolivia	PURF3796	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia brachypodii</i>	Chile	PURF3810	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia brachypodii</i> var. <i>poaememoralis</i>	Chile	PURF3820	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia brachypodii</i> var. <i>poaememoralis</i>	Chile	PURF3821	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia schedonnardi</i>	Bolivia	PURF3862	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia coronata</i>	Bolivia	PURF3865	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia brachypodii</i>	Germany	PURF3874	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia brachypodii</i> var. <i>poaememoralis</i>	Finland	PURF3887	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia aristidae</i>	Bolivia	PURF3897	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia subnitens</i>	Chile	PURF3904	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia subnitens</i>	Bolivia	PURF3905	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia recondita</i>	Bolivia	PURF4088	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia recondita</i>	Chile	PURF4089	Black fruiting bodies. It cannot be identified at species level due to scarce material

Table 2.3 continued

ND	<i>Puccinia recondita</i>	Ecuador	PURF4097	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia recondita</i>	Ecuador	PURF4098	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia recondita</i>	Bolivia	PURF4100	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia recondita</i>	Argentina	PURF4115	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia tornata</i>	Bolivia	PURF4235	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia graminis</i>	Germany	PURF4342	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia graminis</i>	Argentina	PURF4363	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia coronata</i>	Chile	PURF4417	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia coronata</i>	Chile	PURF4418	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia coronata</i>	Bolivia	PURF4443	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia interveniens</i>	Chile	PURF4568	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Uromyces pencanus</i>	Argentina	PURF4600	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia saltensis</i>	Argentina	PURF4604	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia saltensis</i>	Argentina	PURF4605	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia nasellae</i>	Bolivia	PURF4606	Black fruiting bodies. It cannot be identified at species level due to scarce material

Table 2.3 continued

ND	<i>Puccinia saltensis</i>	Bolivia	PURF4611	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia saltensis</i>	Argentina	PURF4614	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia saltensis</i>	Bolivia	PURF4616	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia piptochaetii</i>	Chile	PURF4644	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia piptochaetii</i>	Argentina	PURF4646	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia piptochaetii</i>	Argentina	PURF4647	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia aegopogonis</i>	Bolivia	PURF4649	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia levis</i>	Bolivia	PURF4769	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Phakopsora compressa</i>	Bolivia	PURF4845	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia substriata</i>	Peru	PURF4902	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Phakopsora compressa</i>	Bolivia	PURF4937	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia gymnotrichis</i>	Ecuador	PURF4959	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia gymnotrichis</i>	Ecuador	PURF4960	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia abnormis</i>	Peru	PURF4968	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia inclita</i>	Brazil	PURF4973	Black fruiting bodies. It cannot be identified at species level due to scarce material

Table 2.3 continued

ND	<i>Puccinia posadensis</i>	Ecuador	PURF5022	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia posadensis</i>	Bolivia	PURF5024	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia erianthicola</i>	Brazil	PURF5042	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia purpurea</i>	Philippines	PURF5113	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia polysora</i>	Trinidad	PURF5118	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia arachis</i>	Bolivia	PURF6252	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia oxalidis</i>	Ecuador	PURF6293	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia gouaniae</i>	Trinidad	PURF6425	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia hydrocotyles</i>	Bolivia	PURF6895	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia hydrocotyles</i>	Ecuador	PURF6927	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia menthae</i>	Ecuador	PURF7199	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia aenigmatica</i>	Bolivia	PURF7408	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia melanosora</i>	Argentina	PURF7688	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia eupatorii-columbiani</i>	Trinidad	PURF8038	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia conyzae</i>	Brazil	PURF8092	Black fruiting bodies. It cannot be identified at species level due to scarce material

Table 2.3 continued

ND	<i>Puccinia evadens</i>	Bolivia	PURF8110	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia unicolor</i>	Bolivia	PURF8145	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia salebrata</i>	Bolivia	PURF8153	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia alia</i>	Brazil	PURF8154	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia praeculta</i>	Bolivia	PURF8167	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia cnici-oleracei</i>	Peru	PURF8236	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Melampsora medusae</i>	Argentina	PURF825	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia majuscula</i>	Bolivia	PURF8467	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Uromyces bonariensis</i>	Trinidad	PURF8877	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Melampsora allii-populina</i>	Germany	PURF892	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Uredinopsis pteridis</i>	Brazil	PURF9	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Phragmidiella minuta</i>	Paraguay	PURF9017	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Cerotelium sabiceae</i>	Trinidad	PURF9028	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia punctiformis</i>	Latvia	PURF9456	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Uromyces pencanus</i>	Argentina	PURF9468	Black fruiting bodies. It cannot be identified at species level due to scarce material

Table 2.3 continued

ND	<i>Puccinia graminis</i>	Argentina	PURF9549	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Phakopsora compressa</i>	Venezuela	PURF9792	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia dolosa</i>	Venezuela	PURF9793	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia dolosa</i>	Venezuela	PURF9794	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia substriata</i>	Venezuela	PURF9798	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia substriata</i>	Venezuela	PURF9799	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Phakopsora compressa</i>	Venezuela	PURF9812	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia substriata</i>	Argentina	PURF9842	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Melampsora epitea</i>	Bolivia	PURF9845	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Uromyces crassipes</i>	Bolivia	PURF9847	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Chrysocyclus mikaniae</i>	Ecuador	PURN10077	Pink Mycelium. Macro photograph provided only due to scarce material
ND	<i>Phakopsora compressa</i>	Brazil	PURN10225	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia sp.</i>	Venezuela	PURN10389	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Tranzschelia arthurii</i>	Ecuador	PURN10625	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Sphaerophragmium acaciae</i>	Northern Mariana Islands, Saipan	PURN10854	Black fruiting bodies. It cannot be identified at species level due to scarce material

Table 2.3 continued

ND	<i>Tranzschelia mexicana</i>	Colombia	PURN1090	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia chondrillina</i>	Australia	PURN10906	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia levis</i>	Brazil	PURN11489	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia obliquo-septata</i>	Guyana	PURN15533	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia thaliae</i>	Peru	PURN16397	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Phakopsora meibomia</i>	Brazil	PURN2901	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia lygodii</i>	Brazil	PURN2918	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia thaliae</i>	Brazil	PURN3167	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia thaliae</i>	Brazil	PURN3172	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia thaliae</i>	Brazil	PURN3260	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia sp.</i>	Bolivia	PURN3327	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia sp.</i>	Bolivia	PURN3328	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia sp.</i>	Ecuador	PURN3362	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia bambusarum</i>	Brazil	PURN3684	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Phakopsora melaena</i>	Brazil	PURN3744	Black fruiting bodies. It cannot be identified at species level due to scarce material



Table 2.3 continued

ND	<i>Phakopsora gossypii</i>	India	PURN3868	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Melampsora epitea</i>	Argentina	PURN4000	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Melampsora epitea</i>	USA	PURN4005	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Melampsora larici-populina</i>	Costa Rica	PURN4017	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Chaconia hennenii</i>	Bolivia	PURN4019	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Melampsora larici-populina</i>	Brazil	PURN4113	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Melampsora larici-populina</i>	Argentina	PURN4115	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia brachypodii</i>	Chile	PURN5226	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia cynodontis</i>	Ecuador	PURN5488	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia thaliae</i>	Brazil	PURN5821	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia arachidis</i>	Uruguay	PURN5918	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia arachidis</i>	Argentina	PURN5925	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia hydrocotyles</i>	Ecuador	PURN6111	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia mitrata</i>	Mexico	PURN6156	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia abrupta</i>	Uruguay	PURN6288	Black fruiting bodies. It cannot be identified at species level due to scarce material

Table 2.3 continued

ND	<i>Melampsora yezoensis</i>	Taiwan	PURN6744	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Prospodium appendiculatum</i>	Brazil	PURN6855	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Uredo lueheae</i>	Brazil	PURN6916	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia sp.</i>	Peru	PURN7542	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia neorotundata</i>	Colombia	PURN7734	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia thaliae</i>	Brazil	PURN7884	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia hydrocotyles</i>	Colombia	PURN8267	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia arachidis</i>	Paraguay	PURN8496	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia arachidis</i>	Paraguay	PURN8498	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Uropyxis rickiana</i>	Argentina	PURN8678	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Uropyxis rickiana</i>	Argentina	PURN8681	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia sp.</i>	Ecuador	PURN9457	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia sp.</i>	Colombia	PURN9517	Black fruiting bodies. It cannot be identified at species level due to scarce material
ND	<i>Puccinia polysora</i>	Trinidad	PURF11373	Presence of black fruiting bodies and fungivorous larvae. They cannot be identified at species level due to scarce material
ND	<i>Phakopsora compressa</i>	Bolivia	PURF1209	Presence of black fruiting bodies and fungivorous larvae. They cannot be identified at species level due to scarce material

Table 2.3 continued

ND	<i>Tranzschelia discolor</i>	Ecuador	PURF1335/PU R68235	Presence of black fruiting bodies and fungivorous larvae. They cannot be identified at species level due to scarce material
ND	<i>Puccinia substriata</i>	Trinidad	PURF14940	Presence of black fruiting bodies and fungivorous larvae. They cannot be identified at species level due to scarce material
ND	<i>Uromyces epicampis</i>	Bolivia	PURF2388	Presence of black fruiting bodies and fungivorous larvae. They cannot be identified at species level due to scarce material
ND	<i>Uromyces commelinae</i>	Argentina	PURF2563	Presence of black fruiting bodies and fungivorous larvae. They cannot be identified at species level due to scarce material
ND	<i>Uromyces asclepiadis</i>	Peru	PURF3486	Presence of black fruiting bodies and fungivorous larvae. They cannot be identified at species level due to scarce material
ND	<i>Puccinia brachypoi var. poaememoralis</i>	Chile	PURF3803	Presence of black fruiting bodies and fungivorous larvae. They cannot be identified at species level due to scarce material
ND	<i>Puccinia porphyretica</i>	Bolivia	PURF7401	Presence of black fruiting bodies and fungivorous larvae. They cannot be identified at species level due to scarce material
ND	<i>Puccinia graminis</i>	Australia	PURN10907	Presence of black fruiting bodies and fungivorous larvae. They cannot be identified at species level due to scarce material
ND	<i>Uromyces clarus</i>	Bolivia	PURN2546	Presence of black fruiting bodies and fungivorous larvae. They cannot be identified at species level due to scarce material
ND	<i>Uredinopsis pteridis</i>	Brazil	PURN4036	Presence of black fruiting bodies and fungivorous larvae. They cannot be identified at species level due to scarce material
ND	<i>Puccinia illatabilis</i>	Peru	PURN6215	Presence of black fruiting bodies and fungivorous larvae. They cannot be identified at species level due to scarce material
ND	<i>Puccinia obliquoseptata</i>	Guyana	MCA4170	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Coleosporium ipomoeae</i>	USA	MCA4196a	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Coleosporium ipomoeae</i>	USA	MCA4196b	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Puccinia modiolae</i>	USA	MCA4228	Presence of fungivorous larvae that could not be identified due to dried or scarce material

Table 2.3 continued

ND	<i>Puccinia modiolae</i>	USA	MCA4228-1	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Puccinia kuehnii</i>	Ecuador	ML143	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Coleosporium solidaginis</i>	USA	PAGZ-1 †	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Coleosporium solidaginis</i>	USA	PAGZ-3L †	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Coleosporium solidaginis</i>	USA	PAGZ-4 †	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Uromyces brasiliensis</i>	Trinidad	PUR52270	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Puccinia melampodii</i>	Trinidad	PUR62238	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Phakopsora nishidana</i>	USA	PUR66739	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Crossopsora uleana</i>	Ecuador	PUR66796	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Kuehneola sp.</i>	Ecuador	PUR66834	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Phragmidium fusiforme</i>	USA	PUR7784	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Olivea sp.</i>	Brazil	PUR86966	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Olivea sp.</i>	Brazil	PUR87091	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Olivea sp.</i>	Brazil	PUR87181	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Melampsora euphorbiae</i>	Brazil	PUR87208	Presence of fungivorous larvae that could not be identified due to dried or scarce material

Table 2.3 continued

ND	<i>Coleosporium vernoniae</i>	Brazil	PUR87230	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Melampsora euphorbiae</i>	Brazil	PUR87298	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Coleosporium vernoniae</i>	Brazil	PUR87302	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Melampsora euphorbiae</i>	Brazil	PUR87657	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Chaconia ingae</i>	Brazil	PUR87679	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Melampsora euphorbiae</i>	Brazil	PUR87718	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Coleosporium vernoniae</i>	Brazil	PUR87823	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Olivea sp.</i>	Brazil	PUR87838	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Coleosporium vernoniae</i>	Brazil	PUR87905	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Coleosporium vernoniae</i>	Brazil	PUR87982	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Olivea sp.</i>	Brazil	PUR88988	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Olivea capituliformis</i>	Brazil	PUR89175	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Phakopsora meibomiaae</i>	Colombia	PUR89660	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Phakopsora nishidana</i>	Colombia	PURF10658	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Phakopsora cherimoliae</i>	Colombia	PURF10664	Presence of fungivorous larvae that could not be identified due to dried or scarce material

Table 2.3 continued

ND	<i>Chrysocyclus mikaniae</i>	Venezuela	PURF10670	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Prospodium singeri</i>	Argentina	PURF10907	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Gerwasia holwayi</i>	Peru	PURF11164	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Puccinia roseana</i>	Peru	PURF11211	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Phakopsora tecta</i>	Argentina	PURF1153	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Uromyces bonariensis</i>	Argentina	PURF11712	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Uromyces asclepiadis</i>	Colombia	PURF11728	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Crossopsora mateleae</i>	Grenada	PURF11857	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Puccinia oahuensis</i>	Trinidad	PURF11864	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Phakopsora pavidia</i>	Trinidad	PURF1221	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Endophyllum decoloratum</i>	Trinidad	PURF14688	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Uromyces commelinae</i>	Trinidad	PURF14925	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Uromyces commelinae</i>	Trinidad	PURF14926	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Uromyces dolichosporus</i>	Bolivia	PURF15160	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Uromyces renovatus</i>	Argentina	PURF15276	Presence of fungivorous larvae that could not be identified due to dried or scarce material

Table 2.3 continued

ND	<i>Uromyces hedysari-paniculati</i>	Argentina	PURF15277	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Uromyces columbianus</i>	Colombia	PURF15575	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Alveolaria cordiae</i>	Peru	PURF16284	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Gerwasia variabilis</i>	Venezuela	PURF1661	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Gerwasia clara</i>	Ecuador	PURF1663	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Gerwasia cundinamarcensis</i>	Ecuador	PURF1668	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Gerwasia quitensis</i>	Bolivia	PURF1671	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Chrysocelis lupini</i>	Ecuador	PURF16867	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Mikronegeria fagi</i>	Chile	PURF17323	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Phakopsora zeae</i>	Peru	PURF18138	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Chaconia ingae</i>	Colombia	PURF18645	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Chrysocelis lupini</i>	Ecuador	PURF18654	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Coleosporium vernoniae</i>	Brazil	PURF18869	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Olivea capituliformis</i>	Brazil	PURF19001	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Mikronegeria fagi</i>	Chile	PURF19466	Presence of fungivorous larvae that could not be identified due to dried or scarce material

Table 2.3 continued

ND	<i>Coleosporium tussilaginis</i>	Chile	PURF19576	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Prospodium tuberculatum</i>	Bolivia	PURF1962	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Ravenelia echinata</i>	Bolivia	PURF2063	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Sorataea amiciae</i>	Bolivia	PURF2267	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Uromyces eragrostidis</i>	Bolivia	PURF2357	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Uromyces eragrostidis</i>	Bolivia	PURF2359	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Uromyces hedysari-paniculati</i>	Bolivia	PURF2919	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Uromyces hedysari-paniculati</i>	Bolivia	PURF2923	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Uromyces hedysari-paniculati</i>	Ecuador	PURF2924	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Uromyces viciae-fabae</i>	Bolivia	PURF2978	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Uromyces viciae-fabae</i>	Ecuador	PURF3007	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Uromyces trifolii-repentis</i>	Bolivia	PURF3156	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Uromyces neurocarpi</i>	Ecuador	PURF3283	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Uromyces euphorbiae</i>	Ecuador	PURF3335	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Uromyces euphorbiae</i>	Peru	PURF3353	Presence of fungivorous larvae that could not be identified due to dried or scarce material



Table 2.3 continued

ND	<i>Uromyces euphorbiae</i>	Ecuador	PURF3362	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Uromyces euphorbiae</i>	Peru	PURF3364	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Uromyces asclepiadis</i>	Trinidad	PURF3484	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Uromyces brasiliensis</i>	Ecuador	PURF3497	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Uromyces cestri</i>	Bolivia	PURF3521	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Uromyces cestri</i>	Chile	PURF3525	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Uromyces cestri</i>	Ecuador	PURF3530	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Uromyces cestri</i>	Peru	PURF3539	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Uromyces emmeorrhize</i>	Bolivia	PURF3568	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Uromyces columbianus</i>	Trinidad	PURF3640	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Uromyces bidenticola</i>	Peru	PURF3649	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Uromyces bidenticola</i>	Trinidad	PURF3655	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Uromyces bidentis</i>	Venezuela	PURF3682	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Puccinia poanim</i>	Bolivia	PURF3857	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Puccinia recondita</i>	Chile	PURF4107	Presence of fungivorous larvae that could not be identified due to dried or scarce material

Table 2.3 continued

ND	<i>Puccinia macra</i>	Bolivia	PURF4826	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Puccinia substriata</i>	Peru	PURF4842	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Puccinia abnormis</i>	Bolivia	PURF4966	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Puccinia mogiphanis</i>	Bolivia	PURF5968	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Coleosporium ipomoeae</i>	Ecuador	PURF598	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Coleosporium ipomoeae</i>	Trinidad	PURF603	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Coleosporium ipomoeae</i>	Peru	PURF608	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Puccinia oxalidis</i>	Argentina	PURF6288	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Puccinia sherardiana</i>	Bolivia	PURF6507	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	NA	NA	PURF69289-1	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Puccinia crassipes</i>	Argentina	PURF7138-2	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Puccinia menthae</i>	Bolivia	PURF7194	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Puccinia leonotidicola</i>	Trinidad	PURF7262	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Puccinia hyptidis-mutabilis</i>	Venezuela	PURF7286	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Puccinia perscita</i>	Bolivia	PURF7307	Presence of fungivorous larvae that could not be identified due to dried or scarce material

Table 2.3 continued

ND	<i>Puccinia porphyretica</i>	Bolivia	PURF7400	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Puccinia conturbata</i>	Ecuador	PURF7409	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Coleosporium tussilaginis</i>	NA	PURF756	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Coleosporium tussilaginis</i>	Bolivia	PURF803	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Puccinia ocellifera</i>	Bolivia	PURF8259	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Puccinia ocellifera</i>	Bolivia	PURF8260	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Melampsora medusae</i>	Brazil	PURF827	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Puccinia boliviana</i>	Bolivia	PURF8293	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Puccinia cnici-oleracei</i>	Venezuela	PURF8309	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Puccinia irregularis</i>	Bolivia	PURF8314	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Puccinia subaquila</i>	Ecuador	PURF8350	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Endophyllum decoloratum</i>	Trinidad	PURF8665	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Endophyllum decoloratum</i>	Colombia	PURF8667	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Pucciniosira pallidula</i>	Bolivia	PURF8677-2	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Cionothrix praelonga</i>	Colombia	PURF8725	Presence of fungivorous larvae that could not be identified due to dried or scarce material

Table 2.3 continued

ND	<i>Cionothrix praelonga</i>	Bolivia	PURF8730	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Cionothrix praelonga</i>	Ecuador	PURF8736	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Cionothrix praelonga</i>	Ecuador	PURF8739	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Alveolaria cordiae</i>	Bolivia	PURF8745	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Alveolaria cordiae</i>	Bolivia	PURF8747	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Alveolaria cordiae</i>	Ecuador	PURF8748	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Alveolaria cordiae</i>	Bolivia	PURF8751	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Alveolaria cordiae</i>	Bolivia	PURF8752	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Puccinia hyptidis-mutabilis</i>	Bolivia	PURF9007	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Phragmidiella minuta</i>	Trinidad	PURF9015	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Puccinia dolosa</i>	Venezuela	PURF9795	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Puccinia levis</i>	Venezuela	PURF9796	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Puccinia substriata</i>	Venezuela	PURF9801	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Chrysocyclus mikaniae</i>	Ecuador	PURN10078	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Olivea capituliformis</i>	Brazil	PURN10280	Presence of fungivorous larvae that could not be identified due to dried or scarce material

Table 2.3 continued

ND	<i>Puccinia sorghi</i>	USA	PURN11116	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Chrysocelis lupini</i>	Peru	PURN11562	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Hemileia vastatrix</i>	USA	PURN11725-2	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Melampsora occidentalis</i>	Canada	PURN12881	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Arthuria colombiana</i>	Venezuela	PURN14	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Puccinia caeomariformis</i>	Ecuador	PURN15	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Austropuccinia psidii</i>	Venezuela	PURN15013	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Puccinia psidii</i>	Venezuela	PURN15015-1	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Phakopsora tecta</i>	Venezuela	PURN15016	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Puccinia oxalidis</i>	Venezuela	PURN15017	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Puccinia oxalidis</i>	Venezuela	PURN15019	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Melampsora tremulae</i>	Japan	PURN15091	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Hemileia vastatrix</i>	Bolivia	PURN15328	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Puccinia melampodii</i>	Guyana	PURN15333	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Uromyces hedysari-paniculati</i>	Guyana	PURN15341	Presence of fungivorous larvae that could not be identified due to dried or scarce material

Table 2.3 continued

ND	<i>Uromyces euphorbiae</i>	Guyana	PURN15496	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Coleosporium vernoniae</i>	Guyana	PURN15509	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Melampsora sp.</i>	USA	PURN16034	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Melampsora sp.</i>	Germany	PURN16119-2	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Puccinia oahuensis</i>	Peru	PURN16376	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Coleosporium ipomoeae</i>	USA	PURN16568	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Melampsora sp.</i>	Switzerland	PURN16569	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Cerotelium sabiceae</i>	Guyana	PURN22416	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Hemileia vastatrix</i>	Peru	PURN22445-1	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Hemileia vastatrix</i>	Peru	PURN22445-2	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Hemileia vastatrix</i>	Peru	PURN22448-1	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Hemileia vastatrix</i>	Panama	PURN22625-1	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Coleosporium solidaginis</i>	USA	PURN23056	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Uromyces sp.</i>	USA	PURN23063	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Angiopsora paspalicola</i>	USA	PURN23069	Presence of fungivorous larvae that could not be identified due to dried or scarce material

Table 2.3 continued

ND	<i>Puccinia pelargonii-zonalis</i>	Peru	PURN23083-2	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Puccinia pelargonii-zonalis</i>	Ecuador	PURN24	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Uromyces iresines</i>	Ecuador	PURN2545	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Puccinia sp.</i>	Ecuador	PURN2810	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Puccinia oxalidis</i>	Colombia	PURN2826	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Coleosporium ipomoeae</i>	Brazil	PURN3064	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Coleosporium ipomoeae</i>	Brazil	PURN3065	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Chaconia ingae</i>	Brazil	PURN3099	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Coleosporium ipomoeae</i>	Brazil	PURN3255	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Olivea sp.</i>	Brazil	PURN3267	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Puccinia sp.</i>	Peru	PURN3359	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Gerwasia variabilis</i>	Ecuador	PURN3784	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Gerwasia variabilis</i>	Venezuela	PURN3787	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Gerwasia tenella</i>	Ecuador	PURN3788	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Gerwasia quitensis</i>	Ecuador	PURN3789	Presence of fungivorous larvae that could not be identified due to dried or scarce material

Table 2.3 continued

ND	<i>Gerwasia sp.</i>	Peru	PURN3844	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Gerwasia mayorii</i>	Venezuela	PURN3856	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Puccinosira pallidula</i>	Ecuador	PURN3898	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Trichopsora tournefortiae</i>	Ecuador	PURN3905	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Trichopsora tournefortiae</i>	Ecuador	PURN3906	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Trichopsora tournefortiae</i>	Ecuador	PURN3907	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Trichopsora tournefortiae</i>	Ecuador	PURN3908	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Trichopsora tournefortiae</i>	Ecuador	PURN3909	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Coleosporium vernoniae</i>	Ecuador	PURN3955	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Melampsora sp.</i>	Brazil	PURN4039	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Melampsora sp.</i>	Brazil	PURN4040	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Melampsora sp.</i>	Brazil	PURN4041	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Melampsora sp.</i>	Brazil	PURN4078	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Melampsora sp.</i>	Brazil	PURN4091	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Melampsora sp.</i>	Brazil	PURN4092	Presence of fungivorous larvae that could not be identified due to dried or scarce material



Table 2.3 continued

ND	<i>Uredinopsis pteridis</i>	Brazil	PURN4094	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Puccinia pelargonii-zonalis</i>	Venezuela	PURN5969	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Puccinia inrecta</i>	Paraguay	PURN5992	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Puccinia caeomariformis</i>	Ecuador	PURN6254	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Coleosporium tussilaginis</i>	Argentina	PURN6395	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Coleosporium plumeriae</i>	Colombia	PURN6503	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Puccinia psidii</i>	Brazil	PURN7676	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Uromyces neocarpi</i>	Colombia	PURN8262	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Coleosporium vernoniae</i>	Ecuador	PURN93955/P URN3955	Presence of fungivorous larvae that could not be identified due to dried or scarce material
ND	<i>Coleosporium plumeriae</i>	Brazil	PURN9649	Presence of fungivorous larvae that could not be identified due to dried or scarce material
<i>Ramularia coleosporii</i>	<i>Coleosporium plumeriae</i>	Ecuador	<b>PURN16398</b>	
<i>Ramularia</i> sp.	<i>Melampsora epitea</i>	Brazil	PURN4125	Also symptoms of <i>Sphaerellopsis</i> sp.
<i>Ramularia</i> sp.	<i>Coleosporium plumeriae</i>	Colombia	PURF10667	White Mycelium. Micro and Macro photographs provided. It cannot be identified at species level due to scarce material
<i>Ramularia uredinicola</i>	<i>Melampsora epitea</i>	Argentina	<b>PURN3996-2</b>	
<i>Simplicillium lanosoniveum</i>	<i>Phakopsora</i> sp.	Puerto Rico, USA	<b>PAZ09/PURN23065 †</b>	
<i>Simplicillium lanosoniveum</i>	<i>Puccinia tubulosa</i>	Puerto Rico, USA	<b>PURN23060/P AZ04-8(2-1)</b>	
<i>Simplicillium</i> sp.	<i>Uromyces euphorbiae</i>	USA	PURN11630	White Mycelium. Macro and microphotographs provided

Table 2.3 continued

<i>Sph. filum</i>	<i>Puccinia coronata</i>	Mexico	<b>PUR62890</b>
<i>Sph. filum</i>	<i>Tranzschelia discolor</i>	Ecuador	<b>PURF16864/ PUR83764</b>
<i>Sph. filum</i>	<i>Puccinia brachypodii</i>	Chile	<b>PURF17349</b>
<i>Sph. filum</i>	<i>Puccinia brachypodii</i>	Argentina	<b>PURF19494</b>
<i>Sph. filum</i>	<i>Puccinia brachypodii</i>	Bolivia	<b>PURF3782</b>
<i>Sph. filum</i>	<i>Melampsora ferrinii</i>	Peru	<b>PURN12884</b>
<i>Sph. filum</i>	<i>Puccinia graminis</i>	USA	<b>PURN16937</b>
<i>Sph. filum</i>	<i>Puccinia coronata</i>	Germany	<b>PURN4541a</b>
<i>Sph. filum</i>	<i>Puccinia graminis</i>	Mexico	<b>PURN5297</b>
<i>Sph. filum</i> and <i>Mycodiplosis</i> sp.	<i>Puccinia graminis</i>	Ecuador	<b>PURF4300</b>
<i>Sph. hakeae</i>	<i>Uromyces ehrhartae</i>	Australia	<b>PURF10892</b>
<i>Sph. macroconidialis</i>	<i>Puccinia sorghi</i>	Peru	<b>Eud3.1 †</b>
<i>Sph. macroconidialis</i>	<i>Puccinia graminis</i>	Canada	<b>PUR19637</b>
<i>Sph. macroconidialis</i>	<i>Puccinia recondita</i>	USA	<b>PUR25166</b>
<i>Sph. macroconidialis</i>	<i>Puccinia fuirenicola</i>	Cuba	<b>PUR26871</b>
<i>Sph. macroconidialis</i>	<i>Puccinia incondita</i>	USA	<b>PUR40715</b>
<i>Sph. macroconidialis</i>	<i>Puccinia grindeliae</i>	USA	<b>PUR41914</b>
<i>Sph. macroconidialis</i>	<i>Puccinia eatoniae</i>	USA	<b>PUR55871</b>
<i>Sph. macroconidialis</i>	<i>Ravenelia thornberiana</i>	Mexico	<b>PUR60157</b>
<i>Sph. macroconidialis</i>	<i>Puccinia thaliae</i>	Paraguay	<b>PUR60992</b>
<i>Sph. macroconidialis</i>	<i>Puccinia unica</i>	Spain	<b>PUR62883</b>
<i>Sph. macroconidialis</i>	<i>Ravenelia cassiaeicola</i>	USA	<b>PUR6299</b>
<i>Sph. macroconidialis</i>	<i>Puccinia poarum</i>	Mexico	<b>PUR64476</b>
<i>Sph. macroconidialis</i>	NA	NA	<b>PUR69289</b>
<i>Sph. macroconidialis</i>	<i>Uropyxis diphysae</i>	Guatemala	<b>PUR7334</b>
<i>Sph. macroconidialis</i>	<i>Phragmidium guatemalense</i>	Guatemala	<b>PUR8475</b>
<i>Sph. macroconidialis</i>	<i>Maravalia erythroxyli</i>	Brazil	<b>PUR87630</b>
<i>Sph. macroconidialis</i>	<i>Puccinia recondita</i>	Brazil	<b>PUR88221</b>

Table 2.3 continued

<i>Sph. macroconidialis</i>	<i>Uromyces silphii</i>	Canada	<b>PUR88382</b>
<i>Sph. macroconidialis</i>	<i>Phakopsora coca</i>	Brazil	<b>PUR90210</b>
<i>Sph. macroconidialis</i>	<i>Uromyces striatus</i>	Argentina	<b>PURF10361</b>
<i>Sph. macroconidialis</i>	<i>Puccinia brachypodii</i> var. <i>poaememoralis</i>	Colombia	<b>PURF10651</b>
<i>Sph. macroconidialis</i>	<i>Puccinia spilantheticola</i>	Colombia	<b>PURF10657</b>
<i>Sph. macroconidialis</i>	<i>Puccinia recondita</i>	Australia	<b>PURF10865</b>
<i>Sph. macroconidialis</i>	<i>Phakopsora lenticularis</i>	Venezuela	<b>PURF10996</b>
<i>Sph. macroconidialis</i>	<i>Puccinia graminis</i>	Argentina	<b>PURF11699</b>
<i>Sph. macroconidialis</i>	<i>Puccinia brachypodii</i>	Argentina	<b>PURF17655</b>
<i>Sph. macroconidialis</i>	<i>Puccinia brachypodii</i>	Venezuela	<b>PURF17656</b>
<i>Sph. macroconidialis</i>	<i>Puccinia polypogonis</i>	Brazil	<b>PURF17814</b>
<i>Sph. macroconidialis</i>	<i>Phakopsora coca</i>	Brazil	<b>PURF18990</b>
<i>Sph. macroconidialis</i>	<i>Uromyces epicampis</i>	Ecuador	<b>PURF2397</b>
<i>Sph. macroconidialis</i>	<i>Uromyces pencanus</i>	Chile	<b>PURF2408</b>
<i>Sph. macroconidialis</i>	<i>Uromyces pencanus</i>	Chile	<b>PURF2409</b>
<i>Sph. macroconidialis</i>	<i>Puccinia brachypodii</i> var. <i>poaememoralis</i>	Ecuador	<b>PURF3797</b>
<i>Sph. macroconidialis</i>	<i>Puccinia brachypodii</i> var. <i>poaememoralis</i>	Ecuador	<b>PURF3799</b>
<i>Sph. macroconidialis</i>	<i>Puccinia poarum</i>	Brazil	<b>PURF3854</b>
<i>Sph. macroconidialis</i>	<i>Puccinia molinae</i>	Germany	<b>PURF3879</b>
<i>Sph. macroconidialis</i>	<i>Puccinia aegopogonis</i>	Ecuador	<b>PURF4648</b>
<i>Sph. macroconidialis</i>	<i>Puccinia substriata</i>	Bolivia	<b>PURF4891</b>
<i>Sph. macroconidialis</i>	<i>Puccinia hydrocotyles</i>	Colombia	<b>PURF6912</b>
<i>Sph. macroconidialis</i>	<i>Puccinia praedicata</i>	Brazil	<b>PURF8187</b>
<i>Sph. macroconidialis</i>	<i>Puccinia wedellicola</i>	Brazil	<b>PURF8347</b>
<i>Sph. macroconidialis</i>	<i>Puccinia bonariensis</i>	Argentina	<b>PURF9548</b>
<i>Sph. macroconidialis</i>	<i>Puccinia persistens</i>	USA	<b>PURN11506</b>
<i>Sph. macroconidialis</i>	<i>Phakopsora apoda</i>	Peru	<b>PURN11560</b>
<i>Sph. macroconidialis</i>	<i>Puccinia pygmaea</i>	USA	<b>PURN11633</b>

Table 2.3 continued

<i>Sph. macroconidialis</i>	<i>Tranzschelia mexicana</i>	Peru	<b>PURN16382</b>	
<i>Sph. macroconidialis</i>	<i>Puccinia sorghi</i>	Peru	<b>PURN23084/ SP28PeruCorn †</b>	
<i>Sph. macroconidialis</i>	<i>Puccinia fumosa</i>	Mexico	<b>PURN3032</b>	
<i>Sph. macroconidialis</i>	<i>Chaconia brasiliensis</i>	Brazil	<b>PURN4199</b>	
<i>Sph. macroconidialis</i>	<i>Chaconia brasiliensis</i>	Brazil	<b>PURN4207</b>	
<i>Sph. macroconidialis</i>	<i>Chrysocelis muehlenbeckiae</i>	Colombia	<b>PURN4452</b>	
<i>Sph. macroconidialis</i>	<i>Phakopsora apoda</i>	Ecuador	<b>PUR66593</b>	Also presence of unknown fungivorous larvae.
<i>Sph. macroconidialis</i>	<i>Phakopsora compressa</i>	Bolivia	<b>PURF1211</b>	Also presence of unknown fungivorous larvae.
<i>Sph. macroconidialis</i>	<i>Phakopsora compressa</i>	Bolivia	<b>PURF1212</b>	Also presence of unknown fungivorous larvae.
<i>Sph. macroconidialis</i> and <i>Mycodiplosis</i>	<i>Uromyces iresines</i>	Ecuador	<b>PURN2544</b>	
<i>Sph. melampsoriinae</i>	<i>Melampsora medusae</i>	USA	<b>PUR2041</b>	
<i>Sph. melampsoriinae</i>	<i>Melampsora medusae</i>	USA	<b>PUR2047</b>	
<i>Sph. melampsoriinae</i>	<i>Melampsora medusae</i>	USA	<b>PUR2129</b>	
<i>Sph. melampsoriinae</i>	<i>Puccinia proserpinacae</i>	USA	<b>PUR32274</b>	
<i>Sph. melampsoriinae</i>	<i>Coleosporium helianthi</i>	USA	<b>PUR43744</b>	
<i>Sph. melampsoriinae</i>	<i>Coleosporium helianthi</i>	USA	<b>PUR43798</b>	
<i>Sph. melampsoriinae</i>	<i>Melampsora medusae</i>	USA	<b>PUR47887</b>	
<i>Sph. melampsoriinae</i>	<i>Puccinia recondita</i>	USA	<b>PUR56253</b>	
<i>Sph. melampsoriinae</i>	<i>Coleosporium helianthi</i>	USA	<b>PUR88233</b>	
<i>Sph. melampsoriinae</i>	<i>Melampsora ferrinii</i>	Brazil	<b>PUR90026</b>	
<i>Sph. melampsoriinae</i>	<i>Melampsora larici- populina</i>	Brazil	<b>PUR90183</b>	
<i>Sph. melampsoriinae</i>	<i>Melampsora epitea</i>	Brazil	<b>PUR90242</b>	
<i>Sph. melampsoriinae</i>	<i>Puccinia thaliae</i>	Venezuela	<b>PURF11501</b>	
<i>Sph. melampsoriinae</i>	<i>Puccinia sorghi</i>	Ecuador	<b>PURF14716</b>	
<i>Sph. melampsoriinae</i>	<i>Puccinia phaenospermae</i>	Japan	<b>PURF16121</b>	
<i>Sph. melampsoriinae</i>	<i>Gerwasia holwayi</i>	Peru	<b>PURF1651</b>	

Table 2.3 continued

<i>Sph. melampsoriinae</i>	<i>Puccinia caricis-japonica</i>	Japan	<b>PURF17310</b>
<i>Sph. melampsoriinae</i>	<i>Uromyces wulffiae-stenoglossae</i>	Trinidad	<b>PURF3626</b>
<i>Sph. melampsoriinae</i>	<i>Puccinia recondita</i>	Ecuador	<b>PURF4112</b>
<i>Sph. melampsoriinae</i>	<i>Puccinia boerhaviae</i>	Ecuador	<b>PURF5980</b>
<i>Sph. melampsoriinae</i>	<i>Puccinia inaequata</i>	Ecuador	<b>PURF7943</b>
<i>Sph. melampsoriinae</i>	<i>Melampsora medusae</i>	Bolivia	<b>PURF829</b>
<i>Sph. melampsoriinae</i>	<i>Melampsora humboldtiana</i>	USA	<b>PURN12037</b>
<i>Sph. melampsoriinae</i>	<i>Uromyces minutus</i>	USA	<b>PURN1206</b>
<i>Sph. melampsoriinae</i>	<i>Melampsora medusae</i>	USA	<b>PURN15307(WTHC1)</b>
<i>Sph. melampsoriinae</i>	<i>Uromyces yurimaguensis</i>	Peru	<b>PURN16392</b>
<i>Sph. melampsoriinae</i>	<i>Melampsora ferrinii</i>	USA	<b>PURN16518</b>
<i>Sph. melampsoriinae</i>	<i>Melampsora medusae</i>	USA	<b>PURN16527</b>
<i>Sph. melampsoriinae</i>	NA	NA	<b>PURN21944</b>
<i>Sph. melampsoriinae</i>	<i>Coleosporium montanum</i>	USA	<b>PURN2294</b>
<i>Sph. melampsoriinae</i>	<i>Coleosporium asterum</i>	USA	<b>PURN2303</b>
<i>Sph. melampsoriinae</i>	<i>Coleosporium asterum</i>	USA	<b>PURN2314</b>
<i>Sph. melampsoriinae</i>	<i>Melampsora epitea</i>	USA	<b>PURN2448</b>
<i>Sph. melampsoriinae</i>	<i>Melampsora epitea</i>	Brazil	<b>PURN3993</b>
<i>Sph. melampsoriinae</i>	<i>Melampsora ferrinii</i>	Argentina	<b>PURN3995</b>
<i>Sph. melampsoriinae</i>	<i>Melampsora epitea</i>	Argentina	<b>PURN3996</b>
<i>Sph. melampsoriinae</i>	<i>Melampsora epitea</i>	Argentina	<b>PURN3997</b>
<i>Sph. melampsoriinae</i>	<i>Melampsora epitea</i>	Argentina	<b>PURN3998</b>
<i>Sph. melampsoriinae</i>	<i>Melampsora epitea</i>	Argentina	<b>PURN4001</b>
<i>Sph. melampsoriinae</i>	<i>Melampsora medusae</i>	USA	<b>PURN4010</b>
<i>Sph. melampsoriinae</i>	<i>Melampsora larici-populina</i>	Colombia	<b>PURN4015</b>
<i>Sph. melampsoriinae</i>	<i>Melampsora aecidioides</i>	Brazil	<b>PURN4108</b>
<i>Sph. melampsoriinae</i>	<i>Melampsora larici-populina</i>	Brazil	<b>PURN4109</b>

Table 2.3 continued

<i>Sph. melampsoriinae</i>	<i>Melampsora ferrinii</i>	Brazil	<b>PURN4120</b>	
<i>Sph. melampsoriinae</i>	<i>Melampsora ferrinii</i>	Brazil	<b>PURN4121</b>	
<i>Sph. melampsoriinae</i>	<i>Melampsora epitea</i>	Brazil	<b>PURN4124</b>	
<i>Sph. melampsoriinae</i>	<i>Melampsora medusae</i>	USA	<b>PURN4510</b>	
<i>Sph. melampsoriinae</i>	<i>Melampsora medusae</i>	USA	<b>PURN5424</b>	
<i>Sph. melampsoriinae</i>	<i>Melampsora medusae</i>	USA	<b>PURN6730</b>	
<i>Sph. melampsoriinae</i>	<i>Melampsora epitea</i>	Colombia	<b>PURN8265</b>	
<i>Sph. melampsoriinae</i>	<i>Puccinia vernoniae-mollis</i>	Brazil	<b>PURN9763</b>	
<i>Sph. melampsoriinae</i>	<i>Melampsora aecidioides</i>	Argentina	<b>PURF833</b>	Also presence of unknown fungivorous larvae.
<i>Sph. melampsoriinae</i>	<i>Melampsora ferrinii</i>	Brazil	<b>PURN4127</b>	Also presence of unknown fungivorous larvae.
<i>Sph. paraphysata</i>	<i>Puccinia aframomi</i>	Cameroon	<b>MCA7075</b>	
<i>Sph. paraphysata</i>	NA	Puerto Rico, USA	PAZ14rust-PURN23070 †	
<i>Sph. paraphysata</i>	<i>Puccinia philippinensis</i>	Guam	<b>PP2004 (PURN11661)</b>	
<i>Sph. paraphysata</i>	<i>Uromyces andropogonis</i>	USA	<b>PUR11619</b>	
<i>Sph. paraphysata</i>	<i>Uromyces trifolii-repentis</i>	USA	<b>PUR15359</b>	
<i>Sph. paraphysata</i>	<i>Puccinia sp.</i>	USA	<b>PUR31535</b>	
<i>Sph. paraphysata</i>	<i>Pucciniastrum hydrangeae</i>	USA	<b>PUR3364</b>	
<i>Sph. paraphysata</i>	<i>Crossopsora hymenaeae</i>	Cuba	<b>PUR42807</b>	
<i>Sph. paraphysata</i>	<i>Puccinia levis</i>	USA	<b>PUR48117</b>	
<i>Sph. paraphysata</i>	<i>Uromyces bonariensis</i>	Guatemala	<b>PUR50338</b>	
<i>Sph. paraphysata</i>	<i>Uromyces sp.</i>	USA	<b>PUR50994</b>	
<i>Sph. paraphysata</i>	<i>Phakopsora aurea</i>	Honduras	<b>PUR51302</b>	
<i>Sph. paraphysata</i>	<i>Puccinia arthurella</i>	Trinidad	<b>PUR52253</b>	
<i>Sph. paraphysata</i>	<i>Phakopsora compressa</i>	Costa Rica	<b>PUR52702</b>	
<i>Sph. paraphysata</i>	<i>Puccinia marylandica</i>	USA	<b>PUR56162</b>	
<i>Sph. paraphysata</i>	<i>Puccinia stenotaphri</i>	USA	<b>PUR58549</b>	
<i>Sph. paraphysata</i>	<i>Puccinia unica</i>	Mexico	<b>PUR59371</b>	

Table 2.3 continued

<i>Sph. paraphysata</i>	<i>Puccinia subtilipes</i>	Honduras	<b>PUR60362</b>
<i>Sph. paraphysata</i>	<i>Puccinia unica</i>	USA	<b>PUR62882</b>
<i>Sph. paraphysata</i>	<i>Uromyces costaricensis</i>	Mexico	<b>PUR64079</b>
<i>Sph. paraphysata</i>	<i>Uromyces epicampis</i>	Mexico	<b>PUR64614</b>
<i>Sph. paraphysata</i>	<i>Sorataea baphiae</i>	Nigeria	<b>PUR66889</b>
<i>Sph. paraphysata</i>	<i>Uromyces unioniensis</i>	Brazil	PUR87214
<i>Sph. paraphysata</i>	<i>Puccinia oahuensis</i>	Brazil	PUR88231
<i>Sph. paraphysata</i>	<i>Catenulopsora praelonga</i>	USA	<b>PUR9003</b>
<i>Sph. paraphysata</i>	<i>Crossopsora fici</i>	Uganda	<b>PURF10798</b>
<i>Sph. paraphysata</i>	<i>Puccinia posadensis</i>	Trinidad	<b>PURF11444</b>
<i>Sph. paraphysata</i>	<i>Puccinia polysora</i>	St. Vincent & the Grenadines	<b>PURF11445</b>
<i>Sph. paraphysata</i>	<i>Puccinia purpurea</i>	Venezuela	<b>PURF11502</b>
<i>Sph. paraphysata</i>	<i>Uromyces costaricensis</i>	Trinidad	<b>PURF12985</b>
<i>Sph. paraphysata</i>	<i>Phakopsora clemensiae</i>	India	PURF14478
<i>Sph. paraphysata</i>	<i>Puccinia nakanishikii</i>	India	PURF14635
<i>Sph. paraphysata</i>	<i>Phakopsora africana</i>	Uganda	<b>PURF14757</b>
<i>Sph. paraphysata</i>	<i>Puccinia eleocharidis</i>	Trinidad	<b>PURF14933</b>
<i>Sph. paraphysata</i>	<i>Phakopsora loudetiae</i>	Kenya	<b>PURF14951</b>
<i>Sph. paraphysata</i>	<i>Phakopsora pallescens</i>	Colombia	<b>PURF15450</b>
<i>Sph. paraphysata</i>	<i>Puccinia duthiae</i>	India	PURF17943
<i>Sph. paraphysata</i>	<i>Uromyces manihotis</i>	Brazil	PURF18709c
<i>Sph. paraphysata</i>	<i>Uromyces costaricensis</i>	Brazil	PURF19059
<i>Sph. paraphysata</i>	<i>Uromyces costaricensis</i>	Brazil	PURF19059-2
<i>Sph. paraphysata</i>	<i>Uromyces linearis</i>	Nigeria	<b>PURF19505</b>
<i>Sph. paraphysata</i>	<i>Puccinia cenchri</i>	Nigeria	<b>PURF19703</b>
<i>Sph. paraphysata</i>	<i>Puccinia erythropus</i>	China	PURF19904
<i>Sph. paraphysata</i>	<i>Puccinia paspalina</i>	China	PURF19927

