Resurgence of Less-Studied Smut Fungi as Models of Phytopathogenesis in the Omics Age

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ABSTRACT

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The smut fungi form a large, diverse, and nonmonophyletic group of plant pathogens that have long served as both important pests of human agriculture and, also, as fertile organisms of scientific investigation. As modern techniques of molecular genetic analysis became available, many previously studied species that proved refractive to these techniques fell by the wayside and were neglected. Now, as the advent of rapid and affordable next-generation sequencing provides genomic and transcriptomic resources for even these “forgotten” fungi, several species are making a comeback and retaking prominent places in phytopathogenic research. In this review, we highlight several of these smut fungi, with special emphasis on *Microbotryum lychnidis-dioicae*, an anther smut whose molecular genetic tools have finally begun to catch up with its historical importance in classical genetics and now provide mechanistic insights for ecological studies, evolution of host-pathogen interaction, and investigations of emerging infectious disease.

Additional keywords: genomics, transcriptomics.

Smut fungi of the world. Smut disease is a term that derives from the lower German word “smutt” and standard German “schmutz” meaning “dirt.” This way of characterizing these plant diseases stems from the characteristic dark spore masses observed in or on infected plants, which often break up into a fine powder. In the context of the fungi that cause this disease, when we say “smut fungi,” we do not mean to represent a particular taxonomic unit but, rather, the designation refers to a heterogeneous group of fungi that parasitize plant hosts and produce teliospores as means of reproduction (Bauer et al. 2008; Vánky 2012) and similarity in lifestyle and cellular organization earn them a place in this diverse group. The smut fungi were traditionally divided into the phragmobasidiolate Ustilaginaceae and the holobasidiolate Tilletiaceae families, since publication of Tulasne and Tulasne (1847). Based mainly on host-parasite interactions and the septal pore apparatus, there was a dramatic shift proposed for the systematics of smut fungi (Bauer et al. 1997). As a result, the microbotryales were excluded from the ustilaginomycetes, while the exobasidiales s. l., graphiolales, and microstromatales were included in this group. Cellular interaction (Bauer et al. 1997) and cell-wall

carbohydrate composition (Prillinger et al. 1993) indicate that the class Ustilaginomycetes is monophyletic, and phylogenetic analyses of rDNA sequences (Begerow et al. 1997; Berres et al. 1995; Swann and Taylor 1995) also supports this.

Like the rust fungi, the smut fungi, in general, are members of the division Basidiomycota, although recently a new phylum was established, Entorrhizomycota, which contains smut root pathogens of sedges (Bauer et al. 2015). The smuts do not produce fruiting bodies but do produce basidia and basidiospores (also known as sporidia or yeasts) from germination of their diploid teliospores. Production of the haploid yeast-like form highlights the ability of some of these fungi to grow as saprophytes, thus providing an ecological niche that is lacking in other fungi (Begerow et al. 2014). Whereas rust fungi can be either heteroecious, i.e., requiring an intermediate host as part of their lifecycle, or autoecious (Ono 2016), there are no identified heteroecious smut fungi, i.e., they complete their lifecycle in a single infected species (Bauer et al. 2015).

Smut fungi have been recognized as plant diseases for hundreds, if not thousands, of years. While ancient Greeks (e.g., Aristotle) and Romans (e.g., Virgil, Pliny, Ovid) may not have had names for rust and smut diseases, they were likely observed, and such ancient writings likely referenced these groups of fungi (Fischer and Holton 1957; Littlefield 1981). Ovid, in 8 AD, described an entire ceremony for elimination of the wheat rust outbreak, which later

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became an annual sacred festival, the Robigalia, each April 25 (Fantham 1998). Kavalier (1965) suggests that instead of rust being the only pathogen, smuts as well as other fungi might also have been important in these major destructions of wheat.