Table 2.3 continued

<i>Sph. paraphysata</i>	<i>Puccinia pogonatheri</i>	China	PURF19929
<i>Sph. paraphysata</i>	<i>Puccinia infuscans</i>	Ecuador	<b>PURF2490</b>
<i>Sph. paraphysata</i>	<i>Uromyces bonariensis</i>	Venezuela	<b>PURF2800</b>
<i>Sph. paraphysata</i>	<i>Puccinia cacabata</i>	Brazil	PURF4672
<i>Sph. paraphysata</i>	<i>Puccinia levis</i>	Bolivia	<b>PURF4770</b>
<i>Sph. paraphysata</i>	<i>Uromyces setariae-italicae</i>	Trinidad	<b>PURF4897</b>
<i>Sph. paraphysata</i>	<i>Puccinia neorotundata</i>	Peru	<b>PURF7972</b>
<i>Sph. paraphysata</i>	<i>Puccinia thaliae</i>	Argentina	<b>PURF9841</b>
<i>Sph. paraphysata</i>	<i>Puccinia minuta</i>	Brazil	PURN10179
<i>Sph. paraphysata</i>	<i>Puccinia faceta</i>	Brazil	PURN10369
<i>Sph. paraphysata</i>	<i>Kweilingia divina</i>	USA	<b>PURN10826</b>
<i>Sph. paraphysata</i>	<i>Puccinia invenusta</i>	Guam	<b>PURN10850</b>
<i>Sph. paraphysata</i>	<i>Kweilingia divina</i>	Taiwan	<b>PURN11077</b>
<i>Sph. paraphysata</i>	<i>Mikronegeria fagi</i>	Argentina	<b>PURN1120</b>
<i>Sph. paraphysata</i>	<i>Puccinia purpurea</i>	USA	<b>PURN11634 (PURP)</b>
<i>Sph. paraphysata</i>	<i>Phakopsora sp.</i>	Taiwan	<b>PURN15263</b>
<i>Sph. paraphysata</i>	<i>Uromyces setariae-italicae</i>	Bolivia	<b>PURN15329</b>
<i>Sph. paraphysata</i>	<i>Puccinia duthiae</i>	Guyana	<b>PURN15344</b>
<i>Sph. paraphysata</i>	<i>Uromyces tenuicutis</i>	Guyana	<b>PURN15498</b>
<i>Sph. paraphysata</i>	<i>Puccinia obliquo-septata</i>	Guyana	<b>PURN15511</b>
<i>Sph. paraphysata</i>	<i>Puccinia obliquo-septata</i>	Guyana	<b>PURN16553</b>
<i>Sph. paraphysata</i>	<i>Phakopsora rossmanii</i>	Brazil	PURN2908
<i>Sph. paraphysata</i>	<i>Melampsora epitea</i>	Brazil	PURN4122
<i>Sph. paraphysata</i>	<i>Melampsora epitea</i>	Brazil	PURN4123
<i>Sph. paraphysata</i>	<i>Uromyces rhynchosporae</i>	Papua New Guinea	<b>PURN5064</b>
<i>Sph. paraphysata</i>	<i>Puccinia stenotaphri</i>	Ecuador	<b>PURN5574</b>
<i>Sph. paraphysata</i>	<i>Puccinia arachidis</i>	Brazil	PURN5917
<i>Sph. paraphysata</i>	<i>Chaconia ingae</i>	Brazil	PURN9602



Table 2.3 continued

<i>Sph. paraphysata</i>	<i>Puccinia cf. cyperi tegetiformis</i>	Benin	<b>TA427</b>	
<i>Sph. paraphysata</i>	<i>Puccinia sp.</i>	Venezuela	<b>PURN16743</b>	Also presence of unknown fungivorous larvae.
<i>Sph. paraphysata</i> and <i>Mycodiplosis</i>	<i>Uromyces hedysari-paniculati</i>	Guyana	<b>PURN15342</b>	
<i>Sph. paraphysata</i> and <i>Mycodiplosis</i>	<i>Puccinia commelinae</i>	Guyana	<b>PURN15343</b>	
<i>Sph. paraphysata</i> and <i>Mycodiplosis</i>	<i>Melampsora sp.</i>	China	PURN22990	
<i>Sphaerellopsis melampsoriinae</i>	<i>Uromyces graminicola</i>	USA	<b>PUR11683</b>	
<i>Sphaerellopsis sp.</i>	<i>Puccinia montanensis</i>	USA	<b>PUR23925</b>	
<i>Trochila urediniophila</i>	<i>Cerotelium fici</i>	Trinidad	<b>PURF18316</b>	

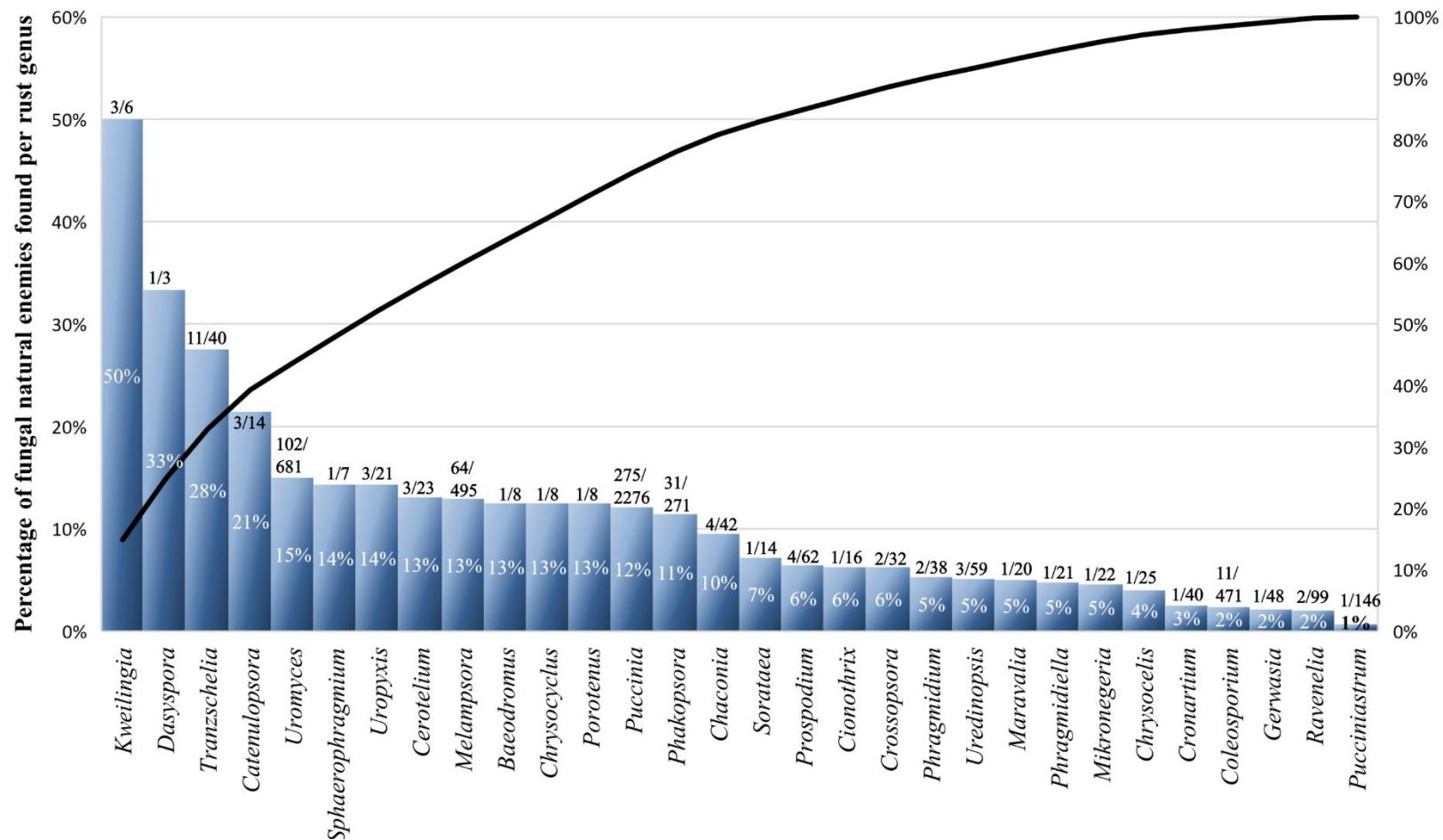


Figure 2.2 Pareto chart. Percentages and proportions of rust genera where we found fungal natural enemies at PUR

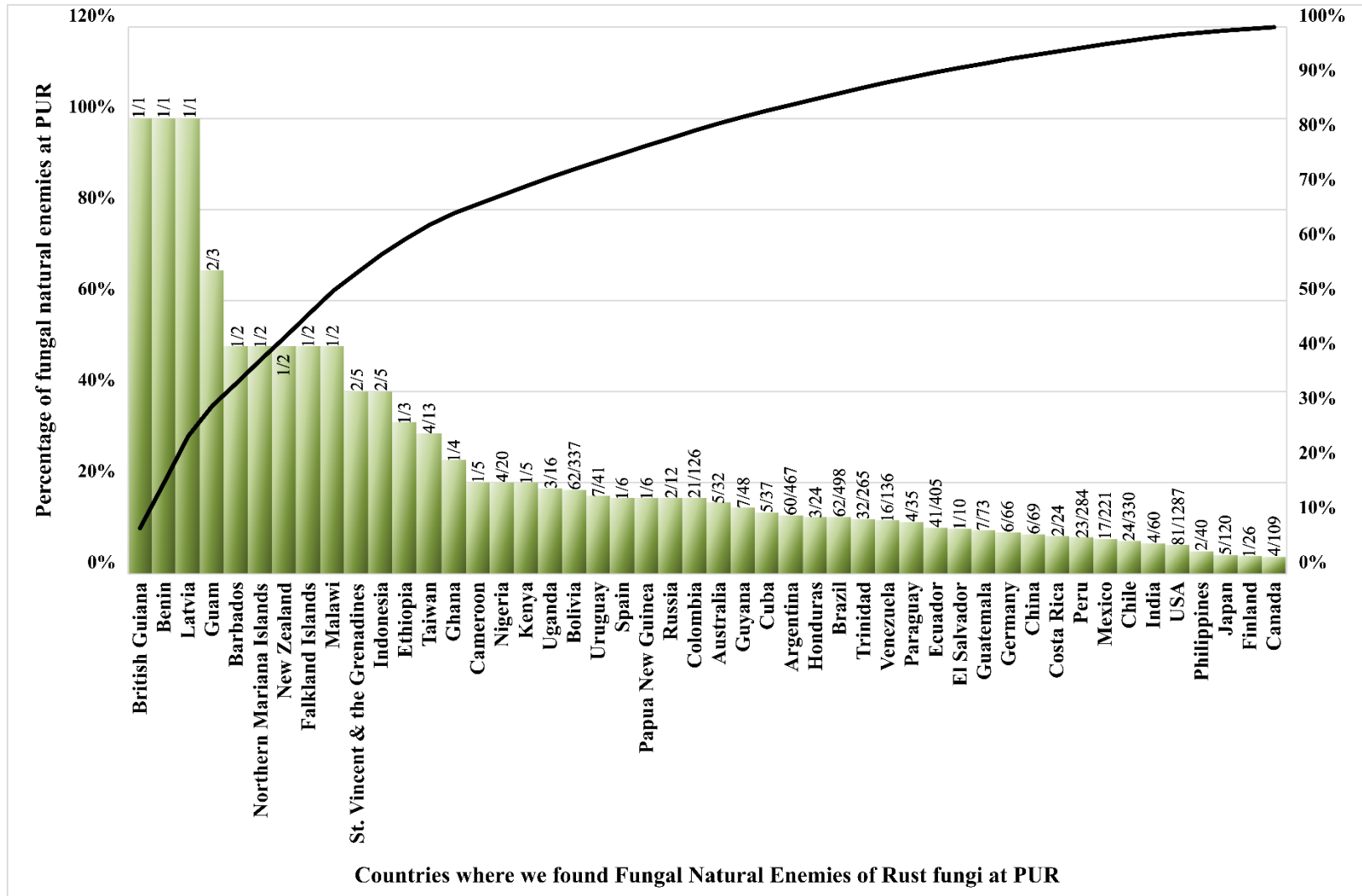


Figure 2.3 Pareto chart. Percentages and proportions of countries where we found fungal natural enemies at PUR

#### 2.4.2.1 The genus *Sphaerellopsis*

Pycnidia solitary or gregarious developed exclusively on rust pustules were the most common fungal structures we observed in our search for natural enemies of rust fungi at PUR (Fig. 2.4 a-g). Of the 523 rust specimens we found with the presence of these fruiting bodies resembling *Sphaerellopsis*, we successfully amplified the ITS region of 195 of them and reconstructed a phylogenetic tree using Maximum Likelihood (ML) analysis. Our dataset consisted of 342 characters, of which 67 were parsimony-informative. JC was the best-fit model selected by ModelFinder according to AICc. We used 211 isolates, of which 15 are reference sequences. *Alternaria consortialis* CBS 104.31 served as the outgroup taxon (Fig. 2.5). Based on our phylogenetic tree, we found five species of *Sphaerellopsis* in association with the rust specimens deposited at PUR: *Sphaerellopsis hakeae*, *Sphaerellopsis filum*, *Sphaerellopsis macroconidialis*, *Sphaerellopsis paraphysata*, and *Sphaerellopsis* sp. Of the 195 isolates of *Sphaerellopsis*, 74 formed a clade with the ext-type and reference sequences of *Sph. paraphysata*, 55 isolates formed a clade with the ext-type and reference sequences of *Sph. macroconidialis*, 12 isolates formed a clade with the ext-type and reference sequences of *Sph. filum*, and one specimen formed a clade with the ext-type of *Sph. hakeae*. We also report 53 specimens as *Sphaerellopsis* sp. whose identifications at the species level are not achieved with the ITS sequence only (see Table 2.3 and supplementary table S2 for more information).

We also found pycnidia of *Sphaerellopsis* restrictively growing on several uredosori of *Puccinia sorghi* infecting corn leaves from live collections of Peru (PUR voucher: N23084). We extracted its DNA and amplified the ITS region to find the closest relative species. Our DNA sequence shared 99% identity (540/545) with *Sph. macroconidialis* (GenBank accession No. MT998445), which was associated with the telial stage of the rust *Uromycladium acaciae* infecting *Acacia mearnsii* in Mpumalanga, South Africa (Fraser et al., 2021). We also added this sequence (N23084) to the phylogenetic tree of all *Sphaerellopsis* specimens we found at PUR. Besides the PUR voucher, we also isolated a strain and stored it in slants and glycerol stocks at Aime Lab's culture collection (Culture collection No. SP28).

Figure 2.4 Morphological features of natural enemies associated with rust fungi from PUR collections. a—d. conidiomata of *Sphaerellopsis* on rust pustules. PUR vouchers: a. PURF10361, b. PURN3997, c. PUR6299, d. PURF11445; e—g. transverse section of conidiomata immersed in rust sori. PUR vouchers: e. PURF19494, f—g. PURF3879; h—k. *Helicobasidium longisporum* on *Puccinia arechavalatae* (PURF6376). h. purple mycelium covering rust sori, i—j. conidiogenous cells with conidia at the tip; k. white globose conidia; l—o. *Ramularia uredinicola* on *Melampsora epitea*. (PURN3996). l. pink mycelium covering rust sori, m—n. conidiophores immersed and protruding from rust sori, o. conidia; p—r. *Ramularia coleosporii* on *Coleosporium plumeriae* (PURN16398). p. white to light pink mycelium covering several uredosori, q—r. conidiophores arising from uredosori. s—u. *Trochila urediniophila* on *Cerotelium fici*. s—t. apothecia growing on uredinia, u. transverse section of ascocarp; v—y. *Simplicillium lanosoniveum* on rust sori of PURN23060 and PURN23065; v, x. white mycelium covering several rust sori, w and y. phialides and conidia; z—ac. *Mycodiplosis* larvae. Scale bars: 2 mm (s); 1 mm (t, u); 400  $\mu$ m (a-d, h, l, p, t, v, x, z—ac); 50  $\mu$ m (f); 40  $\mu$ m (e, g); 20  $\mu$ m (i, m-o, q, r, w, y); 10  $\mu$ m (j); 2  $\mu$ m (k).

Figure 2.4 continued

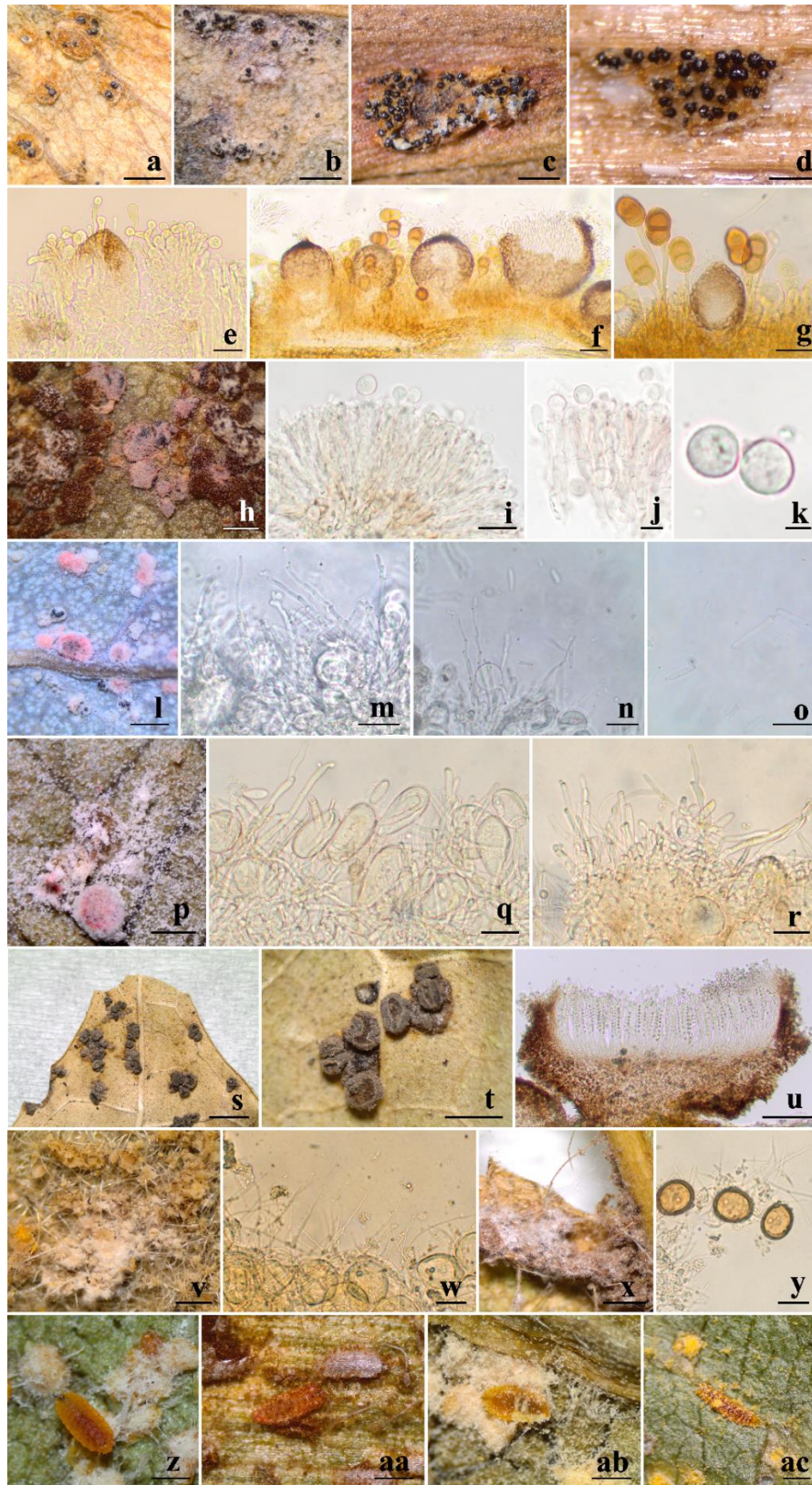


Figure 2.5 Phylogenetic tree of *Sphaerellopsis* specimens resulting from Maximum Likelihood analysis of ITS sequences. Bootstrap support values (>70%) are indicated at the nodes. Type species are written in bold; details of PUR vouchers are provided in Table 2.3. Tree rooted to *Alternaria consortialis* (CBS 104.31)



Figure 2.5 continued

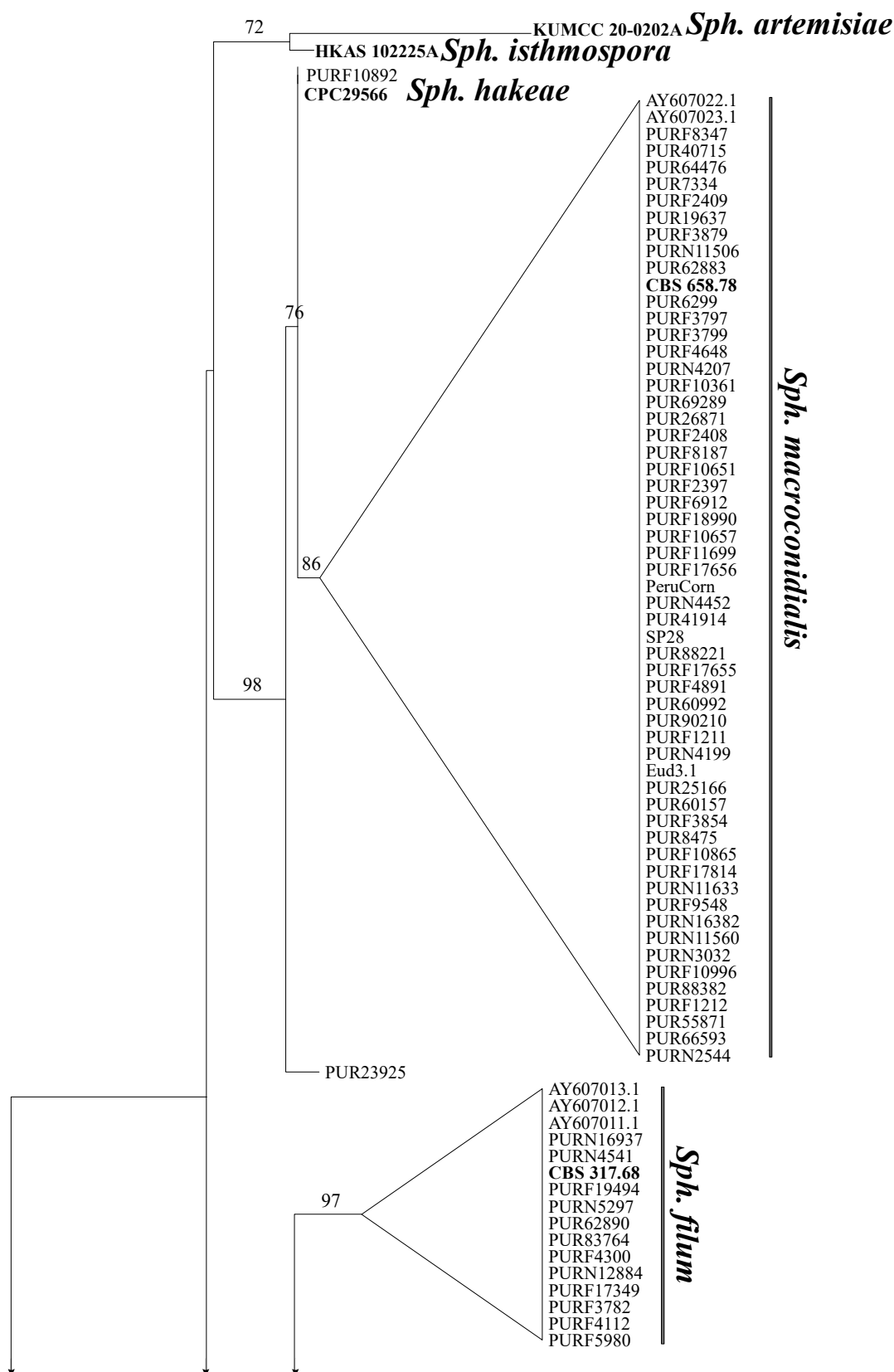
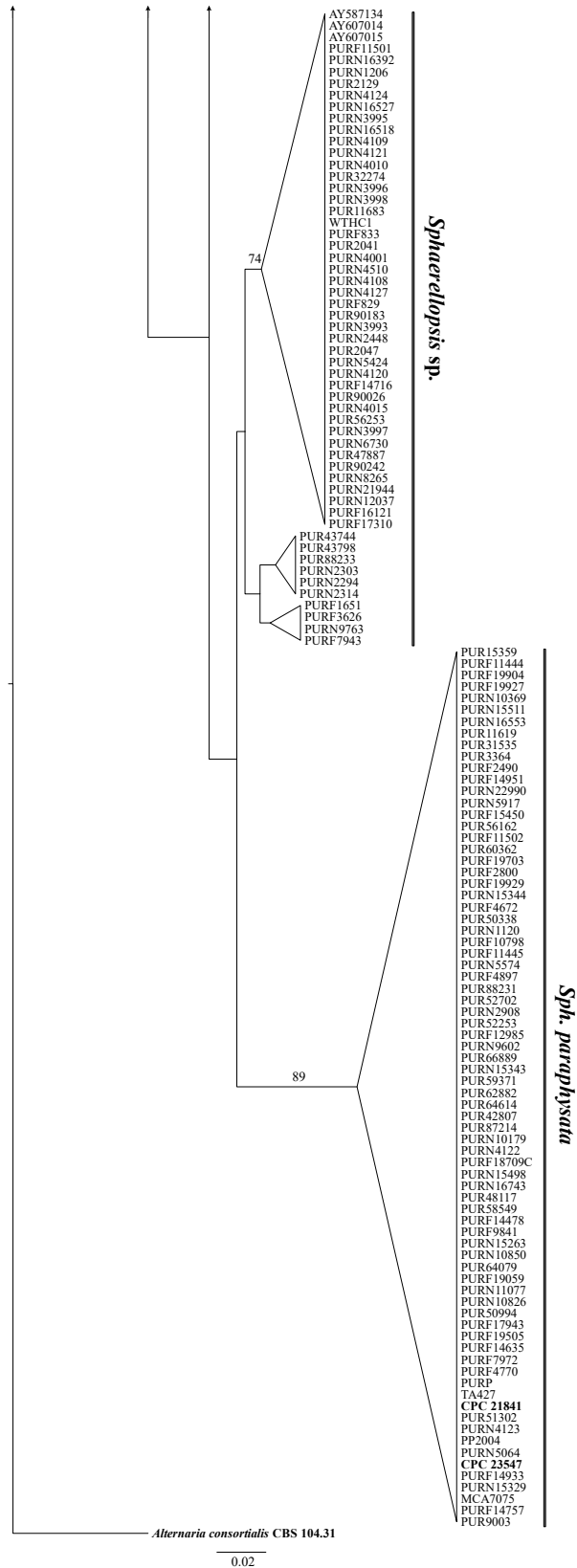




Figure 2.5 continued



#### 2.4.2.2 The genus *Helicobasidium*

Based on both DNA fragments and morphological observations, we found 11 rust specimens associated with the fungal natural enemy *Helicobasidium* (PUR vouchers: F6375, F6376, N11109, F15153, F8203, N6633, F8737, N23064, N4612, F3638, and F11627). From these specimens, we observed a violet mass (sporodochia) covering rust pustules in a palisade arrangement, hyaline conidiogenous cells, and globose, hyaline, smooth conidia (Fig. 2.4. h-k). We reconstructed a phylogenetic tree using Maximum Likelihood (ML) analysis for two *Helicobasidium* specimens whose ITS region amplified successfully (PUR vouchers: F6375, F6376). Our dataset consisted of 611 nucleotide sites, of which 93 were parsimony-informative. TIM2+F+G4 was the best-fit model selected by ModelFinder according to AICc. We used 26 isolates, of which 23 were reference sequences, and *Puccinia chunjei* (GenBank Accession No. HQ012446) served as the outgroup taxon. Based on our phylogenetic tree, *Helicobasidium longisporum* group II is the closest relative species we found as the natural enemy associated with two rust specimens at PUR (Fig. 2.6). Both DNA fragments (F6375 and F6376) shared 100% identity with each other and 92% identity (385/418) with *H. longisporum* group II (GenBank accession No. AY292426). We found both specimens on uredinial sori of *Puccinia arechavaletae* infecting the host plant *Urvillea ulmacea*, collected in Trinidad in 1921. Observations of fungal structures and microphotographs were only possible for specimen F6376 due to the scarce material of specimen F6375. From specimen F6376, we observed a violet mass of hyphae (sporodochia) covering rust pustules of the uredinial stage. This mass consisted of a palisade-like arrangement, hyaline conidiogenous cells, each of which produced one globose and hyaline conidium at the tip, 6.41–10.42  $\mu\text{m}$  (n=20) (Fig. 2.4. h-k).

#### 2.4.2.3 The genus *Ramularia*

We also found four rust specimens associated with the fungal natural enemy *Ramularia* based on DNA fragments and morphological observations (PUR vouchers: F10667, N3996, N4125, and N16398). From these specimens, we observed creamy to pink mycelia covering several rust pustules; caespituli (as turfs of conidiophores) confined to the sori; conidiophores lose to moderately dense arising from pustules, straight, thin-walled, hyaline, unbranched or branched; conidia, fusiform, 0-1 septate, hyaline, thin-walled, smooth, ends rounded to attenuated, and conidial secession schizolytic (Fig. 2.4 l-r), consistent with the description of the genus *Ramularia*.

We reconstructed a phylogenetic tree using Maximum Likelihood (ML) analysis for two *Ramularia* specimens whose ITS region amplified successfully (PUR vouchers: N3996 and N16398). Our dataset consisted of 533 nucleotide sites, of which 21 were parsimony-informative. TVMe+I was the best-fit model selected by ModelFinder according to AICc. We used 34 isolates, of which 31 were reference sequences, and *Zymoseptoria halophila* (GenBank Accession No. JF700876) served as the outgroup taxon. Based on our phylogenetic tree and megablast search using the ITS sequences, we found two species of *Ramularia*, each of which is associated with a rust specimen at PUR (Fig. 2.7).

The first species was found in the uredinial stage of the rust *Melampsora epitea* infecting *Salix babylonica* collected in Argentina in 1993 (PUR voucher: N3996). The DNA fragment of this specimen shared 100% identity (324/324) with *Ramularia uredinicola* (GenBank accession No. NR175043). From this specimen, we observed pink mycelium covering several uredosori of *M. epitea*; caespituli (as turfs of conidiophores) confined to uredosori; conidiophores loose to moderately dense arising from uredosori, straight, thin-walled, hyaline, unbranched or branched; conidia, fusiform, 7.09–17.18 x 2.19–3.94  $\mu\text{m}$  (n=20), 0-1 septate, hyaline, thin-walled, smooth, ends rounded to attenuated, and conidial secession schizolytic (Fig. 2.4 l-o), consistent with the description of the type species (Khodaparast & Braun, 2005).

The second species was found in the uredinial stage of the rust *Coleosporium plumeriae* infecting a host plant in the Apocynaceae family and collected in Ecuador in 2016 (PUR voucher: N16398). The DNA fragment of this specimen shared 99% identity with 17 sequences of *Ramularia coleosporii* in the GenBank database (GenBank accession numbers: KX287384, KX287382, KX287379, X287376, KX287375, KX287372, KX287371, KX287370, KX287368, KX287367, KX287366, KX287365, KX287364, MW470869, GU214692, KX287377, KX287374). From this specimen, we observed white to light pink mycelium covering several uredosori of *C. plumeriae*; caespituli superficial and immersed in uredosori; conidiophores arising from uredosori, straight, subcylindrical, hyaline, unbranched or branched; conidia fusiform, 10.31–26.64 x 2.39–6.35  $\mu\text{m}$  (n=20), catenate, thin-walled, hyaline (Fig. 2.4 p-r), consistent with the description of *R. coleosporii* (Saccardo P.A., 1877; Sun et al., 2017).

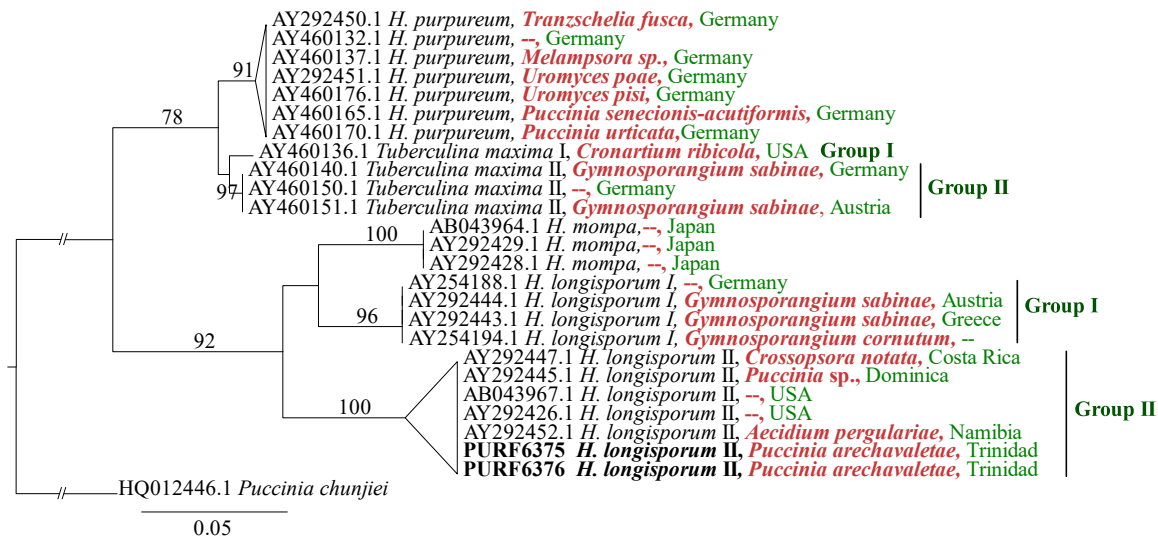


Figure 2.6 Phylogenetic tree of *Helicobasidium* specimens resulting from Maximum Likelihood analysis of ITS sequences. Bootstrap support values (>70%) are indicated at the nodes. Species names are written in black text, rust host species in orange, and country of origin in green. The tree was rooted to *Puccinia chunjei* (GenBank accession No. HQ012446)

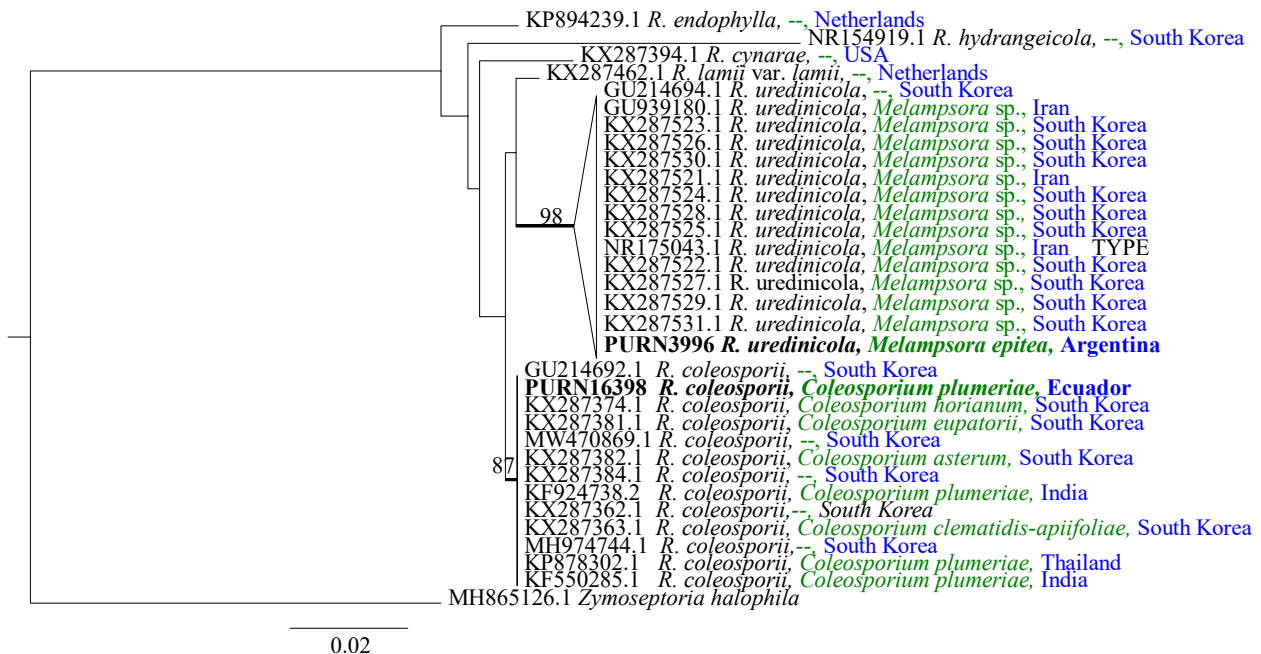


Figure 2.7 Phylogenetic tree of *Ramularia* specimens resulting from Maximum Likelihood analysis of ITS sequences. Bootstrap support values (>70%) are indicated at the nodes. Species names are written in black text, rust host species in green, and country of origin in blue. Samples obtained from this study are written in bold. The tree was rooted to *Zymoseptoria halophila* (GenBank Accession No. JF700876).

#### 2.4.2.4 A new species of *Trochila* associated with rust fungi

We found a new *Trochila* species (PUR voucher: F18316) associated with the rust species *Cerotelium fici* based on morphological characters and amplification of several gene regions. Initially, we observed its fungal structures and noticed they matched within the order Helotiales (Fig. 2.4. s-u) (Leotiomycetes, Ascomycota). Then, we amplified the ITS region and blasted the DNA fragment against the Genbank database to look for the closest relative species. Because the sequence of our specimen shared 90% identity (497/552) with *Trochila viburnicola* (GenBank accession No. NR159070), we suspected this specimen was a new species. Thus, we amplified the large ribosomal subunit (LSU) of the ribosomal DNA to obtain longer DNA fragments and determine if this was a new species. Chapter three includes a revision of the genus *Trochila*, phylogenetic analyses, morphological observations, and the description of the new species we found from this survey, *Trochila urediniophiola*.

#### 2.4.2.5 The genus *Simplicillium*

We found three rust specimens associated with the fungal natural enemy *Simplicillium*. One specimen was found from a preserved rust specimen of *Uromyces euphorbiae* collected in the United States in 2015 (PUR voucher: N11630). The other two were found in Puerto Rico on specimens of *Phakopsora* sp. (PUR voucher: N23065) and *Puccinia* sp. (PUR voucher: N23060). From these three specimens, we observed white mycelia covering several rust pustules; phialides arising from the mycelium, hyaline, and aseptate, tapering toward the apex; and conidia hyaline, aseptate, unicellular, globose, or ovate to ellipsoidal, smooth-walled, adhering as a mass on the tip of phialides (Fig. 2.4 v-y), characteristics that are consistent with *Simplicillium*.

We also reconstructed a phylogenetic tree using Maximum Likelihood (ML) analysis for the two specimens collected in Puerto Rico to confirm their species identity. Our dataset consisted of 617 nucleotide sites, of which 84 were parsimony-informative. TIM+F+G4 was the best-fit model selected by ModelFinder according to AICc. We used 16 isolates, of which 12 were *Simplicillium* reference sequences, and *Cordyceps militaris* and *Lecanicillium lecanii* (GenBank Accession numbers: AF163020, FJ515771) served as the outgroup taxa. Based on our phylogenetic tree, *Simplicillium lanosoniveum* is the closest relative species we found as the natural enemy associated

with these two rust specimens (Fig. 2.8). The DNA fragments of N23060 shared 100% identity with (563/563) with *S. lanosoniveum* (GenBank accession No. MT508802) and DNA fragments of N23065 shared 99.82% identity (562/563) with *S. lanosoniveum* (GenBank accession No. MT508802). Besides the PUR vouchers, we isolated both *Simplicillium* specimens and stored them in slants and glycerol stocks. Culture collections PAZ04-8(2-1): PURN23060 and PAZ09-1(1): PURN23065 can be found at the Aime Lab.

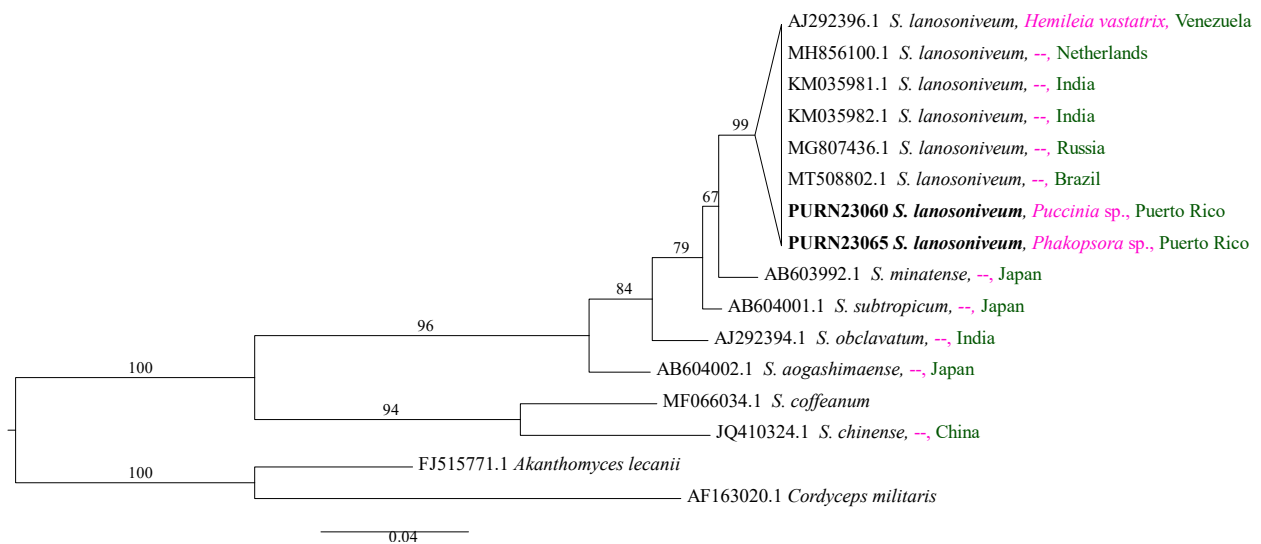


Figure 2.8 Phylogenetic tree of *Simplicillium* specimens resulting from Maximum Likelihood analysis ITS sequences, bootstrap support values (>70%) are indicated at the nodes. Species names are written in black text, rust host species in pink, and country of origin in green. Samples obtained from this study are written in bold. The tree was rooted to *Cordyceps militaris* and *Lecanicillium lecanii* (GenBank Accession numbers: AF163020 and FJ515771).

#### 2.4.2.6 Unidentified fungal specimens associated with rust fungi with morphological descriptions

There are two fungal natural enemies from Brazil that we could not identify either at the species or genus level. The first specimen was found on the uredosori of *Puccinia inclita* collected in 1922 (PUR voucher: F4973). We observed gregarious or solitary black fruiting bodies more prominent than *Sphaerellopsis*' black pycnidia, developed exclusively on the rust pustules; pseudoparenchyma cells violet of textura globulosa, 12.07–23.30 x 9.79–15.48  $\mu\text{m}$  (n=15); and spores hyaline, three-septate, filiform (Fig. 2.9 a–e). The second specimen was found on uredosori

of *Uredinopsis pteridis* collected in 1976 (PUR voucher: N19234). The pycnidia of this specimen were smaller than *Sphaeraellopsis* fruiting bodies, and the conidia were hyaline and unicellular, with the spores size smaller than *Sphaerellopsis* species,  $3.17\text{--}6.97 \times 1.67\text{--}3.25 \mu\text{m}$  ( $n=20$ ) (Fig. 2.6 f–i).

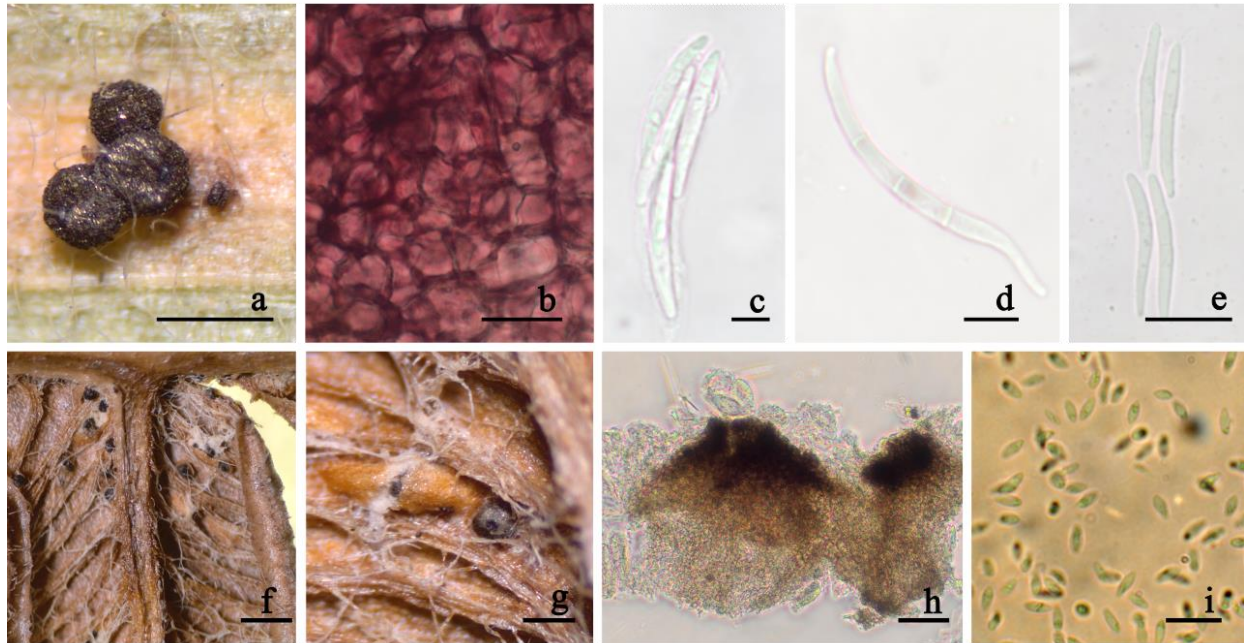


Figure 2.9 Morphological features of unidentified natural enemies associated with rust fungi from PUR collections. a–e fruiting bodies on rust sori of PURF4973. a. stromata, b. pseudoparenchyma cells of stromata, c. ascae, d–e. ascospores; f–i. fruiting bodies on rust sori of PURN19234. i. conidia.

### 2.4.3 Fungivorous larvae associated with rust fungi

Of the 5,618 rust specimens we screened for the presence of natural enemies of rust fungi, we found 287 rust specimens with fungivorous larvae. From these specimens, we collected 335 individual larvae (Fig. 2.4. z-ac) (see Table 2.3 and supplementary table S2 for more information). However, we discarded some of them for DNA extraction due to the highly dried material or low amount of larvae per rust specimen. Of the 335 individual larvae, we successfully amplified the DNA of 126 fungivorous larvae in 91 rust specimens of 12 rust genera whose sequences are in the Cecidomyiidae family and genus *Mycodiplosis* when blasted against the NCBI GenBank. Thus, although in this study, we report the presence of 335 fungivorous larvae on 287 rust specimens, we can only confirm the identity of 126 larvae on 91 rust specimens.

Of the 91 rust specimens confirmed with *Mycodiplosis*, the rust genus containing the largest number of *Mycodiplosis* larvae was *Hemileia*, with 28 specimens due to the 21 fresh larvae collected in coffee farms in Hawaii. Seventeen specimens of *Melampsora* were found to be associated with fungivorous larvae, representing 3% of the 495 *Melampsora* specimens screened for natural enemies. Similarly, only 1% of the *Puccinia* specimens and <1% of the *Uromyces* specimens we screened were found with *Mycodiplosis* larvae (Fig. 2.10). Most of the *Mycodiplosis* larvae were found in the uredinial stage (95%) and collected in 2016. Finally, we found *Mycodiplosis* larvae in 3% of the specimens we screened from the United States, 4% from Peru, and 1% from Brazil and Ecuador (Fig. 2.11).

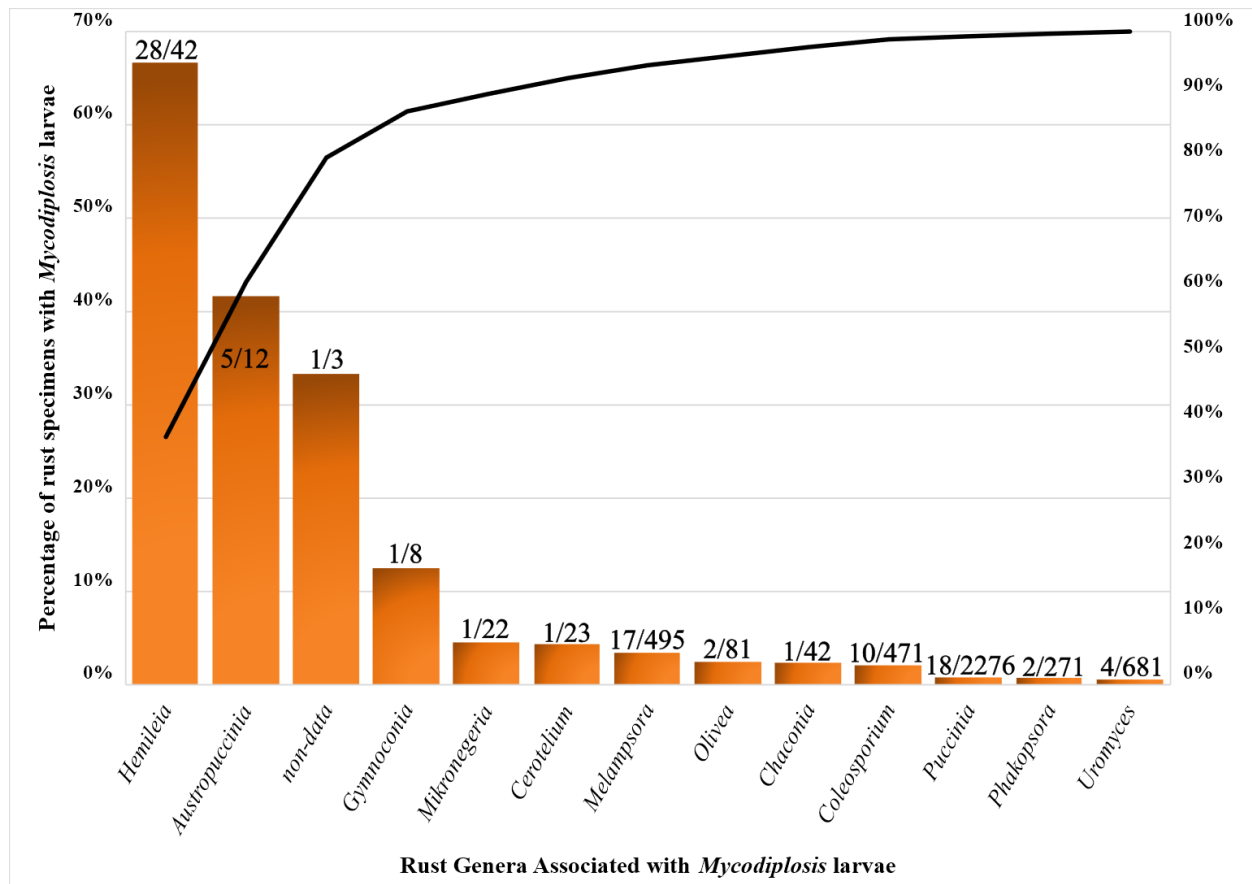


Figure 2.10 Pareto chat. Rust genera associated with *Mycodiplosis* larvae from PUR collections



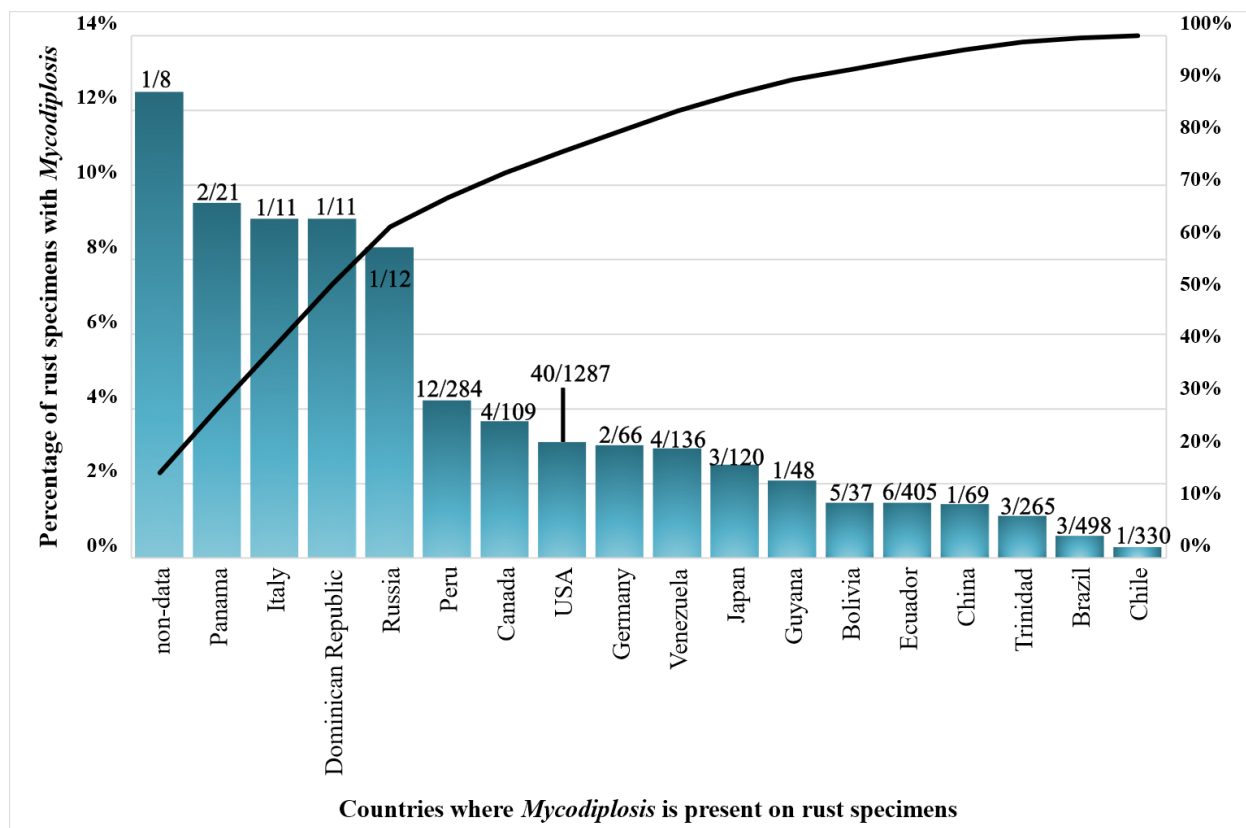


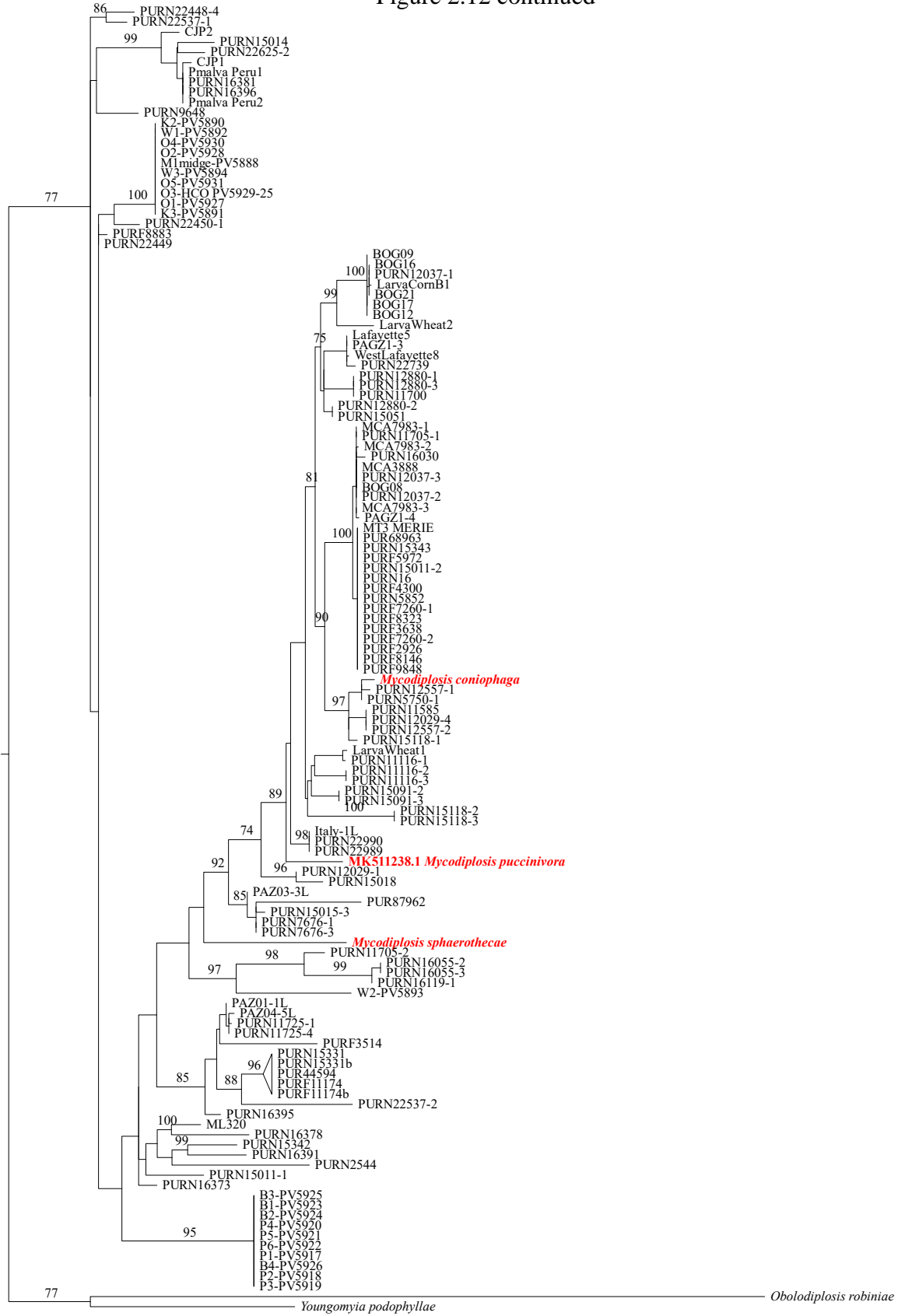
Figure 2.11 Pareto chat. Countries where *Mycodiplosis* larvae were found from PUR rust collections

#### 2.4.4 Phylogenetic inferences of larvae associated with rust fungi

The concatenated two-locus dataset comprised 964 characters, of which 223 were parsimony-informative. The percentage of parsimony-informative characters per locus was 69.96% for CO1 and 30.04% for 16S. We included 129 DNA fragments, of which 82 belonged to rust specimens at PUR, 42 were fresh larvae collected by collaborators and from field trips, three were reference sequences, and two served as outgroup taxa. The following models were selected by ModelFinder (AICc): GTR+F+I+G4 for CO1 and K3Pu+F+I+G4 for 16S (Fig. 2.12). Our phylogenetic tree forms a monophyletic group restricted to *Mycodiplosis* specimens. However, it does not distinguish *Mycodiplosis* members at the species level. We provide information related to larval specimens used in the phylogenetic analysis in Supplementary Table S2.

Figure 2.12 The best-scoring Maximum Likelihood tree of *Mycodiplosis* specimens, reconstructed from a concatenated COI–16Smit dataset. For each node, the ML bootstrap value ( $\geq 70$ ) is presented above the branch leading to that node. Species in red bold are reference sequences. Tree rooted to *Obolodiplosis robiniae* (Accession number: MN201515) and *Youngomyia podophyllae* (Accession number: MN191360).

Figure 2.12 continued



## 2.5 Discussion

This study represents the first catalog of natural enemies of rust fungi from preserved rust collections. The screening and taxonomic identification of these fungal and insect natural enemies are essential to unravel their diversity and geographic distribution for further studies in ecology, phylogenetic relationships, and their potential use as biological control agents for crop protection. This catalog includes 43 rust genera and 349 rust species associated with fungal and insect natural enemies. We provide 588 DNA sequences, five phylogenetic reconstruction trees, nine morphological descriptions, and 1,178 macro and 867 microphotographs of these natural enemies of rust fungi (see Table 2.3, Table S2, and Supplementary data for macro and microphotographs).

The Arthur Fungarium houses a broad diversity of fungi and insects associated with rust specimens. We found 543 rust specimens associated with fungal natural enemies and 287 rust specimens associated with larvae. Most fungal natural enemies belong to the genus *Sphaerellopsis* followed (96%), and most fungivorous larvae, identified with DNA sequences, belong to the genus *Mycodiplosis* (36%). Furthermore, we found more than one natural enemy intimately associated with rust in 28 specimens. For instance, the rust pustules of specimen N3996 contained both pink mycelium of *R. uredinicola* and pycnidia of *Sphaerellopsis* sp. Similarly, other rust specimens contained larvae of *Mycodiplosis* and fungal black fruiting bodies of *Sphaerellopsis* sp. simultaneously.

Notwithstanding our efforts to identify every fungus and fungivorous larva that we found associated with rust specimens, we could not complete this task 100%. Some preserved specimens were significantly dried, and others did not have enough material, such as empty fruiting structures, for proper identification. Nevertheless, we still consider these specimens as repositories of natural enemies of rust fungi and include them in our catalog.

Despite the random strategy we used to screen for natural enemies of rust fungi at PUR, it is important to notice that our results may have inherent biases. Since the primary purpose of Fungarium collections is to preserve mycological specimens, sometimes collectors may prefer a particular geographic region, season, or fungal species. Collections at PUR are not an exception to this behavior. Although PUR contains a high diversity of rust genera collected globally, *Puccinia*

and *Uromyces* have the most significant number of rust specimens and specimens collected from the United States and Brazil in this fungarium. Thus, the results of this study, such as the percentage of natural enemies, rust genera, and countries, are strictly based on raw data with no further statistical analyses.

Interestingly, the most screened rust genera at PUR did not have the highest percentage of fungal natural enemies (Fig. 2.2). For example, specimens of *Puccinia* and *Uromyces* did not reach more than 15% of the presence of fungal natural enemies. Although *Kweilingia*, *Dasyscypha*, and *Tranzschelia* had the highest percentage of specimens associated with fungal natural enemies, the number of specimens we screened was relatively low. Thus, more specimens of these rust genera would be necessary to confirm if they are the most sensitive genera to these natural enemies. Similarly, despite the large number of rust specimens we screened from the United States, the rate was very low (6%) when compared to South American countries such as Bolivia (18%), Colombia (17%), Argentina (13%), and Brazil (12%) (Fig. 2.3). These results suggest a great diversity in the Neotropics compared to North America. Thus, finding more diversity of fungal natural enemies of rust in South America is still possible.

We obtained similar results for fungivorous larvae compared to the fungal natural enemies. Despite the large amount of *Puccinia*, *Uromyces*, and *Melampsora* specimens we screened at PUR for the presence of natural enemies, less than 4% of the specimens were associated with fungivorous larvae. Likewise, we found a low proportion (<4%) of fungivorous larvae in most of the countries where the rust specimens were collected. Nevertheless, it is possible that many of these larvae have been lost while preparing the specimen to be preserved at PUR since these larvae are not attached to rust pustules.

### ***2.5.1 New records of fungal natural enemies associated with rust fungi***

This study increases the records of fungi intimately associated with rust species in the Americas and other regions across the globe (see Table 2.3 and Supplementary Table S2 for more information). Within the fungal genus *Sphaerellopsis*, the species *Sph. macroconidialis* was only reported in South Africa on *Uromycladium acaciae* (Fraser et al., 2021), in Italy on *Uromyces*

*caryophylli*, and in the Netherlands on rust infecting *Carex acutiformis* (Trakunyingcharoen et al., 2014). Herein, we add new host records of *Sph. macroconidialis* from 40 rust species in 53 specimens collected in Argentina (4 specimens), Australia (1 specimen), Bolivia (3 specimens), Brazil (10 specimens), Canada (2 specimens), Chile (2 specimens), Colombia (4 specimens), Cuba (1 specimen), Ecuador (6 specimens), Germany (1 specimen), Guatemala (2 specimens), Mexico (3 specimens), Paraguay (1 specimen), Peru (3 specimens), Spain (1 specimen), The United States (7 specimens) and Venezuela (2 specimens). Each of the rust specimens where we found *Sph. macroconidialis* is also a new report of host rust for this fungal natural enemy.

Similarly, *Sph. paraphysata* was only reported in Australia (Crous et al., 2018), Brazil, South Africa (Trakunyingcharoen et al., 2014), China (Phookamsak et al., 2019), and India (Anandakumar et al., 2019; Ashmitha Sri et al., 2020). Herein, we add new host records of *Sph. paraphysata* on 46 rust species in 56 specimens from 23 countries, including Argentina (2 specimens), Benin (1 specimen), Bolivia (2 specimens), Cameroon (1 specimen), Colombia (1 specimen), Costa Rica (1 specimen), Cuba (1 specimen), Ecuador (2 specimens), Guam (2 specimens), Guatemala (1 specimen), Guyana (6 specimens), Honduras (2 specimens), Kenya (1 specimen), Mexico (3 specimens), Nigeria (3 specimens), Papua New Guinea (1 specimen), Peru (1 specimen), St. Vincent & the Grenadines (1 specimen), Taiwan (2 specimens), Trinidad (5 specimens), Uganda (2 specimens), The United States (12 specimens), and Venezuela (3 specimens). Each of the rust species where we found *Sph. paraphysata* is also a new report of host rust for this fungal natural enemy.

This study also upgraded the records of host rusts associated with *Sph. filum* based on DNA sequences. In 1981, Kranz & Brandenburger wrote a list of 369 host rusts associated with *Sph. filum* based on some morphological characters (Kranz & Brandenburger, 1981). Later, Trakunyingcharoen et al. (2014) used isolates from fresh collections and CBS culture collections named *Sph. filum* to conduct a phylogenetic study of the genus. Their results showed several specimens incorrectly placed in *Sph. filum* and others even belonging to different genera. Thus, records from the list of Kranz & Brandenburger as *Sph. filum* are now uncertain if they belong to this species. Unlike Kranz and Brandenburger, we amplified DNA fragments from our fungal specimens and can confirm that some of them belong to *Sph. filum* based on our phylogenetic

analysis and the reference sequences of Trakunyingcharoen et al . (2014). Hence, we add records of five rust species in 9 rust specimens as host rusts of *Sph. filum* from Argentina (1 specimen), Bolivia (1 specimen), Chile (1 specimen), Ecuador (2 specimens), Mexico (2 specimens), Peru (1 specimen), and The United States (1 specimen). Although *Sph. filum* was previously reported in Germany on *Puccinia deschampsiae* (Trakunyingcharoen et al., 2014), we add a new record for this country on the host rust species *Puccinia coronata*.

*Sphaerellopsis hakeae* has been reported only once in Australia on rust infecting the host plant *Hakea* sp., but also without any association with the rust (Crous et al., 2016). Herein, we add a new record of *Sph. hakeae* on *Uromyces ehrhartae* infecting the host plant *Microlaena stipoides*, also from Australia. Lastly, we report 24 rust species in 54 rust specimens as host rusts of *Sphaerellopsis* sp., which we only identified at the genus level based on the DNA sequences. We found these specimens collected from Argentina (6 specimens), Bolivia (1 specimen), Brazil (11 specimens), Colombia (2 specimens), Ecuador (4 specimens), Japan (2 specimens), Peru (2 specimens), Trinidad (1 specimen), The United States (24 specimens), and Venezuela (1 specimen).

This study also adds new records of the fungal genera *Helicobasidium*, *Ramularia*, and *Simplicillium* associated with rust fungi. *Puccinia arechavaletae* is a new host rust record of *Helicobasidium longisporum*. Although *H. longisporum* was already reported in Trinidad on *Puccinia distinguenda* (Jodhan & Minter, 2006), this is the first record on *P. arechavaletae*. Similarly, *Puccinia* sp. (PUR voucher: N23060) is a new record associated with *S. lanosoniveum* from Puerto Rico. *Simplicillium lanosoniveum* had been previously reported on *Phakopsora pachyrhizi* (Ward et al., 2011, 2012) in Florida and Louisiana, USA, and on *Hemileia vastatrix* in Costa Rica and Venezuela (García-Nevárez & Hidalgo-Jaminson, 2019; Nonaka et al., 2013). *Ramularia coleosporii* (PUR voucher N16398) is a new report in Ecuador on the host rust *Coleosporium plumeriae*. This species was previously reported in India and Thailand on the same host rust (Baiswar et al., 2014; Sun et al., 2017) but never in the Americas. Lastly, *R. uredinicola* (PUR voucher: N3996) is a new record from Argentina and the Americas associated with *Melampsora epitea*. Interestingly, all host rusts reported in association with *R. coleosporii* belong to the genus *Coleosporium* and *R. uredinicola* to the rust genus *Melampsora* (Videira et al., 2016), which may suggest a pattern of rust host rust preference in these two species.

### **2.5.2 New records of *Mycodiplosis* associated with rust fungi in the Americas**

Although the genus *Mycodiplosis* is known to be cosmopolitan, most specimens are reported from Asia and Europe (Gagné & Jaschhof, 2021). *Mycodiplosis* had only been reported in seven countries in the Americas (Gagné & Jaschhof, 2021). However, this study adds ten more countries from the Americas to the records of *Mycodiplosis*. We thus contribute 48 specimens of *Mycodiplosis* in the following countries: six specimens from Bolivia, five specimens from Canada, one specimen from Chile, one from the Dominican Republic, six from Ecuador, one from Guyana, three from Panama, 15 from Peru, four from Trinidad, and six from Venezuela. In addition, we add 31 rust species as new host rusts to the list of new records. (see Table 2.3 and Supplementary Table S2 for more information). Lastly, this study adds 124 DNA sequences of *Mycodiplosis* from two mitochondrial genes: the barcoding section of CO1 and ribosomal 16S.

### **2.5.3 Phylogeny of the genus *Mycodiplosis***

Identification of *Mycodiplosis* members at the species level is not an easy task. Since the current classification of *Mycodiplosis* members is morphology-based, the adult male is always necessary for accurate species identification, which is usually not present on the rust. Thus, DNA sequences seem to be the most feasible option to unravel the relationships between these larvae and rust fungi. In this study, we contribute 124 DNA fragments of two mitochondrial genes: the barcoding section of CO1 and ribosomal 16S. Although our multi-locus phylogenetic reconstruction tree forms a monophyletic group with our sequences and the *Mycodiplosis* reference sequences we downloaded from the NCBI, these two loci did not have the resolution to elucidate the species diversity within the genus. Thus, other gene regions are necessary to amplify to resolve the complexity of these species.

### **2.5.4 *Mycodiplosis* and *Sphaerellopsis*, the most common natural enemies of rust fungi at PUR**

*Mycodiplosis* and *Sphaerellopsis* are the two most common genera that we found associated with rust fungi at PUR. Thus, besides reporting them in our catalog, we studied their distribution patterns, amplified more gene regions to resolve their phylogenetic relationships, and unraveled their specificity for their host rusts in Chapters four and five. In addition, since we isolated a strain of *Sph. macroconidialis* from southern corn rust, we showed the first interaction events between



this strain with rust spores of *Puccinia polysora*, the causal agent of southern corn rust, through in-vitro essays.

### **2.5.5 Importance of biological collections to study natural enemies of rust fungi**

This study emphasizes the importance of preserved biological collections in fungaria for biodiversity inventories and applied biological sciences, such as biological control of rust fungi. Thanks to the well-preserved rust specimens at the Arthur Fungarium, we found 543 rust specimens associated with fungal natural enemies and 280 associated with larvae. We could amplify 588 DNA sequences from these natural enemies and provide new records of host rusts in several geographic regions, emphasizing the Americas. In addition, we could amplify gene regions of 49 natural enemies of rust fungi collected for more than 100 years, with the oldest specimen collected in 1883 of the fungal natural enemy *Sphaerellopsis* sp. on *Melampsora medusae* from the United States (PUR voucher: 2041). Our study proves the great potential of preserved biological collections, providing a source not only for morphological studies but also for genetic information and the discovery of natural enemies of rusts collected incidentally.

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## CHAPTER 3. NOTES ON *TROCHILA* (ASCOMYCOTA, LEOTIOMYCETES), WITH NEW SPECIES AND COMBINATIONS.

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### 3.2 Abstract

Studies of *Trochila* (Leotiomyces, Helotiales, Cenangiaceae) are scarce. Here, we describe two new species based on molecular phylogenetic data and morphology. *Trochila bostonensis* was

collected at the Boston Harbor Islands National Recreation Area, Massachusetts. It was found on the stem of *Asclepias syriaca*, representing the first report of any *Trochila* species from a plant host in the family Apocynaceae. *Trochila urediniophila* is associated with the uredinia of the rust fungus *Cerotelium fici*. It was discovered during a survey for rust hyperparasites conducted at the Arthur Fungarium, in a single sample from 1912 collected in Trinidad. Macro- and micromorphological descriptions, illustrations, and molecular phylogenetic analyses are presented. The two new species are placed in *Trochila* with high support in both our six-locus (SSU, ITS, LSU, rpb1, rpb2, tef1) and two-locus (ITS, LSU) phylogenetic reconstructions. In addition, two species are combined in *Trochila*: *Trochila colensoi* (formerly placed in *Pseudopeziza*) and *T. xishuangbanna* (originally described as the only species in *Calycellinopsis*). This study reveals new host plant families, a new ecological strategy, and a new country record for the genus *Trochila*. Finally, our work emphasizes the importance of specimens deposited in biological collections such as fungaria.

### 3.3 Keywords

4 new taxa, biological collections, Boston Harbor Islands, fungarium specimens, fungicolous fungi, South America, taxonomy, Trinidad

### 3.4 Introduction

The genus *Trochila* Fr. (Ascomycota, Leotiomyces) was erected by Fries (1849) to accommodate four species previously placed in *Phacidium* Fr., *Sphaeria* Haller, and *Xyloma* Pers. *Trochila craterium* (DC) Fr. was the first species listed by Fries, based on *Sphaeria craterium* DC., which was later selected by Clements and Shear (1931) as the type species of *Trochila*. The other three species included by Fries (1849) were: *T. ilicis* (Fr.) Fr. [= *Sphaeria ilicis* Fr.], *T. laurocesari* (Desm.) Fr. [= *Phacidium laurocerasi* Desm.], and *T. taxi* (Fr.) Fr. [= *Xyloma taxi* Fr.]. Only the genus and one species (*T. laurocerasi*) were briefly described by Fries (1849). However, the type species, *T. craterium*, was well described macromorphologically by Lamarck and de Candolle (1805). The description can be translated loosely from French as “a fungus growing on the lower surface of ivy leaves, initially forming a flat white disc, then turning blackish and concave opening by a split along radial lines, the disc usually surrounded by a whitish membrane” (Lamarck and de

Candolle 1805). Later, the generic concept was expanded to include other types of apothecial opening. Rehm (1896) remarked that the covering layer of the apothecia could also open completely like a lid depending on host characters such as cuticle thickness. After the inclusion of this new character describing the genus, *Stegia ilicis* (Chevall.) Gillet was transferred as *Trochila ilicina* (Nees ex Fr.) Courtec (Crouan and Crouan 1867; Rehm 1896).

In our current circumscription of the genus *Trochila*, apothecia are sunken in the host tissues and hymenia are exposed either by splitting along radial lines or by splitting into a number of lobes that roll outward exposing the hymenium. The excipulum is composed of dark, globose-angular cells; asci contain eight ellipsoid, hyaline ascospores with oil guttules (except *T. substrictica* Rehm and *T. tetraspora* E. Müll. & Gamundí, which both have asci containing four ascospores); and paraphyses possess yellowish guttules (Dennis 1978; Baral and Marson 2005). Thirty-three names have been applied in the genus (Index Fungorum 2021). Jaklitsch et al. (2016) suggest that only ca. 10 names should be accepted.

Fries (1849) included *Trochila* in “Patellariacei” (= Patellariaceae). Later, it was transferred to Dermateaceae, Helotiales (Fuckel 1869; Karsten 1869; Saccardo 1884; Lambotte 1888). *Trochila* remained in this family (Korf 1973; Dennis 1978) into the molecular era (Lumbsch and Huhndorf 2010). Jaklitsch et al. (2016) placed *Trochila* in the resurrected family Cenangiaceae based on morphological and molecular data. Later, the relationships among genera in this family were supported in another, 5–15-locus phylogeny of Leotiomyces (Johnston et al. 2019).

Most species of *Trochila* have been described from their sexual morph. The asexual morph has the characteristics of the form-genus *Cryptocline* Petr. (Morgan-Jones 1973; Kiffer and Morelet 2000; Hyde et al. 2011). Two species of *Trochila* have been linked to their asexual morphs: *T. craterium* to *C. paradoxa* (De Not.) Arx and *T. laurocerasi* to *C. phacidiella* (Grove) Arx (von Arx 1957). The paucity of culture and molecular data of both *Cryptocline* and *Trochila* species has hindered the linkage of sexual and asexual morphs for most species. *Trochila viburnicola* Crous & Denman was the first species of the genus to be described based on the combination of morphology and molecular data, but only its asexual morph is known (Crous et al. 2018). The species was named referring to its host, *Viburnum* sp. (Dipsacales, Adoxaceae). In addition to *T. viburnicola*, two

other species have been reported on this host genus, but only from their sexual morph, *T. ramulorum* Feltgen and *T. tini* (Duby) Quél. [currently *Pyrenopeziza tini* (Duby) Nannf.]. Due to the lack of sequences or cultures of these two species, a comparison with *T. viburnicola* is impossible (Feltgen 1903; Crous et al. 2018).

Most *Trochila* members have a restricted record of geographical distribution and ecological strategy. *Trochila* records typically originate from the Northern Hemisphere limited to temperate regions in Europe and North America (Ziolo et al. 2005; Stoykov and Assyov 2009; Crous et al. 2018; Stoykov 2019; Global Biodiversity Information Facility 2020). Nonetheless, a number of putative *Trochila* reports are known from the Southern hemisphere (Spegazzini 1888, 1910, 1921; Rehm 1909; Gamundí et al. 1978). In addition, species of *Trochila* are typically recorded as saprotrophs on dead leaves and branches of both herbaceous plants and trees. However, a few species have been found infecting living plant tissues. *Trochila ilicina* is reported as both a weak parasite and a saprotroph because of its presence on living, decaying, and fallen leaves of *Ilex aquifolium* (Aquifoliales, Aquifoliaceae) (Ziolo et al. 2005), *T. laurocerasi* as a parasite of living leaves of *Prunus laurocerasus* (Rosales, Rosaceae) (Gregor 1936), and *T. symploci* as a pathogen of living leaves of *Symplocos japonica* (Ericales, Symplocaceae) (Hennings 1900; Stevenson 1926).

Here, we describe two new species, *T. bostonensis* and *T. urediniophila*, collected at the Boston Harbor Islands National Recreation Area, Massachusetts and at Port of Spain, Trinidad, respectively. We also make two new combinations in *Trochila* based on morphological studies and phylogenetic analyses. We reveal two new host plant families (Apocynaceae and Asparagaceae) and a new ecological strategy (fungicolous symbiont) for the genus. Finally, we provide a comparative table of characters, based on literature review, for all currently accepted species of *Trochila* (sensu Index Fungorum 2021).

### 3.5 Materials and Methods

#### 3.5.1 Collected samples

Samples were collected in the field and from fungaria. One collection of *Trochila* was discovered during the Boston Harbor Islands (BHI) National Recreation Area fungal ATBI (Haelewaters et al. 2018a). In this project, above-ground, ephemeral fruiting bodies of non-lichenized fungi were collected. In the field, specimens were placed in plastic containers or brown paper bags. BHI-F collection numbers were assigned. Date, specific locality when applicable, GPS coordinates, substrate, and habitat notes were recorded. Specimens were dried using a Presto Dehydro food dehydrator (National Presto Industries, Eau Claire, Wisconsin) set at 35 °C for 7–9 hours. Collections were packaged, labeled, and deposited at FH. A second *Trochila* collection came to our attention during a survey for hyperparasites of rust fungi at PUR. The specimen was found on the uredinia of the rust fungus *Cerotelium fici* on the underside of *Ficus maxima* leaves. Fungarium acronyms follow Thiers (continuously updated).

#### 3.5.2 Morphological studies

Methods to study the morphological characteristics of the *Trochila* specimens followed the process given in Baral (1992). Macro- and micromorphological features were examined on both fresh and dried apothecia for the specimen collected at the BHI and on dried apothecia for the specimen found at PUR. Apothecia from the BHI specimen were observed under an EZ4 stereomicroscope (Leica, Wetzlar, Germany) and studied under a B1 compound microscope (Motic, Barcelona, Spain). Apothecia from the PUR specimen were examined on an SZ2-ILTS dissecting microscope (Olympus, Center Valley, Pennsylvania) and studied using a BH2-RFCA compound microscope (Olympus). Sections of apothecia were cut free-hand and mounted in water or pre-treated in 5% KOH. Sections were also mounted in Melzer's reagent with and without KOH-pretreatment to determine dextrinoid or amyloid reactions. At least 10 measurements were made for each structure at 400–1000× magnification. Measurements for each character are given as (a–)b–c (–d), with b–c indicating the 95% confidence interval and a and d representing the smallest and large single measurement, respectively. Macro- and microphotographs were taken with a USB Moticam 2500 camera (Motic) (BHI specimen) or an Olympus SC30 camera (PUR specimen). Measurements were made using the following software suites: Motic Images Plus 2.0 and cellSens Standard 1.18

Imaging Software (Olympus). Color coding refers to Kelly (1965). Abbreviations were adopted from Baral (1992) and Baral and Marson (2005) as follows:

\* living state;

† dead state;

IKI Lugol's solution;

KOH potassium hydroxide;

LBs lipid bodies;

MLZ Melzer's reagent;

OCI oil content index;

VBs refractive vacuolar bodies.

### ***3.5.3 DNA isolation, PCR amplifications, sequencing***

Genomic DNA was isolated from 1–3 apothecia per specimen using the E.Z.N.A. HP Fungal DNA Kit (Omega Bio-Tek, Norcross, Georgia), QIAamp DNA Micro Kit (Qiagen, Valencia, California), following the manufacturer's instructions, and the Extract-N-Amp Plant PCR Kit (Sigma-Aldrich, St. Louis, Missouri), following Haelewaters et al. (2018a). We amplified the following loci: nuclear small and large ribosomal subunits (SSU and LSU), internal transcribed spacer region of the ribosomal DNA (ITS), RNA polymerase II second largest subunit (*rpb2*), and translation elongation factor 1- $\alpha$  (*tef1*). Primer combinations were as follows: NS1/NS2 and NS1/NS4 for SSU (White et al. 1990); LR0R/LR5 for LSU (Vilgalys and Hester 1990; Hopple 1994); ITS1F/ITS4, ITS9mun/ITS4A, and ITS5/ITS2 for ITS (White et al. 1990; Gardes and Bruns 1993; Egger 1995); RPB2-5F2/fRPB2-7cR for *rpb2* (Liu et al. 1999; Sung et al. 2007); and EF1-983F/EF1-1567R and EF1-983F/EF1-2218R for *tef1* (Rehner and Buckley 2005). All 25- $\mu$ l PCR reactions were conducted on a Mastercycler ep gradient Thermal Cycler (Eppendorf model #5341, Hauppauge, New York) and consisted of 12.5  $\mu$ l of 2 $\times$  MyTaq Mix (Bioline, Swedesboro, New Jersey), 1  $\mu$ l of each 10  $\mu$ M primer, and 10.5  $\mu$ l of 1/10 diluted DNA extract. Amplifications of rDNA and *rpb2* loci were run under the following conditions: initial denaturation at 95 °C for 5 min (94 °C for LSU); followed by 40 cycles of denaturation at 95 °C for 30 sec (94 °C for LSU), annealing at 45 °C (ITS) / 50 °C (LSU) / 55 °C (SSU, *rpb2*) for 45 sec, and elongation at 72 °C for 45 sec (1 min for LSU); and final extension at 72 °C for 7 min (1 min for SSU). Amplification of *tef1* was done with a touchdown PCR as follows: initial denaturation at 95 °C for 10 min; followed



by 30 cycles of 95 °C for 1 min, 62 °C for 1 min (decreasing 1 °C every 3 cycles), 72 °C for 90 sec; then 30 cycles of 95 °C for 30 sec, 55 °C for 30 sec, and 72 °C for 1 min; and final extension at 72 °C for 7 min (Don et al. 1991; Haelewaters et al. 2018b). PCR products were visualized by gel electrophoresis. Purification of successful PCR products and subsequent sequencing in both directions were outsourced to Genewiz (South Plainfield, New Jersey). Raw sequence reads were assembled and edited using Sequencher version 5.2.3 (Gene Codes Co., Ann Arbor, Michigan).

### **3.5.4 Sequence alignment and phylogenetic analysis**

Edited sequences were blasted against the NCBI GenBank nucleotide database (<http://ncbi.nlm.nih.gov/blast/Blast.cgi>) to search for closest relatives. For phylogenetic placement of our isolates, we downloaded SSU, ITS, LSU, *rpb1*, *rpb2*, and *tef1* sequences of *Trochila* from GenBank. We also downloaded sequence data of selected clades of Helotiales, mainly from Pärtel et al. (2017) but also other sources (details in Table 3.1), as a basis for our six-locus phylogenetic analysis. We selected representative taxa of Cenangiaceae, Cordieritidaceae, Rutstroemiaceae, and Sclerotiniaceae, with taxa in the family Chlorociboriaceae serving as outgroups (Johnston et al. 2019). Alignment of DNA sequences was done for each locus separately using MUSCLE version 3.7 (Edgar 2004), available on the Cipres Science Gateway 3.3 (Miller et al. 2010). The aligned sequences for each locus were concatenated in MEGA7 (Kumar et al. 2016). Maximum likelihood (ML) inference was performed using IQ-TREE from the command line (Nguyen et al. 2015) under partitioned models (Chernomor et al. 2016). Nucleotide substitution models were selected under Akaike's information criterion corrected for small sample size (AICc) with the help of the built-in program ModelFinder (Kalyaanamoorthy et al. 2017). Ultrafast bootstrap analysis was implemented with 1000 replicates (Hoang et al. 2017).

For the purpose of species delimitation, we constructed a second dataset of ITS–LSU consisting of isolates of *Trochila* and closely related taxa in the family Cenangiaceae. We included *Trochila* spp., *Calycellinopsis xishuangbanna*, and *Pseudopeziza colensoi*, with *Cenangiopsis* spp. serving as outgroup. In this analysis, we included *T. ilicina*, for which only a single ITS sequence is available. The same methods as above were applied: alignment using MUSCLE (Edgar 2004), selection of nucleotide substitution models with the help of ModelFinder (Kalyaanamoorthy et al.

2017), ML using IQ-TREE (Nguyen et al. 2015; Chernomor et al. 2016; Hoang et al. 2017). Phylogenetic reconstructions with bootstrap values (BS) were visualized in FigTree version 1.4.3 (<http://tree.bio.ed.ac.uk/software/figtree/>).

Table 3.1 Sequences used in phylogenetic analyses. Accession numbers in boldface indicate sequences that were generated during the course of this study.

Isolate	Species	Family	SSU	ITS	LSU	<i>rpb1</i>	<i>rpb2</i>	<i>tef1</i>	Reference
KL391	<i>Ameghiniella australis</i>	Cordieritidaceae	KX090893		KX090841	KX090787		KX090690	Pärtel et al. (2017)
AD283531 <sup>T</sup>	<i>Annabella australiensis</i>	Cordieritidaceae		MK328475	MK328476				Fryar et al. (2019)
AFTOL-ID 59	<i>Botryotinia fuckeliana</i>	Sclerotiniaceae	AY544695		AY544651	DQ471116	DQ247786	DQ471045	Spatafora et al. (2006)
HMAS:187063	<i>Calycellinopsis xishuangbanna</i>	Cenangiaceae	GU936124		KR094163	MH729338	MH729345		W.Y. Zhuang et al. (unpubl.)
KL375	<i>Cenangiosis alpestris</i>	Cenangiaceae			KX090837	KX090784	KX090736		Pärtel et al. (2017)
KL378	<i>Cenangiosis alpestris</i>	Cenangiaceae	KX090891	LT158470	KX090839	KX090786	KX090738		Pärtel et al. (2017)
KL157	<i>Cenangiosis alpestris</i>	Cenangiaceae	KX090858	LT158421	KX090806		KX090709		Pärtel et al. (2017)
KL174	<i>Cenangiosis quercicola</i>	Cenangiaceae	KX090862	LT158425	KX090811	KX090760	KX090713	KX090663	Pärtel et al. (2017)
KL377	<i>Cenangiosis</i> sp.	Cenangiaceae	KX090890	KX090900	KX090838	KX090785	KX090737		Pärtel et al. (2017)
KL276	" <i>Cenangium</i> " <i>acuum</i>	<i>Piceomphale</i> clade	KX090879	LT158445	KX090828		KX090727	KX090680	Pärtel et al. (2017)
KL243	" <i>Cenangium</i> " <i>acuum</i>	<i>Piceomphale</i> clade	KX090873	LT158439	KX090822	KX090767	KX090720	KX090674	Pärtel et al. (2017)
KL390	<i>Cenangium ferruginosum</i>	Cenangiaceae	KX090892	LT158471	KX090840		KX090739		Pärtel et al. (2017)
KL167	<i>Chlorencoelia torta</i>	Cenangiaceae		LT158424	KX090810	KX090759			Pärtel et al. (2017)
KP606	<i>Chlorencoelia versiformis</i>	Cenangiaceae	KX090894			KX090788	KX090740	KX090692	Pärtel et al. (2017)
KL21	<i>Chlorencoelia versiformis</i>	Cenangiaceae	KX090846	LT158427	KX090795				Pärtel et al. (2017)
KL152	<i>Chlorociboria aeruginascens</i>	Chlorociboriaceae		LT158419		KX090752	KX090706	KX090657	Pärtel et al. (2017)
KL247	<i>Chlorociboria aeruginella</i>	Chlorociboriaceae	KX090875			KX090769	KX090722	KX090676	Pärtel et al. (2017)

Table 3.1 continued

KL238	<i>Chlorociboria glauca</i>	Chlorociboriaceae	KX090872	LT158438	KX090821	KX090766		KX090673	Pärtel et al. (2017)
KL212	<i>Ciboria viridifusca</i>	Sclerotiniaceae	KX090863	LT158429	KX090812				Pärtel et al. (2017)
KL254	<i>Crumenulopsis sororia</i>	Cenangiaceae		LT158442	KX090826		KX090725		Pärtel et al. (2017)
KL317	<i>Diplocarpa bloxamii</i>	Cordieritidaceae	KX090885		KX090834	KX090778	KX090745	KX090688	Pärtel et al. (2017)
SK80	<i>Diplolaeviopsis ranula</i>	Cordieritidaceae	KX090896	KP984782		KX090790			Etayo et al. (2015), Pärtel et al. (2017)
TU:109263	<i>Dumontinia tuberosa</i>	Sclerotiniaceae	KX090897	LT158412	KX090843	KX090792		KX090697	Pärtel et al. (2017)
KL111	<i>Encoelia fimbriata</i>	Cenangiaceae	KX090852		KX090800		KX090703	KX090655	Pärtel et al. (2017)
KL108	<i>Encoelia furfuracea</i>	Cenangiaceae	KX090851		KX090799		KX090702	KX090654	Pärtel et al. (2017)
KL107	<i>Encoelia furfuracea</i>	Cenangiaceae	KX090850	LT158416	KX090798	KX090749	KX090701	KX090653	Pärtel et al. (2017)
KL106	<i>Encoelia furfuracea</i>	Cenangiaceae	KX090849	LT158415		KX090748		KX090652	Pärtel et al. (2017)
KL92	<i>Encoelia furfuracea</i>	Cenangiaceae	KX090847	LT158482	KX090796			KX090651	Pärtel et al. (2017)
KL164	<i>Encoelia heteromera</i>	Cenangiaceae	KX090861		KX090809	KX090758	KX090712	KX090662	Pärtel et al. (2017)
KL304	<i>Encoelia heteromera</i>	Cenangiaceae	KX138404			KX138400			Pärtel et al. (2017)
KL244	Helotiales sp.	Cenangiaceae	KX090874	LT158440	KX090823	KX090768	KX090721	KX090675	Pärtel et al. (2017)
KL20	<i>Heyderia abietis</i>	Cenangiaceae	KX090845	LT158426		KX090747	KX090699	KX090650	Pärtel et al. (2017)
HMAS:71954	<i>Heyderia abietis</i>	Cenangiaceae	AY789295	AY789297	AY789296				Wang et al. (2005)
KL216	<i>Heyderia pusilla</i>	Cenangiaceae	KX090865	LT158430		KX090762	KX090715	KX090665	Pärtel et al. (2017)
KL299	<i>Ionomidotis frondosa</i>	Cordieritidaceae	KX090882			KX090775		KX090685	Pärtel et al. (2017)

Table 3.1 continued

KL231	<i>Ionomidotis fulvotogens</i>	Cordieritidaceae	KX090870		KX090819	KX090765	KX090719	KX090671	Pärtel et al. (2017)
KL239	<i>Ionomidotis fulvotogens</i>	Cordieritidaceae	KX138403		KX138407	KX138399	KX138401		Pärtel et al. (2017)
KL154	<i>Ionomidotis irregularis</i>	Cordieritidaceae	KX090856		KX090804	KX090754		KX090658	Pärtel et al. (2017)
KL301	<i>Ionomidotis olivascens</i>	Cordieritidaceae	KX090883		KX090833	KX090776	KX090732	KX090686	Pärtel et al. (2017)
CBS:811.85	<i>Lambertella subrenispora</i>	Rutstroemiaceae	KF545416	AB926097	MH873604				Zhao et al. (2016), Pärtel et al. (2017), Vu et al. (2019)
LL95	<i>Llimoniella terricola</i>	Cordieritidaceae	KX090895		KX090842	KX090789	KX090741	KX090693	Pärtel et al. (2017)
AFTOL-ID 169	<i>Monilinia laxa</i>	Sclerotiniaceae	AY544714		AY544670	FJ238425	DQ470889	DQ471057	Spatafora et al. (2006)
KL374	<i>Piceomphale bulgarioides</i>	Piceomphale clade	KX090889	LT158469	KX090836	KX090783			Pärtel et al. (2017)
KL98	<i>Piceomphale bulgarioides</i>	Piceomphale clade	KX090848	LT158483	KX090797		KX090700		Pärtel et al. (2017)
PDD:112240	<i>Pseudopeziza colensoi</i>	Cenangiaceae		MH921874	MH985297	MH986706	MH986705		P.R. Johnston and D. Park (unpubl.)
KL267	<i>Pycnopeziza sejournei</i>	Sclerotiniaceae	KX090878	LT158443	KX090827	KX090772	KX090726	KX090679	Pärtel et al. (2017)
AFTOL-ID 907	<i>Rhabdocline laricis</i>	Cenangiaceae	DQ471002		DQ470954	DQ471146	DQ470904	DQ471073	Spatafora et al. (2006)
KL292	<i>Rutstroemia firma</i>	Rutstroemiaceae	KX090881	LT158450	KX090832	KX090774	KX090731	KX090684	Pärtel et al. (2017)
KL291	<i>Rutstroemia firma</i>	Rutstroemiaceae		LT158449	KX090831		KX090730	KX090683	Pärtel et al. (2017)
KL290	<i>Rutstroemia firma</i>	Rutstroemiaceae			KX090830		KX090729	KX090682	Pärtel et al. (2017)
KL222	<i>Rutstroemia firma</i>	Rutstroemiaceae	KX138402		KX138406			KX138397	Pärtel et al. (2017)

Table 3.1 continued

KL310	<i>Rutstroemia johnstonii</i>	Rutstroemiaceae	KX090884	LT158454		KX090777	KX090733	KX090687	Pärtel et al. (2017)
KL234	<i>Rutstroemia juniperi</i>	Rutstroemiaceae	KX090871		KX090820			KX090672	Pärtel et al. (2017)
KL217	<i>Rutstroemia luteovirescens</i>	Rutstroemiaceae		LT158431	KX090814	KX090763	KX090716	KX090666	Pärtel et al. (2017)
KL160	<i>Rutstroemia tiliacea</i>	Rutstroemiaceae	KX090860	LT158423	KX090808	KX090757	KX090711	KX090661	Pärtel et al. (2017)
KL393	Rutstroemiaceae sp.	Rutstroemiaceae	KX138405	LT158472	KX138408		KX138398	KX090691	Pärtel et al. (2017)
KL288	Rutstroemiaceae sp.	Rutstroemiaceae	KX090880	LT158446	KX090829	KX090773	KX090728	KX090681	Pärtel et al. (2017)
CBS:273.74 <sup>T</sup>	<i>Sarcotrichila longispora</i>	Cenangiaceae		KJ663836	KJ663877		KJ663918		Crous et al. (2014)
KL347	<i>Sclerencoelia fascicularis</i>	Sclerotiniaceae				KX090782			Pärtel et al. (2017)
KL156	<i>Sclerencoelia fraxinicola</i>	Sclerotiniaceae	KX090857		KX090805	KX090755	KX090708	KX090659	Pärtel et al. (2017)
KL344	<i>Sclerencoelia pruinosa</i>	Sclerotiniaceae	KX090888			KX090781	KX090735		Pärtel et al. (2017)
CBS:499.50	<i>Sclerotinia sclerotiorum</i>	Sclerotiniaceae	DQ471013		DQ470965		DQ470916		Spatafora et al. (2006)
NY:01231276	<i>Skyttea radiatilis</i>	Cordieritidaceae		KJ559538	KJ559560	KX090791	KX090742	KX090694	Suija et al. (2015), Pärtel et al. (2017)
TH90	<i>Thamnogalla crombiei</i>	Cordieritidaceae	KJ559583	KJ559535	KJ559557		KX090743	KX090695	Pärtel et al. (2017)
BHI-F974a <sup>T</sup>	<i>Trochila bostonensis</i>	Cenangiaceae	<b>MT873949</b>	<b>MT873947</b>	<b>MT873952</b>		<b>MT861181</b>	<b>MT861183</b>	This study
BHI-F974b <sup>T</sup>	<i>Trochila bostonensis</i>	Cenangiaceae	<b>MT873950</b>	<b>MT873948</b>	<b>MT873948</b>		<b>MT861182</b>	<b>MT861184</b>	This study
KL332	<i>Trochila craterium</i>	Cenangiaceae	KX090886			KX090779			Pärtel et al. (2017)
KL336	<i>Trochila laurocerasi</i>	Cenangiaceae	KX090887	LT158460	KX090835	KX090780	KX090734	KX090689	Pärtel et al. (2017)
F18316 <sup>T</sup>	<i>Trochila urediniophila</i>	Cenangiaceae		<b>MT873946</b>	<b>MT873951</b>				This study

Table 3.1 continued

CBS:144206 <sup>T</sup>	<i>Trochila</i> <i>viburnicola</i>	Cenangiaceae		MH107921	MH107967		MH108011	MH108031	Crous et al. (2018)
KL253	<i>Velutarina rufo-</i> <i>olivacea</i>	Cenangiaceae	KX090877		KX090825	KX090771	KX090724	KX090678	Pärtel et al. (2017)

### 3.6 Results

#### 3.6.1 Nucleotide alignment dataset and phylogenetic inferences

The concatenated six-locus dataset consisted of 11343 characters, of which 2655 were parsimony-informative. The percentage of parsimony-informative characters per locus was 9.3% for SSU, 48.1% for ITS, 21.4% for LSU, 48.9% for *rpb1*, 30.0% for *rpb2*, and 19.2% for *tef1*. A total of 71 isolates were included, of which *Chlorociboria aeruginascens* (Nyl.) Kanouse ex C.S. Ramamurthi, Korf & L.R. Batra, *C. aeruginella* (P. Karst.) Dennis, and *C. glauca* (Dennis) Baral & Pärtel (Helotiales, Chlorociboriaceae) served as outgroup taxa. The following models were selected by ModelFinder (AICc): TNe+R3 (SSU,  $-\ln L = 23478.796$ ); GTR+F+I+G4 (ITS,  $-\ln L = 18385.043$ ); TN+F+R4 (LSU,  $-\ln L = 28398.591$ ); SYM+I+G4 (*rpb1*,  $-\ln L = 41387.214$ ); GTR+F+R10 (*rpb2*,  $-\ln L = 57025.083$ ); and GTR+F+R8 (*tef1*,  $-\ln L = 35467.940$ ). Our ML analysis reveals five high to maximum-supported clades (Fig. 3.1): Cenangiaceae, Cordieritidaceae, Rutstroemiaceae, Sclerotiniaceae, and a clade with *Piceomphale bulgarioides* (P. Karst.) Svrček and “*Cenangium*” *acuum* Cooke & Peck (Piceomphale clade sensu Pärtel et al. 2017). As previously reported (e.g., Pärtel et al. 2017; Johnston et al. 2019), several genera in their current circumscription are polyphyletic: *Encoelia* (Fr.) P. Karst. in Cenangiaceae and Rutstroemiaceae, *Ionomidotis* E.J. Durand ex Thaxt. in Cordieritidaceae, *Rutstroemia* P. Karst. in Rutstroemiaceae, and *Trochila* in Cenangiaceae. *Trochila laurocerasi* is placed as a sister taxon to *Calycellinopsis xishuangbanna* W.Y. Zhuang and *Pseudopeziza colensoi* (Berk.) Masee. The other species of *Trochila*, including the type species *T. craterium* and the here described species, form a monophyletic clade (BS = 81).