Unlike the history of human battles with rusts, the plant pathologists' war against the smuts has mostly been successful, due to the use of pesticides or resistant cultivars. For example, this has been a strategy for maize, using cultivars resistant or less susceptible to infection with *Ustilago maydis* (Christensen and Johnson 1935; Cornell University College of Agriculture and Life Sciences fact sheet, <http://plantclinic.cornell.edu/factsheets/cornsmut.pdf>) and extensive use of fungicides. Moreover, as will be discussed later, the smuts acting on nonagricultural species (e.g., on wild grasses or on wildflower species) provide an alternative model to examine modes of pathogenesis in situations in which host genetic diversity likely informs the genetic diversity and strategies of their corresponding smut species. After first providing background on some historically important smut fungi, this review will focus on less-studied smuts, with a special emphasis on how modern genomics and transcriptomics methodologies have brought these smuts into resurgence as models of investigation.

Some history: Smuts on agriculturally important hosts.

First, let us examine some smuts of agricultural importance. *Ustilago maydis* (previously also known variously by many names, such as *Lycoperdon zaeae*, *Uredo maydis* DC, *Uredo zaeae*, *Caeoma zaeae*, *Ustilago zaeae*, *Ustilago maydis-zaeae* [DC.]) is a smut native to Mexico but is now found essentially wherever its host, *Zea mays* (maize or corn), is cultivated. The galls characteristic of maize smut were undoubtedly well known to the Aztecs long before the invasions of the Spanish conquistadors in the 16th century (León-Ramírez et al. 2014). Maize smut is the oldest plant disease illustrated by drawings, with infected maize plants represented in the Florentine Codex manuscript prepared soon after the Spanish conquest of Mexico in 1519 (Valverde et al. 1995), not to mention images of Aztecs in heaven enjoying the fruits of maize infection as a delicacy (discussed below) and portrayals in early Mexican mural paintings (Valverde et al. 1995). In 1768 in Europe, the maize smut was referred to as *Lycoperdon zaeae*, the generic name now applied to puff-balls (Fischer and Holton 1957). It was first described scientifically in 1815 in France by de Candolle as *Uredo maydis* (de Candolle and Lamarck 1815). In Britain, it was first recorded in Dorset in 1850, an observation published by Miles J. Berkeley in the *Gardener's Chronicle* (Massee 1913). We now know *U. maydis* as the causative agent of gall formation and subsequent "corn smut disease" on maize, and it is the leading model for studying the fungal plant pathogens that collectively are responsible for huge annual losses of marketable food and fiber. *U. maydis* is a well-established model for molecular studies of fungal mating-type regulation, dimorphism (morphogenesis), and biotrophic pathogenesis (Andrews et al. 2004; Banuett and Herskowitz 1996; Bölker 2001; Brefort et al. 2009; Fernández-Álvarez et al. 2013; García-Pedrajas et al. 2010; Horst et al. 2010; Kämper et al. 2006; Klosterman et al. 2007; Steinberg and Perez-Martin 2008). The economic importance of this species has led to an extensive literature on its biology, and it serves as a useful pathosystem, being a pathogen capable of infecting all above-ground parts of the plant in its program of gall formation. Interestingly, the swollen corn kernels containing *U. maydis* teliospores have long been an important food item, known as huitlacoche in Central America, and is becoming increasingly so in North America. In traditional cuisine and now among "foodies" and more adventuresome connoisseurs, huitlacoche is used especially as soup and in quesadillas and enchiladas. It is of increasing economic importance in North America and is grown more extensively as a cash crop that may be more valuable than the maize itself. It is especially important in certain regions of Mexico such as Guanajuato (Juárez-Montiel et al. 2011; Valverde et al. 1993, 1995) and as a nutraceutical (Juárez-Montiel et al. 2015), and most recently, efforts are being made in Canada to turn this into an important industry

(Toronto Star 2015, http://www.thestar.com/life/food_wine/2015/02/05/is-corn-smut-canadas-new-truffle.html). It may also provide certain nutritional benefits, as it contains carbohydrates, fats, and proteins and has high levels of essential amino acids, oleic and linoleic acids, and vitamins (Valverde et al. 1995). Since it produces ustilagine, it proved useful to Native Americans for inducing labor. It is also valued as a homeopathic remedy, primarily for diseases of the female reproductive organs (Boericke, 1987).