The second two-locus dataset consisted of 2284 characters (ITS: 924, LSU: 1360), of which 2040 were parsimony-informative (ITS: 782, LSU: 1258). A total of 13 isolates were included, of which *Cenangiosis alpestris* (Baral & B. Perić) Baral, B. Perić & Pärtel, *C. quercicola* (Romell) Rehm, and *Cenangiosis* sp. served as outgroup taxa. The following models were selected by ModelFinder (AICc): GTR+F+I+G4 (ITS,  $-\ln L = 5810.483$ ) and TIM+F+R2 (LSU,  $-\ln L = 5595.374$ ). *Calycellinopsis xishuangbanna*, *Pseudopeziza colensoi*, and all *Trochila* species form a monophyletic clade with high support (BS = 96) (Fig. 3.2). Both new species of *Trochila* are



distinct from previously described species. The undescribed *Trochila* species found on uredinia of *Cerotelium fici* is retrieved as sister to *T. viburnicola* (BS = 90).

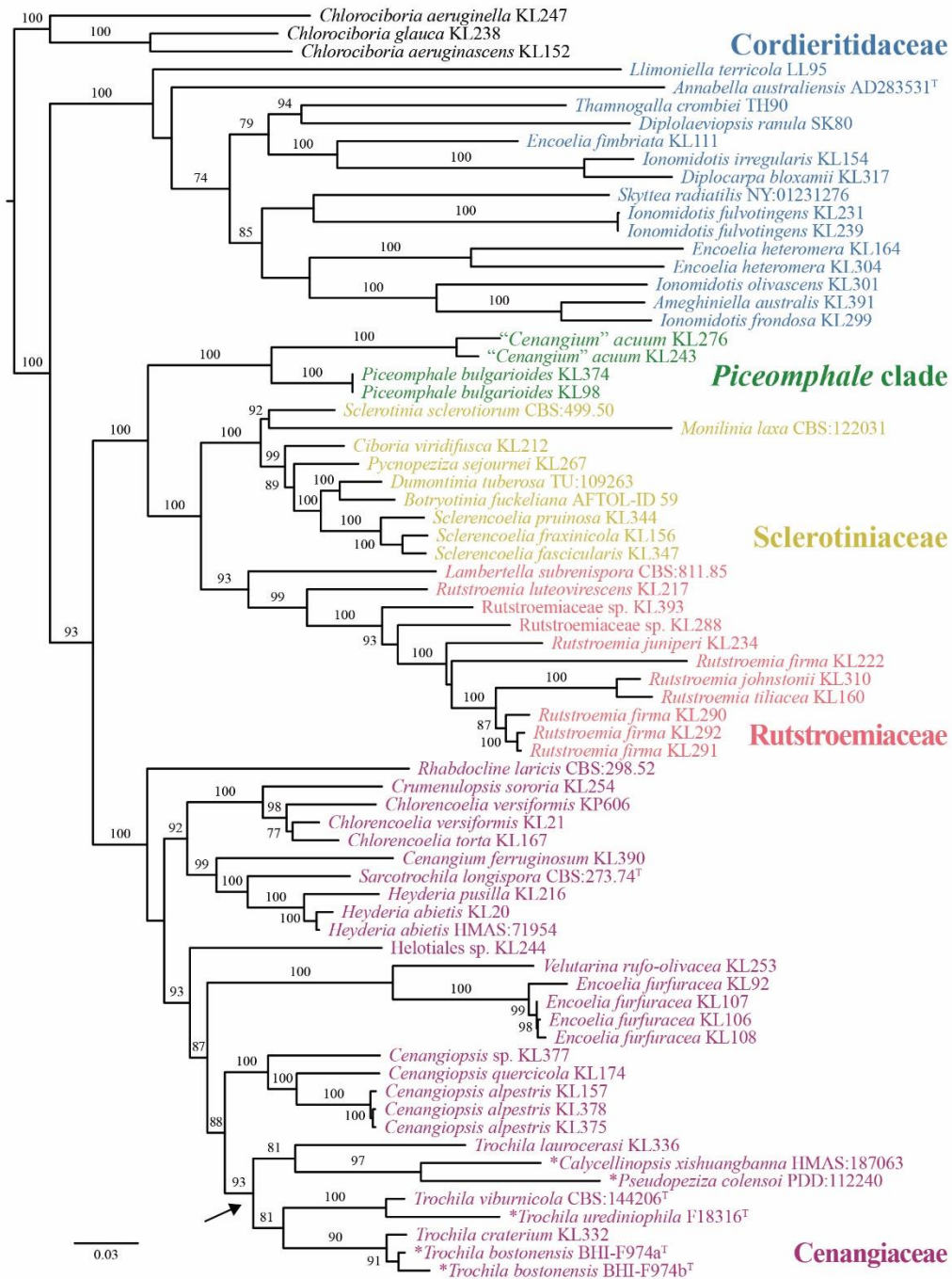


Figure 3.1 The best-scoring ML tree ( $-\ln L = 87544.854$ ) of Cenangiaceae, Cordieritidaceae, Rutstroemiaceae, Sclerotiniaceae, and the Piceomphale clade, reconstructed from a concatenated six-locus dataset (SSU, ITS, LSU, rpb1, rpb2, and tef1). For each node, the ML bootstrap value (if  $\geq 70$ ) is presented above or in front of the branch leading to that node. The arrow denotes the genus *Trochila*. Species with an asterisk (\*) are treated in the Taxonomy section.

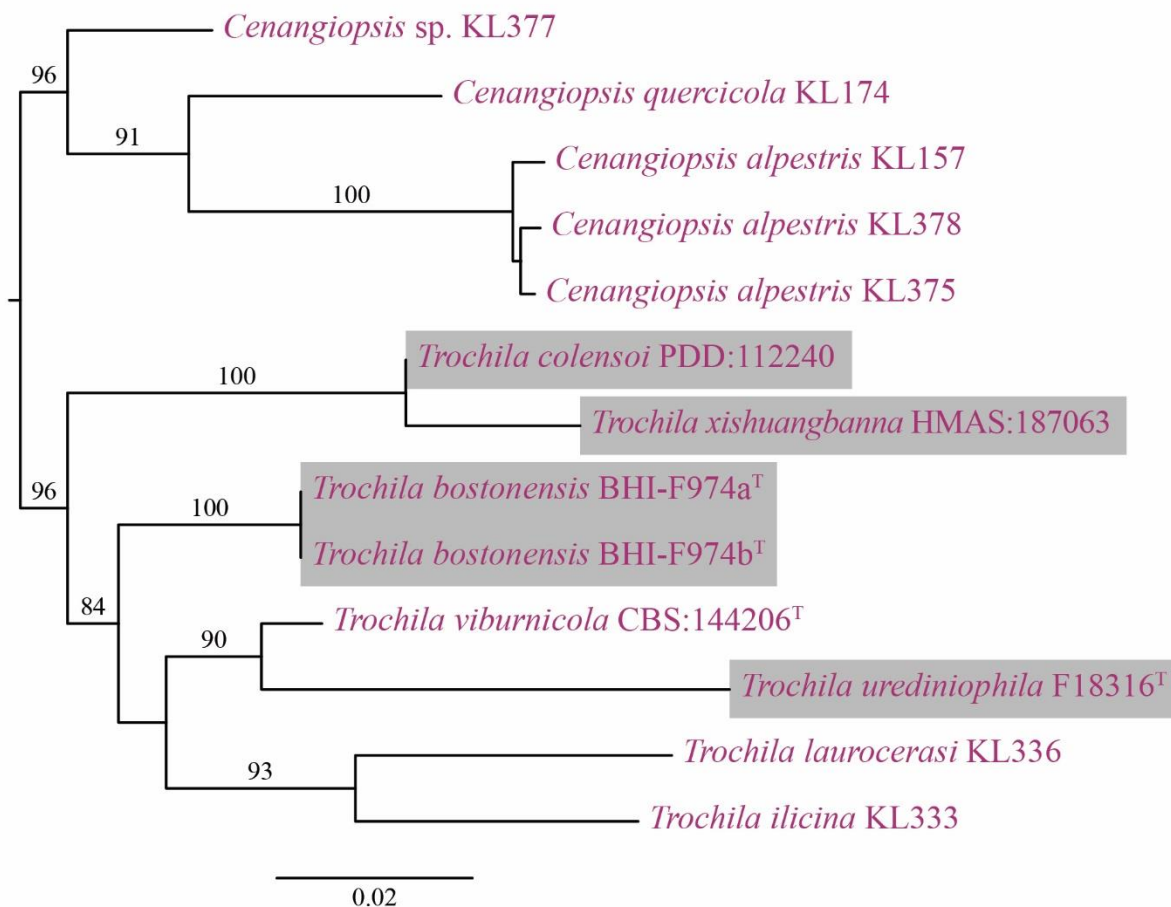


Figure 3.2 The best-scoring ML tree ( $-\ln L = 5225.551$ ) of Cenangiaceae, reconstructed from a concatenated ITS–LSU dataset. For each node, the ML bootstrap value (if  $\geq 70$ ) is presented above the branch leading to that node. Species treated in the Taxonomy section are highlighted with gray shading.

### 3.6.2 Taxonomy

Leotiomyces O.E. Erikss. & Winka

Helotiales Nannf. ex Korf & Lizoň

Cenangiaceae Rehm

#### 3.6.2.1 *Trochila bostonensis* Quijada & Haelew, sp. nov.

MycoBank No: 836582

Fig. 3.3

## Diagnosis

Differs from *Trochila craterium* and *T. laurocerasi* in its host (Apocynaceae), sizes of asci ( $57\text{--}65.5 \times 5\text{--}6 \mu\text{m}$ ) and ascospores ( $6.2\text{--}7.2 \times 2.6\text{--}2.8 \mu\text{m}$ ), and the inamyloidity of its ascus apex.

## Type

Holotype: USA, Massachusetts, Boston Harbor Islands National Recreation Area, Plymouth County, Great Brewster Island,  $42.3310722^\circ\text{N}$ ,  $70.8977667^\circ\text{W}$ , alt. 10 m a.s.l., 16 Oct 2017, leg. D. Haelewaters, J.K. Mitchell & L. Quijada, on hollow dead stem of *Asclepias syriaca* (Gentianales, Apocynaceae), FH:BHI-F0974. Ex-holotype sequences: isolates BHI-F0974a (1 apothecium, SSU: MT873949, ITS: MT873947, LSU: MT873952, rpb2: MT861181, tef1: MT861183) and BHI-F0974b (1 apothecium, SSU: MT873950, ITS: MT873948, LSU: MT873953, rpb2: MT861182, tef1: MT861184).

## Etymology

*bostonensis* – referring to Boston, Massachusetts, the locality of the type collection.

## Description

Apothecia erumpent singly or in groups of 2–3, protruding from the bark by lifting and rolling outward the host periderm, sessile on a broad base, closed and barely visible when dry, rehydrated 0.4–1.1 mm diam., 0.1–0.2 mm thick; mature flat to slightly cupulate, dark grayish red brown (47.D.gy.r.Br) to black (267.Black). Margin toothed and lighter than the disc, apothecia star-shaped, with 3–6 teeth of 0.1–0.3 mm in length, each tooth deep yellowish brown (75.deeepyBr). Asci  $*(46.5\text{--})55.5\text{--}66.5(-73) \times (5.5\text{--})6.0\text{--}6.5(-7.0) \mu\text{m}$ ,  $\dagger(50.5\text{--})57\text{--}65.5(-66) \times (4.5\text{--})5.0\text{--}6.0 \mu\text{m}$ , 8-spored, cylindrical, pars sporifera  $*30\text{--}52 \mu\text{m}$ ; apex rounded to subconical, inamyloid (IKI, KOH-pretreated or not), slightly thick-walled at apex, lateral walls thin; base slightly tapered and arising from croziers. Ascospores  $*(6.3\text{--})6.7\text{--}7.7(-8.6) \times 2.7\text{--}3.4 \mu\text{m}$ ,  $\dagger(5.8\text{--})6.2\text{--}7.2 \times 2.6\text{--}2.8 \mu\text{m}$ , ellipsoid-cuneate, inequilateral, ends rounded or subacute, aseptate, hyaline, smooth, thick-walled, oligoguttulate, containing 2–5 grayish yellow (90.gy.Y) oil drops (LBs), 1–2.4  $\mu\text{m}$  diam., OCI =  $(45\text{--})60\text{--}75(-90)\%$ . Paraphyses slightly to medium clavate, terminal cell  $*(17.5\text{--})18\text{--}23(-29.5) \times 3\text{--}4 \mu\text{m}$ , secondary cells  $*(8\text{--})9\text{--}10(-11) \times 2.5\text{--}3 \mu\text{m}$ , lower cells  $*(7.5\text{--})8.5\text{--}10.5(-11.5) \times 2.5\text{--}3 \mu\text{m}$ , unbranched, thin-walled, smooth, with one or several cylindric to globose refractive

drops (VBs, not present after KOH-pretreated),  $*3.5\text{--}14 \times 2\text{--}3.5 \mu\text{m}$ . Medullary excipulum  $17.5\text{--}54 \mu\text{m}$  thick, grey yellowish brown (80.gy.yBr), upper part of textura porrecta, lower part dense textura intricata, cells with tiny globose deep yellow (85.deepY) refractive drops (VBs). Ectal excipulum of thin-walled textura globulosa–angularis at base and lower flanks, dark yellowish brown (78.d.yBr) to dark brown (59.d.Br),  $(40\text{--})55\text{--}78 \mu\text{m}$  thick, cells  $*(7.0\text{--})9.5\text{--}13(\text{--}15.5) \times (3.0\text{--})5.0\text{--}8.5(\text{--}10) \mu\text{m}$ ; at upper flanks and margin of textura prismatica,  $30\text{--}40 \mu\text{m}$  thick, cells  $*(5.5\text{--})6.5\text{--}7.5(\text{--}8.5) \times 2.5\text{--}3.5 \mu\text{m}$ , entirely without drops and slightly gelatinized, cells slightly thick-walled with irregular patches of dark brown exudates in areas of mutual contact, cortical cells in flanks covered by amorphous refractive deep yellow (88.d.Y) granular exudates, at margin some cells protruding like short hairs ( $*6.5\text{--}14 \times 2.5\text{--}3.5 \mu\text{m}$ ). Asexual state unknown.

#### Notes

*Trochila bostonensis* is the only species of the genus found on a member of Apocynaceae (Table 3.2). It was growing in the outer layer of a dead stem of *Asclepias syriaca*, which had fallen on the ground. The host was close to the shore in a shrubby thicket of *Rhus*. There are two similar species. *Trochila laurocerasi* has wider asci ( $6.0\text{--}8.0 \mu\text{m}$  vs.  $4.5\text{--}6.0 \mu\text{m}$ ) and larger ascospores ( $6.3\text{--}10 \times 2.5\text{--}4.6 \mu\text{m}$  vs.  $5.8\text{--}7.2 \times 2.6\text{--}2.8 \mu\text{m}$ ) compared to *T. bostonensis*. Ascus and ascospore length are similar in *T. bostonensis* and *T. craterium*, although ascospores are slightly larger in *T. craterium*. The two species mostly differ in the width of their asci ( $7\text{--}12 \mu\text{m}$  in *T. craterium* vs.  $4.5\text{--}6.0 \mu\text{m}$  in *T. bostonensis*). We used the measurements in dead state to compare *T. bostonensis* with other species in the genus (see Table 3.2).



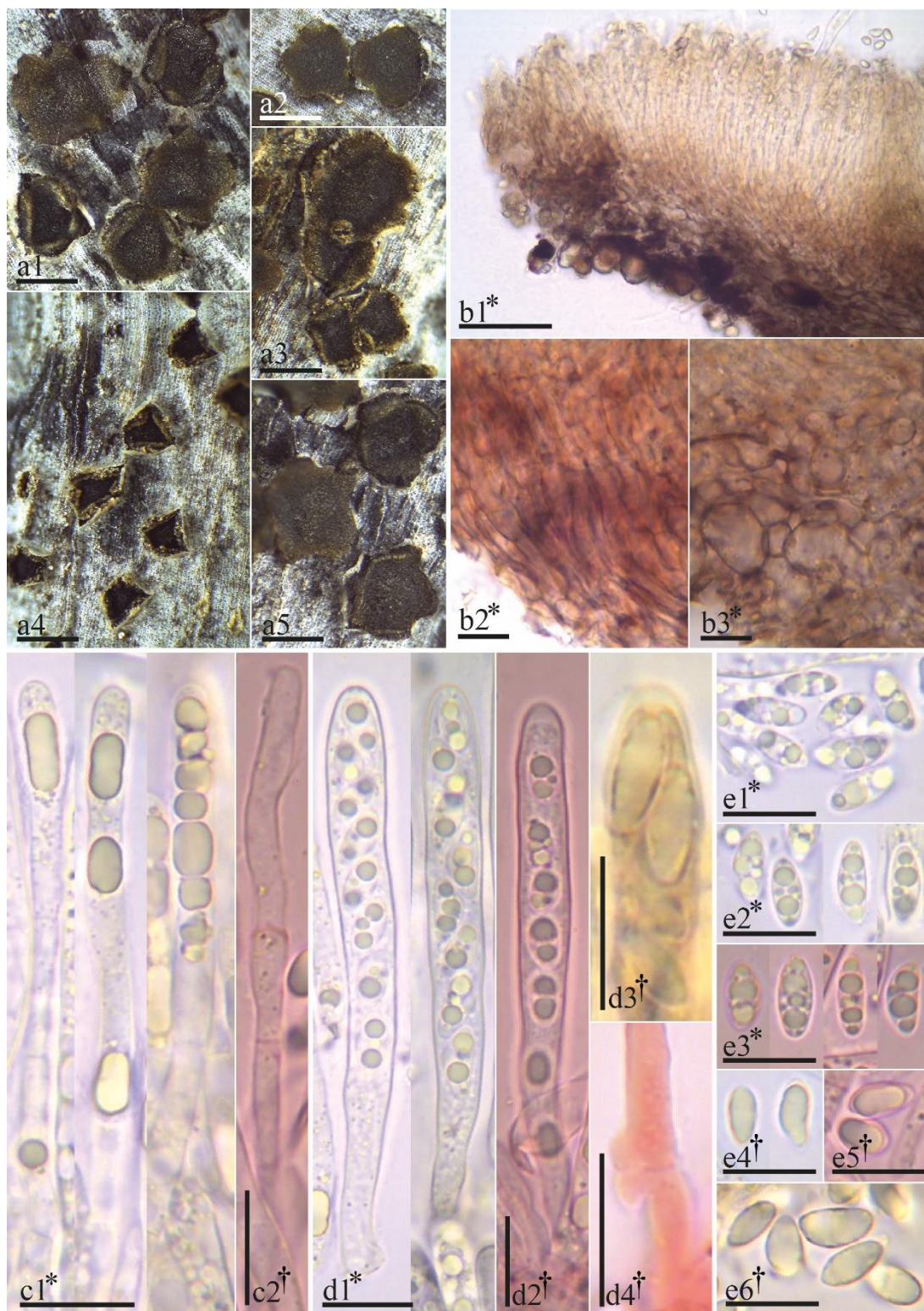


Figure 3.3 Morphological features of *Trochila bostonensis* (holotype collection FH:BHI-F0974) a1–3, a5 fresh apothecia a4 dried apothecia b1 excipular tissues in median section b2 cells at the base b3 cells at the upper and lower flank c1, c2 paraphyses d1, d2 asci d3 ascus pore with inamyloid reaction d4 crozier at ascus base e1–e6 ascospores. Mounted in: Congo Red (c2, d2, d4, e3, e5), H<sub>2</sub>O (b1–b3, c1, d1, e1, e2), KOH (e4), MLZ (d3, e6). Scale bars: 500 μm (a1–a5); 50 μm (b1); 10 μm (b2, b3, c1, c2, d1–d4, e1–e6).

Table 3.2 Comparative table of currently accepted species of *Trochila* (except *T. viburnicola*). For each species, the following characters are presented: host plant, host family, measurements of asci and ascospores (dead state). The asterisk (\*) indicates a fungal host.

Species	Host Plant	Host Family	Asci (µm)		Ascospores (µm)		Reference
			Length	Width	Length	Width	
<i>T. andromedae</i>	<i>Andromeda polifolia</i>	Ericaceae	80	12	15–18	4–5	Karsten (1871)
<i>T. astragali</i>	<i>Astragalus glycyphyllos</i>	Fabaceae	50–60	6–7	8	4	Rehm (1896)
<i>T. atrosanguinea</i>	<i>Carex rigida</i>	Cyperaceae	45–68	7–8	7–8	2–3	Rostrup (1885)
	<i>Carex vulgaris</i>	Cyperaceae					
<i>T. bostonensis</i>	<i>Asclepias syriaca</i>	Apocynaceae	(50.5)57–65.5(66)	(4.5)5–6	(5.8)6.2–7.2	2.6–2.8	This study
<i>T. chilensis</i>	<i>Lardizabala bitemata</i>	Lardizabaleae	70–80	8–9	14–15	4	Spegazzini (1910)
<i>T. cinerea</i>	<i>Pyrola</i> sp.	Ericaceae	no data	no data	6–7	1.5	Patouillard (1886)
<i>T. colensoi</i>	<i>Cordyline</i> sp.	Asparagaceae	60–70	8–10	9–12.5	3.5–5	Dennis (1961)
<i>T. conioselini</i>	<i>Conioselinum</i> sp.	Apiaceae	38–40	6–7	10–13	3	Rostrup (1886)
	<i>Gmelina</i> sp.	Apiaceae					
<i>T. craterium</i>	<i>Cassiope tetragona</i>	Araliaceae	50–60	8–12	6–8	4–5	Rehm (1896)
	<i>Hedera algeriensis</i>	Araliaceae	no data	7	6–8.2	3–4.5	Greenhalgh and Morgan-Jones (1964)
	<i>Hedera helix</i>	Araliaceae					
<i>T. epilobii</i>	<i>Epilobium angustifolium</i>	Onagraceae	75–95	17–20	15–17	8	Karsten (1871)
<i>T. exigua</i>	<i>Nardus stricta</i>	Poaceae	32	6	8–10	0.8	Rostrup (1888)
<i>T. fallens</i>	<i>Salix</i> sp.	Salicaceae	50–60	7–9	9–14	3.5–4.5	Karsten (1871)
<i>T. ilicina</i>	<i>Ilex aquifolia</i>	Aquifoliaceae	75–80	9–10	9–11	3.5–4.5	Rehm (1896)
	<i>Ilex aquifolium</i>	Aquifoliaceae	60–76	8.5–10	10–12.5	3.5–4.5	Greenhalgh and Morgan-Jones (1964)
	<i>Ilex colchica</i>	Aquifoliaceae					
	<i>Ilex platyphylla</i>	Aquifoliaceae					
	<i>Lapageria rosea</i>	Philesiaceae	50–70	25	13–14	6–7	Spegazzini (1921)
<i>T. jaffuelii</i>	<i>Juncus compressus</i>	Juncaceae	40–45	5–6	8–9	1–1.5	Rostrup (1886)
<i>T. laurocerasi</i>	<i>Laurocerasus officinalis</i>	Rosaceae	45–60	8–9	7–10	3.5–4	Rehm (1896)
	<i>Photinia serrulata</i>	Rosaceae	50–65	6–9	7.5–10	3–3.75	Greenhalgh and Morgan-Jones (1964)
	<i>Prunus laurocerasus</i>	Rosaceae					
	<i>Prunus lusitanica</i>	Rosaceae					
	<i>Nectandra rigida</i>	Lauraceae	45–50	7	8–9	3	Rehm (1909)
<i>T. majalis</i>	<i>Fagus sylvatica</i>	Fagaceae	38–45	7–8	7–9	3–3.5	Kirschstein (1944)

Table 3.2 continued

<i>T. molluginea</i>	<i>Galium molluginis</i>	Rubiaceae	55–60	7	10–12	2.5	Mouton (1900)
<i>T. oleae</i>	<i>Olea europaea</i>	Oleaceae	no data	no data	no data	no data	Fries (1849)
<i>T. oxycoccos</i>	<i>Vaccinium oxycoccos</i>	Ericaceae	60–70	11–14	14–18	5	Karsten (1871)
<i>T. perexigua</i>	<i>Hippophae rhamnoides</i>	Elaeagnaceae	80	15	14	7	Spegazzini (1881)
<i>T. perseae</i>	<i>Persea lingue</i>	Lauraceae	50–60	10	9–10	3	Spegazzini (1910)
<i>T. plantaginea</i>	<i>Plantago major</i>	Plantaginaceae	42–50	12–16	18–25	4–4.5	Karsten (1871)
<i>T. prominula</i>	<i>Juniperus sabina</i>	Cupressaceae	65–70	10–12	18–20	6	Saccardo (1878)
<i>T. puccinioidea</i>	<i>Carex</i> sp.	Cyperaceae	no data	no data	no data	no data	De Notaris (1863)
<i>T. ramulorum</i>	<i>Viburnum opulus</i>	Viburnaceae	40–55	5.5–7	5–7	1.5–2	Feltgen (1903)
<i>T. rhodiolae</i>	<i>Rhodiola</i> sp.	Crassulaceae	40	5–6	10	1–1.5	Rostrup (1891)
<i>T. staritziana</i>	<i>Ailanthus glandulosa</i>	Simaroubaceae	no data	no data	no data	no data	Kirschstein (1941)
	<i>Rhus glabra</i>	Anacardiaceae					
<i>T. substricta</i>	<i>Solidago virgaurea</i>	Asteraceae	60	9	12–14	6	Rehm (1884)
<i>T. symploci</i>	<i>Symplocos japonica</i>	Symplocaceae	65–85	5–7	8–11	4–5	Hennings (1900)
<i>T. tami</i>	<i>Tamus communis</i>	Dioscoreaceae	40–55	6–7	5–8	2.5–4	Grelet and de Crozals (1928)
<i>T. tetraspora</i>	<i>Nothofagus dombeyi</i>	Nothofagaceae	58–72	7.7–9.6	12–15	3.4–4.8	Gamundí et al. (1978)
<i>T. urediniophila</i>	<i>Cerotelium fici</i> *	Phakopsoraceae*	(86.4)102.4– 111.2(121.8)	(9.1)10.5– 11.6(13.1)	(7.6)9.0– 9.7(10.9)	(5.1)6.3– 7.1(8.1)	This study
<i>T. xishuangbanna</i>	no data	no data	55–60	3.5–4	8–11	1.2–1.7	Zhuang et al. (1990)
<i>T. winteri</i>	<i>Drymis Winteri</i>	Winteraceae	40–50	10–12	12–13	5	Spegazzini (1888)

3.6.2.2 *Trochila urediniophila* Gomez-Zap., Haelew. & Aime, sp. nov.

MycoBank No: 836583

Fig. 3.4

Diagnosis

Differs from *Trochila ilicina* in ecological strategy (fungicolous symbiont); sizes of asci (102.4–111.2 × 10.5–11.6 µm), ascospores (9.0–9.7 × 6.3–7.1 µm), paraphyses (3.2–3.6 µm wide); and the inamyloidity of its ascus apex.

Type

Holotype: Reliquiae Farlowiana No. 723; Trinidad and Tobago, Port of Spain, Trinidad, Maraval Valley, ca. 10.5°N, 61.25°W, alt. ±301 m a.s.l., 1 Apr 1912, leg. R. Thaxter, on uredinia of *Cerotelium fici* [as *Phakopsora nishidana*] (Pucciniales, Phakopsoraceae) on the underside of *Ficus maxima* (Rosales, Moraceae) leaves, PUL F27668 (ex-PUR F18316). Ex-holotype sequences: isolate F18316 (3 apothecia, ITS: MT873946, LSU: MT873951).

Etymology

Referring to the intimate association of the fungus with the uredinia of *Cerotelium fici*.

Description

Apothecia protruding from uredinia of *Cerotelium fici*, gregarious in small groups or rarely solitary, discoid to irregular-ellipsoid when crowded, 0.4–1.0 mm diam., subsessile on a broad base, flat to slightly concave at maturity, dark grayish yellow brown (81.d.gy.yBr) to dark grayish brown (62.d.gy.Br), margin marked and lighter than hymenium, light grayish yellow brown (79.l.gr.yBr) to medium yellow brown (77.m.yBr), receptacle concolor with margin and surface slightly pruinose. Asci †(86.4–)102.4–111.2(–121.8) × (9.1–)10.5–11.6(–13.1) µm, 8-spored, cylindrical, †uniseriate; apex rounded to subconical, inamyloid (IKI, KOH-pretreated or not), base arising from croziers. Ascospores †(7.6–)9.0–9.7(–10.9) × (5.1–)6.3–7.1(–8.1) µm, ovoid to ellipsoid, aseptate, hyaline, smooth-walled, guttulate, containing †one to two pale yellow (89.p.Y) to yellow gray (93.y Gray) oil drops (LBs), 2–5 µm diam., OCI = (40–)55.1–66.9(–81)%. Paraphyses cylindrical to slightly or medium clavate-spathulate, unbranched, smooth, septate, hyaline, †(2.3–)3.2–3.6(–4.1) µm wide, apex up to 6.8 µm wide. Medullary excipulum †17.4–79.4



μm thick, textura intricata strong brown (55.s.Br) to deep brown (56.deepBr). Ectal excipulum of textura globulosa–angularis at base and lower flanks, strong yellow brown (74.s.yBr) to dark brown (59.d.Br), †32.8–93.5 μm thick, cells †(7.3–)9.0–10.8(–15.3) × (6.0–)7.5–8.7(–11.5) μm; at upper flanks and margin cells vertically oriented of textura prismatica, 17–34 μm thick, at margin and upper flank cells protruding like short hairs, hyaline, aseptate, cylindrical, †(9.5–)16–20.6(–29.1) × (3.0–)3.9–4.5(–5.8) μm. Asexual state unknown.

#### Notes

*Trochila urediniophila* is the first known fungicolous member of the genus. The specimen described here was discovered during a survey of hyperparasites of rust fungi at PUR. Apothecia of *T. urediniophila* were never observed in direct contact with the plant tissue; instead, they grew directly on the uredinia of *Cerotelium fici* on the underside of *Ficus maxima* leaves. *Trochila ilicina* is most similar to *T. urediniophila*, but *T. urediniophila* differs from *T. ilicina* in its distinctly wider ascospores, larger asci, inamyloid ascus apex, and wider apex of the paraphyses. The uredinia of the host fungus, *C. fici*, become a solidified mass that changes in color from dark orange yellow (72.d.OY) without apothecia of *Trochila* to brownish black (65.brBlack) where apothecia are present.

A second duplicate of the Reliquiae Farlowiana No. 723 is also deposited at PUR (accession PUR F1098). However, no apothecia were present on this specimen, nor could additional specimens of *T. urediniophila* be found on any of the other specimens of *C. fici* housed at PUR. At least eight other duplicates are housed at BPI, CINC, CUP, F, ISC, MICH, and UC (MyCoPortal 2020). It is unknown whether any of them may host *T. urediniophila*.

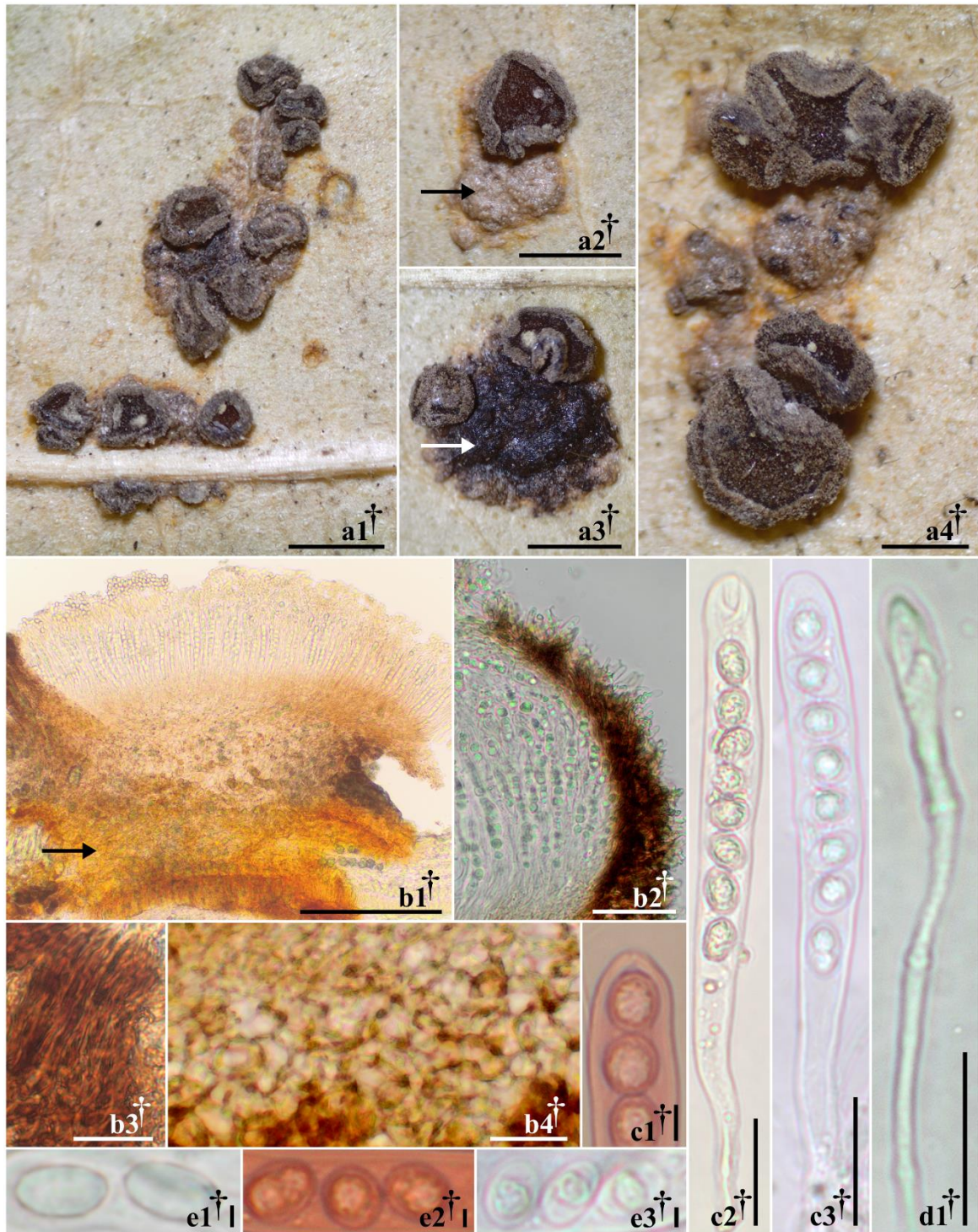


Figure 3.4 Morphological features of *Trochila urediniophila*, holotype collection (PUL F27668) a1–a4 dried apothecia growing on uredinia of *Cerotelium fici* a2, a3 substrate (uredinia) on which the apothecia grow (arrows) b1 transverse section of apothecia; arrow pointing out the substrate b2, b3 details of excipulum at margin and upper flanks b4 cells at base c1–c3 asci d1 paraphyses e1–e3 ascospores e2, e3 oil drops (LBs) inside ascospores. Mounted in: Congo Red (c1, e2), H<sub>2</sub>O (b2, c3, d1, e1, e3), KOH (b1, b3, b4, c2). Scale bars: 1 mm (a1–a3); 500 μm (a4); 200 μm (b1); 50 μm (b2); 20 μm (b3, b4, c2, c3, d1); 2 μm (c1, e1–e3).

### 3.6.2.3 New combinations

*Trochila colensoi* (Berk.) Quijada, comb. nov.

MycoBank No: 836591

≡ *Cenangium colensoi* Berk., Hooker, Bot. Antarct. Voy. Erebus Terror 1839–1843, II, Fl. Nov.-Zeal.: 201 (1855). [Basionym]

= *Pseudopeziza colensoi* (Berk.) Massee, J. Linn. Soc., Bot. 31: 468 (1896)

#### Notes

*Cenangium colensoi* is described from dead leaves of *Cordyline* sp. (Asparagales, Asparagaceae) in New Zealand (Hooker 1855). The host had been mistakenly reported as *Phormium* (Asparagales, Asphodelaceae) by Berkeley in Hooker (1855) and only recently corrected after re-study of the type collection (Landcare Research 2020). *Cenangium colensoi* was later combined in *Pseudopeziza* and described in more detail by Massee (1896). Both authors commented on the watery-grey disc and brownish receptacle of the apothecia. The apothecia develop among the rigid vascular bundles of the epidermis, first covered by the cuticle, then erumpent and opening by a narrow slit, becoming discoid when mature (Hooker 1855; Massee 1896). The habit of this fungus fits well with typical macromorphological features of the genus *Trochila* – a dark brown to black receptacle, which develops beneath the host tissues and eventually becomes erumpent to expose the hymenium by splitting along radial lines or by its splitting into lobes (von Höhnelt 1917; Greenhalgh and Morgan-Jones 1964; Dennis 1978; Baral and Marson 2005). Microscopically, *P. colensoi* was described with a parenchymatous excipulum (angular-globose or isodiametric cells), hyaline under the hymenium and dark brown at the cortex (Berkeley in Hooker 1855; Massee 1896), which is also in agreement with the excipular features of *Trochila* species. Finally, the hymenium of *P. colensoi* was described as composed of inamyloid, 8-spored asci with elliptical hyaline ascospores and slender paraphyses (op. cit.).

In 2018, P.R. Johnston collected two specimens (PDD:112240, PDD:112242, Landcare Research 2020) on leaves of *Cordyline australis* (Asparagaceae). The morphology, ecology (host), and locality of these new collections agree with *P. colensoi*. The photographs of both specimens reveal

features such as guttules in ascospores and paraphyses, protruding hyaline cells in the cortical layer of the upper flank and margin, and hyaline gelatinized hyphae covering the dark globose-angular cells of the ectal excipulum at the base and lower flanks. The latter excipular feature of the receptacle is reminiscent of Zhuang's (1990) description of *Calycellinopsis xishuangbanna*. An ITS sequence of this species was generated from the recent material (PDD:112240) and included in the Leotiomycetes-wide ITS phylogeny of Johnston et al. (2019). Their results and those in this study (Figs 3.1, 3.2) show that *P. colensoi* is placed among species of *Trochila*.

*Trochila xishuangbanna* (W.Y. Zhuang) Quijada, comb. nov.

MycoBank No: 836592

≡ *Calycellinopsis xishuangbanna* W.Y. Zhuang, Mycotaxon 38: 121 (1990). [Basionym]

#### Notes

The genus *Calycellinopsis* was proposed with a single species, *C. xishuangbanna*, which is a petiole-inhabiting fungus (Zhuang 1990). The genus was placed in Dermateaceae because of its isodiametric dark brownish excipular cells (Zhuang 1990). In 2002, a second collection of the same species was sampled (HMAS:187063), which was sequenced (Zhuang et al. 2010). Additional morphological details were provided, and the genus was placed in Helotiaceae (Zhuang et al. 2010). *Trochila* was treated in Dermateaceae until recently because of its excipular features (Fuckel 1869; Karsten 1869; Saccardo 1884; Lambotte 1888; Lumbsch and Huhndorf 2010). Collections of *Calycellinopsis* have a well-developed excipulum, with an outer layer of angular to isodiametric cells with brownish walls and cortical cells at flanks and margin with protruding hyaline cells. The medullary excipulum is subhyaline and composed of textura angularis to textura intricata (Zhuang 1990; Zhuang et al. 2010).

Species in *Trochila* usually have a poorly developed excipulum. For example, *T. bostonensis* and *T. craterium* produce only a thin layer of globose to angular dark excipular cells (von Höhnelt 1917; Greenhalgh and Morgan-Jones 1964; Baral and Marson 2005). However, other species, such as *T. laurocerasi* and *T. urediniophila*, have a well-developed excipulum (op. cit.). The excipulum of *Calycellinopsis* is very similar to those species of *Trochila* with a well-developed excipulum, composed of an outer layer of dark textura globulosa-angularis and an inner layer of hyaline

medulla made of textura angularis–porrecta–intricata. At the flanks and margin of the excipulum, *Calycellinopsis* has protruding hyaline cells similar to *Trochila* species with a well-developed excipulum (Fig. 3.4). Although limited details about the living features can be obtained from the original description of *Calycellinopsis*, its hymenial features are consistent with *Trochila*. The ascospores of *Calycellinopsis* are described with several guttules, a feature that is also observed in species of *Trochila*.

### 3.7 Discussion

#### 3.7.1 Taxonomy of *Trochila*

This study represents the first attempt to investigate the systematics of *Trochila* using both morphological features and DNA sequences. We have added four species to *Trochila*, bringing the total number of species described in the genus to 37. Most *Trochila* species have been delimited based on the size of asci and ascospores, but we find that amyloidity of ascus apex, excipular features, details of the paraphyses, and presence vs. absence of guttules are also diagnostic (Table 3.2). For this study, we also applied a two-dataset approach for phylogenetic analyses (e.g., Aime and Phillips-Mora 2005; Haelewaters et al. 2019). Our phylogenetic reconstruction of a six-locus dataset resolved *Trochila* as polyphyletic with respect to *C. xishuangbanna* and *P. colensoi* (Fig. 3.1). Because morphological data of these two taxa agree with *Trochila*, we recombined them in this genus. The second, two-locus dataset was used for species delimitation, which showed *T. bostonensis* and *T. urediniophila* as distinct from the other *Trochila* species. Our molecular phylogenetic results (Figs 3.1, 3.2) and morphological comparisons of *Trochila* species (Table 3.2) will facilitate future taxonomic studies in the genus.

#### 3.7.2 Host associations

Thus far, members of *Trochila* have been reported from 31 families of both monocots and dicots (Table 3.2). In this study, we add two plant family hosts, Apocynaceae (for *T. bostonensis*) and Asparagaceae (for *T. colensoi*). In addition, we reveal a new ecological niche (for *T. urediniophila*) – a species that associates with uredinia of the rust species *Cerotelium fici*. This sample was collected in 1912 as a rust specimen and deposited in the Arthur Fungarium (PUR) at Purdue University. More than a century later, the exsiccatae sample was scanned for the presence of

hyperparasites of rust fungi from South America. Apothecia of *T. urediniophila* were found exclusively on uredinia without any direct contact with the host plant. Due to the age and limited available material, ultrastructural examinations of the interaction between these two fungi could not be made. However, *T. urediniophila* is the first species in the genus that fruits exclusively from another fungus, hinting at more complex associations among *Trochila* species and other fungi on which they might act as mycoparasites.

### **3.7.3 *Trochila* in the Neotropics**

South America is known to be one of the most biodiverse continents in the world (Dourojeanni 1990; Hawksworth 2001). However, its fungal communities are thought to be severely understudied (Mueller and Schmit 2007). Members of *Trochila* are no exception to this. Six species of *Trochila* have been described from South America. These are *T. chilensis* Speg., *T. jaffuelii* Speg., and *T. perseae* Speg. from Chile; *T. leopoldina* Rehm from Brazil; and *T. tetraspora*, and *T. winteri* Speg. from Argentina (Spegazzini 1888, 1910, 1921; Rehm 1909; Gamundí et al. 1978). Their type collections need to be re-examined to determine if these species are in fact members of *Trochila*. One of our new species, *T. urediniophila*, was collected in Port of Spain, Trinidad. Little data are available regarding the Funga (sensu Kuhar et al. 2018) of Trinidad and Tobago (Baker and Dale 1951; Dennis 1954a, b). The most recent work on the fungal diversity from this country was published online (Jodhan and Minter 2006) derived from reference collections and data from scientific literature. Based on the available literature, no records of *Trochila* are known in Trinidad. As a result, *T. urediniophila* represents the first published report of the genus from Trinidad, and by extension from the Caribbean (Minter et al. 2001).

*Trochila* species are likely more broadly distributed than generally thought, and certainly not limited to the Northern Hemisphere. This is often the case for many fungi that are based on limited regional collecting and thus may not represent the full extent of their distributional ranges due to, for example, the lack of studies in subtropical and tropical ecosystems (Groombridge 1992; Hawksworth and Mueller 2005; Mueller and Schmit 2007; Aime and Brearley 2012; Cheek et al. 2020).

### 3.7.4 The importance of biological collections

Our work emphasizes the importance of specimens preserved in biological collections – such as fungaria and herbaria – for studies of biodiversity and applied biological sciences, and for climate change research (Hawksworth and Lücking 2017; Andrew et al. 2019; Lang et al. 2019; Ristaino 2020; Wijayawardene et al. 2020). Because of the well-preserved specimens deposited at PUR, the genus *Trochila* is now known to be present in Trinidad and to form fungicolous associations. Another interesting example of the use of collections is *Trochila colensoi*. Known only from the type specimen for more than 100 years, additional specimens were only reported following the correction of the host substrate (as *Cordyline* rather than *Phormium*), which was based on re-examination of the type specimen preserved at K. Biological collections are not only important for morphological studies, but also as sources of genetic and genomic information (Bruns et al. 1990; Brock et al. 2009; Redchenko et al. 2012; Dentinger et al. 2016; this study). The single-oldest fungal specimen used for DNA extraction and sequencing was the type of *Hygrophorus cossus* (Sowerby) Fr. (Agaricales, Hygrophoraceae), collected in 1794 and deposited at K (Larsson and Jacobsson 2004). Our material of *T. urediniophila* gathered by Roland Thaxter in 1912 proves again that old samples can be used successfully for modern molecular phylogenetic analyses.

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## **CHAPTER 4. SPECIES DIVERSITY, HOST RANGE AND BIOGEOGRAPHY OF *MYCODIPLOSIS* LARVAE (DIPTERA, CECIDOMYIIDAE); A FLY INSECT INTIMATELY ASSOCIATED WITH RUST FUNGI (BASIDIOMYCOTA, PUCCINIALES)**

### **4.1 Introduction**

*Mycodiplosis* (Diptera: Cecidomyiidae) is a fly genus erected by Rübsaamen in 1895 to accommodate fungus-feeding midge species (Rübsaamen, 1895). *Cecidomyia coniophaga*, described by Winnertz in 1853, was the first species listed by Rübsaamen and placed as the type species of the genus *Mycodiplosis* (Winnertz, 1853). Currently known as *Mycodiplosis coniophaga*, this species was found feeding on rust spores of *Phragmidium mucronatum* (Pers.) Schltdl., on leaves of *Rosa* sp., in Germany. Kieffer, in 1913, listed 48 species of *Mycodiplosis* in his monograph of Cecidomyiidae (Kieffer, 1913). Later, Holz, in 1970, wrote a thorough systematic revision of the genus *Mycodiplosis* and also listed the *Mycodiplosis* species accepted at that time (Holz, 1970). There are 49 currently accepted *Mycodiplosis* species, most of which feed on rust or mildew fungi (Gagné & Jaschhof, 2021; Kolesik et al., 2022).

Species identification of members of *Mycodiplosis* is a complex task. All members are identified based on the morphology of the adult male, whose body length is usually less than 2 mm (Gagné & Jaschhof, 2021; Rübsaamen, 1895). Since these flies are fragile and difficult to manipulate, several adult males are required for correct species identification (Gagné, personal communication, November 5, 2018). In addition, molecular data are scarce. There are currently four *Mycodiplosis* species with molecular data reported in the NCBI nucleotide database (<http://ncbi.nlm.nih.gov/blast/Blast.cgi>) out of the 49 currently accepted species, which restricts any species identification through DNA barcoding or phylogenetic analysis of the genus.

The life cycle of *Mycodiplosis* is short and lasts about two weeks. Adults feed little and have a short life. The primary role of adults is to mate and lay eggs on the host plant. Females emerge from the pupal stage in the soil with their eggs fully developed. The larval stage is the one that lasts longer in the life cycle and does most of the feeding (Gagné, 1994). Larvae are predominantly

fungivorous, feeding on spores of several rust species (Pucciniales, Basidiomycota) and powdery mildews (Erysiphales, Ascomycota).

Rust fungi and powdery mildews are plant pathogens that cause diseases to economically important plants. Rust fungi can also cause diseases in native plants threatening extinction, as is occurring in Australian ecosystems (Fensham & Radford-Smith, 2021; Fernandez Winzer et al., 2018). Members of both orders are obligate pathogens, meaning they cannot grow in artificial culture media, but only on their host plant (Hückelhoven, 2005; Kolmer et al., 2009). Fungicides are commonly used to control these fungi, but some species are becoming resistant to these recurrent chemical applications, especially for rust diseases (Godoy, 2011; Kikuhara et al., 2019; Simões et al., 2018). Thus, natural enemies such as *Mycodiplosis* may offer another alternative to control these fungal diseases. However, Molecular identification, host range, and other characterization studies for *Mycodiplosis* are unknown. Hence, the insufficient knowledge of this insect refrains its use as a biological control agent and part of integrated pest management (IPM) of fungal diseases.

*Mycodiplosis* is considered a cosmopolitan genus. However, most *Mycodiplosis* species have a restricted geographical record. Records of *Mycodiplosis* species are typically from the Northern Hemisphere in Asia, Europe, and North America, most of which are predominantly from Asia and Europe. In contrast, only six out of 49 species of *Mycodiplosis* have been reported in the Neotropics (Gagné & Jaschhof, 2021). *Mycodiplosis coniophaga* is the only species that seems to be widely distributed in several European countries, Mexico, The United States, and Egypt (Gagné & Jaschhof, 2021). However, most of these records were published before 1970. Since then, few other records of known *Mycodiplosis* species have been published, and four new species have been described (*M. ampla*, *M. constricta*, *M. puccinivora*, and *M. vernalis*) (Jiao et al., 2019; Kolesik et al., 2022; Plakidas, 2019). From these, *M. vernalis* is the only one reported from the Americas in the United States. Thus, besides the scarce characterization studies of the genus *Mycodiplosis*, information on this natural enemy is even less in the Americas.

This study aims to fill a knowledge gap in characterization studies of *Mycodiplosis* larvae that feed on rust fungi, emphasizing the Americas. Phylogenetic relationships of *Mycodiplosis* members,

specificity range for host rust species, and geographical distribution are some aspects we will cover. The results of this study are intended to be a baseline for further studies in the biological control of rust diseases to be implemented in IPM of rust diseases.

## **4.2 Materials and Methods**

### ***4.2.1 Collected samples from PUR collections and field trips***

We collected fungivorous larvae, presumably of *Mycodiplosis*, from preserved rust specimens at PUR and on field trips in several regions. When collecting larvae at PUR, we randomly screened rust specimens for the presence of fungivorous larvae by selecting the top rust specimens in each folder. Although we screened rust specimens collected across the globe, we emphasized collections from the Americas to improve knowledge of this geographic region. Each rust-infected leaf of every rust specimen was screened under a stereoscope Olympus Model SZ2-ILST by screening for these larvae. Because fungivorous larvae are often dislodged from the rust sori during sample pressing and processing, we collected any larvae of 2 mm in body size that were present in the herbarium packet. When collecting live fungivorous larvae from field trips (Table 4.1), we first collected rust-infected plant leaves. Then, we screened those specimens under a stereoscope to select larvae feeding directly on rust spores. Four fungivorous larvae were removed at the most from each rust specimen with a fine tweezer and placed individually in microcentrifuge tubes. Before collecting the larvae of another rust specimen, we cleaned the tweezers with 70% ethanol to prevent cross-contamination. Each tube was labeled either with the PUR barcode of the rust specimen or with a serial collection number. Additionally, some fungivorous larvae collected from Celery Bog, Indiana, USA, were brought with rust-infected leaves to the Aime Lab and placed in plastic bags to complete their life cycle and obtain the adults. These adults were sent for morphological identification to Raymond Gagné, a taxonomist expert in the Cecidomyiidae family.

Once we found a fungivorous larva, we manually recorded the following information for each rust specimen: the PUR barcode, year of collection, rust species name, host plant, country of origin, and rust spore stage. We also recorded two videos of fungivorous larvae feeding on rust spores from Peru and Puerto Rico. Finally, we took photographs of some of the collected larvae with an

Olympus SC30 camera and image software Olympus cellSens entry version 1.14 under a stereoscope Olympus Model SZ2-ILST at PUR.

Table 4.1 Field sites screened for natural enemies of rust fungi

Site	System	Year of collection
National Forest El Yunque , Puerto Rico, USA	Natural	2018
Celery Bog, Indiana, USA	Natural	2018
Beck Agricultural Center, Indiana, USA	Wheat crop	2021
Southwest Purdue Agricultural Center, Indiana, USA	Maize crop	2022
Coffee farms, Hawaii, USA	Coffee crop	2022
Calabria, Italy	Natural	2018
Peru	Natural	2019

#### ***4.2.2 Species identification of fungivorous larvae feeding on rust fungi***

Species identification of fungivorous insects is traditionally based on the morphological traits of the adult male (Gagné & Jaschhof, 2021). Since the adult does not feed on rust fungi, this stage is usually absent in preserved rust specimens. Thus, the only possibility to identify dried fungivorous larvae is through DNA sequences. Below we describe the methodology used for species identification of these larvae based on DNA sequences.

##### ***4.2.2.1 DNA isolation of fungivorous larvae feeding on rust fungi***

The genomic DNA from each larva was extracted using InstaGene Matrix catalog #732-6030: DNA preparation from whole blood with several modifications as follows: skip steps 1 through 4 from the manufacturer's protocol. Add a single larva into a microtube. Then, add 50 uL of InstaGene Matrix to the tube containing the larva. Macerate the larva using autoclaved pestles or 200 uL micropipette tips. After that, place the microtube at 56 °C for 30 minutes. Mix the tube to maintain the matrix in suspension and vortex at high speed for 10 seconds. Then, place the tube in a 100 °C heat block for 8 minutes and vortex it at high speed for 10 seconds. Spin the tube at 15,000 rpm for 3 minutes and store it at -20 °C for PCR reaction.

#### 4.2.2.2 PCR amplifications of fungivorous larvae feeding on rust fungi

We amplified several loci of the mitochondrial and nuclear ribosome DNA as follows: the "barcoding" section of the Cytochrome Oxidase unit 1 (CO1) (3' positions 1514-2173), typically used for species delimitation of insects (Folmer et al., 1994); a conserved section of CO1 for mid- to low phylogenetic inferences at genus or family level (3; positions 2090-2395) (Lunt et al., 1996); one section of the mitochondrial large subunit 16S rDNA (Tóthová, 2006); the internal transcribed spacer 1 (ITS1) region of the ribosomal DNA (Dai et al., 2012), and the large subunit (LSU) of the ribosomal DNA (Belshaw et al., 2001). Their respective primer pairs are referenced in Table 4.2. We conducted all 25- $\mu$ l PCR reactions on a Mastercycler ep gradient Thermal Cycler (Eppendorf model #5341, Hauppauge, New York) that consisted of 12.5  $\mu$ l of 2 $\times$  MyTaq Mix (Bioline, Swedesboro, New Jersey), 1.25  $\mu$ l of each 10  $\mu$ M primer, and 10  $\mu$ l of either 1/10 diluted DNA extract. Amplifications were run under the following conditions: initial denaturation at 94°C for 2 min (4 min for conserved CO1 region, 1 min for 'barcoding' CO1); followed by 40 cycles of denaturation at 94°C for 30 sec (20 sec for ITS1, 95°C for 1 min for 'barcoding CO1', and 95°C for 40 sec for conserved CO1 region), annealing at 40°C for 1:30 min for 'barcoding CO1' / 41°C for 1 min for conserved CO1 region / 53°C for 30 sec for Mit16S / 51°C for 30 sec for ITS1/ 50°C for 30 sec for LSU; and elongation at 72°C for 1:30 min for 'barcoding' CO1/ 67°C for 1 min for conserved CO1 region/ 15 sec for ITS1/ 1 min for LSU); and final extension at 72°C for 7 min (8 min for conserved CO1 region/ 10 min for ITS1).

Table 4.2 Primers used to amplify mitochondrial and nuclear DNA fragments

Gene	Primer name	Sequences (5' to 3')	Reference
<b>Barcoding CO1 region</b>	LCO1490	GGTCAACAAATCATAAAGATATTGG	Folmer et al., 1994
	HCO2198	TAAACTTCAGGGTGACCAAAAATCA	Folmer et al., 1994
<b>Conserved CO1 region</b>	UEA5	AGTTTTAGCAGGAGCAATTACTAT	Lunt et al., 1996
	UEA6	TTAATWCCWGTWGGNACNGCAATRATTAT	Lunt et al., 1996
<b>Mit16S</b>	CER-1	TAATCCAACATCGAGGTC	Tóthová, 2006
	CER-3	CGAAGGTAGCATAATCAGTAG	Tóthová, 2006
<b>ITS1</b>	18SF1	TACACACCGCCCGTCGCTACTA	Dai et al., 2012
	5.8SB1d	ATGTGCGTTTCRAAATGTCGATGTTCA	Dai et al., 2012
<b>LSU</b>	D2	AGAGAGAGTTCAAGAGTACGTG	Belshaw et al., 2001
	D3R	TAGTTCACCATCTTTCGGGTC	Belshaw et al., 2001

#### *4.2.2.3 Electrophoresis and sequencing*

We ran the PCR products in 1% agarose and stained them with GelRed (RGB4102, Phoenix Research Products) for 35 min at 110 V in a Bio-Rad electrophoresis tank to visualize PCR products. PCR products of samples that showed bands were sent to Genewiz (South Plainfield, New Jersey) for purification and subsequent sequencing in both directions. Raw sequence reads were edited manually and assembled using Sequencher version 5.2.3 (Gene Codes Co., Ann Arbor, Michigan).

#### *4.2.3 Sequence alignment and phylogenetic trees*

The edited sequences were blasted against the NCBI GenBank nucleotide database (<http://ncbi.nlm.nih.gov/blast/Blast.cgi>) to search for the closest relative species. We downloaded reference sequences from peer-reviewed articles that included morphological descriptions and phylogenetic analyses of fungivorous larvae of rust fungi (Table 4.3). We also downloaded DNA sequences of selected genera in the Cecidomyiidae family (Dorchin et al., 2019) to use them as outgroups for our multi-locus phylogenetic analysis. Sequences were aligned using MUSCLE version 3.7 (Edgar, 2004) in MEGA7 (S. Kumar et al., 2016). Then, the aligned sequences were trimmed using trimAl version 1.2 (Capella-Gutiérrez et al., 2009) with a minimum percentage of positions to conserve [0-100]: 50, and gap threshold, the fraction of positions without gaps in a column [0-1]: 0.6. We performed maximum likelihood (ML) inference using IQ-TREE (Minh et al., 2020) under partitioned models (Chernomor et al., 2016) and selected the best nucleotide substitution model under Akaike's information criterion corrected for small sample size (AICc) using ModelFinder using ModelFinder (Kalyaanamoorthy et al., 2017). An ultrafast bootstrap analysis was implemented with 1,000 replicates (Hoang et al., 2018). The "-bnni" option was used to reduce the risk of overestimating branch supports with UFBoot due to severe model violations. Finally, phylogenetic reconstructions with bootstrap values were visualized in FigTree version 1.4.3 (<http://tree.bio.ed.ac.uk/software/figtree/>) and colored in Inkscape (<https://inkscape.org>).

#### *4.2.4 Geographical distribution*

The localities of fungivorous larvae with successfully amplified gene regions were used to build a geographic map and observe their distributions. We used the GPS coordinates of each of these

fungivorous larvae when present. Otherwise, we generated approximated coordinates according to the locality description following a geocoding Python Script in the GitHub repository (Lynn, 2017). We plotted the geographic data of each larva on a map and colored each point by the clades formed in the multi-locus phylogenetic tree using the package Geopandas in Python (Jordahl, 2014).

## 4.3 Results

### 4.3.1 *Mycodiplosis* dataset

Of the 5,618 rust specimens screened, we collected 335 individual fungivorous larvae on 287 rust specimens (see Supplementary Table S2). From these larvae, we successfully amplified several gene regions of 131 larval specimens confirmed to be in the Cecidomyiidae family or genus *Mycodiplosis* (Table 4.3) when blasted against the NCBI nucleotide database (<http://ncbi.nlm.nih.gov/blast/Blast.cgi>). Although we successfully amplified the five gene regions for some larvae, it was not possible to get all DNA fragments for all of them. We successfully amplified 279 DNA sequences of 131 *Mycodiplosis* specimens collected from preserved rust specimens at the Arthur Fungarium and live collections from field trips in the Americas. Sixty-one DNA sequences are from the barcoding CO1 gene, 57 are from the conserved CO1 region, 91 are from mit16S, 34 are from ITS1 rDNA, and 36 are from LSU rDNA. 66% of these *Mycodiplosis* specimens were collected from preserved rust specimens at PUR, while 16% were larvae collected from the coffee leaf rust, *Hemileia vastatrix*, in coffee farms in Hawaii. The oldest larva we successfully amplified one gene region (mit16S) was collected in 1890 on *Phakopsora cherimoliae* in Ecuador (PUR voucher: F8883). However, despite the large number of larval sequences we obtained from the preserved specimens at PUR, we could not identify 104 individual larvae at the genus level due to the dried or scarce material available.

Furthermore, we recorded two videos of live larvae to provide evidence of their fungivory behavior. One video was recorded in 2018 from a larva feeding on uredospores of *Coleosporium* sp., in Puerto Rico (PUR voucher: N23057). The other video was from a larva feeding on uredospores of *Puccinia* sp., from Peru in 2019. These videos clearly show how both larvae feed exclusively on rust spores and not on plant tissue. Both larvae have 2 mm body size length approximately and are the same color as the rust spores they feed. They are legless, cylindrical, and tapered at both ends.



Their head is small and robust but has articulated mandibles that grasp several rust spores to ingest them through the esophagus (watch the videos in Supplementary data).

We obtained five adults from the live larvae we collected at Celery Bog, Indiana, USA, and brought them to Aime Lab to complete their life cycles. According to Raymond Gagné, a taxonomist expert in the Cecidomyiidae family, the specimens consisted of one adult male and female of *Mycodiplosis* sp., and two adult males and one female of *Lestodiplosis* sp. *Lestodiplosis* are predators as larvae and may have been present on rust-infected plant leaves by feeding on other Cecidomyiidae larvae, including *Mycodiplosis* (Gagné & Jaschhof, 2021). Due to the scarce adult males we obtained, it was impossible to use morphology to identify the *Mycodiplosis* specimens at the species level. Since adult males measure 2 mm in body length on average, it is necessary to collect several of them for a good slide mounting and compare their morphological traits to specimens of known *Mycodiplosis* species in Gagné's collection. As previously mentioned, larvae and females are not identifiable. Finally, the genomic DNA of this larva was also extracted, and two loci were successfully amplified: the barcoding CO1 region and mit16S. Although we did not include the DNA sequences of this larva in our multi-locus phylogenetic tree, they can be found in the Supplementary data together with all the sequences used in this study (voucher: PAGZ1\_1).

Table 4.3 Sequences used in phylogenetic analyses. Reference sequences in bold red; the asterisk (\*) indicates available DNA sequences obtained from this study; a dagger (†) indicates outgroups; NA: data not available.

Isolate	Species	Host Rust	CO1 mitDNA. Primers: LCO1490/ HCO2198	CO1 mitDNA. Primers: UEA5/UEA6	16S mitDNA	ITS rDNA	LSU rDNA	Reference
PURN11585	<i>M. coniophaga</i>	<i>Melampsora epiphylla</i>			*			This study
PURN5750-1	<i>M. coniophaga</i>	<i>Melampsora larici-populina</i>			*			This study
PURN12029-4	<i>M. coniophaga</i>	<i>Melampsora</i> sp.			*			This study
PURN15118-1	<i>M. coniophaga</i>	<i>Melampsora</i> sp.		*	*	*	*	This study
PURN12557-1	<i>M. coniophaga</i>	<i>Melampsora warburgii</i> aff.			*			This study
PURN12557-2	<i>M. coniophaga</i>	<i>Melampsora warburgii</i> aff.		*	*	*		This study
<b>Myccon70</b>	<b><i>M. coniophaga</i></b>	Several rust species	<b>MN191325.1</b>		<b>MN20151 3.1</b>	<b>MN20140 7.1</b>	<b>MN201284.1</b>	Dorchin et al., 2019
PURN22989	<i>M. melampsorae</i>	<i>Melampsora</i> sp.			*			This study
PURN22990	<i>M. melampsorae</i>	<i>Melampsora</i> sp.			*			This study
Italy-1L	<i>M. melampsorae</i>	<i>Melampsora gelmii</i>		*	*	*	*	This study
<b>2606509</b>	<b><i>M. puccinivora</i></b>	<i>Maravalia pterocarpi</i>	<b>MK511238.1</b>					Jiao & Kolesik, 2019
PURN15091-2	<i>M. puccinivora</i>	<i>Melampsora</i> sp.			*			This study
PURN15091-3	<i>M. puccinivora</i>	<i>Melampsora</i> sp.			*	*	*	This study
PURN15118-2	<i>M. puccinivora</i>	<i>Melampsora</i> sp.		*	*			This study
PURN15118-3	<i>M. puccinivora</i>	<i>Melampsora</i> sp.		*	*	*		This study
PURN11116-1	<i>M. puccinivora</i>	<i>Puccinia sorghi</i>	*	*	*	*	*	This study
PURN11116-2	<i>M. puccinivora</i>	<i>Puccinia sorghi</i>	*	*	*	*	*	This study
PURN11116-3	<i>M. puccinivora</i>	<i>Puccinia sorghi</i>	*	*	*	*	*	This study
larvawheat1	<i>M. puccinivora</i>	<i>recondita</i> f. sp. <i>tritici</i>	*	*	*	*	*	This study
<b>Mycsph71</b>	<b><i>M. sphaerothecae</i></b>	Powdery mildews	<b>MN191326.1</b>					Dorchin et al., 2019
larvacornb1	<i>Mycodiplosis</i> sp.	<i>Puccinia polysora</i>	*	*	*	*	*	This study
PURN23059/PA Z03-3L	<i>Mycodiplosis</i> sp.	<i>Austropuccinia</i> sp.		*	*			This study

Table 4.3 continued

BOG02	<i>Mycodiplosis</i> sp.	<i>Coleosporium solidaginis</i>				*	*	This study
PURN15011	<i>Mycodiplosis</i> sp.	<i>Austropuccinia psidii</i>		*				This study
PURN15011-1	<i>Mycodiplosis</i> sp.	<i>Austropuccinia psidii</i>	*	*	*			This study
PURN15011-2	<i>Mycodiplosis</i> sp.	<i>Austropuccinia psidii</i>			*			This study
PURN15014	<i>Mycodiplosis</i> sp.	<i>Austropuccinia psidii</i>	*	*	*	*	*	This study
PURN15015-3	<i>Mycodiplosis</i> sp.	<i>Austropuccinia psidii</i>		*	*	*	*	This study
PURN7676-1	<i>Mycodiplosis</i> sp.	<i>Austropuccinia psidii</i>			*			This study
PURN7676-2	<i>Mycodiplosis</i> sp.	<i>Austropuccinia psidii</i>		*				This study
PURN7676-3	<i>Mycodiplosis</i> sp.	<i>Austropuccinia psidii</i>			*			This study
BOG03	<i>Mycodiplosis</i> sp.	<i>Coleosporium solidaginis</i>		*				This study
ML320	<i>Mycodiplosis</i> sp.	<i>Cerotelium sabiceae</i>	*	*	*			This study
PURN16391	<i>Mycodiplosis</i> sp.	<i>Chaconia ingae</i>	*	*	*	*	*	This study
PURN16395	<i>Mycodiplosis</i> sp.	<i>Coleosporium plumeriae</i>	*	*				This study
PURN16396	<i>Mycodiplosis</i> sp.	<i>Coleosporium plumeriae</i>			*		*	This study
PURN9648	<i>Mycodiplosis</i> sp.	<i>Coleosporium plumeriae</i>		*	*			This study
BOG08	<i>Mycodiplosis</i> sp.	<i>Coleosporium solidaginis</i>		*	*	*		This study
BOG09	<i>Mycodiplosis</i> sp.	<i>Coleosporium solidaginis</i>	*	*	*	*	*	This study
BOG12	<i>Mycodiplosis</i> sp.	<i>Coleosporium solidaginis</i>		*	*	*	*	This study
BOG16	<i>Mycodiplosis</i> sp.	<i>Coleosporium solidaginis</i>	*		*		*	This study
BOG17	<i>Mycodiplosis</i> sp.	<i>Coleosporium solidaginis</i>			*			This study
BOG21	<i>Mycodiplosis</i> sp.	<i>Coleosporium solidaginis</i>			*			This study
lafayette5	<i>Mycodiplosis</i> sp.	<i>Coleosporium solidaginis</i>	*	*	*	*	*	This study
PAGZ 1-3	<i>Mycodiplosis</i> sp.	<i>Coleosporium solidaginis</i>	*	*	*			This study

Table 4.3 continued

PAGZ 1-4	<i>Mycodiplosis</i> sp.	<i>Coleosporium solidaginis</i>	*	*		*		This study
West Lafayette8	<i>Mycodiplosis</i> sp.	<i>Coleosporium solidaginis</i>	*	*	*	*	*	This study
PURN23057/PA Z01-1L	<i>Mycodiplosis</i> sp.	<i>Coleosporium</i> sp.	*	*				This study
B1_PV5923	<i>Mycodiplosis</i> sp.	<i>Hemileia vastatrix</i>	*					This study
MCA7983-1	<i>Mycodiplosis</i> sp.	<i>Coleosporium</i> sp.	*	*	*			This study
MCA7983-2	<i>Mycodiplosis</i> sp.	<i>Coleosporium</i> sp.	*	*	*	*		This study
MCA7983-3	<i>Mycodiplosis</i> sp.	<i>Coleosporium</i> sp.	*	*				This study
B2_PV5924	<i>Mycodiplosis</i> sp.	<i>Hemileia vastatrix</i>	*					This study
PURN22411/MC A3888	<i>Mycodiplosis</i> sp.	<i>Gymnoconia</i> sp.		*	*			This study
B3_PV5925	<i>Mycodiplosis</i> sp.	<i>Hemileia vastatrix</i>	*					This study
B4_PV5926	<i>Mycodiplosis</i> sp.	<i>Hemileia vastatrix</i>	*					This study
K2_PV5890	<i>Mycodiplosis</i> sp.	<i>Hemileia vastatrix</i>	*					This study
K3_PV5891	<i>Mycodiplosis</i> sp.	<i>Hemileia vastatrix</i>	*					This study
M1midge_PV5888	<i>Mycodiplosis</i> sp.	<i>Hemileia vastatrix</i>	*					This study
O1_PV5927	<i>Mycodiplosis</i> sp.	<i>Hemileia vastatrix</i>	*					This study
O2_PV5928	<i>Mycodiplosis</i> sp.	<i>Hemileia vastatrix</i>	*					This study
O3_HCO_PV5929_25	<i>Mycodiplosis</i> sp.	<i>Hemileia vastatrix</i>	*					This study
O4_PV5930	<i>Mycodiplosis</i> sp.	<i>Hemileia vastatrix</i>	*					This study
O5_PV5931	<i>Mycodiplosis</i> sp.	<i>Hemileia vastatrix</i>	*					This study
P1_PV5917	<i>Mycodiplosis</i> sp.	<i>Hemileia vastatrix</i>	*					This study
P2_PV5918	<i>Mycodiplosis</i> sp.	<i>Hemileia vastatrix</i>	*					This study
P3_PV5919	<i>Mycodiplosis</i> sp.	<i>Hemileia vastatrix</i>	*					This study
P4_PV5920	<i>Mycodiplosis</i> sp.	<i>Hemileia vastatrix</i>	*					This study
P5_PV5921	<i>Mycodiplosis</i> sp.	<i>Hemileia vastatrix</i>	*					This study
P6_PV5922	<i>Mycodiplosis</i> sp.	<i>Hemileia vastatrix</i>	*					This study
W1_PV5892	<i>Mycodiplosis</i> sp.	<i>Hemileia vastatrix</i>	*					This study
W2_PV5893	<i>Mycodiplosis</i> sp.	<i>Hemileia vastatrix</i>	*					This study
PURN11725-1	<i>Mycodiplosis</i> sp.	<i>Hemileia vastatrix</i>	*					This study
PURN11725-3	<i>Mycodiplosis</i> sp.	<i>Hemileia vastatrix</i>		*				This study
PURN11725-4	<i>Mycodiplosis</i> sp.	<i>Hemileia vastatrix</i>	*					This study

Table 4.3 continued

PURN15328/ PURF3514	<i>Mycodiplosis</i> sp.	<i>Hemileia vastatrix</i>	*	*					This study
PURN22448-4	<i>Mycodiplosis</i> sp.	<i>Hemileia vastatrix</i>	*		*				This study
PURN22449	<i>Mycodiplosis</i> sp.	<i>Hemileia vastatrix</i>			*				This study
PURN22450-1	<i>Mycodiplosis</i> sp.	<i>Hemileia vastatrix</i>	*	*	*		*		This study
PURN22537-1	<i>Mycodiplosis</i> sp.	<i>Hemileia vastatrix</i>	*						This study
PURN22537-2	<i>Mycodiplosis</i> sp.	<i>Hemileia vastatrix</i>			*				This study
PURN22625-2	<i>Mycodiplosis</i> sp.	<i>Hemileia vastatrix</i>	*						This study
W3_PV5894	<i>Mycodiplosis</i> sp.	<i>Hemileia vastatrix</i>	*						This study
p.malva jorge.peru1	<i>Mycodiplosis</i> sp.	<i>Puccinia pelargonii- zonalis</i>		*	*		*		This study
PURN16055-2	<i>Mycodiplosis</i> sp.	<i>Melampsora epitea</i> complex			*				This study
PURN16055-3	<i>Mycodiplosis</i> sp.	<i>Melampsora epitea</i> complex			*				This study
PURN12037-1	<i>Mycodiplosis</i> sp.	<i>Melampsora humboldtiana</i>	*	*	*	*	*		This study
PURN12037-2	<i>Mycodiplosis</i> sp.	<i>Melampsora humboldtiana</i>			*				This study
PURN12037-3	<i>Mycodiplosis</i> sp.	<i>Melampsora humboldtiana</i>			*	*	*		This study
PURN12880-1	<i>Mycodiplosis</i> sp.	<i>Melampsora humboldtiana</i>	*		*	*	*		This study
PURN12880-2	<i>Mycodiplosis</i> sp.	<i>Melampsora humboldtiana</i>			*	*	*		This study
PURN12880-3	<i>Mycodiplosis</i> sp.	<i>Melampsora humboldtiana</i>			*				This study
PURN15051	<i>Mycodiplosis</i> sp.	<i>Melampsora humboldtiana</i>			*	*	*		This study
PURN11700	<i>Mycodiplosis</i> sp.	<i>Melampsora medusae</i>			*	*			This study
PURN11705-1	<i>Mycodiplosis</i> sp.	<i>Melampsora occidentalis</i>	*	*	*	*	*		This study
PURN11705-2	<i>Mycodiplosis</i> sp.	<i>Melampsora occidentalis</i>	*	*	*	*	*		This study
MT3-merie	<i>Mycodiplosis</i> sp.	<i>Melampsora</i> sp.	*	*	*		*		This study
PURN12029-1	<i>Mycodiplosis</i> sp.	<i>Melampsora</i> sp.		*	*	*	*		This study
PURN16030	<i>Mycodiplosis</i> sp.	<i>Melampsora</i> sp.			*				This study

Table 4.3 continued

PURN16119-1	<i>Mycodiplosis</i> sp.	<i>Melampsora</i> sp.	*	*	*	This study
PURN22739	<i>Mycodiplosis</i> sp.	<i>Melampsora</i> sp.		*		This study
PURN16373	<i>Mycodiplosis</i> sp.	<i>Mikronegeria alba</i>	*	*	*	This study
PUR68963	<i>Mycodiplosis</i> sp.	N.A.		*		This study
PUR87962	<i>Mycodiplosis</i> sp.	<i>Olivea</i> sp.		*		This study
PURN15331	<i>Mycodiplosis</i> sp.	<i>Olivea tectonae</i>	*	*		This study
PURN15331b	<i>Mycodiplosis</i> sp.	<i>Olivea tectonae</i>	*	*	*	This study
PURF8883	<i>Mycodiplosis</i> sp.	<i>Phakopsora cherimoliae</i>		*		This study
PUR44594	<i>Mycodiplosis</i> sp.	<i>Phakopsora nishidana</i>		*		This study
PURN16381	<i>Mycodiplosis</i> sp.	<i>Puccinia pelargonii-zonalis</i>		*		This study
PURN16378	<i>Mycodiplosis</i> sp.	<i>Puccinia arundinariae</i>	*	*	*	This study
PURN16	<i>Mycodiplosis</i> sp.	<i>Puccinia caeomatiformis</i>		*	*	This study
PURN15343	<i>Mycodiplosis</i> sp.	<i>Puccinia commelinae</i>		*	*	This study
PURF6270	<i>Mycodiplosis</i> sp.	<i>Puccinia distenta</i>	*			This study
PURF4300	<i>Mycodiplosis</i> sp.	<i>Puccinia graminis</i>		*		This study
PURF7260-1	<i>Mycodiplosis</i> sp.	<i>Puccinia leonotidicola</i>		*		This study
PURF7260-2	<i>Mycodiplosis</i> sp.	<i>Puccinia leonotidicola</i>		*		This study
PURF5972	<i>Mycodiplosis</i> sp.	<i>Puccinia mogiphanis</i>		*		This study
PURN5852	<i>Mycodiplosis</i> sp.	<i>Puccinia mogiphanis</i>		*		This study
PURN15018	<i>Mycodiplosis</i> sp.	<i>Puccinia oxalidis</i>	*	*	*	This study
p.malva jorge.peru2	<i>Mycodiplosis</i> sp.	<i>Puccinia pelargonii-zonalis</i>		*	*	This study
PURN23084-1/CJP1	<i>Mycodiplosis</i> sp.	<i>Puccinia sorghi</i>	*	*		This study
PURN23084-2/CJP2	<i>Mycodiplosis</i> sp.	<i>Puccinia sorghi</i>	*		*	This study
larvawheat2	<i>Mycodiplosis</i> sp.	<i>recondita</i> f. sp. <i>tritici</i>	*	*	*	This study
PURF6438	<i>Mycodiplosis</i> sp.	<i>Puccinia triumfettae</i>		*		This study
PURN23060/PA Z04-5L	<i>Mycodiplosis</i> sp.	<i>Puccinia tubulosa</i>	*	*		This study
PURF8146	<i>Mycodiplosis</i> sp.	<i>Puccinia unicolor</i>		*		This study
PURF8323	<i>Mycodiplosis</i> sp.	<i>Puccinia verbesinae-dentatae</i>		*		This study

Table 4.3 continued

PURF9848	<i>Mycodiplosis</i> sp.	<i>Uromyces cestri</i>		*	*				This study
PURF11174	<i>Mycodiplosis</i> sp.	<i>Uromyces blainvilleae</i>			*				This study
PURF11174B	<i>Mycodiplosis</i> sp.	<i>Uromyces blainvilleae</i>			*				This study
PURF3638	<i>Mycodiplosis</i> sp.	<i>Uromyces colombianus</i>			*				This study
PURF2926	<i>Mycodiplosis</i> sp.	<i>Uromyces hedysari-paniculati</i>			*				This study
PURN15342	<i>Mycodiplosis</i> sp.	<i>Uromyces hedysari-paniculati</i>	*	*	*		*		This study
PURN2544	<i>Mycodiplosis</i> sp.	<i>Uromyces iresines</i>	*		*				This study
		†	MN191328.1†		MN20151 5.1†	MN20140 8.1†	MN201286.1 †		Dorchin et al., 2019
		†	MN191360.1†			MN20144 4.1†	MN201323.1 †		Dorchin et al., 2019

#### **4.3.2 Nucleotide alignment dataset and phylogenetic inferences**

The concatenated five gene-region datasets consisted of 2,565 characters, of which 383 were parsimony-informative. The percentage of parsimony-informative characters per gene region was 6% for the barcoding CO1 region, 2.6% for the conserved CO1 region and mit16S, 0.6% for LSU, and 3% for ITS1. A total of 131 individual larvae were included, of which *Obolodiplosis robiniae* and *Youngomyia podophyllae* served as outgroup taxa. The following models were selected by ModelFinder (AICc): GTR+F+I+G4 for barcoding CO1, TIM+F+I+G4 for conserved CO1 region, K3Pu+F+I+G4 for mit16S, TIM+F+I for LSU, and TPM2+F+G4 for ITS1. Our maximum likelihood analysis reveals 14 clades (Fig. 4.1), from which only two have bootstrap support lower than 70%. In addition, all DNA sequences of these fungivorous larvae form a monophyletic group.

#### **4.3.3 Diversity of *Mycodiplosis* larvae associated with rust fungi**

Based on our phylogenetic reconstruction of *Mycodiplosis* members, we identified 17 individuals at the species level. Six larvae belong to *Mycodiplosis coniophaga* (clade 3), three to *M. melampsorae* (clade 4), and eight to *M. puccinivora* (clade 5). *Mycodiplosis sphaerothecae* was also used as a reference sequence in our phylogenetic reconstruction. However, none of the sequences of fungivorous larvae collected in this study fitted in the same clade of this species. *Mycodiplosis sphaerothecae* is usually found feeding on fungal powdery mildews (Erysiphales) and has never been reported on rust fungi (Gagné & Jaschhof, 2021). Due to the few available sequences of *Mycodiplosis* species, 11 clades from our phylogenetic tree could not be determined at the species level. Thus, it is uncertain if there are new species of *Mycodiplosis* from our data or which other currently accepted *Mycodiplosis* species are present in this study.

#### **4.3.4 Host-specificity of *Mycodiplosis* larvae associated with rust fungi at PUR and field trips from the Americas**

The multi-locus phylogenetic tree built from this study shows that most of the *Mycodiplosis* members did not have a preference to feed on specific rust genera. For example, *Mycodiplosis* members in clade one were recovered feeding on willow rusts and weed rusts from the genera *Melampsora* and *Coleosporium* (Fig. 4.1). Similarly, in clade 10, *Mycodiplosis* larvae can feed on rusts from six genera, including *Coleosporium*, *Hemileia*, *Olivea*, *Phakopsora*, *Puccinia*, and



*Uromyces*. The same behavior was observed in 12 of the 14 clades. Nevertheless, *Mycodiplosis* larvae from clades 5 and 14 showed different behavior. In clade five, we found larvae feeding only on *Melampsora* specimens, and in clade 14, larvae feeding only on the coffee leaf rust, *Hemileia vastatrix*. We also found several larvae from different clades in a single rust specimen packet. For example, the rust specimen packet of *Melampsora occidentalis* (PUR voucher: N11705) contained two larvae, each fitting in clades two and eight. The same results were shown in five more specimen packets (PUR vouchers: N12029, N12037, N15011, N15118, and N22537).

#### **4.3.5 Geographic distribution**

This study includes fungivorous larvae of rust fungi collected in 17 countries across the globe. Twelve larvae are from Asia, three from Europe, 65 from North America, and 51 from South America (Fig. 4.2). Based on our results, we found two distribution patterns. In the first pattern, *Mycodiplosis* members from seven clades (2, 3, 4, 5, 7, 8, and 13) are cosmopolitan and distributed in different continents or climate regions (Fig. 4.3). In the second pattern, other *Mycodiplosis* members from six clades (1, 6, 10, 11, 12, and 14) are geographically restricted by country or climate region (Fig. 4.4). Since the taxon of clade 9 served as a reference sequence (*M. sphaerothecae*) and none of the larvae from this study were part of this clade, we did not plot this specimen on the maps.

### **4.4 Discussion**

#### **4.4.1 Phylogenetic inference of the genus *Mycodiplosis***

This study is the first to use five datasets for phylogenetic analyses of *Mycodiplosis* larvae providing high bootstrap supports. From Chapter 2, the two-locus datasets that we used (barcoding CO1 region and mit16S) did not have the resolution to resolve the complexity of *Mycodiplosis* larvae associated with rust fungi. In this chapter, we added three more loci that significantly improved the phylogenetic inferences of *Mycodiplosis*. Although two more *Mycodiplosis* sequences of a section of the protein-encoding CAD gene (Carbamoyl-phosphate synthetase 2, aspartate transcarbamylase, and dihydroorotase) are available in the NCBI nucleotide database (Dorchin et al., 2019), perhaps due to our dried material, we could not amplify this gene region in our collected larvae. However, our multi-locus phylogenetic tree showed clear delimitations for

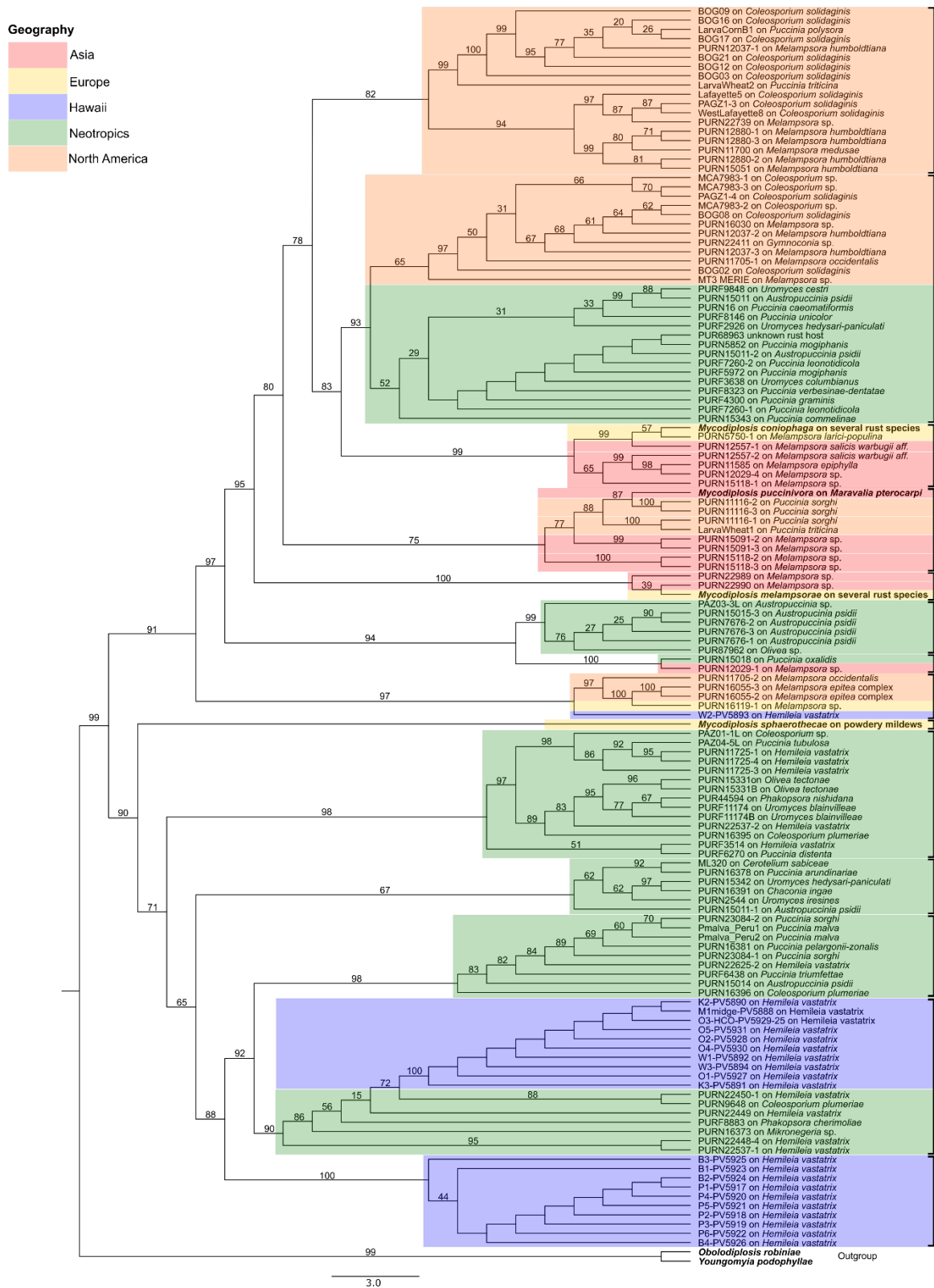
most clades with high bootstrap support (>70%). The four *Mycodiplosis* species used as references formed different clades with high bootstrap support, consistent with the morphological traits that differentiate them from each other. We also compared each phylogenetic tree reconstructed from individual loci to the multi-locus tree for congruences in their topologies (see Supplementary data).. However, none of the topologies of the single locus trees were congruent with the multi-locus tree and each other, and most of their bootstrap supports were lower than 70%. Therefore, our results suggest that several gene regions are necessary to unravel the phylogenetic relationships of the genus *Mycodiplosis*.

#### ***4.4.2 Comparison of datasets between this study and two other studies of Mycodiplosis larvae associated with rust fungi.***

Two other studies of fungivorous larvae associated with rust fungi have been made in the last decade (Henk et al., 2011; Nelsen, 2013). Although both studies attempted to amplify some DNA fragments of these larvae for phylogenetic inferences of the genus *Mycodiplosis*, none succeeded. In the first study (Henk et al., 2011), DNA extractions included up to 15 individual larvae per tube, which we proved may generate a mix of DNA fragments from larvae that may not belong to the genus *Mycodiplosis*. In addition, they designed two primers to amplify the large ribosomal subunit (LSU) of the DNA for larval identification at the genus level. Nevertheless, the amplified gene region has not been used in other phylogenetic studies of the Cecidomyiidae family, which restricted its use in our multi-locus phylogenetic analysis. In the second study (Nelsen, 2013), single larvae were used for DNA extraction, and two loci were amplified for phylogenetic analyses. One locus was the same LSU region amplified in the first study (Henk et al., 2011), and the other was a conserved region of the mitochondrial CO1, different than the one we used in this study. Similar to the first study, we did not use those DNA fragments in our multi-locus phylogenetic analysis for two reasons. Both loci are conserved gene regions that do not resolve the species diversity of *Mycodiplosis* and do not align with any available *Mycodiplosis* sequence confirmed through morphological identification of the adult male.

Figure 4.1 The best-scoring ML cladogram tree (-10998.4224) of the genus *Mycodiplosis*, reconstructed from concatenated five-locus datasets (barcoding CO1, conserved CO1, mit16S, LSU, and ITS1). The ML bootstrap value is presented above each branch. Brackets delimit clades, each labeled with a number on the right side of the bracket. Taxa labels are written on the tree as "larva code" on "the host rust where the larva was found." The origin/locality of each larva is colored by the color code shown on the top left of the figure. Reference sequences and outgroup taxa are written in bold. The tree was rooted to *Obolodiplosis robiniae* and *Youngomyia podophyllae*.

Figure 4.1 continued



#### ***4.4.3 The importance of collaborative research for taxonomic studies of Mycodiplosis***

This study highlights the importance and need for collaborative projects with entomological collections housing *Mycodiplosis* specimens identified at the species level. Of the 49 currently accepted species of *Mycodiplosis*, only four are represented by DNA sequences and are identified at the species level through the identification of the adult male. Most of the *Mycodiplosis* sequences deposited in the NCBI nucleotide database are not morphologically confirmed to belong to this genus. Collectors assume that larvae found in association with rust fungi belong to *Mycodiplosis*. Nevertheless, we show the importance of morphological identification of the adult male to discriminate *Mycodiplosis* species and even other genera within the Cecidomyiidae family, as we found a member of *Lestodiplosis*, a predator of *Mycodiplosis*, on rust-infected plant tissue. Thus, it is not surprising that some DNA sequences from the NCBI nucleotide database reported as *Mycodiplosis* sp. are misidentified.

#### ***4.4.4 Host preference of Mycodiplosis larvae for rust genera***

The multi-locus phylogenetic tree reconstructed in this study suggests that members of *Mycodiplosis* do not have a host preference for specific rust genera. Although members of *Mycodiplosis melampsorae* (clade five) from this study preferred rust specimens of the genus *Melampsora*, this *Mycodiplosis* species has been previously reported on several rust genera (Gagné & Jaschhof, 2021). In addition, *Mycodiplosis* members from clade 14 are restricted to feeding the coffee leaf rust spores of *Hemileia vastatrix* from coffee farms in Hilo and Kona in Hawaii. However, because coffee leaf rust was the only rust species collected from these places, we cannot confirm the host preference of these *Mycodiplosis* members only for *Hemileia*. Rust specimens from other rust genera must be collected in the same areas in Hawaii to confirm if *Mycodiplosis* members of clade 14 are specific to the genus *Hemileia* or feed other rust genera.