Another plant pathogen with demonstrated importance as a food item is *Ustilago esculenta*, a smut fungus that infects the Manchurian wild rice *Zizania latifolia*. The fungally infected host has been cultivated as a vegetable for more than 1,500 years across Asia but, especially, in China and Japan (Yu et al. 2015). The plants have been grown for the edible "galls" (enlarged culms) resulting from infection by the fungus and are eaten raw and in stir-fry; the nutritional value of such products of infected plants is currently being investigated (Yu et al. 2015).

Other examples of smuts on agricultural species are those that infect wheat. In France, Mathieu Tillet, a keeper of the mint, was also interested in problems affecting farmers. In 1755, while observing smuts on wheat, Tillet discovered that there were two types of smut present. The first he called *la carie* or what the English called "common bunt" or "stinking smut." Grains infected with this type of smut seemed healthy, but grains were actually filled with brownish balls (sori) full of a black, foul-smelling powder. Tillet called the second type of smut *le charbon* (literally charcoal); the plants looked healthy but were covered with a black powder that readily blew away. Louis and Charles Tulasne later verified the distinction between the two types of smut in 1847 (Hudler 1998; Sprott and Carefoot 1969; Tulasne and Tulasne 1847). To honor Tillet, they created a new genus, *Tilletia*, and named the stinking smut *Tilletia caries*. After experiments he conducted on his land and discussions with local farmers, Tillet concluded that the disease was spread by infected seeds, since the seeds covered with black powdery spores appeared to cause the disease on wheat plants regardless of the weather conditions in which they were planted or types of fertilizer used. Work later by Prévost demonstrated that spores placed in water led to germination of the teliospores, an observation he confirmed microscopically (Prévost 1807). He guessed that the germinating spores penetrated the soft tissue of the germinating wheat seedling and destroyed the grain. However, Prévost never saw the mycelium growing into the plant. By the mid- to late-1800s additional species of "loose" smuts, including *Ustilago nuda* (on barley) and its close cousin *U. tritici* (Obs. syn. *Ustilago nuda* var. *tritici* Schaffnit), affecting wheat, were named for the pathogens that replace the grain heads in their respective hosts with their developing teliospores (Martens et al. 1984).

Since these early studies, other pathogenic *Tilletia* species have been identified and characterized. *T. indica* (karnal bunt) was first discovered in India (Bonde et al. 1997) in 1931 and was first detected in the United States in 1996. The fungus replaces part or all of the infected wheat kernels with its teliospores and is capable of infecting common wheat, durum wheat, and triticale (Bonde et al. 1997). While strict quarantine measures were put in place to prevent its spread, it is found in the Southwestern United States and in parts of Alabama, Florida, Georgia, and Tennessee. Although economic losses in the United States are kept relatively low (Goates 2010), *T. indica* is still economically important. As of 2008, there were 37 countries that banned import of wheat contaminated with *T. indica* and, for example, it is estimated that introduction of this fungus into Australia could result in \$1.3 billion lost revenue (Stansbury et al. 2002). Similarly, dwarf bunt of wheat, caused by *T. controversa*, is important less for actual losses to wheat but due to the politics of the international wheat trade (Mathre 1996; Trione 1982). *T. horrida* Takah, which causes kernel smut in rice, is distantly related but morphologically similar to *T. indica* (Carris et al. 2006). More recent molecular genetic data highlight the importance of such

approaches in proper phylogenetic placement. Error in the identification of sporidium morphology had supported the proposal to place *T. horrida* and *T. indica* in *Neovossia* instead of *Tilletia* (Joshi et al. 1983; Khanna and Payak 1968; Singh and Pavgi 1979) but Vánky (2008) maintained that only *N. molinae* should belong to the genus *Noevassia*, since it causes localized infection, and its important characteristics are long hyaline appendages on the teliospores and the absence of sterile cells and trimethylamine smell. This distinction is supported by recent molecular work (Castlebury et al. 2005).