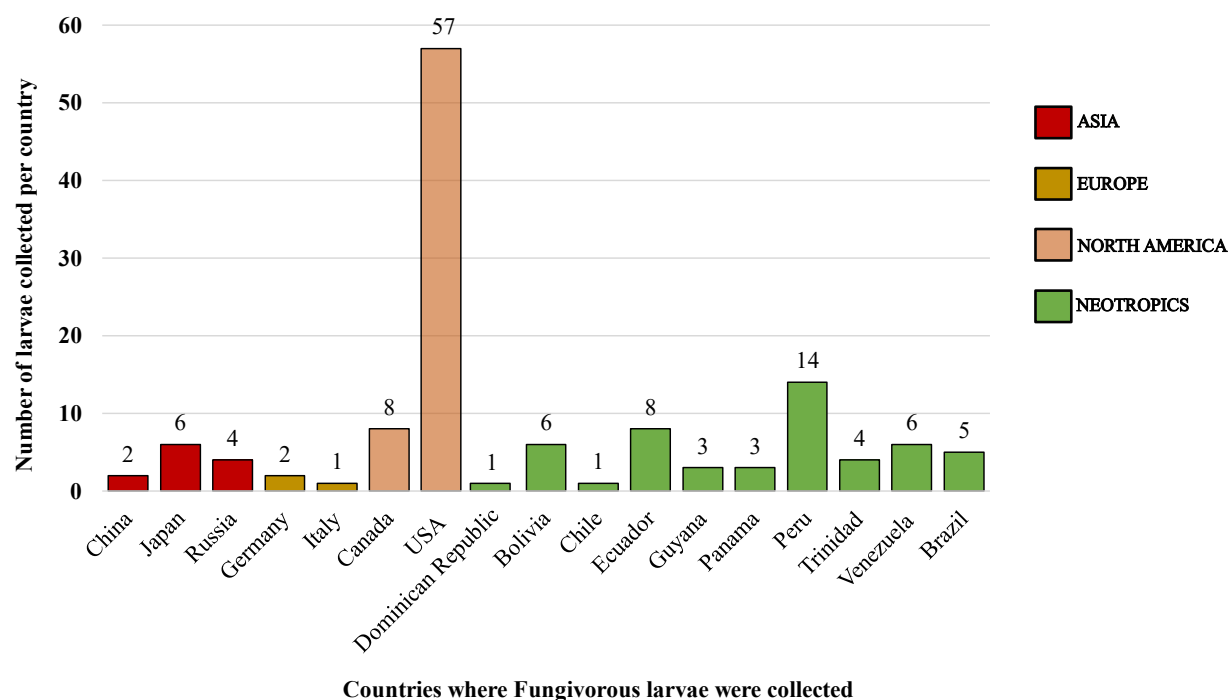


Figure 4.2 Origin/localities of fungivorous larvae associated with rust fungi. The number of individual larvae collected per country is written above the bar. Countries are colored by continent.

Host range is one of the critical steps in the risk assessment process of a natural enemy as a biological control agent (Loomans, 2021; van Lenteren et al., 2003). In risk assessment, the term "generalist" refers to an organism that feeds or hyperparasites a wide variety of prey species. However, this term includes all feeding strategies, such as oligophagy, polyphagy, omnivorous, facultative hyperparasitism, or phytophagy (Loomans, 2021). Feeding strategies that do not precisely match each other (Loomans, 2021). Despite the "generalist" strategy of *Mycodiplosis* members for any rust genus, this insect is actually monophagous because its host range is restricted to fungi. In fact, none of the *Mycodiplosis* members from this study are part of the clade of *Mycodiplosis sphaerothecae* (Fig. 4.1), a species intimately associated with fungal powdery mildews (Gagné & Jaschhof, 2021). Thus, some *Mycodiplosis* species may feed exclusively on rust spores. Monophagous biological control agents are not expected to create severe environmental risks when their establishment and dispersion are controlled (van Lenteren et al., 2003). Therefore, the following step to discard other feeding strategies for *Mycodiplosis* members is testing their host range on non-target species, such as plants and other insects in the ecosystem

where *Mycodiplosis* is planned to release. A host range assay on non-target species would confirm the monophagous habit of particular *Mycodiplosis* species for rust fungi only, or it will serve to discard them as potential biological control agents of rust fungi.

#### ***4.4.5 Geographic distribution of Mycodiplosis larvae feeding on rust fungi***

This study revealed two distribution patterns of *Mycodiplosis* members associated with rust fungi. The first pattern is cosmopolitan, having several *Mycodiplosis* members distributed on several continents and climate regions. This pattern is seen in the specimens of *M. coniophaga*, *M. puccinivora*, and *M. melampsorae*. *Mycodiplosis coniophaga* was already reported as a cosmopolitan species, but *M. puccinivora* was only found in Asia (Gagné & Jaschhof, 2021). This study extends then the distribution of *M. puccinivora* to North America, with three larvae feeding on *Puccinia sorghi* and one larva on *Puccinia triticina*. Similarly, *M. melampsorae* was primarily distributed in Europe and Turkey (Gagné & Jaschhof, 2021). However, this study extends the *M. melampsorae* to Asia, reporting two specimens from China on *Melampsora* sp. *Mycodiplosis* members, non-identified at the species level from other clades, also show this cosmopolitan pattern. For example, taxa from clade 13 are distributed in several South American countries and Hawaii, with a preference for warmer weather. Finally, clades 2, 7, and 8 are distributed in four continents with different climate conditions: Asia, Europe, North America, and South America. The second pattern of these larvae is locally restricted. This pattern is seen only in *Mycodiplosis* members from the Americas. *Mycodiplosis* members of clade one are unique to North America; taxa from clades 6, 10, 11, and 12 are restrictively distributed in the Neotropics, and members of clade 14 to Hawaii. Since members of these clades cannot be identified at the species level, it is unknown if any of these represent undescribed *Mycodiplosis* species.

*Mycodiplosis* species do not naturally disperse over long distances. Larvae are legless, and adults are weak fliers, especially newly emerging females that have to hold several eggs (Freeman & Geoghgen, 1987). Once these flies find their host plant, they only actively fly toward the plant (Sylvén, 1979), so they can complete their life cycle in the same host without traveling long distances. Thus, these flies seem to be more restricted geographically than having a cosmopolitan distribution by nature. Although it is unsure why some *Mycodiplosis* species are cosmopolitan,

such as *M. coniophaga*, *M. puccinivora*, and *M. Melampsora*, external sources of dispersion, such as global trade in crops, could help them to disperse over long distances.

The restricted geographical distribution of a natural enemy can make it a biological control candidate for pest control. For example, the environmental risk of a locally restricted natural enemy is usually low compared to an exotic one because the local natural enemy is already present in the ecosystem. Thus, it is already adapted to the climate conditions and has a balanced ecological interaction with other species in the local ecosystem. Then, it is unlikely to cause adverse effects on non-target species (van Lenteren et al., 2003). In addition, Since these natural enemies do not disperse over long distances, once their food resources decrease, it is also expected that their population also decrease. Finally, these natural enemies have a high chance of remaining in the ecosystem because it is their natural habit.

#### ***4.4.6 Mycodiplosis larvae on rust fungi that have caused disease outbreaks***

Although this study did not select specific rust genera to screen for the presence of fungivorous larvae, we found some economically important rust species associated with *Mycodiplosis*. For example, several larvae were found in rust specimens of *Austropuccinia psidii*, the causal agent of myrtle rust disease, which threatens the extinction of plants in the Myrtaceae family in several regions, including Australia, New Zealand, Hawaii, and New Caledonia (Fensham et al., 2020; Fensham & Radford-Smith, 2021; Toome-Heller et al., 2020). Because these host plants are commonly found in natural ecosystems, traditional methods to control rust diseases, such as fungicide applications and rust-resistant plant varieties, are not applicable in this case. *Mycodiplosis constricta* was recently reported on *A. psidii* in New Zealand (Kolesik et al., 2022), but DNA sequences were not available to include them in our multi-locus phylogenetic tree. The *Mycodiplosis* larvae we found on *A. psidii* were collected in Brazil and Venezuela. These larvae fit in two different clades, two and six, which may suggest that one or more than one species of *Mycodiplosis* can feed on rust spores of *Austropuccinia psidii*.



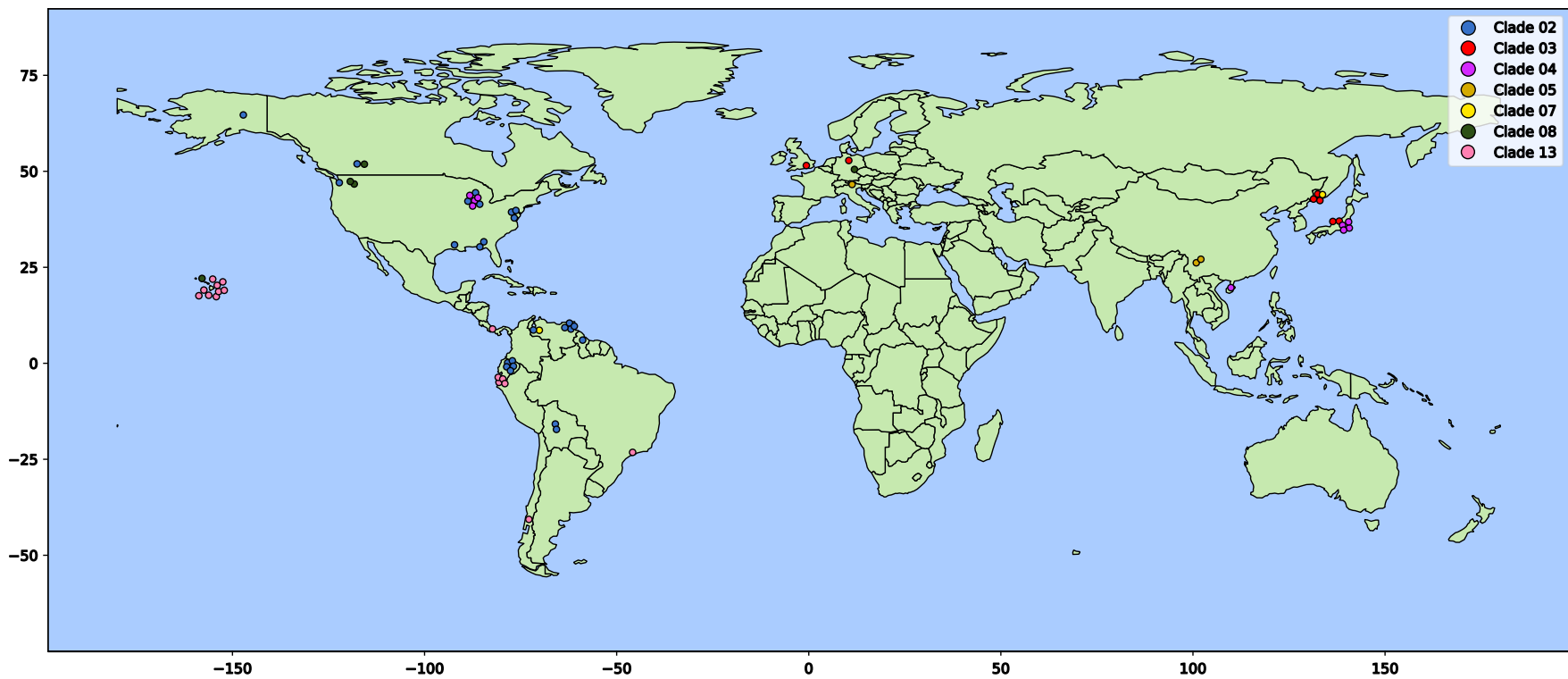


Figure 4.3 Cosmopolitan distribution of *Mycodiplosis* larvae feeding on rust fungi. Each circle represents an individual larva. Circles are colored by clades of the phylogenetic tree shown in Fig. 4.1.

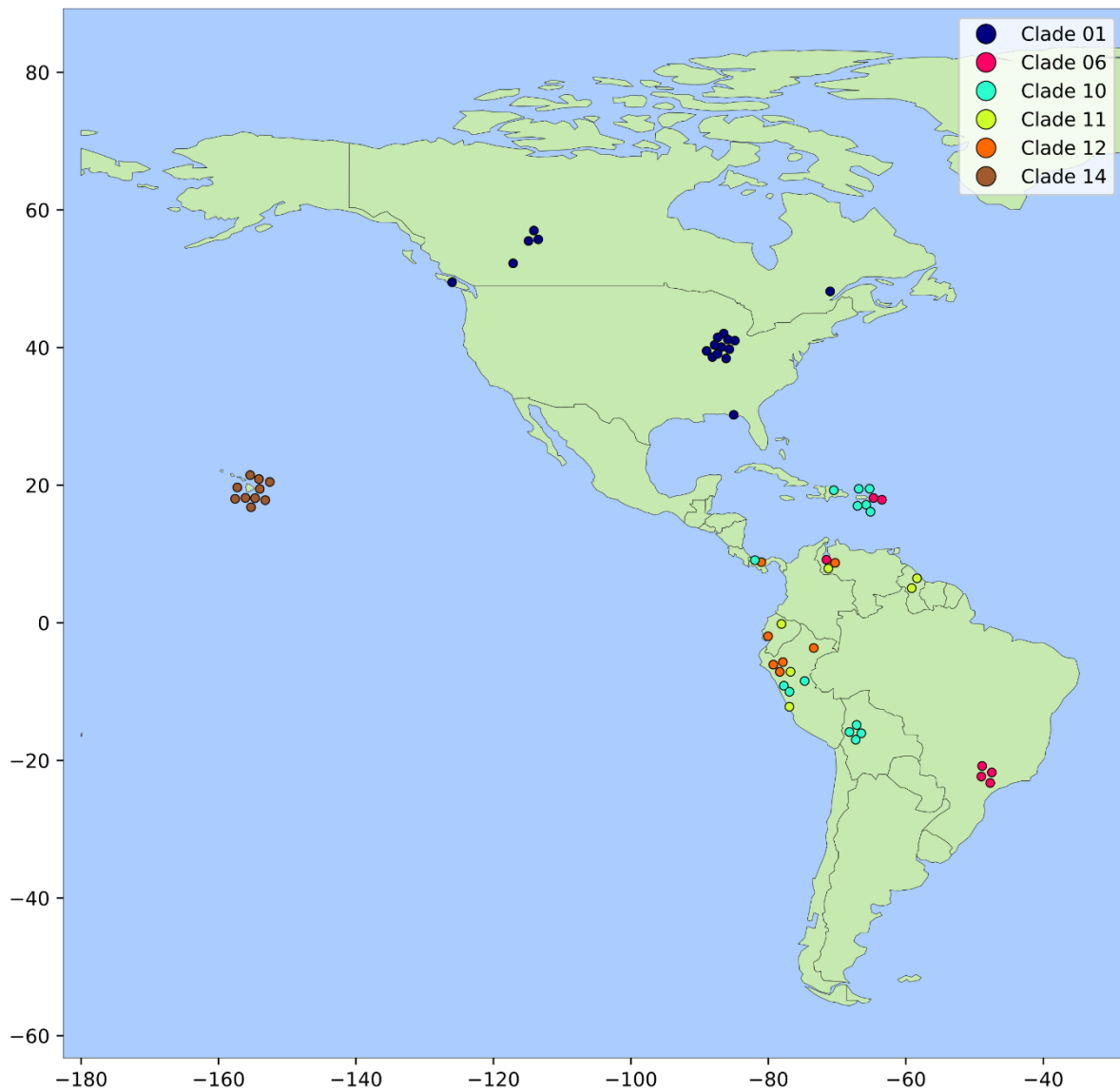


Figure 4.4 *Mycodiplosis* larvae that feed on rust fungi regionally restricted to either a specific country or climate region. Each circle represents an individual larva. Circles are colored by clades of the phylogenetic tree shown in Fig. 4.1.

Larvae feeding on spores of coffee leaf rust were also found in this study. Coffee leaf rust caused by *Hemileia vastatrix* is another economically important disease in all coffee-producing countries (Arneson, 2000; Kolmer et al., 2009; Ristaino et al., 2021) that causes up to 40% annual yield losses in Latin-American countries (Cressey, 2013; Cristancho et al., 2012; A. Kumar et al., 2016;

McCook, 2006). Although *Mycodiplosis* larvae have already been reported to feed spores of coffee leaf rust (Gagné & Jaschhof, 2021; Milne, 1975; Santiago-Elena et al., 2020), only one specimen has been described as *Mycodiplosis hemileiae* from Tanzania (Barnes, 1939). Most of them remain unknown at the species level. Based on our multi-locus phylogenetic analysis, five clades (8,10,12,13, and 14) contain at least one larvae feeding on coffee leaf rust. Two of these clades were locally restricted (10 and 12). This finding suggests a high diversity of *Mycodiplosis* larvae for controlling coffee leaf rust. However, further taxonomic studies are required to identify these specimens at the species level.

We also found *Mycodiplosis* larvae feeding on southern and common corn rust spores. These rust diseases are distributed worldwide and have caused severe epidemics in maize crops with yield losses of up to 80% (Groth et al., 1983; Liu & Wang, 1999; Mueller et al., 2020; Reyes, 1953; Rhind et al., 1952). Several larvae were found on the southern corn rust, *Puccinia polysora*, in maize crops at Southwest Purdue Agricultural Center (SWPAC), Indiana, USA. However, we only used one in this study, which fitted clade 1 in our multi-locus phylogenetic tree. Five other larvae were found on rust specimens of common corn rust, *Puccinia sorghi*. Two larvae were collected in Peru in 2019 and three in the United States in 2014. Larvae from Peru fitted on clade 12 and larvae from the United States in clade 4. Although we only collected larvae in SWPAC once, it is interesting that fungicides are regularly applied to these maize crops. Thus further studies on the fungicide resistance level of these larvae could help elucidate their potential as biological control agents in IPM.

Finally, at Beck Agricultural Center (BECK), Indiana, USA, we collected two *Mycodiplosis* larvae from organic wheat crops infected by *Puccinia recondita* f. sp. *tritici*, the causal agent of wheat leaf rust. Both larvae were collected in 2021 from the same field, but interestingly, each larva fitted on a different clade (clades 1 and 4). These larvae also fitted on the same clades as the larvae collected from rust-infected corn in SWPAC. The distance between BECK and SWPAC is ca. 250 km. It is also interesting to notice that taxa from clade four were collected in different years: 2014 and 2021. These results may suggest two things: several *Mycodiplosis* species remain and co-habit in that area, and these larvae are dispersed through external sources, such as humans.

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## **CHAPTER 5. CHARACTERIZATION OF THE FUNGAL GENUS SPHAERELLOPSIS ASSOCIATED WITH RUST FUNGI: SPECIES DIVERSITY, HOST RANGE, BIOGEOGRAPHY, AND IN-VITRO MYCOPARASITIC EVENTS OF S. MACROCONIDIALIS ON THE SOUTHERN CORN RUST, PUCCINIA POLYSORA.**

### **5.1 Introduction**

*Sphaerellopsis* Cooke (Leptosphaeriaceae, Ascomycota) is the most commonly reported fungal genus associated with rust fungi. It has been reported on 369 rust species and 30 genera in more than 50 countries across the globe (Kranz & Brandenburger, 1981). The fungus is usually described as solitary or gregarious spherical black pycnidia that develop on sori and, thus, presumably infect rust spores and prevent their dispersion (Eriksson, 1966). These black pycnidia are usually found on uredinia, the spore stage most frequently associated with severe rust disease epidemics and long-distance dispersal across continents. However, these pycnidia have also been found in association with other rust spore stages. Due to the intimate association of *Sphaerellopsis* with rust fungi, this fungal genus is tentatively considered a potential biological control agent for rust diseases, which have caused devastating epidemics and yield losses worldwide (Chen et al., 2002; Kolmer et al., 2009; Lidwell-Durnin & Laphorn, 2020).

The type species *Sphaerellopsis filum* was initially described as *Sphaeria filum* by Bivona-Bernadi in 1815 on rusts infecting *Convolvulus sepium* and *Populus nigra* in Sicily (Bivona-Bernardi, 1815). Then, Fries transferred the species to the genus *Phoma* as *Phoma filum* in 1823 (Fries, 1823). Later, Castagne erected the genus *Darluca* and treated *Sphaeria filum* as a synonym of *Darluca vagans* (Castagne, 1851). However, in 1966 Eriksson considered the epithet "*vagans*" superfluous and prioritized "*filum*" over "*vagans*" (Eriksson, 1966). In 1908, Spegazzini considered *Eudarluca caricis* the teleomorph of *Darluca filum* (Spegazzini, 1908). Later in 1951, Keener proved the connection between these two genera experimentally (Keener, 1951), and Yuan et al., 1998 confirmed this connection by obtaining the anamorphic stage from the teleomorph in culture studies (Z. W. Yuan et al., 1998). Lastly, in 1977, Sutton transferred *Darluca filum* to the genus *Sphaerellopsis* as *S. filum* (Sutton, 1977). The fungus is commonly found in its anamorphic state, and the teleomorph is rarely observed. Although *Sphaerellopsis* and *Eudarluca* are now

known to be congeneric, it is still uncertain which *Sphaerellopsis* species is conspecific with *Eudarluca caricis*.

Although most scientific publications posit *Sphaerellopsis* as a mycoparasite of rust fungi, its relationship with these plant pathogens is still poorly understood. While there is some evidence of direct interaction between the type species *Sphaerellopsis filum* and several rust species, the interaction is not consistent between *S. filum* strains. For instance, some researchers argue that *S. filum* can colonize rust spores by penetrating nonspecialized hyphae and disrupting cytoplasm (Carling, D.E. Brown, M.F. Millikan, 1976; Plachecka, 2005; Sappin-Trouffy, 1896; Whelan et al., 1997). However, other studies report no evident cytoplasmic disruptions of rust spores when *S. filum* is present (D'Oliveira, 1941; Hulea, 1939). In-vitro assays demonstrated fast growth of the mycelium and conidioma of *S. filum* when cultured with intact or ruptured rust spores (Rambo & Bean, 1970). However, changes in fungal growth rate do not necessarily demonstrate that *S. filum* can infect rust fungi. Similarly, lab and field experiments have shown a significant reduction in rust infection when *S. filum* is present (Black, 2012; Gordon & Pfender, 2012; X. Yuan & Han, 2000), but these conclusions are contradicted by other studies (Z. W. Yuan et al., 1999). In recent years, through phylogenetic analyses, several isolates named *S. filum* have turned out to be incorrectly placed in the genus *Sphaerellopsis* (Trakunyingcharoen et al., 2014). Thus, new genera were allocated, and new species within *Sphaerellopsis* were introduced. Hence, the previous interaction tests of *S. filum* with rust fungi are in question, as the *Sphaerellopsis* specimens used in those studies may no longer be placed in the genus.

Based on morphology and DNA sequence data, there are currently seven accepted *Sphaerellopsis* species. Five are reported as mycoparasites of rust fungi: *Sphaerellopsis anomala*, *S. filum*, *S. hakeae*, *S. macroconidialis*, and *S. paraphysata* (Crous et al., 2016; Nag Raj, 1993; Trakunyingcharoen et al., 2014); and two are considered saprobic: *S. artemisiae* and *S. isthmospora* (Doilom et al., 2021; Phookamsak et al., 2019). Although *S. hakeae* and *S. paraphysata* were reported to be associated with rust sori and plant tissue (Crous et al., 2016, 2018), it is unclear if the association with the host plant is parasitic or saprobic. Furthermore, it is yet not proven that all *Sphaerellopsis* species associated with rust fungi are mycoparasites. This intimate association might even be commensal or mutualistic. Therefore, while significant improvements

have been made to the taxonomy of *Sphaerellopsis* (Trakunyingcharoen et al., 2014), the relationship between members with rust fungi is still unclear.

Among the five *Sphaerellopsis* species associated with rust fungi, *S. paraphysata* is the only one confirmed to have a mycoparasitic strategy. Secondary metabolites obtained from *S. paraphysata* disrupted the urediniospores cell wall of *Puccinia substriata*, leading to cellular component leakage (Ashmitha Sri et al., 2020). Inoculation of the conidia of *S. paraphysata* on the uredinia of *P. substriata* reduced rust spore germination by up to 76% (Anandakumar et al., 2019). In addition, the disease severity of the rust was 13% when *S. paraphysata* was present compared to the control of 86% (Anandakumar et al., 2019). However, because the species was also found in plant tissue, further studies are needed to discard a plant pathogenic strategy or reconsider it as a potential biological control agent.

Although interaction studies between members of *Sphaerellopsis* and rust fungi are crucial to confirm their mycoparasitic strategy, other ecological studies are equally essential to determine the potential of *Sphaerellopsis* species as biological control agents. For example, biogeography can explain the distribution of species of *Sphaerellopsis* across the globe, which environmental conditions those species are adapted to, and how easily they disperse. Furthermore, host-specificity studies would help clarify whether/which *Sphaerellopsis* species are generalists or host-specific on rust species or genera. Knowing the host range of a natural enemy is crucial for environmental risk assessments to prevent the release of new diseases. Nevertheless, biogeography and the host range of *Sphaerellopsis* species are unknown due to the few records of the currently accepted species. Most of these records are primarily from temperate regions (Ashmitha Sri et al., 2020; Crous et al., 2016; Trakunyingcharoen et al., 2014) with insufficient knowledge of the Americas. Thus, this study has two aims: 1) to characterize the fungal genus *Sphaerellopsis* through its geographic distribution, specificity to rust genera, and species diversity. This will be achieved by screening for *Sphaerellopsis* specimens on rust fungi collected primarily in the Americas and conducting phylogenetic analyses; and 2) to elucidate the strategy of *S. macroconidialis* when interacting with rust fungi, using the urediniospores of *Puccinia polysora* as a model system.

## 5.2 Materials and Methods

### 5.2.1 Collected samples from PUR collections and field trips

We collected black fruiting bodies of *Sphaerellopsis* from preserved rust specimens in the Arthur Fungarium (PUR) supplemented with newly collected material from Peru in 2019 and Puerto Rico in 2018 (see Supplementary data Table S2). The PUR was chosen to screen for *Sphaerellopsis* samples because it contains one of the world's largest collections of rust fungi, with approximately 110,000 collections (Purdue Herbaria, 2022). Rust specimens at PUR are stored in folders sorted by rust species in host plant families and geographic regions. When collecting *Sphaerellopsis* samples at PUR, we randomly screened these rust specimens by selecting the top, middle, and bottom specimens from the Americas in each rust species folder. When collecting natural enemies from other geographic regions, we randomly selected one rust specimen per folder. Although we screened rust specimens collected across the globe, the Americas was our preferred geographic region. We screened each rust-infected leaf of every rust specimen under a stereoscope Olympus Model SZ2-ILST. We looked for visible signs of *Sphaerellopsis*-type fruiting bodies developed only on the sori. Only specimens fruiting exclusively on rust sori but not on surrounding host tissue were removed for further analyses, as the ability to also fruit on host plant tissues would indicate a non-rust-specific pathogen. Then, we removed one *Sphaerellopsis*-infected sorus with a razor blade per rust specimen. Only one razor blade was used per specimen to prevent cross-contamination. Each infected sorus was placed in a microcentrifuge tube labeled with the PUR barcode of the rust specimen and a serial number.

When collecting *Sphaerellopsis* specimens from field trips, we first collected rust-infected plant leaves. Then, we looked for black fruiting bodies developed only on sori under a stereoscope. If *Sphaerellopsis* was present, we isolated it by cutting a piece of the plant tissue containing both the sorus and *Sphaerellopsis* with a razor blade. Then, the plant tissue was sterilized with 1/10 chlorine dilution bleach for one minute and washed three times with sterile water. The piece of plant tissue was inoculated onto Petri Dishes containing Potato Dextrose Agar (PDA) and 50 mg/mL Chloramphenicol. Petri Dishes were shipped to Aime Lab at Purdue University for pure isolation. Once the Petri Dishes arrived at Aime lab, we subcultured them and made pure isolations on PDA and 2% malt extract agar (MEA) with 50 mg/mL Chloramphenicol. Pure isolations were stored

long-term in PDA slants at 4°C and 15% glycerol at -80 °C. Finally, we pressed, dried, and deposited the collected rust specimens at PUR.

Once we found a sample of *Sphaerellopsis* in the rust specimens at PUR, we manually recorded the following information for each of them: the PUR barcode, year of collection, rust species name, host plant, country of origin, and rust spore stage (see Supplementary data Table S2). Finally, we took macro and micro photographs of some of the collected *Sphaerellopsis* samples with an Olympus SC30 camera and image software Olympus cellSens entry version 1.14 under a stereoscope Olympus Model SZ2-ILST and a compound microscope Olympus BH2-RFCA at PUR. Measurements of fungal structures were made using cellSens Standard 1.18 Imaging Software (Olympus).

### **5.2.2 Species identification of *Sphaerellopsis* specimens associated with rust fungi**

The collected *Sphaerellopsis* samples were identified based on morphological characters and DNA sequences. Original descriptions of the currently accepted *Sphaerellopsis* species were used as references for morphological comparison (Cooke M.C., 1883; Crous et al., 2016; Doilom et al., 2021; Nag Raj, 1993; Phookamsak et al., 2019; Trakunyingcharoen et al., 2014). We also amplified several gene regions to identify the specimens at the species level. Below we describe the methodology used for species identification of *Sphaerellopsis* specimens based on DNA sequences.

### **5.2.3 DNA isolation and PCR amplification**

The genomic DNA of each fungal natural enemy was extracted using the EZNA HP Fungal DNA kit (Omega Bio-Tek, Norcross, Georgia), following the manufacturer's instructions and modifying only the incubation time in the third step. Instead of 30 min, we incubated the samples overnight to ensure complete lysis of cells in the suspension. Leaving the specimen for a long time helped to make DNA accessible easily. We amplified the following loci: internal transcribed spacer region of the ribosomal DNA (5.8S and ITS2), large ribosomal subunit (LSU), translation elongation factor 1- $\alpha$  (*tef1*), and RNA polymerase II second largest subunit (*rpb2*) (Table 5.1). All 25- $\mu$ l PCR reactions were conducted on a Mastercycler ep gradient Thermal Cycler (Eppendorf model #5341,

Hauppauge, New York) that consisted of 12.5 µl of 2× MyTaq Mix (Bioline, Swedesboro, New Jersey), 1.25 µl of each 10 µM primer, and 10 µl of either 1/10 or 2/10 diluted DNA extract. Amplifications of rDNA and *rpb2* loci were run under the following conditions: initial denaturation at 94°C for 5 min (95°C for *rpb2*/96°C for 2 min for *tef1*); followed by 40 cycles of denaturation (45 cycles for ITS) at 94°C for 30 sec (95°C for *rpb2*), annealing at 51.8 °C for 45 sec for ITS/54°C for 45 sec for LSU/56°C for 30 sec for *tef1*/55°C for 45 sec for *rpb2*, and elongation at 72°C for 45 sec (1 min for LSU and 30 sec for *tef1*); and final extension at 72°C for 7 min.

Table 5.1 Primers for PCR amplification and sequencing used in this study

Gene	Primer name	Orientation	Sequences (5' to 3')	Reference
ITS	EudITS2F	F	AACTTTCAACAACGGATCTCTTGGT	This study
	EudITS4R	R	ATGCTTAAGTTCAGCGGGTA	This study
	EuSP_ITS_R2	R	ATGTGTCYRMGMTYCAGGC	This study
LSU	Spha_28sf1	F	GAGTGAAGCGGCAACAGCTC	This study
	Spha_28sr1	R	CGATTTGCACGTCAGAACCGC	This study
TEF-1	EF1-728F	F	CATCGAGAAGTTCGAGAAGG	Carbone & Kohn, 1999
	EF1-986R	R	TACTTGAAGGAACCCTTACC	Carbone & Kohn, 1999
RPB2	RPB2-5F2	F	GGGGWGAYCAGAAGAAGGC	Sung et al., 2007
	fRPB2-7CR	R	CCCATRGCT TGYTTR CCCAT	Liu et al., 1999

#### 5.2.4 Electrophoresis and sequencing

We ran the PCR products in 1% agarose and stained them with GelRed (RGB4102, Phoenix Research Products) for 35 min at 110 V in a Bio-Rad electrophoresis tank to visualize PCR products. PCR products of samples that showed bands were sent to Genewiz (South Plain- field, New Jersey) for purification and subsequent sequencing in both directions. Raw sequence reads were edited manually and assembled using Sequencher version 5.2.3 (Gene Codes Co., Ann Arbor, Michigan).

#### 5.2.5 Sequence alignment and phylogenetic trees

The edited sequences were blasted against the NCBI GenBank nucleotide database (<http://ncbi.nlm.nih.gov/blast/Blast.cgi>) to search for the closest species. We downloaded DNA sequences of the type species as references for our phylogenetic analyses (Table 5.2). Sequences were aligned using MUSCLE version 3.7 (Edgar, 2004) in MEGA7 (Kumar et al., 2016). Then,

the aligned sequences were trimmed using trimAl version 1.2 (Capella-Gutiérrez et al., 2009) with a minimum percentage of positions to conserve [0-100]: 50, and gap threshold, the fraction of positions without gaps in a column [0-1]: 0.6. We performed maximum likelihood (ML) inference using IQ-TREE (Minh et al., 2020) under partitioned models (Chernomor et al., 2016) and selected the best nucleotide substitution model under Akaike's information criterion corrected for small sample size (AICc) using ModelFinder using ModelFinder (Kalyaanamoorthy et al., 2017). An ultrafast bootstrap analysis was implemented with 1,000 replicates (Hoang et al., 2018). The "-bnni" option was used to reduce the risk of overestimating branch supports with UFBoot due to severe model violations. Finally, phylogenetic reconstructions with bootstrap values were visualized in FigTree version 1.4.3 (<http://tree.bio.ed.ac.uk/software/figtree/>) and colored in Inkscape (<https://inkscape.org>).

#### **5.2.6 Geographical distribution**

The localities of *Sphaerellopsis* specimens with successfully amplified gene regions were used to build a geographic map. We used the GPS coordinates of each of these specimens when present. Otherwise, we generated approximated coordinates according to the locality description following a geocoding Python Script in the GitHub repository (Lynn, 2017). We plotted the geographic data of each specimen on a map and colored each point by the clades formed in the multi-locus phylogenetic tree using the package Geopandas in Python (Jordahl, 2014).

#### **5.2.7. Setup of interaction test between conidia of *Sphaerellopsis macroconidialis* and urediniospores of *Puccinia polysora***

*P. polysora* was chosen in this study because it is the original rust host from which the strain of *S. macroconidialis* was collected. *Puccinia polysora* is an agriculturally important fungus that causes one of the most devastating maize diseases with significant yield losses worldwide (Sun et al., 2021).



Table 5.2 Reference sequences used in phylogenetic analyses.

NCBI Reference	Species Name	Gene region	Reference
MH855147.1	<i>Alternaria consortialis</i>	ITS	Woudenberg et al. (2013)
MT957065.1	<i>Sphaerellopsis artemisiae</i>	ITS	Doilom et al. (2021)
NR_171717.1	<i>Sphaerellopsis filum</i>	ITS	Trakunyingcharoen et al. (2014)
AY607011	<i>S. filum</i>	ITS	Lieseback & Zaspel (2004)
AY607012	<i>S. filum</i>	ITS	Lieseback & Zaspel (2004)
AY607013	<i>S. filum</i>	ITS	Lieseback & Zaspel (2004)
NR_155859.1	<i>Sphaerellopsis hakeae</i>	ITS	Crous et al. (2016)
MK387925.1	<i>Sphaerellopsis isthmospora</i>	ITS	Phookamsak et al. (2019)
KP170659.1	<i>Sphaerellopsis macroconidialis</i>	ITS	Trakunyingcharoen et al. (2014)
AY607023	<i>S. macroconidialis</i>	ITS	Lieseback & Zaspel (2004)
AY607022	<i>S. macroconidialis</i>	ITS	Lieseback & Zaspel (2004)
NR_137956.1	<i>Sphaerellopsis paraphysata</i>	ITS	Trakunyingcharoen et al. (2014)
KP170661.1	<i>S. paraphysata</i>	ITS	Trakunyingcharoen et al. (2014)
AY607015	<i>Sphaerellopsis sp.</i>	ITS	Lieseback & Zaspel (2004)
AY607014	<i>Sphaerellopsis sp.</i>	ITS	Lieseback & Zaspel (2004)
AY587134	<i>Sphaerellopsis sp.</i>	ITS	Nischwitz et al. (2005)
MH866597.1	<i>A. consortialis</i>	LSU	Woudenberg et al. (2013)
NG_088168.1	<i>S. artemisiae</i>	LSU	Doilom et al. (2021)
NG_067290.1	<i>S. filum</i>	LSU	Trakunyingcharoen et al. (2014)
KY173555.1	<i>S. hakeae</i>	LSU	Crous et al. (2016)
MK387963.1	<i>S. isthmospora</i>	LSU	Phookamsak et al. (2019)
KP170727.1	<i>S. macroconidialis</i>	LSU	Trakunyingcharoen et al. (2014)
NG_067291.1	<i>S. paraphysata</i>	LSU	Trakunyingcharoen et al. (2014)
KC584742.1	<i>A. consortialis</i>	<i>tef-1</i>	Woudenberg et al. (2013)
KP170684.1	<i>S. macroconidialis</i>	<i>tef-1</i>	Trakunyingcharoen et al. (2014)
KP170685.1	<i>S. paraphysata</i>	<i>tef-1</i>	Trakunyingcharoen et al. (2014)
KC584482.1	<i>A. consortialis</i>	<i>rpb2</i>	Woudenberg et al. (2013)
MH108009.1	<i>S. paraphysata</i>	<i>rpb2</i>	Crous et al. (2018)

Table 5.3 Sequences used in multi-locus phylogenetic analysis. Reference sequences in bold red; the asterisk (\*) indicates available DNA sequences obtained from this study; NA: data not available.

Isolate	Species	Host Rust	Locality	5.8S-ITS2 rDNA	LSU rDNA	TEF-1	RPB2
<b>CBS 104.31 KUMCC 20-0202A</b>	<b><i>Alternaria consortialis</i></b>	<b>NA</b>	<b>NA</b>	<b>MH855147.1</b>	<b>MH866597.1</b>	<b>KC584742.1</b>	<b>KC584482.1</b>
<b>CBS 317.68</b>	<b><i>Sph. artemisiae</i></b>	<b><i>Artemisia argyi</i></b>	<b>China</b>	<b>MT957065.1</b>	<b>NG_088168.1</b>		
PUR62890	<i>Sph. filum</i>	<i>Puccinia coronata</i>	Mexico	*	*		
PURF16864/ PUR83764	<i>Sph. filum</i>	<i>Tranzschelia discolor</i>	Ecuador	*			
PURF17349	<i>Sph. filum</i>	<i>Puccinia brachypodii</i>	Chile	*			
PURF19494	<i>Sph. filum</i>	<i>Puccinia brachypodii</i>	Argentina	*	*		
PURF3782	<i>Sph. filum</i>	<i>Puccinia brachypodii</i>	Bolivia	*			
PURF4300	<i>Sph. filum</i>	<i>Puccinia graminis</i>	Ecuador	*			
PURN12884	<i>Sph. filum</i>	<i>Melampsora ferrinii</i>	Peru	*			
PURN16937	<i>Sph. filum</i>	<i>Puccinia graminis</i>	USA	*			
PURN4541a	<i>Sph. filum</i>	<i>Puccinia coronata</i>	Germany	*			
PURN5297	<i>Sph. filum</i>	<i>Puccinia graminis</i>	Mexico	*			
<b>s15</b>	<b><i>Sph. filum</i></b>	<b><i>Melampsora sp.</i></b>	<b>Germany</b>	<b>AY607011</b>			
<b>s27</b>	<b><i>Sph. filum</i></b>	<b><i>Melampsora sp.</i></b>	<b>Germany</b>	<b>AY607012</b>			
<b>s45</b>	<b><i>Sph. filum</i></b>	<b><i>Melampsora sp.</i></b>	<b>Germany</b>	<b>AY607013</b>			
<b>CPC 29566</b>	<b><i>Sph. hakeae</i></b>	<b><i>Hakea sp.</i></b>	<b>Australia</b>	<b>NR_155859.1</b>	<b>KY173555.1</b>		
PURF10892	<i>Sph. hakeae</i>	<i>Uromyces ehrhartae</i>	Australia	*			
<b>HKAS 102225A</b>	<b><i>Sph. isthmospora</i></b>	<b>Dead branches</b>	<b>China</b>	<b>MK387925.1</b>	<b>MK387963.1</b>		
PURN11633	<i>Sph. macroconidialis</i>	<i>Puccinia pygmaea</i>	USA	*	*	*	*
PURN23084/ PeruCorn	<i>Sph. macroconidialis</i>	<i>Puccinia sorghi</i>	Peru	*	*	*	*
SP28	<i>Sph. macroconidialis</i>	<i>Puccinia sorghi</i>	Peru	*	*	*	*
<b>CBS 658.78</b>	<b><i>Sph. macroconidialis</i></b>	<b><i>Puccinia allii</i></b>	<b>Netherlands</b>	<b>KP170659.1</b>	<b>KP170727.1</b>	<b>KP170684.1</b>	
Eud3.1	<i>Sph. macroconidialis</i>	<i>Puccinia sorghi</i>	Peru	*	*	*	
PUR19637	<i>Sph. macroconidialis</i>	<i>Puccinia graminis</i>	Canada	*			
PUR25166	<i>Sph. macroconidialis</i>	<i>Puccinia recondita</i>	USA	*			
PUR26871	<i>Sph. macroconidialis</i>	<i>Puccinia fuirenicola</i>	Cuba	*			

Table 5.3 continued

PUR40715	<i>Sph. macroconidialis</i>	<i>Puccinia incondita</i>	USA	*		
PUR41914	<i>Sph. macroconidialis</i>	<i>Puccinia grindeliae</i>	USA	*		
PUR55871	<i>Sph. macroconidialis</i>	<i>Puccinia eatoniae</i>	USA	*	*	*
PUR60157	<i>Sph. macroconidialis</i>	<i>Ravenelia thornberiana</i>	Mexico	*		
PUR60992	<i>Sph. macroconidialis</i>	<i>Puccinia thaliae</i>	Paraguay	*		
PUR62883	<i>Sph. macroconidialis</i>	<i>Puccinia unica</i>	Spain	*		
PUR6299	<i>Sph. macroconidialis</i>	<i>Ravenelia cassiaecola</i>	USA	*		
PUR64476	<i>Sph. macroconidialis</i>	<i>Puccinia poarum</i>	Mexico	*		
PUR66593	<i>Sph. macroconidialis</i>	<i>Phakopsora apoda</i>	Ecuador	*		
PUR69289	<i>Sph. macroconidialis</i>	NA	NA	*	*	*
PUR7334	<i>Sph. macroconidialis</i>	<i>Uropyxis diphysae</i>	Guatemala	*		
PUR8475	<i>Sph. macroconidialis</i>	<i>Phragmidium guatemalense</i>	Guatemala	*		
PUR87630	<i>Sph. macroconidialis</i>	<i>Maravalia erythroxyli</i>	Brazil		*	
PUR88221	<i>Sph. macroconidialis</i>	<i>Puccinia recondita</i>	Brazil	*		*
PUR88382	<i>Sph. macroconidialis</i>	<i>Uromyces silphii</i>	Canada	*		
PUR90210	<i>Sph. macroconidialis</i>	<i>Phakopsora coca</i>	Brazil	*	*	*
PURF10361	<i>Sph. macroconidialis</i>	<i>Uromyces striatus</i>	Argentina	*		
PURF10651	<i>Sph. macroconidialis</i>	<i>Puccinia brachypodii</i> var. <i>poaememoralis</i>	Colombia	*		
PURF10657	<i>Sph. macroconidialis</i>	<i>Puccinia spilanthicola</i>	Colombia	*		
PURF10865	<i>Sph. macroconidialis</i>	<i>Puccinia recondita</i>	Australia	*		
PURF10996	<i>Sph. macroconidialis</i>	<i>Phakopsora lenticularis</i>	Venezuela	*		
PURF11699	<i>Sph. macroconidialis</i>	<i>Puccinia graminis</i>	Argentina	*		
PURF1211	<i>Sph. macroconidialis</i>	<i>Phakopsora compressa</i>	Bolivia	*		
PURF1212	<i>Sph. macroconidialis</i>	<i>Phakopsora compressa</i>	Bolivia	*		
PURF17655	<i>Sph. macroconidialis</i>	<i>Puccinia brachypodii</i>	Argentina	*		
PURF17656	<i>Sph. macroconidialis</i>	<i>Puccinia brachypodii</i>	Venezuela	*		
PURF17814	<i>Sph. macroconidialis</i>	<i>Puccinia polypogonis</i>	Brazil	*		
PURF18990	<i>Sph. macroconidialis</i>	<i>Phakopsora coca</i>	Brazil	*		
PURF2397	<i>Sph. macroconidialis</i>	<i>Uromyces epicampis</i>	Ecuador	*	*	*
PURF2408	<i>Sph. macroconidialis</i>	<i>Uromyces pencanus</i>	Chile	*		
PURF2409	<i>Sph. macroconidialis</i>	<i>Uromyces pencanus</i>	Chile	*		

Table 5.3 continued

PURF3797	<i>Sph. macroconidialis</i>	<i>Puccinia brachypoi</i> var.	Ecuador	*	*	*
PURF3799	<i>Sph. macroconidialis</i>	<i>Puccinia brachypoi</i> var.	Ecuador	*		
PURF3854	<i>Sph. macroconidialis</i>	<i>Puccinia poarum</i>	Brazil	*		
PURF3879	<i>Sph. macroconidialis</i>	<i>Puccinia molinae</i>	Germany	*		
PURF4648	<i>Sph. macroconidialis</i>	<i>Puccinia aegopogonis</i>	Ecuador	*		
PURF4891	<i>Sph. macroconidialis</i>	<i>Puccinia substriata</i>	Bolivia	*		
PURF6912	<i>Sph. macroconidialis</i>	<i>Puccinia hydrocotyles</i>	Colombia	*		
PURF8187	<i>Sph. macroconidialis</i>	<i>Puccinia praedicata</i>	Brazil	*		
PURF8347	<i>Sph. macroconidialis</i>	<i>Puccinia wedellicola</i>	Brazil	*		
PURF9548	<i>Sph. macroconidialis</i>	<i>Puccinia bonariensis</i>	Argentina	*	*	*
PURN11506	<i>Sph. macroconidialis</i>	<i>Puccinia persistens</i>	USA	*		
PURN11560	<i>Sph. macroconidialis</i>	<i>Phakopsora apoda</i>	Peru	*	*	*
PURN16382	<i>Sph. macroconidialis</i>	<i>Tranzschelia mexicana</i>	Peru	*	*	*
PURN2544	<i>Sph. macroconidialis</i>	<i>Uromyces iresines</i>	Ecuador	*		*
PURN3032	<i>Sph. macroconidialis</i>	<i>Puccinia fumosa</i>	Mexico	*		
PURN4199	<i>Sph. macroconidialis</i>	<i>Chaconia brasiliensis</i>	Brazil	*	*	*
PURN4207	<i>Sph. macroconidialis</i>	<i>Chaconia brasiliensis</i>	Brazil	*		
PURN4452	<i>Sph. macroconidialis</i>	<i>Chrysocelis muehlenbeckiae</i>	Colombia	*	*	*
<b>s101</b>	<b><i>Sph. macroconidialis</i></b>	<b><i>Puccinia obscura</i></b>	<b>Germany</b>	<b>AY607023</b>		
<b>s13</b>	<b><i>Sph. macroconidialis</i></b>	<b><i>Puccinia abrupta</i></b>	<b>Ethiopia</b>	<b>AY607022</b>		
MCA7075	<i>Sph. paraphysata</i>	<i>Puccinia aframomi</i>	Cameroon	*	*	*
PURN11634 (PURP)	<i>Sph. paraphysata</i>	<i>Puccinia purpurea</i>	USA	*	*	*
PURN15263	<i>Sph. paraphysata</i>	<i>Phakopsora</i> sp.	Taiwan	*		*
PURN15329	<i>Sph. paraphysata</i>	<i>Uromyces setariae-italicae</i>	Bolivia	*	*	*
<b>CPC 21841</b>	<b><i>Sph. paraphysata</i></b>	<b>NA</b>	<b>Brazil</b>	<b>NR_137956.1</b>	<b>NG_067291.1</b>	<b>KP170685.1</b>
<b>CPC 23547</b>	<b><i>Sph. paraphysata</i></b>	<b><i>Ravenelia macowania</i></b>	<b>South Africa</b>	<b>KP170661.1</b>		
PAZ14rust- PURN23070	<i>Sph. paraphysata</i>	NA	Puerto Rico, USA		*	*
PP2004 (PURN11661)	<i>Sph. paraphysata</i>	<i>Puccinia philippinensis</i>	Guam	*		*

Table 5.3 continued

PUR11619	<i>Sph. paraphysata</i>	<i>Uromyces andropogonis</i>	USA	*		
PUR15359	<i>Sph. paraphysata</i>	<i>Uromyces trifolii-repentis</i>	USA	*		
PUR31535	<i>Sph. paraphysata</i>	<i>Puccinia</i> sp.	USA	*		
PUR3364	<i>Sph. paraphysata</i>	<i>Pucciniastrum hydrangeae</i>	USA	*		
PUR42807	<i>Sph. paraphysata</i>	<i>Crossopsora hymenaeae</i>	Cuba	*		
PUR48117	<i>Sph. paraphysata</i>	<i>Puccinia levis</i>	USA	*	*	*
PUR50338	<i>Sph. paraphysata</i>	<i>Uromyces bonariensis</i>	Guatemala	*		
PUR50994	<i>Sph. paraphysata</i>	<i>Uromyces</i> sp.	USA	*		
PUR51302	<i>Sph. paraphysata</i>	<i>Phakopsora aurea</i>	Honduras	*	*	*
PUR52253	<i>Sph. paraphysata</i>	<i>Puccinia arthurella</i>	Trinidad	*		
PUR52702	<i>Sph. paraphysata</i>	<i>Phakopsora compressa</i>	Costa Rica	*		
PUR56162	<i>Sph. paraphysata</i>	<i>Puccinia marylandica</i>	USA	*		
PUR58549	<i>Sph. paraphysata</i>	<i>Puccinia stenotaphri</i>	USA	*		
PUR59371	<i>Sph. paraphysata</i>	<i>Puccinia unica</i>	Mexico	*		
PUR60362	<i>Sph. paraphysata</i>	<i>Puccinia subtilipes</i>	Honduras	*		
PUR62882	<i>Sph. paraphysata</i>	<i>Puccinia unica</i>	USA	*		
PUR64079	<i>Sph. paraphysata</i>	<i>Uromyces costaricensis</i>	Mexico	*		
PUR64614	<i>Sph. paraphysata</i>	<i>Uromyces epicampis</i>	Mexico	*		
PUR66889	<i>Sph. paraphysata</i>	<i>Sorataea baphiae</i>	Nigeria	*		
PUR87214	<i>Sph. paraphysata</i>	<i>Uromyces unioniensis</i>	Brazil	*	*	*
PUR88231	<i>Sph. paraphysata</i>	<i>Puccinia oahuensis</i>	Brazil	*		
PUR9003	<i>Sph. paraphysata</i>	<i>Catenulopsora praelonga</i>	USA	*		
PURF10798	<i>Sph. paraphysata</i>	<i>Crossopsora fici</i>	Uganda	*		
PURF11444	<i>Sph. paraphysata</i>	<i>Puccinia posadensis</i>	Trinidad	*		
PURF11445	<i>Sph. paraphysata</i>	<i>Puccinia polysora</i>	St. Vincent & the Grenadines	*		
PURF11502	<i>Sph. paraphysata</i>	<i>Puccinia purpurea</i>	Venezuela	*		
PURF12985	<i>Sph. paraphysata</i>	<i>Uromyces costaricensis</i>	Trinidad	*		
PURF14478	<i>Sph. paraphysata</i>	<i>Phakopsora clemensiae</i>	India	*	*	*
PURF14635	<i>Sph. paraphysata</i>	<i>Puccinia nakanishikii</i>	India	*		
PURF14757	<i>Sph. paraphysata</i>	<i>Phakopsora africana</i>	Uganda	*		

Table 5.3 continued

PURF14933	<i>Sph. paraphysata</i>	<i>Puccinia eleocharidis</i>	Trinidad	*		
PURF14951	<i>Sph. paraphysata</i>	<i>Phakopsora loudetiae</i>	Kenya	*		
PURF15450	<i>Sph. paraphysata</i>	<i>Phakopsora pallescens</i>	Colombia	*		
PURF17943	<i>Sph. paraphysata</i>	<i>Puccinia duthiae</i>	India	*		
PURF18709c	<i>Sph. paraphysata</i>	<i>Uromyces manihotis</i>	Brazil	*		
PURF19059	<i>Sph. paraphysata</i>	<i>Uromyces costaricensis</i>	Brazil	*		*
PURF19059-2	<i>Sph. paraphysata</i>	<i>Uromyces costaricensis</i>	Brazil			*
PURF19505	<i>Sph. paraphysata</i>	<i>Uromyces linearis</i>	Nigeria	*		
PURF19703	<i>Sph. paraphysata</i>	<i>Puccinia cenchri</i>	Nigeria	*		
PURF19904	<i>Sph. paraphysata</i>	<i>Puccinia erythropus</i>	China	*		
PURF19927	<i>Sph. paraphysata</i>	<i>Puccinia paspalina</i>	China	*		
PURF19929	<i>Sph. paraphysata</i>	<i>Puccinia pogonatheri</i>	China	*		
PURF2490	<i>Sph. paraphysata</i>	<i>Puccinia infuscans</i>	Ecuador	*		
PURF2800	<i>Sph. paraphysata</i>	<i>Uromyces bonariensis</i>	Venezuela	*		
PURF4672	<i>Sph. paraphysata</i>	<i>Puccinia cacabata</i>	Brazil	*		
PURF4770	<i>Sph. paraphysata</i>	<i>Puccinia levis</i>	Bolivia	*		
PURF4897	<i>Sph. paraphysata</i>	<i>Uromyces setariae-italicae</i>	Trinidad	*		*
PURF7972	<i>Sph. paraphysata</i>	<i>Puccinia neorotundata</i>	Peru	*	*	*
PURF9841	<i>Sph. paraphysata</i>	<i>Puccinia thaliae</i>	Argentina	*		
PURN10179	<i>Sph. paraphysata</i>	<i>Puccinia minuta</i>	Brazil	*		
PURN10369	<i>Sph. paraphysata</i>	<i>Puccinia faceta</i>	Brazil	*		
PURN10826	<i>Sph. paraphysata</i>	<i>Kweilingia divina</i>	USA	*	*	*
PURN10850	<i>Sph. paraphysata</i>	<i>Puccinia invenusta</i>	Guam	*		
PURN11077	<i>Sph. paraphysata</i>	<i>Kweilingia divina</i>	Taiwan	*		
PURN1120	<i>Sph. paraphysata</i>	<i>Mikronegeria fagi</i>	Argentina	*		
PURN15342	<i>Sph. paraphysata</i>	<i>Uromyces hedysari-paniculati</i>	Guyana		*	*
PURN15343	<i>Sph. paraphysata</i>	<i>Puccinia commelinae</i>	Guyana	*		
PURN15344	<i>Sph. paraphysata</i>	<i>Puccinia duthiae</i>	Guyana	*	*	*
PURN15498	<i>Sph. paraphysata</i>	<i>Uromyces tenuicutis</i>	Guyana	*		
PURN15511	<i>Sph. paraphysata</i>	<i>Puccinia obliquo-septata</i>	Guyana	*	*	*
PURN16553	<i>Sph. paraphysata</i>	<i>Puccinia obliquo-septata</i>	Guyana	*	*	*

Table 5.3 continued

PURN16743	<i>Sph. paraphysata</i>	<i>Puccinia</i> sp.	Venezuela	*	*	*
PURN22990	<i>Sph. paraphysata</i>	<i>Melampsora</i> sp.	China	*		
PURN2908	<i>Sph. paraphysata</i>	<i>Phakopsora</i> <i>rossmanii</i>	Brazil	*		
PURN4122	<i>Sph. paraphysata</i>	<i>Melampsora</i> <i>epitea</i>	Brazil	*	*	*
PURN4123	<i>Sph. paraphysata</i>	<i>Melampsora</i> <i>epitea</i>	Brazil	*	*	
PURN5064	<i>Sph. paraphysata</i>	<i>Uromyces</i> <i>rhynchosporae</i>	Papua New Guinea	*		
PURN5574	<i>Sph. paraphysata</i>	<i>Puccinia</i> <i>stenotaphri</i>	Ecuador	*		
PURN5917	<i>Sph. paraphysata</i>	<i>Puccinia</i> <i>arachidis</i>	Brazil	*		
PURN9602	<i>Sph. paraphysata</i>	<i>Chaonia</i> <i>ingae</i>	Brazil	*		
TA427	<i>Sph. paraphysata</i>	<i>Puccinia</i> cf. <i>cyperi</i> <i>tegetiformis</i>	Benin	*	*	
<b>CBS 143579</b>	<b><i>Sph. paraphysata</i></b>	<b>Leaves of <i>Phragmites</i> sp.</b>	<b>Australia</b>			<b>MH108009.1</b>
PURN15307 (WTHC1)	<i>Sphaerellopsis</i> sp.	<i>Melampsora</i> <i>medusae</i>	USA	*	*	*
<b>ATCC</b>						
<b>MYA-2847</b>	<b><i>Sphaerellopsis</i> sp.</b>	<b><i>Melampsora</i> <i>medusae</i></b>	<b>USA</b>	<b>AY587134</b>		
PUR11683	<i>Sph. melamporiinae</i> .	<i>Uromyces</i> <i>graminicola</i>	USA	*		
PUR2041	<i>Sph. melamporiinae</i>	<i>Melampsora</i> <i>medusae</i>	USA	*		
PUR2047	<i>Sph. melamporiinae</i>	<i>Melampsora</i> <i>medusae</i>	USA	*		*
PUR2129	<i>Sph. melamporiinae</i>	<i>Melampsora</i> <i>medusae</i>	USA	*		
PUR23925	<i>Sphaerellopsis</i> sp.	<i>Puccinia</i> <i>montanensis</i>	USA	*		
PUR32274	<i>Sph. melamporiinae</i>	<i>Puccinia</i> <i>proserpinacae</i>	USA	*		
PUR43744	<i>Sph. melamporiinae</i>	<i>Coleosporium</i> <i>helianthi</i>	USA	*		
PUR43798	<i>Sph. melamporiinae</i>	<i>Coleosporium</i> <i>helianthi</i>	USA	*		
PUR47887	<i>Sph. melamporiinae</i>	<i>Melampsora</i> <i>medusae</i>	USA	*		
PUR56253	<i>Sph. melamporiinae</i>	<i>Puccinia</i> <i>recondita</i>	USA	*		
PUR88233	<i>Sph. melamporiinae</i>	<i>Coleosporium</i> <i>helianthi</i>	USA	*		
PUR90026	<i>Sph. melamporiinae</i>	<i>Melampsora</i> <i>ferrinii</i>	Brazil	*		*
PUR90183	<i>Sph. melamporiinae</i>	<i>Melampsora</i> <i>larici-populina</i>	Brazil	*	*	
PUR90242	<i>Sph. melamporiinae</i>	<i>Melampsora</i> <i>epitea</i>	Brazil	*	*	
PURF11501	<i>Sph. melamporiinae</i>	<i>Puccinia</i> <i>thaliae</i>	Venezuela	*	*	
PURF14716	<i>Sph. melamporiinae</i>	<i>Puccinia</i> <i>sorghi</i>	Ecuador	*	*	*

Table 5.3 continued

PURF16121	<i>Sph. melamporiinae</i>	<i>Puccinia phaenospermae</i>	Japan	*		*
PURF1651	<i>Sph. melamporiinae</i>	<i>Gerwasia holwayi</i>	Peru	*		
PURF17310	<i>Sph. melamporiinae</i>	<i>Puccinia caricis-japonica</i>	Japan	*		
PURF3626	<i>Sph. melamporiinae</i>	<i>Uromyces wulffiae-stenoglossae</i>	Trinidad	*		
PURF4112	<i>Sph. filum</i>	<i>Puccinia recondita</i>	Ecuador	*	*	
PURF5980	<i>Sph. filum</i>	<i>Puccinia boerhaviae</i>	Ecuador	*		
PURF7943	<i>Sph. melamporiinae</i>	<i>Puccinia inaequata</i>	Ecuador	*		
PURF829	<i>Sph. melamporiinae</i>	<i>Melampsora medusae</i>	Bolivia	*		
PURF833	<i>Sph. melamporiinae</i>	<i>Melampsora aecidioides</i>	Argentina	*	*	*
PURN12037	<i>Sph. melamporiinae</i>	<i>Melampsora humboldtiana</i>	USA	*	*	
PURN1206	<i>Sph. melamporiinae</i>	<i>Uromyces minutus</i>	USA	*		
PURN16392	<i>Sph. melamporiinae</i>	<i>Uromyces yurimaguensis</i>	Peru	*	*	
PURN16518	<i>Sph. melamporiinae</i>	<i>Melampsora ferrinii</i>	USA	*	*	
PURN16527	<i>Sph. melamporiinae</i>	<i>Melampsora medusae</i>	USA	*		
PURN21944	<i>Sph. melamporiinae</i>	NA	NA	*	*	
PURN2294	<i>Sph. melamporiinae</i>	<i>Coleosporium montanum</i>	USA	*		
PURN2303	<i>Sph. melamporiinae</i>	<i>Coleosporium asterum</i>	USA	*		
PURN2314	<i>Sph. melamporiinae</i>	<i>Coleosporium asterum</i>	USA	*		
PURN2448	<i>Sph. melamporiinae</i>	<i>Melampsora epitea</i>	USA	*		
PURN3993	<i>Sph. melamporiinae</i>	<i>Melampsora epitea</i>	Brazil	*	*	
PURN3995	<i>Sph. melamporiinae</i>	<i>Melampsora ferrinii</i>	Argentina	*		
PURN3996	<i>Sph. melamporiinae</i>	<i>Melampsora epitea</i>	Argentina	*	*	
PURN3997	<i>Sph. melamporiinae</i>	<i>Melampsora epitea</i>	Argentina	*	*	*
PURN3998	<i>Sph. melamporiinae</i>	<i>Melampsora epitea</i>	Argentina	*		
PURN4001	<i>Sph. melamporiinae</i>	<i>Melampsora epitea</i>	Argentina	*	*	
PURN4010	<i>Sph. melamporiinae</i>	<i>Melampsora medusae</i>	USA	*		
PURN4015	<i>Sph. melamporiinae</i>	<i>Melampsora larici-populina</i>	Colombia	*	*	
PURN4108	<i>Sph. melamporiinae</i>	<i>Melampsora aecidioides</i>	Brazil	*	*	*
PURN4109	<i>Sph. melamporiinae</i>	<i>Melampsora larici-populina</i>	Brazil	*		
PURN4120	<i>Sph. melamporiinae</i>	<i>Melampsora ferrinii</i>	Brazil	*		
PURN4121	<i>Sph. melamporiinae</i>	<i>Melampsora ferrinii</i>	Brazil	*	*	



Table 5.3 continued

PURN4124	<i>Sph. melamporiinae</i>	<i>Melampsora epitea</i>	Brazil	*	*	
PURN4127	<i>Sph. melamporiinae</i>	<i>Melampsora ferrinii</i>	Brazil	*		
PURN4510	<i>Sph. melamporiinae</i>	<i>Melampsora medusae</i>	USA	*	*	
PURN5424	<i>Sph. melamporiinae</i>	<i>Melampsora medusae</i>	USA	*	*	*
PURN6730	<i>Sph. melamporiinae</i>	<i>Melampsora medusae</i>	USA	*	*	*
PURN8265	<i>Sph. melamporiinae</i>	<i>Melampsora epitea</i>	Colombia	*		
PURN9763	<i>Sph. melamporiinae</i>	<i>Puccinia vernoniae-mollis</i>	Brazil	*	*	
<b>s18</b>	<b><i>Sph. melamporiinae</i></b>	<b><i>Melampsora sp.</i></b>	<b>Germany</b>	<b>AY607014</b>		
<b>s21</b>	<b><i>Sph. melamporiinae</i></b>	<b><i>Melampsora sp.</i></b>	<b>Germany</b>	<b>AY607015</b>		

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#### 5.2.7.1 collection and identification of urediniospores of *Puccinia polysora* from maize crops

In the summer of 2021, rust-infected maize leaves of *P. polysora* were harvested from field-grown maize plants at SWPAC, Indiana, USA, and brought to the Aime Lab. Urediniospores were collected using a mini cyclone spore collector (Tallgrass solutions, INC; Manhattan, KS) and stored in gelatin capsules at -80°C until further use. To confirm the identity of the rust at the species level, we used both morphological observations and molecular identification. For molecular identification, we extracted the genomic DNA of urediniospores from a single sorus using the EZNA HP Fungal DNA kit (Omega Bio-Tek, Norcross, Georgia), following the manufacturer's instructions. We amplified the large subunit (LSU) rDNA sequence, which is the most informative gene for rust species identification (M. C. Aime et al., 2017), using the primers LR5 and LR0R (Vilgalys & Hester, 1990) under the following conditions: initial denaturation at 94°C for 5 min; followed by 40 cycles of denaturation at 94°C for 30 sec, annealing at 50 °C for 45 sec, and elongation at 72°C for 1 min; and final extension at 72°C for 7 min. Electrophoresis and sequencing of the PCR products followed the same methodology in section 5.2.4. The resulting DNA fragment was blasted against the NCBI nucleotide database for the closest related rust species.

#### 5.2.7.2 Cultivation of corn plants in greenhouse and installation of humidity chamber

Healthy corn plants were cultivated in the greenhouse facility at Lily Hall of Sciences, Purdue University, to infect them with urediniospores of *Puccinia polysora* previously collected in the field. We planted at least seven 3-gallon pots with two corn seeds per pot. Following germination, we removed the weakest seedling and left only one plant per pot. Plants were maintained at a temperature range between 24 and 30 °C and watered and fertilized as needed. While corn plants grew until the tassel apparition, we installed a humidity chamber for rust inoculation in the same room where plants grew. This chamber consisted of a simple cubic structure (30 cm<sup>3</sup>) made of PVC pipes and covered with a white 4 mil plastic sheeting. A door was installed to the chamber for easy access and manipulation of the corn plants once these were inside. A PVC pipe (2cm diam. and 20 cm length) was also inserted in one side of the chamber to connect a 2.2 L humidifier (AquaOasis<sup>TM</sup>) placed outside the chamber. Finally, we placed a hygrometer inside the chamber to measure temperature and humidity.

#### 5.2.7.3 Rehydration of urediniospores of *P. polysora* for inoculation

Before inoculating healthy corn plants with *P. polysora*, we took the urediniospores stored at -80 and rehydrated them in two steps as follows: we thawed the spores at 4°C for 16 hours; then we took a sterile plastic container and put it inside the gelatin capsules containing urediniospores and a beaker with 23.5% KOH, as a source of water vapor. This concentration of KOH gives approx. 80% of relative humidity inside the container and avoid water condensation (Rowell, 1984). Then, we closed the container with a lid and let the urediniospores rehydrate for 12 hours at room temperature. Once urediniospores were rehydrated, we added them into a sterile glass vial containing 0.1% tween 20. We slightly mixed the spores with the solution to resuspend them and ensure no clumps were formed. The solution then turned light brown.

#### 5.2.7.4 Inoculation of corn plants with urediniospores of *Puccinia polysora* in the greenhouse

We used a spore inoculator (Tallgrass Solutions, Manhattan, Kansas) attached to an air compressor (California Air Tools CAT-1P060S) operating in the 2-5 psi range to inoculate healthy corn plants with urediniospores immersed in 0.1% tween 20. Each healthy corn leaf was sprayed with the spore solution at 2cm from the leaf. Once each plant was covered entirely with the spore solution, we placed them in the humidity chamber and did not close the chamber completely to ensure air circulation. Inoculations were done late afternoon to keep cool and low temperatures, which help moisture to stay longer on the leaf surface and facilitate spore germination for successful infection (Borlaug Global Rust, 2017). During all infecting time, we kept temperatures between 23 to 30 °C and continuously filled the humidifier with distilled water to keep relative humidity between 50 to 80%. We used 16 daylight hours and eight-night hours. Under optimal conditions, we observed rust symptoms on corn leaves between 7 to 15 days after inoculation.

#### 5.2.7.5 Harvesting of fresh conidia of *S. macroconidialis* and urediniospores of *P. polysora* for the in-vitro interaction test

Conidia of *S. macroconidialis* (strain SP28) from a two-week-old PDA Petri dish were harvested for the interaction test. We added 1 mL of autoclaved water to the medium, then slightly moved the Petri Dish to let the water mix with the conidia for about a minute. Once the water turned light white, we collected the conidia solution with a micropipette and transferred it to a 2mL tube. Fresh

urediniospores of *P. polysora* infecting corn plants in the greenhouse were also harvested for the interaction test. We collected urediniospores from open and pulverent sori to ensure the urediniospores were mature and ready to germinate. We gently tapped the rust-infected leaf against a 2mL tube containing 0.1% tween 20 to make the spores fall into it. Once the tween 20 solution turned light brown, we closed the lid. The concentration of conidia and urediniospores was measured with a hemocytometer to reach a dilution of  $10^4$  spores per mL. The viability of the spores was checked with Trypan Blue. We used more than 80% viable spores for the interaction test. Conidia and urediniospores were harvested on the same day before the interaction test.

#### 5.2.7.6 *In-vitro* interaction between spores of *S. macroconidialis* and *P. polysora*

We poured 1 mL of 1% water agar with 50 mg/mL Chloramphenicol into small Petri Dishes (50 mm diam.) to set up the interaction test. Then, we added 40  $\mu$ L of the urediniospores suspension to at least five Petri Dishes. To trace the urediniospores during the interaction test, we drew two points on each side of the bottom of each Petri Dish with a marker. Each Petri Dish was sealed with parafilm and incubated in the dark at 25°C overnight to facilitate urediniospores germination. The next day, we observed each Petri Dish under a compound microscope Olympus BH2-RFCA using a 20X objective. Petri Dishes containing at least 70% of the urediniospores germinated were kept for the next step. Otherwise, those Petri Dishes were discarded. We made sure to have at least three Petri dishes with more than 70% of the urediniospores germinated for the interaction test. Then, we added the conidia of *Sphaerellopsis* suspended in water to the same Petri dishes containing germinated urediniospores at a 1mm distance from the urediniospores. Petri Dishes were sealed again with parafilm and remained for 24 hours at room temperature. After 24 hours of co-inoculation, daily observations were made for the next 12 days to elucidate the first interaction events between these two fungal species. Because the Petri Dishes only have 17 mm in height, we could observe interaction events under a microscope using the 20X objective without opening the lid to avoid contamination. Lids were only removed on the last day of observation to use a 40x objective and take final pictures. Two negative controls were also used in this interaction test. The first one consisted of three Petri dishes containing urediniospores only. The second consisted of having three Petri dishes containing conidia of *S. macroconidialis* only. The experiment was repeated three times.

## 5.3 Results

### 5.3.1 *Sphaerellopsis* dataset

We randomly screened 5,618 rust specimens in 99 rust genera, which represents 58% of the total collections at PUR and 58% of the accepted rust genera (Berndt & Aime, n.d.), for the presence of *Sphaerellopsis* that was incidentally co-collected with rust specimens (see Supplementary data Table S1). Of these 5,618 specimens, we collected 523 to be intimately associated with black fruiting bodies resembling the fungal genus *Sphaerellopsis* (see Supplementary Data Table S2). Of these 523 collections, 199 were confirmed as *Sphaerellopsis* members through both phylogenetic analyses and morphological observations (see Supplementary Data Table S2 and Table 5.3). Five *Sphaerellopsis* species were recovered, infecting 122 rust species in 18 genera from 34 countries (Table 5.3).

We successfully amplified 195 DNA sequences of *Sphaerellopsis* from the 5.8S and ITS2 rDNA, 58 sequences from LSU rDNA, 47 from *tef-1*, and eight from *rpb2*. Although we amplified the four gene regions for some *Sphaerellopsis* specimens, it was impossible to get all specimens' DNA fragments due to the typical degraded DNA of preserved biological collections. Nevertheless, we successfully amplified the ITS region of 163 specimens collected between 1883 and 1998. The oldest *Sphaerellopsis* specimen whose ITS region was successfully amplified was collected in 1883 on *Melampsora medusae* from the United States (PUR voucher: 2041). Lastly, we isolated one strain of *S. macroconidialis* from fresh specimens collected on field trips. This strain was used to observe the first interaction events between the conidia of *S. macroconidialis* and germinated urediniospores of *Puccinia polysora*.

### 5.3.2 Nucleotide alignment dataset and phylogenetic inferences

Our multi-locus phylogenetic analysis consisted of four concatenated gene-region datasets of 1968 characters, of which 322 were parsimony-informative. The percentage of parsimony-informative characters per gene region was 3.4% for ITS, 1.3% for LSU, 5.7% for *tef-1*, and 5.9% for *rpb2*. We included 217 taxa, of which 17 were *Sphaerellopsis* reference taxa, and *Alternaria consortialis* served as an outgroup taxon (Table 5.3). The following models were selected by ModelFinder (AICc): JC for ITS, GTR+F+R2 for LSU, TNe+G4 for *tef-1*, and TIM2e+I for *rpb2*. Our maximum

likelihood analysis revealed eight supported clades (Fig. 5.1), from which all have bootstrap support greater than 70%, and all DNA sequences form a monophyletic group.

### **5.3.3 Species Diversity of *Sphaerellopsis* associated with rust fungi**

Five species of *Sphaerellopsis* were recovered from our sampling; four of the seven previously accepted species are represented, with the addition of one that appears to be undescribed (Fig. 5.1). *Sphaerellopsis paraphysata* was the most common species, found on 77 rust specimens, followed by *S. macroconidialis* on 56 and *S. filum* on 12. *Sphaerellopsis hakeae* was found in one rust specimen, and *S. artemisiae* and *S. isthmospora* were not found in this study. *Sphaerellopsis anomala* was not included in our phylogenetic analysis due to the unavailable DNA sequences for this species. Two other well-supported clades were found in the phylogeny without any currently accepted referenced *Sphaerellopsis* species. One of these consisted of a single specimen found on sori of *Puccinia montanensis* from the United States collected in 1896 (PUR voucher: 23925). We amplified the ITS region of this specimen and took macro photographs. However, due to the scarce and dry material, the specimen's morphology and amplification of other gene regions were impossible. Thus, in this study, we do not consider it an undescribed species. The other clade comprised 55 taxa from rust specimens collected between 1883 and 2016. One taxon contained the teleomorph of *Sphaerellopsis*, whose morphology was described, and the ITS and LSU gene regions were successfully amplified. Since we could describe the anamorph and the teleomorph of the specimens within this clade and obtain molecular data, we confirm that these taxa represent a previously undescribed *Sphaerellopsis* species.

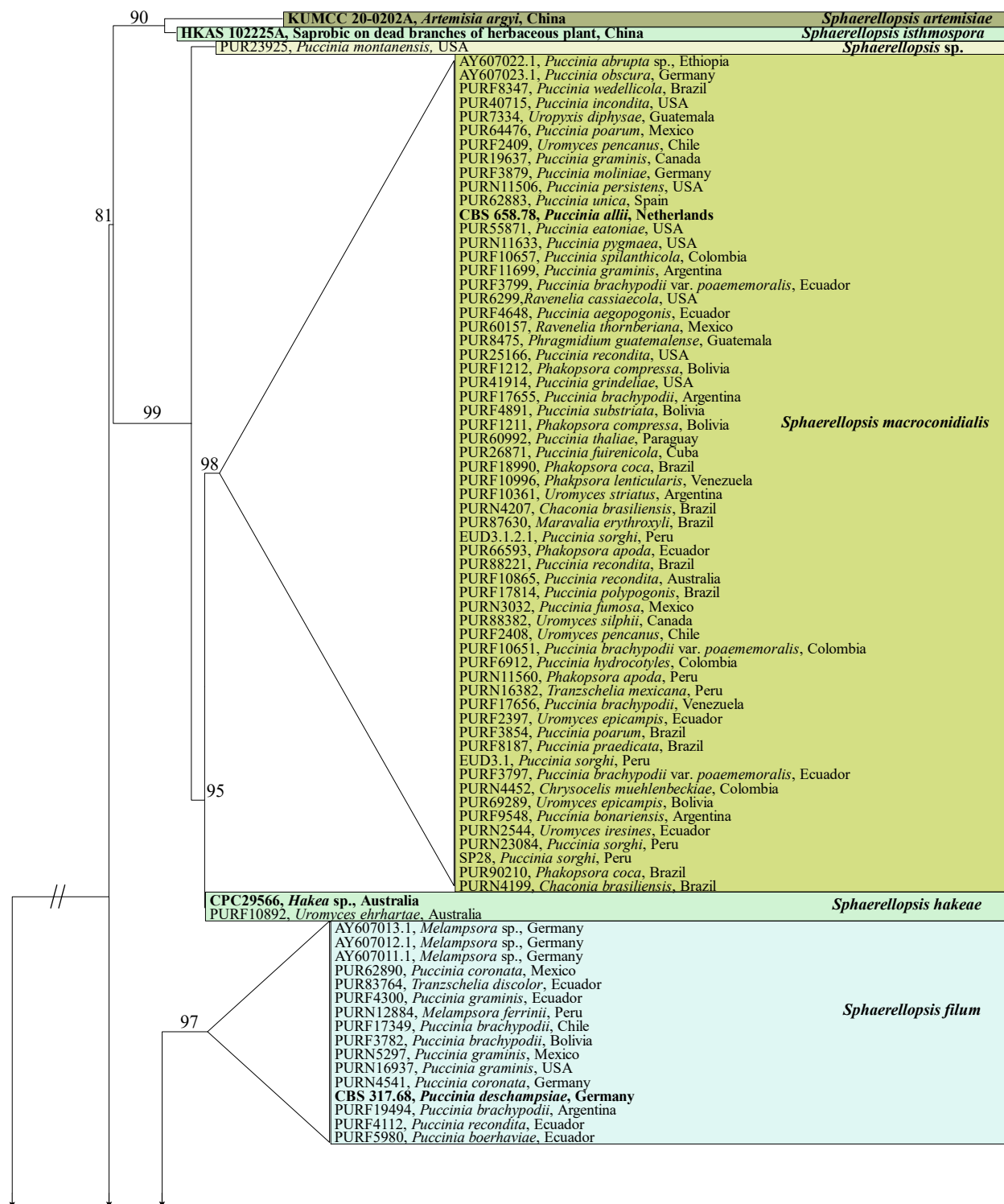
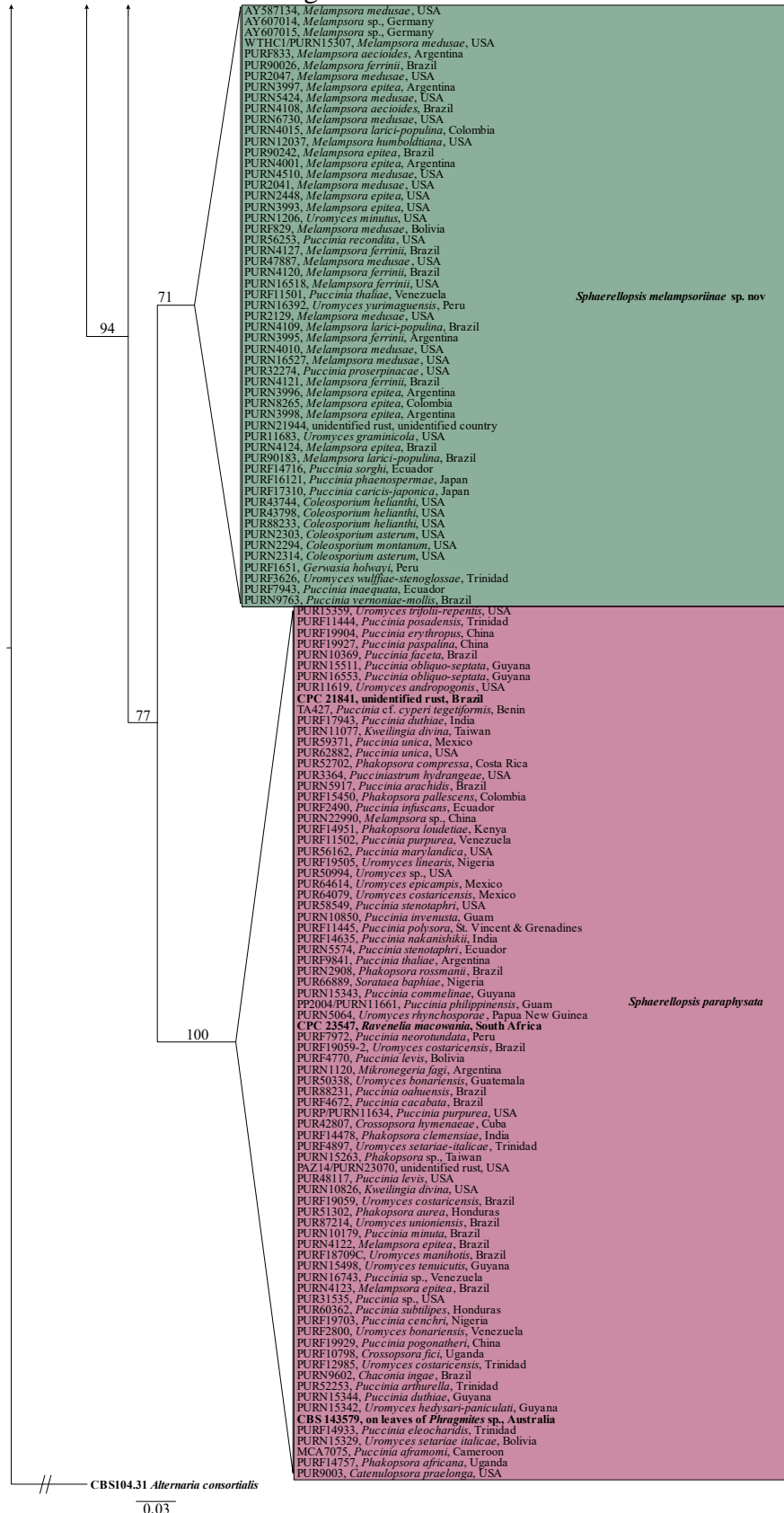


Figure 5.1 The best-scoring ML phylogenetic tree of the genus *Sphaerellopsis* reconstructed from concatenated four-locus datasets (ITS, LSU, *tef-1*, and *rpb2*). The ML bootstrap value is presented above each branch. Colors delimit clades, each labeled with the *Sphaerellopsis* species. Taxa labels are written on the tree as "PUR voucher," "the host rust where *Sphaerellopsis* was found," and "the origin/locality of each specimen." Reference sequences and outgroup taxa are written in bold. The tree was rooted to *Alternaria consortialis* CBS 104.31.

Figure 5.1 continued





#### 5.3.3.1 Taxonomy

##### ***Sphaerellopsis melampsoriinae* Gomez-Zap. & Aime, sp. nov.**

Fig. 5.2

Mycobank No: pending

**Etymology:** Named after the large number of rust hosts that belong to the suborder *Melampsoriineae*

**Diagnosis:** Similar to *S. filum* but differs in conidiomata size (up to 107 µm diam.), conidia 1-septate, and conidia length (9.1–)10.3–14.3(–16.6) µm.

**Type: Holotype:** United States, Indiana, on urediniospores of *Melampsora medusae* infecting *Populus deltoides*, 19 September 2015, M. Catherine Aime, collection number: sn. Anamorph: PUL F29362 (ex-PURN15307), ex-neotype sequences available: ITS, LSU, *tef-1*, *rpb2*. **Epitype designated here:** Brazil, São Paulo, on urediniospores of *Puccinia vernoniae-mollis* infecting leaves of *Vernonia* sp., 17 February 1989, Anibal de Carvalho, collection number: 89-7. Teleomorph: PUL F29361 (ex-PURN9763), ex-epitype sequences available: ITS and LSU.

**Description:** Conidiomata associated with rust sori. Conidiomata pycnidial, erumpent, aggregated, globose, 48–107 µm, with central ostiole, outer layers dark brown cells textura angularis, 3.8–6.92 µm diam. Paraphyses not seen. Conidiophores reduced to conidiogenous cells. Conidiogenous cells lining the inner cavity, smooth, hyaline, globose to ampulliform. Conidia fusoid, hyaline, smooth, guttulate, 1-septate, slightly constricted at the septum, apex subobtuse, tapering to truncate hilum, (9.1–)10.3–14.3(–16.6) × (3–5) µm. Stromata associated with rust sori, 76–162 µm diam., solitary or gregarious; loci immersed, brown in outer zone consisting of two to three rows of dark cells, hyaline in inner part, subglobose to ampulliform, with protruding papillate neck and ostiole. Pseudoparaphyses filiform, septate, hyaline. Asci numerous, 8-spored, bitunicate, cylindrical-clavate, short stipitate, 59.6–101.3 × 8.5–10.4 µm. Ascospores irregularly biserial, fusiform, hyaline to pale yellow, 15.2–21.3 × 3.8–6.0, 1-septate, slightly constricted at the septum, surrounded by a mucous sheath not easily perceived.

**Substrate/Host:** on rust sori of several rust species.

**Distribution:** Argentina, Bolivia, Brazil, Colombia, Ecuador, Germany, Japan, Peru, Trinidad, United States of America, Venezuela.

**Additional materials examined:** **Colombia**, Antioquia, on urediniospores of *Melampsora larici-populina*, infecting *Populus nigra*, 20 March 1989, V.M Pardo-Cardona, collection number: unavailable. Voucher: PUL F29359 (ex-PURN4015). **Peru**, Ucayali, on urediniospores of *Uromyces yurimaguasensis*, 22 October 2016, M. Catherine Aime, collection number: MCA6471. Voucher: PUL F29363 (ex-PURN16392). **United States of America:** Illinois, on urediniospores of *Melampsora* sp. infecting *Populus* sp., 22 September 2012, M. Catherine Aime, collection number: 5030. Voucher: PUL 29360 (ex-PURN6730); Georgia, on urediniospores of *Coleosporium helianthi*, infecting *Silphium compositum*, 24 August 1977, Yoshitaka Ono, John McCain & Joe F. Hennen, collection number: 10185. Voucher: PUL F29357 (ex-PUR88233).

**Notes.** The conidiomata and length of conidia of *S. melampsoriinae* are smaller than any other *Sphaerellopsis* species. However, the width of the conidia of *S. melampsoriinae* is similar to *S. anomala*, *S. filum*, and *S. macroconidialis*. In addition, *S. melampsoriinae* is distributed worldwide and seems not to prefer any rust genus to be associated with. However, most rust hosts of *S. melampsoriinae* belonged to the suborder *Melampsorineae*, with 41 out of 55 taxa within the clade. Lastly, since the anamorph and teleomorph of *S. melampsoriinae* were found in different specimens, we designated here the epitype, including the teleomorph.

#### 5.3.3.2 The teleomorph of *S. macroconidialis*

Like all *Sphaerellopsis* species, *S. macroconidialis* was described from the anamorph only. However, in this study, we recovered one specimen containing the teleomorph of *S. macroconidialis* (Fig. 5.2). This specimen was found in Brazil, Rio de Janeiro, associated with teliosori of *Puccinia wedellicola* infecting the host plant *Wedelia trichostephia*, collected on 7 May 1922, by E.W.D Holway, collection number: 1822, Voucher PUL F29358 (ex-PURF8347) (Fig. 5.3). The ITS sequence obtained from this specimen shared 100% identity (239/239 no gaps) with *S. macroconidialis* CBS 233.51 (GenBank Accession No. MH856836.1). In addition, due to the well-preserved specimen, herein we describe its morphology as follows: Stromata were observed

developed on rust sori, up to 123  $\mu\text{m}$  diam., brown in outer zone, cells textura parenchymatic, hyaline in inner part, erumpent, gregarious; loci subglobose to ampulliform. Pseudoparaphyses filiform, septate, hyaline. Asci numerous, 8-spored, bitunicate, cylindrical-clavate, short stipitate,  $68.2\text{--}106.7 \times 7.3\text{--}11.1 \mu\text{m}$ . Ascospores irregularly biseriate, fusiform, hyaline to pale yellow,  $17.2\text{--}23 \times 4.8\text{--}6.0$ , 1-septate, slightly constricted at the septum, surrounded by a hyaline mucous sheath not easily perceived. Additionally, we made morphological comparisons between the teleomorph of *S. macroconidialis* and *S. melampsoriinae*. However, their asci and ascospores differed in size. To our knowledge, this is the first report of the teleomorph of *S. macroconidialis*.

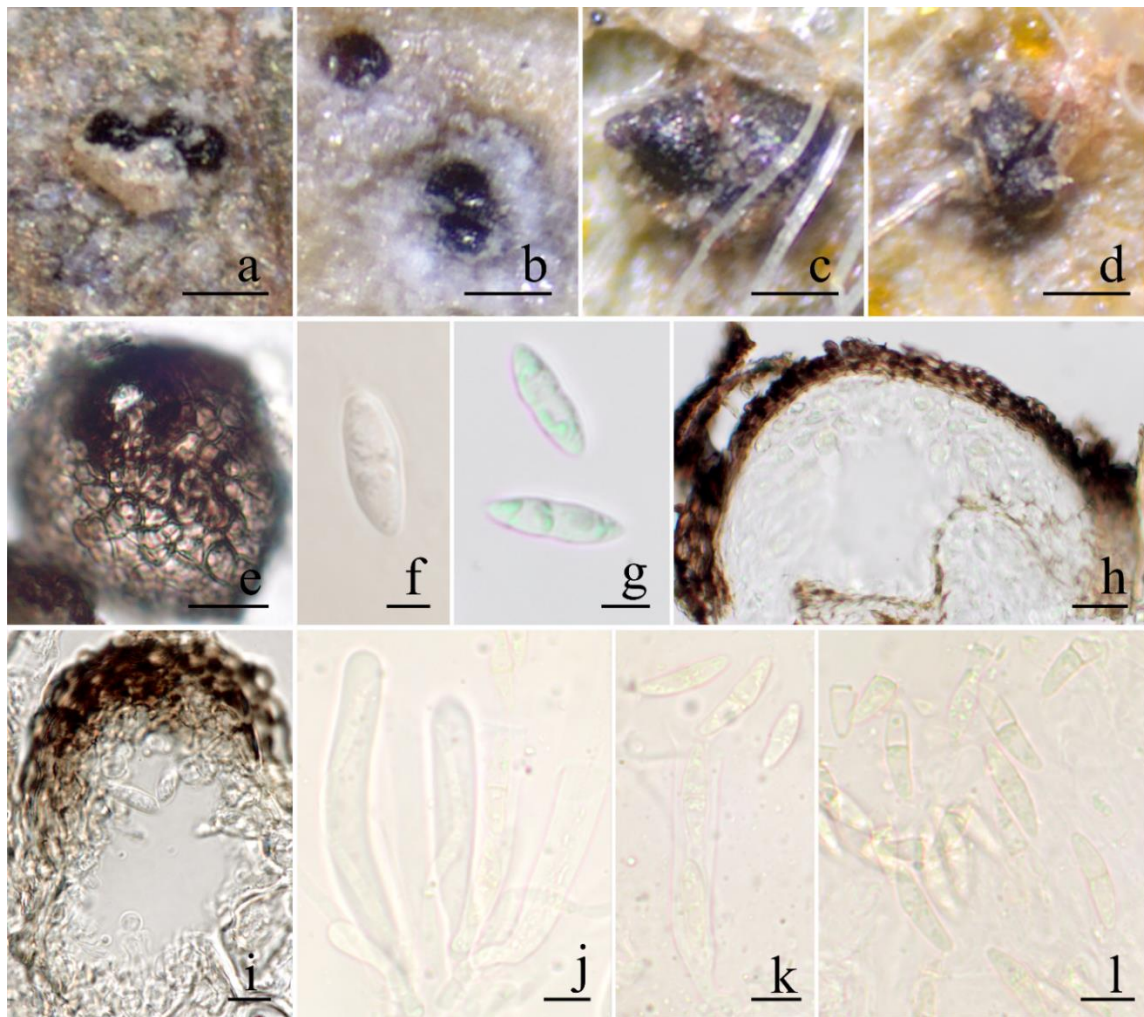


Figure 5.2 *Sphaerellopsis melampsoriinae* (PURN15307, PURN6730, PURN9763). A, B. Conidiomata developed on sori. C, D. Stromata developed on sori. E. Outer layers of conidioma, textura angularis. F, G. Conidia. H. Vertical section through ascomata. I. Conidiogenous cells. J, K. Asci and pseudoparaphyses. K, L. Ascospores. Bars: A–D = 100  $\mu\text{m}$ , E–G = 10  $\mu\text{m}$ , H = 20  $\mu\text{m}$ , I–L = 10  $\mu\text{m}$ .

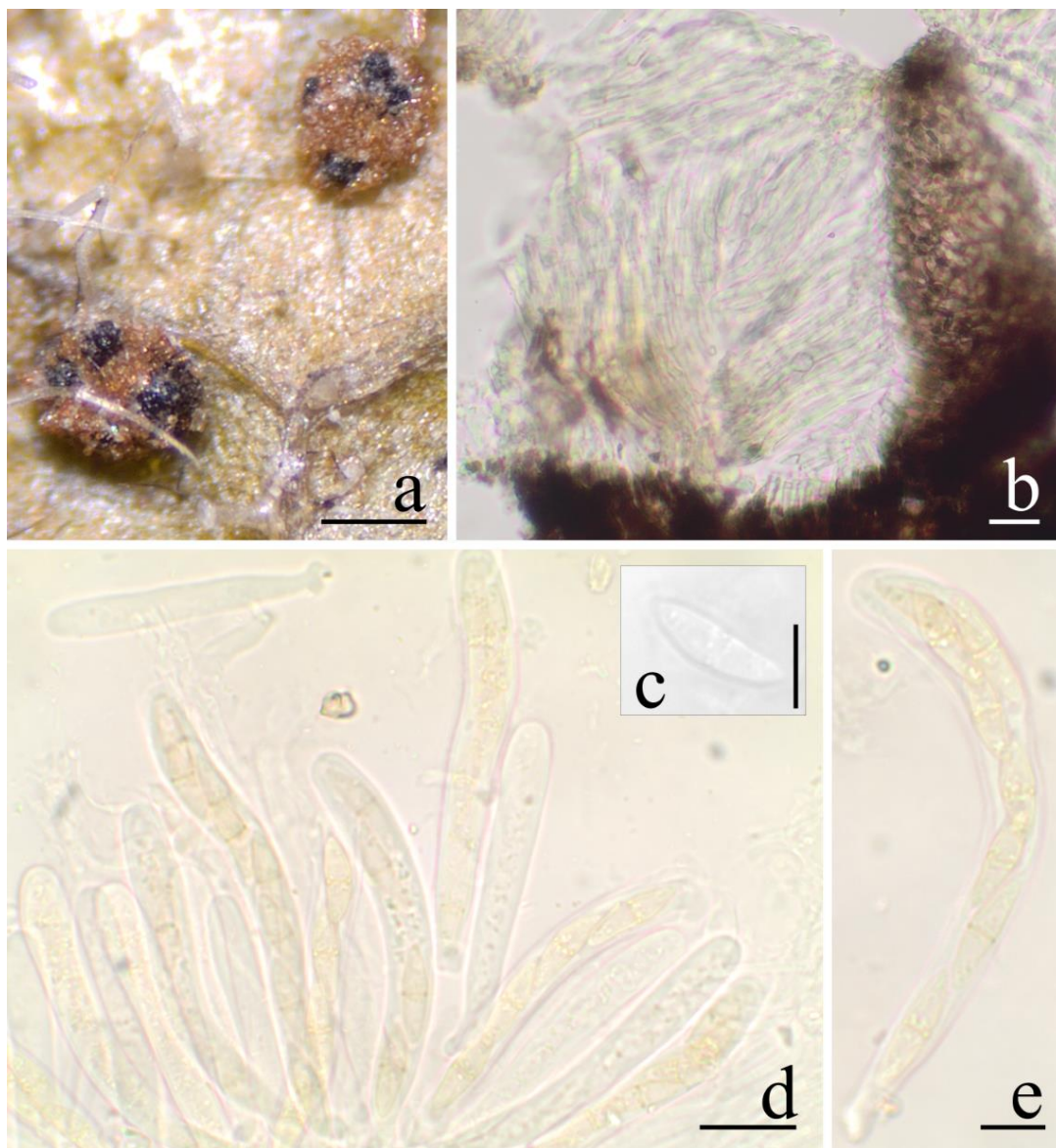


Figure 5.3 Teleomorph of *S. macroconidialis* (PURF8347).

A. Stromata. B. Vertical section through ascomata. C. Ascospore. D. Asci and Pseudoparaphyses. E. Ascae and ascospores. Bars: A= 200  $\mu\text{m}$ , B, C = 20  $\mu\text{m}$ , D, E = 10  $\mu\text{m}$ .

#### 5.3.4 Host-specificity of *Sphaerellopsis* associated with rust fungi at PUR

Our work does not show that *Sphaerellopsis* species prefer specific rust genera. For example, *S. macroconidialis* was found to be associated with several rust genera such as *Chaconia*, *Phakopsora*, *Phragmidium*, *Puccinia*, *Ravenelia*, and *Uropyxis*, among others (Fig. 5.1). Similarly, *S. paraphysata* was associated with several rust genera, including *Crossopsora*, *Kweilingia*,

*Melampsora*, *Mikronegeria*, *Phakopsora*, *Puccinia*, *Sorataea*, and *Uromyces*, among others. The same behavior was observed for *S. filum* and *S. hakeae*. Although the new species *S. melampsoriinae* did not prefer a specific rust genus, most of its rust hosts belonged to the suborder *Melampsorineae*, with 41 out of 55 taxa within the clade.

### **5.3.5 Geographic distribution**

This study includes *Sphaerellopsis* specimens associated with rust fungi collected in 35 countries across the globe. Ten specimens are from Africa, 13 from Asia, 11 from Europe, 56 from North America, 116 from the Neotropics, and seven from Oceania (Fig. 5.4). Our results suggest that *S. macroconidialis*, *S. paraphysata*, *S. filum*, and *S. melampsoriinae* have a cosmopolitan distribution and are adapted to different environmental conditions in temperate and tropical regions (Fig. 5.5). However, *S. paraphysata* is more abundant in the tropics. Since we only found one specimen of *S. hakeae* in our sampling collected in Australia, and this species only has one record, also from Australia (Crous et al., 2016), *S. hakeae* is so far restricted to that country. Similar results are for *S. artemisiae* and *S. isthmospora*. Although we did not find any rust specimens associated with these two species, both were previously reported in China (Doilom et al., 2021; Phookamsak et al., 2019). Thus, their geographic distribution may also be restricted.

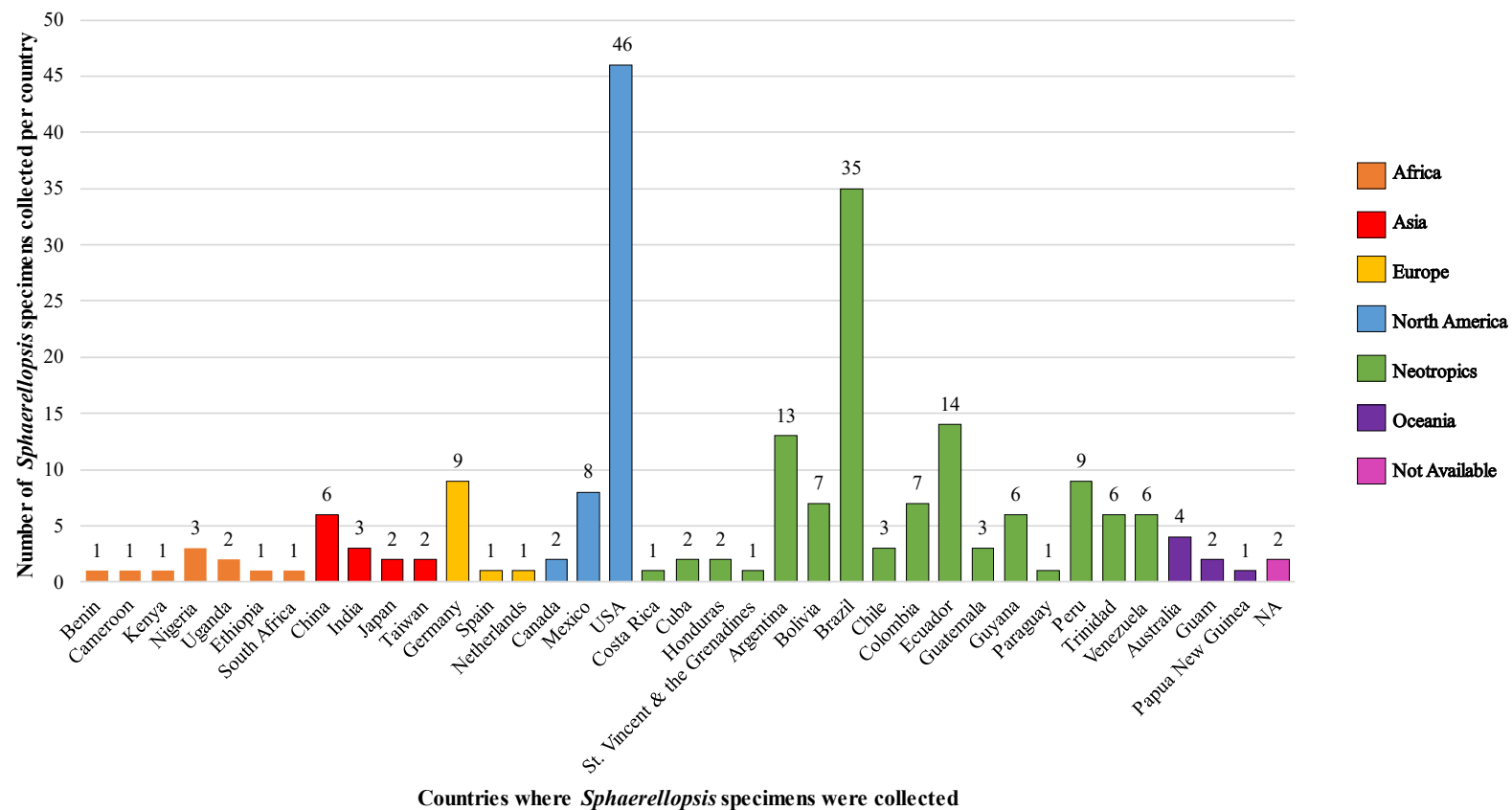


Figure 5.4. Origin/localities of *Sphaerellopsis* specimens associated with rust fungi. The number of specimens collected per country is written above the bar. Countries are colored by geographic regions.



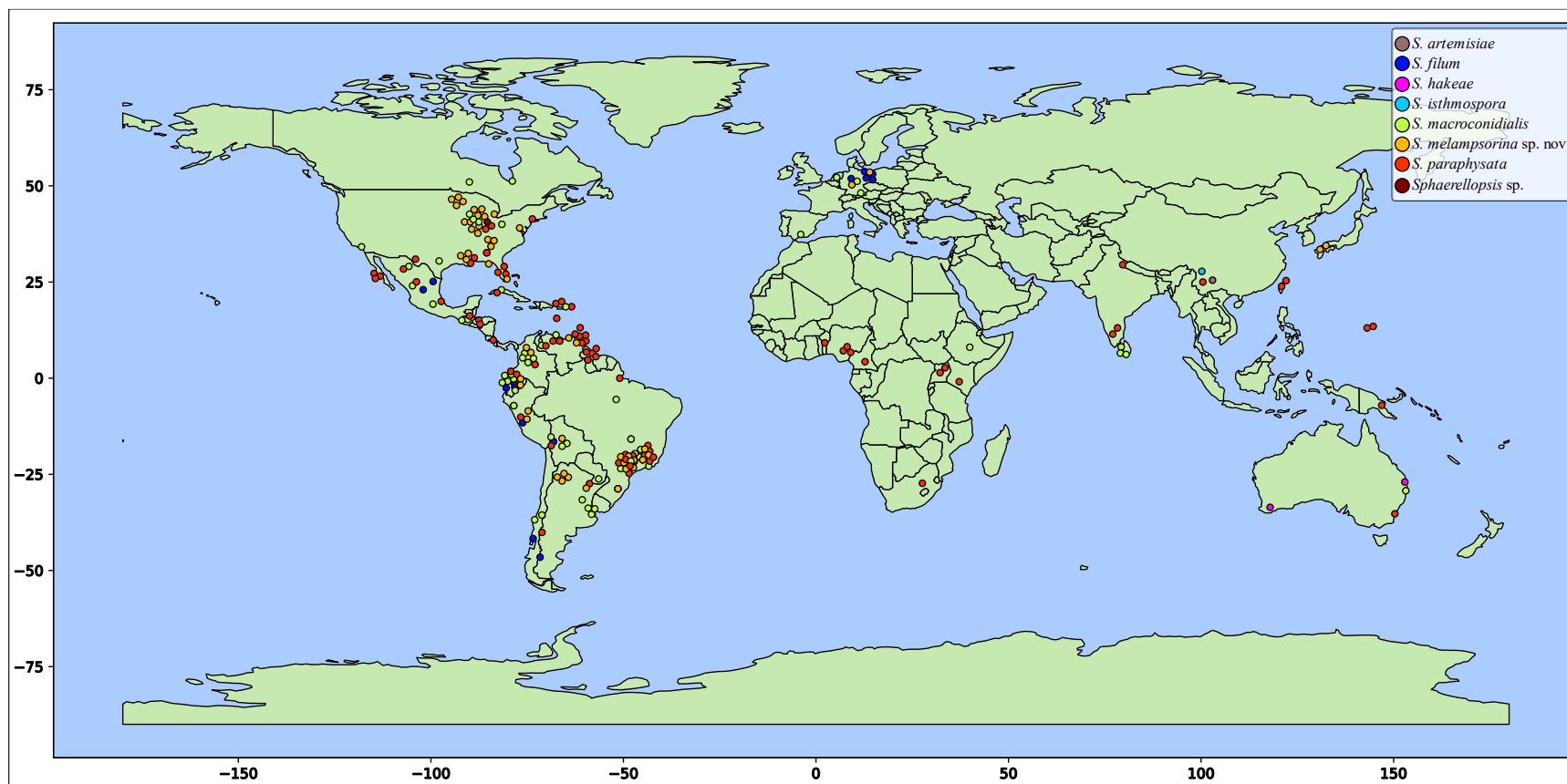


Figure 5.5 Cosmopolitan distribution of *Sphaerellopsis* species. Each circle represents one specimen. Circles are colored by species.

### **5.3.6 *In vitro* Interaction test between *S. macroconidialis* and *P. polysora***

The interaction test of this study confirms the mycoparasitic strategy of *S. macroconidialis* to rust fungi. Five days after the co-cultivation, we observed hyphae of *S. macroconidialis* growing along the germinative tubes of *P. polysora* by coiling around them (Fig. 5.6). The germinative tubes of *P. polysora* measured 6.5  $\mu\text{m}$  in diam., while those of *S. macroconidialis* measured 1.8  $\mu\text{m}$  in diam., making them easy to distinguish. During the first day after co-cultivation, we observed the first contact between germinated conidia of *S. macroconidialis* and germinative tubes of urediniospores of *P. polysora*. Then, during the next four days, hyphae of *S. macroconidialis* started to grow over the urediniospores and their germinative tubes, but without clear evidence of antagonism. However, on the fifth day of co-cultivation, we noticed several germinative tubes of urediniospores coiled by *S. macroconidialis* hyphae. Coils tightly encircled the germinative tubes, clearly showing a mycoparasitic interaction. However, the cell wall of germinative tubes was not disrupted. Such coils were not seen on *S. macroconidialis* hyphae inoculated alone, which means that the presence of germinative tubes of *P. polysora* induces the formation of coils. During the next six days, we did not notice any new sign of mycoparasitic mechanism against *P. polysora*. Nonetheless, on day 12, we noticed the formation of an appressorium attached to a urediniospore and turgor loss of a few germinative tubes already coiled by *S. macroconidialis*. Such mycoparasitic signs were not seen on germinated urediniospores inoculated alone. After 12 days of observations, *S. macroconidialis* hyphae grew abundantly, and no other antagonistic events could be viewed.



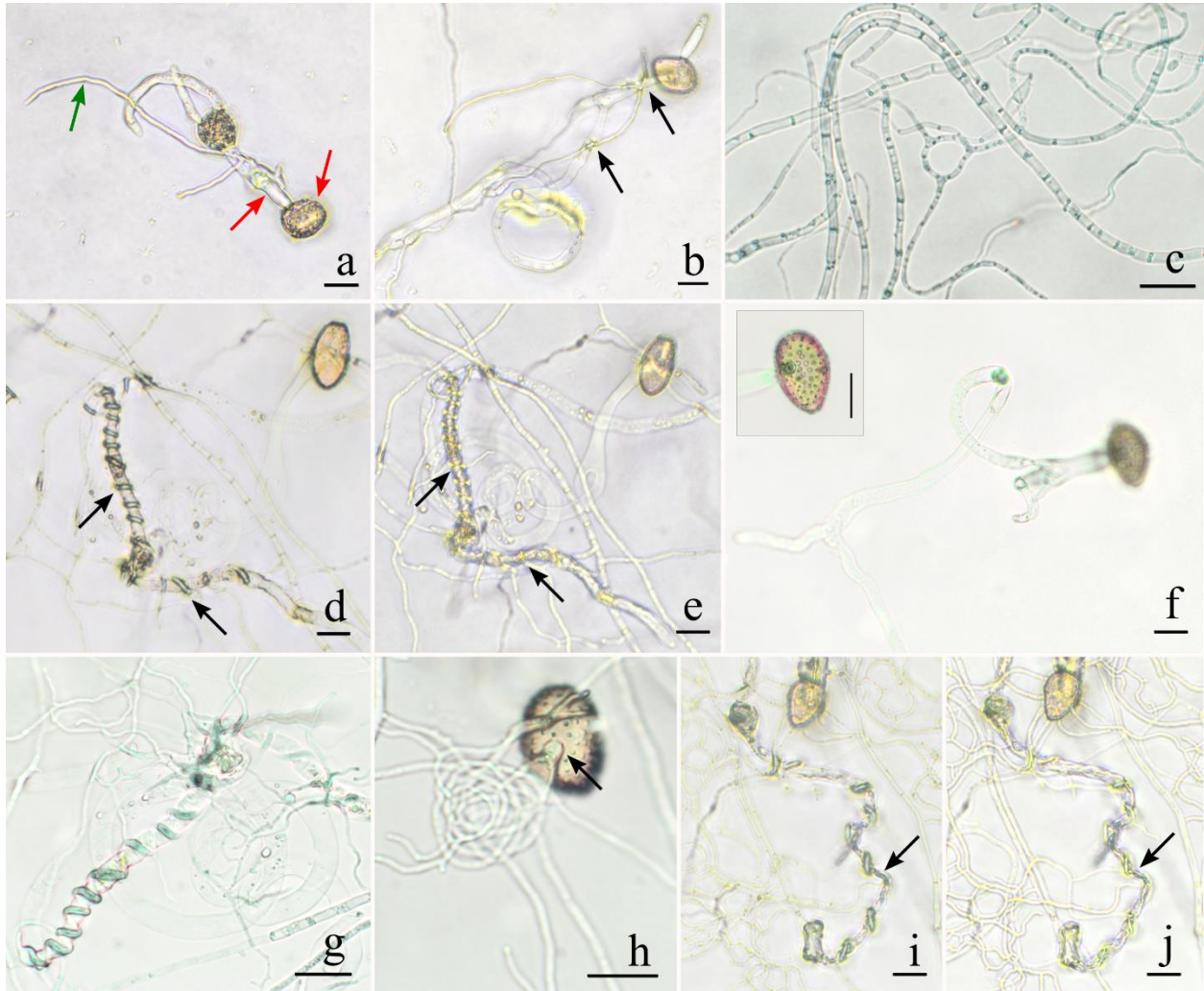


Figure 5.6 Light micrographs of *Sphaerellopsis macroconidialis* interacting with germinated urediniospores of *Puccinia polysora* *in-vitro*. A–B. Day one after co-cultivation. A. Red arrows point to the urediniospore and its germinative tube, and the green arrow points to *S. macroconidialis* hypha. B. black arrows point to the first contact. C. Negative control, hyphae of *S. macroconidialis* alone on day 12. D–E. Day five after co-cultivation. Hyphae of *S. macroconidialis* form coils and tightly encircle germinative tubes of *P. polysora*. Black arrows point to dense coils. F. Negative control, urediniospores, and germinative tubes alone on day 12. G–J. Day 12 after co-cultivation. G. Dense coils around a germinative tube of *P. polysora*. H. Appressorium attached to urediniospore. I and J. Loss of turgor of germinative tube of *P. polysora*. Scale bars: 20  $\mu\text{m}$  (A–B, D–J); 50  $\mu\text{m}$  (C).

## 5.4 Discussion

### 5.4.1 Species diversity of the genus *Sphaerellopsis* associated with rust fungi.

Results from this study suggest that *S. macroconidialis* and *S. paraphysata* are the most commonly collected species associated with rust fungi. *S. macroconidialis* was found on ten rust genera and *S. paraphysata* on 12 rust genera (Fig. 5.1). Surprisingly, the type species, *S. filum*, was not as common as expected based on the previous rust hosts list made by Kranz & Brandenburger, who reported *S. filum* on 30 rust genera and 369 rust species (Kranz & Brandenburger, 1981). In contrast, we found *S. filum* in only three rust genera: *Melampsora*, *Puccinia*, and *Tranzschelia*. These results are consistent with the phylogenetic analyses of *Sphaerellopsis* by (Trakunyingcharoen et al., 2014), which showed that several isolates were misidentified as *S. filum*. Thus, this species may not be the most commonly associated with rust fungi.

Our extensive dataset also shows that *S. artemisiae* and *S. isthmospora* may not be associated with rust fungi because they were not found in any rust specimen of our extensive sampling. These results are consistent with previous observations by several mycologists suggesting that *Sphaerellopsis* may have several nutrition strategies. For example, Hulea (1939) and Eriksson (1966) did not consider all *Sphaerellopsis* members exclusively mycoparasites. They posited that *Sphaerellopsis* could also be a commensal, saprobic, or plant pathogen. Nicolas & Villanueva (1965) sustained that the anamorph of *Sphaerellopsis* might utilize a large number of carbon compounds, and it likely needed only one nitrogen compound to survive. Then, Eriksson (1966) considered that *Sphaerellopsis* might feed on plant tissue and also hypothesized that other factors, such as specific compounds secreted from the rust, might be required for *Sphaerellopsis* to develop its fruiting bodies. This hypothesis is related to recent studies of some natural enemies attracted by volatiles secreted by plant tissue once a pathogen infects them (Eberl et al., 2018). Therefore, we do not discard that this can be a way to attract *Sphaerellopsis* to infect rust fungi, either by the metabolites secreted by the rust infecting the plant tissue or the plant tissue secreting some metabolites after being infected by the rust. Thus, a tri-trophic relationship may still be possible, where the *Sphaerellopsis* species and the host plant can have a mutualistic interaction against the rust. However, based on our multi-locus phylogenetic tree, *S. artemisiae* and *S. isthmospora* appear to be the earliest diverging members of the genus. So another possible hypothesis is that

*Sphaerellopsis* began as a plant associate, and then species evolved to specialize in rust fungi. Further studies are then needed to confirm each of these hypotheses.

#### **5.4.2 Phylogenetic inference of *Sphaerellopsis***

This study contributes with the largest dataset of *Sphaerellopsis* sequences providing a multi-locus phylogenetic tree. Although in Chapter 2, the ITS phylogenetic tree of *Sphaerellopsis* provided helpful information about the phylogenetic relationships of the currently accepted *Sphaerellopsis* species, some clades did not have high bootstrap support. Thanks to additional DNA fragments amplified in this study, we significantly improved the phylogenetic inferences of *Sphaerellopsis*, which also let us describe a new species. Furthermore, we compared each phylogenetic tree reconstructed from individual loci to the multi-locus tree for congruence in their topologies (see supplementary data). The ITS, *tef-1*, and *rpb2* phylogenetic reconstructions shared the same topology as the multi-locus phylogenetic tree, but the topology of the LSU phylogenetic tree was different. These results are consistent with previous phylogenetic studies of *Sphaerellopsis* (Trakunyingcharoen et al., 2014). In addition, Trakunyingcharoen et al. (2014) demonstrated that the ITS gene region provided a high resolution to distinguish members of *Sphaerellopsis* at the species level. However, because most of our specimens come from fungarium collections, we could only amplify the 5.8S and ITS2 gene regions and thus needed other DNA fragments for a higher-resolution phylogenetic tree. Our results also suggest that although the barcoding ITS region provides enough confidence to identify a *Sphaerellopsis* member at the species level, amplifying the gene regions *tef-1* or *rpb2* is also helpful to confirm the ITS output, especially when the ITS fragment is short (250 bp).

#### **5.4.3 The conspecific *Sphaerellopsis* species with *Eudarluca caricis* remains uncertain**

*Eudarluca* and *Sphaerellopsis* are considered congeneric (Keener, 1951; Z. W. Yuan et al., 1998). *Eudarluca* has been described in the teleomorph state, and *Sphaerellopsis* in the anamorph state. Both genera are found intimately associated with the sori of several rust species across the globe. The genus *Eudarluca* was erected in 1908 by Spegazzini to place "a new pyrenomycete" associated with the uredosori of an unknown rust, infecting *Canna* sp. in the Botanical Garden in São Paulo, Brazil (Spegazzini, 1908). It is when Spegazzini named *Eudarluca australis* as the type species of

the genus. However, later in 1966, Eriksson combined several species with *E. australis* into *Eudarluka caricis* based on an overview of the taxonomy, nomenclature, and ecology of *E. caricis* (Eriksson, 1966). The specific epithet "caricis" was kept based on the basionym *Sphaeria caricis* described by Fries in 1823 (Fries, 1823). The original specimen of *Sphaeria caricis* was collected from uredosori of a rust species on *Carex* sp. We thus tried to track the teleomorph of *Sphaerellopsis* in similar specimens whose hosts were *Carex* sp. However, none of the *Sphaerellopsis* specimens that we identified through morphological descriptions and phylogenetic analyses were collected on *Carex* sp. The teleomorph of *S. macroconidialis* was collected on teliosori of *Puccinia wedellicola* infecting the host plant *Wedelia trichostephia*, while the teleomorph of *S. melampsoriinae* was collected on uredosori on *Puccinia vernoniae-mollis* on the leaves of *Vernonia* sp. Therefore, the application of the specific epithet of *E. caricis* still remains uncertain.

#### **5.4.4 The teleomorph of *Sphaerellopsis* seems to prefer tropical environments**

The two teleomorphic specimens of *Sphaerellopsis* obtained from this study were collected in the Neotropics. Interestingly, Eriksson (1966), Ramakrishnan & Narasimhalu (1941), and Sebesta (1963) sustained that the teleomorph preferred environmental conditions with high humidity, as in the tropics. Similarly, when Västerbotten found the teleomorphic state of *Sphaerellopsis* in the Summer of 1962 in northern Sweden, the locality was a hollow in a compost heap, a few meters from a rivulet giving microclimate conditions "similar to the tropics" (Eriksson, 1966). Furthermore, the host plant may play an important role in helping develop the teleomorph of *Sphaerellopsis*. For example, Eriksson (1966) sustained that this state was commonly found on plants in Poaceae and Cyperaceae due to their continuous growth and formation of high-humidity microclimates. However, this hypothesis has not been proven yet. Nonetheless, the latter observations of the presence of the teleomorph in environmental conditions at high humidity are consistent with our findings of the two teleomorphic specimens collected in Brazil.

#### **5.4.5 Our analyses reject the hypothesis that *Sphaerellopsis* species are specific to rust species or genera**

Based on our multi-locus phylogenetic tree, none of the species of *Sphaerellopsis* have a preference for specific rust genera. Although Liesebach & Zaspel (2004), Nischwitz et al. (2005),

and Kajamuhan et al. (2015) showed strong levels of host specificity for *Puccinia* species on grass hosts separated from *Melampsora* on poplars, our large dataset refutes those results. Overall, the sampling of the previous studies was limited to the genera *Melampsora* and *Puccinia*. The dataset of Liesebach & Zaspel (2004) and Nischwitz et al. (2005) did not exceed 20 isolates, and the sampling of Kajamuhan et al. (2015) comprised 82 isolates but of *Puccinia* species only. In contrast, our dataset covered 19 rust genera and 216 specimens. Thanks to the inclusion of these genera, we noticed that the *Sphaerellopsis* isolates collected from *Puccinia* and *Melampsora* from previous studies that used to be clustered in separate clades are now grouped with other rust genera. Although *S. paraphysata* and *S. macroconidialis* are predominantly associated with *Puccinia* specimens and *S. melampsoriinae* to *Melampsora* specimens, the association of some other rust genera with these *Sphaerellopsis* species discards any possibility of rust host specificity of *Sphaerellopsis*. Furthermore, after including specimens from the Neotropics in the multi-locus phylogenetic tree, our results emphasized the generalist behavior of *Sphaerellopsis* for the rusts. Thus, this study highlights the importance of broad coverage, both geographically and at species diversity, when sampling and analyzing the host range of a fungal genus.

Because members of *Sphaerellopsis* did not show a preference for any rust genus, we also checked if there was a preference at the family level. We used the most recent phylogenetic analyses of higher-rank classification rust fungi as a reference (M. C. C. Aime & McTaggart, 2020). However, we found different rust families clustered in single *Sphaerellopsis* clades. For example, *S. paraphysata* included rust hosts of the families Zaghouniaceae, Melampsoraceae, Phakopsoraceae, and Pucciniaceae, which are not phylogenetically closely related. Similar results were obtained for *S. macroconidialis*, *S. filum*, and *S. melampsoriinae*. Thus, we confirm that *Sphaerellopsis* species associated with rust fungi do not have a particular preference for any rust.

#### ***5.4.6 Geographic distribution of Sphaerellopsis associated with rust fungi***

This study confirms that members of *Sphaerellopsis* associated with rust fungi are cosmopolitan and distributed in temperate and tropical regions. Although Kuhlman et al. (1978) sustained that conidia of *Sphaerellopsis* did not disperse over long distances because they spread through water splashing to nearby hosts, our results suggest that *Sphaerellopsis* likely use other dispersers. Like *Sphaerellopsis*, rust fungi are cosmopolitan. Rust spores are dispersed through wind currents and

can cross continents. Due to the close relationship between *Sphaerellopsis* and rust fungi, we do not discard that the small conidia of *Sphaerellopsis*, compared to the size of rust spores, use rust spores as dispersers or are also dispersed by wind currents. However, further studies in the dispersion mode of *Sphaerellopsis* are necessary to prove this hypothesis.

Our extensive sampling in the Americas also suggests that *Sphaerellopsis* do not follow any latitudinal diversity gradient (LDG), wherein species richness increases from the poles to the tropics (Hillebrand, 2004). Contrary to LDG, our results are consistent with the distribution of other microorganisms, such as bacteria and other fungi, whose propagules are microscopic and thus easily dispersed (De Wit & Bouvier, 2006). In addition, the fact that all our *Sphaerellopsis* specimens had fruiting bodies implies that they were adapted to those environments to develop such structures and were not only latently present. This broad distribution suggests that *Sphaerellopsis* can easily adapt to different ecosystems in temperate and tropical regions, which is also promising for its potential use as a biological control agent.

Due to the cosmopolitan distribution of *Sphaerellopsis* species associated with rust fungi, our results contradict the ones from Yuan et al. (1999). They suggested that the *Sphaerellopsis* species had allopatric speciation due to the geographically isolated specimens they found in their sampling. However, we can confirm that such allopatric speciation is unlikely for *Sphaerellopsis* species associated with the rust fungi. However, the *Sphaerellopsis* species with a saprobic strategy could show a more geographically restricted pattern. Nevertheless, because a few specimens of these species have been reported so far, we refrain from making any conclusion yet.

#### **5.4.7 The absence of *Sphaerellopsis* on coffee leaf rust**

We found the presence of *Sphaerellopsis* on several economically important plants infected by rust species, such as maize, wheat, and poplars. However, we did not find *Sphaerellopsis* associated with coffee leaf rust caused by *Hemileia vastatrix*. Kranz & Brandenburger (1981) did not register the rust genus *Hemileia* on their rust hosts list either. Keener (1934) suggested that a possible limiting factor of *Sphaerellopsis* infection to specific rust genera could be attributed to the type of sorus produced by certain species. Based on his results, Keener suggested that cushion-shaped sori were less sensitive to host fruiting bodies of *Sphaerellopsis* and mentioned some

species, such as *Puccinia poculiformis* and a few *Coleosporium* species. Contrary to Keener's opinion, our dataset includes some *Coleosporium* species associated with *Sphaerellopsis* and several species of *Puccinia*. Although we think Keener was right when he suggested that the shape of the sori may have some effect on the presence of *Sphaerellopsis*, we disagree with the shape of the sorus he described. The sori of *Hemileia*, for example, protrude through the stoma like a "bouquet" (McCain, 1983) and do not tear the epidermis of the host plant as it occurs with *Coleosporium* and *Puccinia*. The urediniospores of *Hemileia* are then exposed and are not protected by any plant tissue. We thus think that the torn epidermis may play a role in protecting the fruiting bodies of *Sphaerellopsis* within the rust sori. However, further studies evaluating different shapes of sori from several rust families could help understand why some rust genera are most sensitive than others to *Sphaerellopsis*.

#### **5.4.8 Antagonistic interaction between *S. macroconidialis* and *P. polysora*.**

This study confirms *S. macroconidialis* as a mycoparasite of rust fungi. Coiling and appressorium formation by *S. macroconidialis* and turgor loss of germinative tubes of *P. polysora* are evident signs of antagonistic relationships between these two fungi. Appressorium formation and coiling are the most common mechanisms of mycoparasites to attack their host pathogens. For example, *Trichoderma harzianum* and *Trichoderma atroviridae* show the exact mechanism, coiling around its host, *Rhizoctonia solani*, and forming appressoria as an early event before hyphal damage (Benhamou & Chet, 1993; Benítez et al., 2004; Chet et al., 1981; Rocha-Ramírez et al., 2002). Similarly, *Simplicillium lanosoniveum* and *Cladosporium tenuissimum* form appressoria and helix-shaped hyphae around urediniospores of the soybean rust, *Phakopsora pachyrhizi* (Ward et al., 2011) and aeciospores of the two-needle pine stem rusts, *Cronartium flaccidum*, and *Peridermium pini* (Moricca et al., 2001), respectively. The congeneric species, *Sphaerellopsis paraphysata*, was also found coiling around urediniospores of *Puccinia substriata*, but appressorium formation was not seen (Anandakumar et al., 2019).

The formation of helix-shaped hyphae of mycoparasites around the structures of their fungal hosts is a phenomenon usually dependent on lectins recognition. Fungal lectins are carbohydrate-binding proteins located on the fungal surface, which play a role in the recognition and defense of other organisms (Lebreton et al., 2021). Once the mycoparasite recognizes the lectins of the fungal host

upon first physical contact, the mycoparasite hyphae start coiling around the fungal host for colonization and further infection (Omann & Zeilinger, 2010). Thus, since *S. macroconidialis* was observed coiling around germinative tubes of *P. polysora* on day five after co-cultivation, genes coding for lectins-binding proteins were likely up-regulated during the first four days, where signs of mycoparasitism were not noticed. Many lectins have been identified in filamentous fungi and yeasts (Lebreton et al., 2021), but information on rust fungi is scarce. While this study did not identify the lectins of *P. polysora* or lectins-binding proteins of *S. macroconidialis*, we found how long this first host-recognition event lasted. Thus, our results can help identify these proteins through transcriptomic analyses already knowing the duration of the first interaction event.

Although we observed appressorium formation by *S. macroconidialis* in the interaction test, this structure was atypical. We only observed one appressorium-like structure attached to a urediniospore on day 12 of co-cultivation (Fig. 5.6. h). This appressorium was not formed over any germinative pore of the urediniospore, and the spore showed no signs of turgor loss. Because we stopped our observations on day 12 due to the overgrowth of *S. macroconidialis* hyphae, it is impossible to know if the appressorium had any mycoparasitic effect on the rust. Appressorium formation was also not observed on *Sphaerellopsis paraphysata* infecting urediniospores of *P. substriata* (Anandakumar et al., 2019). Because this structure was atypical in our strain and absent in another *Sphaerellopsis* species, it seems that members of *Sphaerellopsis* do not form appressoria as the primary structure to penetrate and infect rust fungi. However, further studies with other strains and *Sphaerellopsis* species must be done to confirm this hypothesis. In contrast, *Sphaerellopsis* species are likely to secrete lytic enzymes, such as chitinases, glucanases, and proteases, to infect host rusts once their hyphae coiled around rust structures; as we noticed on day 12, where some germinative tubes of *P. polysora* lost turgor (Fig. 5.6. i, j). Although we did not detect those enzymes in this study, our experimental design can then be helpful for future secretome analyses to identify the enzymes that *S. macroconidialis* secrete to infect rust fungi.

Overall, this study contributes with some advances in the characterization of the fungal genus *Sphaerellopsis*. The mycoparasitic strategy confirmed here gives *S. macroconidialis* the potential to be a biological control agent for rust diseases. Phylogenetic analyses helped identify *S. melampsoriinae* as a new species and the teleomorph of *S. macroconidialis*. Our results also



suggest that species of *Sphaerellopsis* have a cosmopolitan distribution and do not prefer specific rust genera. We hope these results serve as a baseline for further assessments of the biology of *Sphaerellopsis* and its potential use as a biological control agent of rust fungi.

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## CHAPTER 6. CONCLUDING REMARKS

### 6.1 Conclusions and future work

In this work, I utilized multiple approaches to characterize several natural enemies of rust fungi. Such characterization studies included morphological descriptions, phylogenetic analyses, geographic distributions, and host range. I also conducted in-depth antagonistic studies between conidia of *Sphaerellopsis macroconidialis* and urediniospores of *Puccinia polysora* to determine their mode of interaction (Chapter 5). In addition, I contributed to the first catalog of natural enemies of rust fungi from the Arthur Fungarium (PUR) collections, one of the largest and most diverse rust collections across the globe (Chapter 2).

This study emphasizes the importance of preserved biological collections for biodiversity inventories, fundamental science, and applied biological sciences. Thanks to the well-preserved specimens at PUR, I found more than 500 rust specimens associated with fungal natural enemies and more than 200 associated with the fungivorous larvae of *Mycodiplosis* (Chapter 2). I also amplified more than 500 DNA sequences from specimens collected more than 100 years ago. Thanks to these biological collections, I provided geographic distributions of the two most common natural enemies of rust fungi previously undocumented (Chapters 4 and 5). Results from this study demonstrate the great potential of preserved biological collections as a resource for genetic information, morphological studies, and the discovery of natural enemies of rust fungi for further studies in biological control.

The great proportions of rust specimens associated with natural enemies in the Neotropics suggest that this geographic region is rich in natural enemies of rusts. Of the 878 specimens found as natural enemies of rust fungi, 621 were collected in the Neotropics. In countries such as Argentina, Ecuador, and Guyana, I found several rust specimens, each associated with multiple natural enemies, including several fungal species and larvae of *Mycodiplosis*. In addition, thanks to a specimen collected in Trinidad, the fungal genus *Trochila* revealed a new strategy, potentially mycoparasitic, due to the intimate association of its ascocarps with the rust sori of *Cerotelium fici* (Chapter 3). Although not all natural enemies collected were successfully identified due to the

scarce or dried material of preserved collections, I do not discard the possibility that some may be new species. More efforts must be made to thoroughly document the incredible biodiversity of the natural enemies of rust fungi in the Neotropics. Results from this and future studies can significantly contribute to applied biological sciences to develop more sustainable agricultural practices controlling rust diseases.

The results from this thesis suggest that the fungal genus *Sphaerellopsis* is the most common natural enemy of rust fungi. In addition, analyses reject the hypothesis that *Sphaerellopsis* species are specific to rust species or genera (Chapter 4). Although generalist natural enemies are undesirable for biological control, the broad host range of the *Sphaerellopsis* species associated with rust fungi does not yet preclude them as potential biological control agents for rust diseases. They can be generalists for any rust genus but also can be restricted exclusively to infecting rust. Thus, further interaction studies on non-target species, such as host plants and other organisms inhabiting an ecosystem of interest, are required to determine how broad the host range of these *Sphaerellopsis* species is.

Our multi-locus phylogenetic analyses provide evidence of a possible host jumping among *Sphaerellopsis* species (Chapter 5). Two of the eight currently accepted species of *Sphaerellopsis* have been reported exclusively on dead plant tissue (*S. artemisiae* and *S. isthmospora*), and other two species have been found in both plant tissue and rust sori (*S. hakeae* and *S. paraphysata*). The two species reported exclusively as saprobic were the only ones not found in our dataset, perhaps because our screening focused on collecting fungal natural enemies developed directly on rust sori and not on plant material. Analyses show that *S. artemisiae* and *S. isthmospora* are sister species that form the earliest diverging lineage in the genus (Chapter 4). This raises the intriguing question of whether the mycoparasitic strategy is derived in *Sphaerellopsis* from saprobic ancestors. Thus, to prove this hypothesis, I could have performed a different screening process by not discarding the fungal natural enemies developed on plant tissue. Then, I might have been able to collect saprobic or opportunistic *Sphaerellopsis* specimens by unraveling how the strategy of this genus has evolved through time.

The six *Sphaerellopsis* species associated with rust fungi have a cosmopolitan distribution (Chapter 5). Although it is tempting to suggest that members of *Sphaerellopsis* quickly adapt to several environmental conditions, the rust sorus may likely provide similar microenvironments no matter the geographic region. Thus, two hypotheses arise from the cosmopolitan distribution of *Sphaerellopsis*. The first hypothesis is that *Sphaerellopsis* members can easily adapt to several macro environments. Consequently, they might be used to control any rust-infected crop across the globe. To support this hypothesis, I would need to analyze our sampling data by climate conditions, such as arid, seasonally wet, and tropical weather, to confirm that *Sphaerellopsis* is adapted to any environmental condition. On the other hand, the second hypothesis is that the shape of the rust sorus is a decisive variable that provides an adequate microenvironment for *Sphaerellopsis* to survive and develop. Then, to test this hypothesis, I would need to collect my data based on the sorus shape to compare which sori are more sensitive to the presence of *Sphaerellopsis* no matter the macro environment conditions.

Our extensive screening for the presence of *Sphaerellopsis* on rust specimens suggests that the teleomorph state prefers tropical environments (Chapter 5). The two teleomorphs we found were collected in Brazil. However, other specimens containing the teleomorph have also been reported from temperate countries in Asia, Europe, and North America (Eriksson, 1966). Thus, this preference is not based on geographic regions but on specific weather conditions, such as high and constant humidity through time. One could hypothesize that the anamorph is more commonly found because it does not require high humidity conditions to survive and develop on rust sori. Thus, it could extend to any geographic region as rust fungi do. This hypothesis could be proven by selecting our sampling data by specific climate conditions, such as arid, seasonally wet, and tropical weather, to determine if the teleomorph formation is directly related to tropical environments.

Findings from *in-vitro* interaction tests between *Sphaerellopsis macroconidialis* and *P. polysora* confirm that *S. macroconidialis* is a mycoparasite (Chapter 5). The first antagonistic events observed in our test were similar to those of *Sphaerellopsis paraphysata* against *Puccinia substriata* (Anandakumar et al., 2019) and several mycoparasitic species against other plant pathogens (Benhamou & Chet, 1993; Benítez et al., 2004; Chet et al., 1981; Quandt et al., 2016;

Rocha-Ramírez et al., 2002; Ward et al., 2011). Genomics and transcriptomic analyses of some mycoparasitic species have been performed to identify genes involved in mycoparasitic mechanisms to infect plant pathogens (Faure et al., 2020; Quandt et al., 2016; Zhao et al., 2020). However, no *Sphaerellopsis* genomes or transcriptomic analyses are yet available. An essential next step to determine which genes are involved in these mycoparasitic events is to generate a genome and a transcriptomic analysis for *Sphaerellopsis* to search, for instance, orthologous to the gene families agglutinin-like sequence (ALS) and MAD1, which encode for Adhesin proteins (Quandt et al., 2016). These proteins are involved in cell surface adhesion playing an essential role in pathogenesis. In addition, since chitins and glucans are the main components of the urediniospores wall (Silva et al., 1999), orthologous proteins to chitinases and glucanases would be expected to be highly upregulated in the transcriptomic analysis. Lastly, I could expect the overexpression of genes involved in redox reactions and transmembrane transport (Quandt et al., 2016) to degrade rust spores and their germinative tubes. Thus, comparative genome sequence and transcriptomic analyses between *Sphaerellopsis* and rust hosts can help reveal which genes of *Sphaerellopsis* are involved when interacting with rust fungi.

Our results contributed significantly to the characterization of fungivorous larvae of *Mycodiplosis* through molecular data. Like *Sphaerellopsis* members, *Mycodiplosis* larvae are commonly associated with rust fungi and do not prefer any particular rust genera. However, our results revealed that these larvae may be regionally adapted (Chapter 4). Adaptation to specific environments has advantages in conservation biological control where resident natural enemies can be stimulated to increase their populations and control targeted pests. Thus, *Mycodiplosis* larvae can be locally used to control rust diseases without introducing non-native invasive species to new environments.

Despite our thorough characterization of *Mycodiplosis* and the contribution of hundreds of DNA sequences to resolve phylogenetic relationships within the genus, there is an urgent need for collaborative research with taxonomist experts in morphological descriptions of the Cecidomyiidae family. Since morphological characters of adult males are the only way to identify *Mycodiplosis* species, determining the species from dried larvae was impossible. Characterization studies of natural enemies are never complete without proper identification of the organism at the

species level. Once collaborative research is possible, these fungivorous larvae could be tested in greenhouses and field trials to test their potential as biological control agents.

Like *Sphaerellopsis*, there is great potential to study *Mycodiplosis* larvae through genomics and transcriptomic analyses. Since these larvae digest rust spores, they likely produce lytic enzymes that disrupt the spores' cell walls and degrade their cytoplasm. The genes that encode those proteins may be identified through "omics" analyses and potentially used for plant defense or as bioproducts in integrated pest management. Thus, genome sequencing of natural enemies can open a new window to applied biological science for rust disease control.

There is a great potential to use *Mycodiplosis* larvae in conventional farming where fungicides are applied regularly. Interestingly, I found larvae of *Mycodiplosis* on maize crops in Indiana, USA, where fungicides were commonly applied to control southern corn rust caused by *P. polysora*. Although I did not follow the schedule of those chemical applications and the synchrony with the life cycle of *Mycodiplosis* in that crop, it was still interesting to find these larvae in a monocrop feeding on urediniospores of *P. polysora*. Biological control agents complemented with chemicals are more attractive for commercial use in integrated pest management. Thus, further studies of these larvae in greenhouses assessing their control over southern corn rust together with occasional fungicide applications could help determine how resistant and efficient these larvae can be as biological control agents for southern corn rust in non-organic maize crops.

Overall, this work highlights the importance of studying the natural enemies of rust fungi to develop sustainable agronomical practices in controlling rust diseases. It is true that fungicide applications and some rust-resistant plant varieties can mitigate the yield losses caused by rust diseases nowadays. However, the recent emergence of new rust strains that resist current fungicides and infect commercial crop varieties threatens our future food production. Biological control can be promising to keep low yield losses caused by rust diseases. However, significant efforts in multidisciplinary research are also required to make big steps in this control method. Since characterization studies of natural enemies of rust fungi are the baseline for further biological

control studies, I anticipate that the results of this work will contribute to developing other alternatives for controlling rust diseases.

## **6.2 Developing a transformation system for *Sphaerellopsis***

During the course of my Ph.D. studies, I also attempted to perform a fungal transformation using fluorescent proteins (FP) on the strain SP28 of *S. macroconidialis* to observe its mycoparasitic mechanism against the rust *P. polysora* on plant tissue. However, after several efforts, the FP transformation was unsuccessful. Herein, I share the methodology we used for FP transformation on *S. macroconidialis* in case any other researcher may be interested in performing similar essays.

### **6.2.1 *Rhizobium radiobacter* preparation**

The plasmids pCBCT (Gorfer et al., 2007) and pBHt2-tdTom (Caasi et al., 2010) carried by *Rhizobium radiobacter* strain AGL1 (Lazo et al. 1991) were kindly provided by Dr. Markus Gorfer. Both plasmids were used separately to transform *S. macroconidialis* strain SP28 with the Green fluorescent protein (GFP) and Red fluorescent protein (RFP). Upon receipt of *R. radiobacter* carrying the binary vectors pCBCT and pBHt2-tdTom separately, we cultured both bacterial strains on Tryptic Soy Agar (TSA) supplemented with 50 µg/mL of Kanamycin at 28°C. After observing individual bacteria colonies on the plates, we isolated single colonies using a sterile toothpick and inoculated them on 6 mL LB broth supplemented with 50µg/ml of Kanamycin. The liquid cultures were placed in an incubating shaker under continuous agitation (180-200 rpm) at 28°C. The negative control consisted of LB broth and 50µg/ml of Kanamycin without the bacteria. Once the glass tubes had an OD concentration of approximately 0.6, we aliquoted 1,000 uL from each culture for plasmid extraction and PCR amplification of the target genes. The plasmids were extracted using the UltraClean 6-minute Mini Plasmid Prep kit (Mobio Labs), following the manufacturer's instructions. We used the primers hph200f (5' GAGCGGGTTCGGCCCATTCG 3') and hph3R (5' GATGTTGGCGACCTCGTATT 3') to target the Hygromycin (hph) resistance gene on both plasmids. In addition, the primers GFPF/GFPR (Sarrocco et al., 2006) and mcherry01F (5' ATGGTGAGCAAGGGCGAGGAG 3') and tdTom01R (5' TTA CTTGTACAGCTCGTCCATGCCGTA 3') were used to amplify the green (sGFP) and red (tdTomato) fluorescent protein genes on the plasmids pCBCT and pBHt2-tdTom, respectively.

Twenty-five µl PCR reactions were conducted on a Mastercycler ep gradient Thermal Cycler (Eppendorf model #5341, Hauppauge, New York). They consisted of 12.5 µl of 2× MyTaq Mix (Bioline, Swedesboro, New Jersey), 1 µl of each 10 µM primer, and 10.5 µl of 1/10 diluted DNA extract. Amplifications of hph, sGFP, and RFP regions were run under the following conditions: initial denaturation at 98 °C for 2 min; followed by 35 cycles of denaturation at 98 °C for 10 sec, annealing at 60 °C (hph) / 66 °C (sGFP) / 63.5 °C (TdTomato) for 10 sec, and elongation at 72 °C for 30 sec; and final extension at 72 °C for 5 min.

### **6.2.2 Transformation of *S. macroconidialis***

The fungal transformation of *S. macroconidialis* strain SP28 was based on a combination of the protocols by Gorfer et al. (2007) and Németh et al., (2019), as follows. Since both plasmids contained the Hygromycin resistance gene hph, we initially used the antibiotic Hygromycin B as a selection agent to perform the fungal transformation. Eight different concentrations (1.5, 2.5, 3.5, 5, 10, 25, 50, and 100 mg/L) of Hygromycin B were added to Petri dishes containing PDA to determine the lowest concentration that caused fungal growth inhibition. The test was repeated twice using three Petri dishes per concentration. After inoculation, we made observations every 72 hours for 30 days to observe fungal growth inhibition. Our results showed that hyphal growth was inhibited entirely at 2.5 mg/L of Hygromycin B and conidial germination at 50 mg/L. Thus, we used 2.5 mg/L of hygromycin B to perform the co-cultivation using hyphae and 50 mg/L using fresh conidia.

In the first attempt to perform an FP transformation, we used hyphae of *S. macroconidialis*. We first inoculated conidia on autoclaved dialysis membrane sheets placed on PDA or 2% MEA and let them germinate at room temperature until a layer of mycelium was observed. The day before adding the bacteria containing the plasmids to the mycelium, we grew the bacteria on TSB medium containing 200 µM of Acetosyringone. We added aliquots of the bacteria over the mycelium, covering it completely. We tried several OD<sub>600</sub> of the bacteria: 0.3, 0.4, 0.5, and 0.6, and co-cultivation times: 30 min, 1 hour, 2 hours, 6 hours, 12 hours, one day, and two days. Co-cultivation plates were incubated at room temperature and 26 °C. After co-cultivation, we transferred the dialysis membranes to new plates containing selective media (PDA, 2.5 mg/L of Hygromycin B as a selection agent for transformants, and 100 mg/L of Cefotaxime to kill *R. radiobacter* cells).

We let these plates at room temperature for six weeks until visible fungal growth emerged. Observations and photographs of the fungal growth were taken every other day. However, we did not observe any new mycelium emerging from the dialysis membrane. Instead, we observed an overgrowth of bacteria. We then washed the bacteria from the dialysis membrane sheets with 100 mg/L of Cefotaxime aliquots and autoclaved water, but new mycelium never emerged, and overgrowth of the bacteria was observed again.

In the second attempt to perform an FP transformation, we used fresh conidia harvested from two weeks old plates. We added aliquots of autoclaved water to the PDA medium where *S. macroconidialis* strain SP28 was growing to harvest the conidia. We agitated the Petri dishes gently to release the spores of *S. macroconidialis* from their fruiting bodies and then collected the spore suspension using a micropipette. The day before co-cultivation, we grew the bacteria on TSB medium containing 200 uM of Acetosyringone. Then, an equal volume of the bacteria and the spores were added to a glass tube. We used a concentration of  $10^6$  conidia per mL and the same concentrations of the bacteria and co-cultivation times as in the first attempt. Since the co-cultivation of the second attempt was in liquid media, we placed the glass tubes in an incubating shaker under continuous agitation (180-200 rpm) at 25°C. After co-cultivation, we took aliquots of 1mL and transferred them to selective media (same as the first attempt). These plates remained at room temperature for six weeks until visible fungal growth emerged. Observations were made every other day for six weeks, but again no mycelium or any visible fungal structure was observed.

### 6.3 Literature cited

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