Modern classification of smut fungi. The last comprehensive compilation of taxonomy for smut fungi was produced in the publication “Smut Fungi of the World” by K. G. Vánky (2012). In it, Vánky listed 1,650 recognized species of smut fungi, which he believed to only represent one third of all basidiomycetous smut fungi (Vánky 2008), and the book also stated that 25% of the known species were collected only once (Vánky 2012). These species within basidiomycota were found across two subphyla, four classes, eight orders, 26 families, and 93 genera (Vánky 2012). Much of the systematic delineation of smuts was greatly influenced by over 30 years of ultrastructural studies by R. Bauer (Bauer et al. 1997; Begerow et al. 2014). The classification system in use today applies classical methods of taxonomic classification relying on morphological characteristics of teliospores and basidiospores but has been augmented by molecular techniques, including DNA and, especially, comparative genome analyses.

This latter assertion highlights the fact that, with the increasing availability of genome sequences, comparisons between characteristic groups of fungi have become possible. Some studies focused on differences between animal and plant hosts (Baxter et al. 2010; Kämper et al. 2006; Laurie et al. 2012; Que et al. 2014; Schirawski et al. 2010), while others focused on the lifestyle and host characteristics. (Baxter et al. 2010; Doehlemann et al. 2014; Duplessis et al. 2011; Lévesque et al. 2010; O’Connell et al. 2012; Sharma et al. 2014; Tyler et al. 2006). Often the less-studied smut species have been included as outgroups for identifying important genes and functions in economically important groups of such pathogens (Sharma et al. 2014, 2015).

Toolboxes for less-studied smut fungi. The tool chest for molecular genetic analyses and manipulations is well stocked for *U. maydis* and a number of other phytopathogenic species of agricultural importance. However, many tools that were effective in models such as *U. maydis* (and for the more traditional fungal models such as *Saccharomyces cerevisiae*, *Neurospora crassa*, and *Aspergillus nidulans*) have not been easily transferrable or adapted for use in other fungi, especially the less-studied smut fungi. Hence, the notion arises that these other less-studied smuts are “recalcitrant” or difficult to study at the molecular genetic level. For example, members of the *Microbotryum violaceum* species complex were historically treated as a single species. Methods for protoplasting *Microbotryum* cells and for transformation have been inefficient and not reproducible (Bej and Perlin 1989a and b; Perlin 1996; also discussed below). Nevertheless, several additional molecular genetic techniques were developed over the years for characterization of fungi, and these have also been applied to smut fungi, including those that had been ignored previously. For example, polymerase chain reaction (PCR)-based techniques were developed for DNA fingerprinting and genetic mapping (e.g., random amplified polymorphic DNA [RAPD], amplified fragment length polymorphisms [AFLPs], PCR restriction fragment length polymorphisms [RFLPs], comparative sequencing of internal transcribed spacer [ITS], microsatellites); these were also useful for population genetic studies and phylogenetic analysis (Giraud et al. 2002, 2008; Karwasra et al. 2012; Pimentel et al. 1998; Shi et al. 1996; Stoll et al. 2003). In several instances, e.g., AFLP analysis, such techniques proved instrumental in distinguishing gene expression in sensitive vs. resistant hosts of some smuts of economic importance as well (Borrás-Hidalgo et al. 2005).

Moreover, the development of techniques for whole chromosome analysis via pulse-field gel electrophoresis using clamped homogeneous electric field gels allowed for the production of electrophoretic karyotypes (EKs) that were particularly effective in characterization of smut fungi in terms of individual strains but, also, to distinguish different species of the fungi that might otherwise overlap in terms of morphological aspects and features of their pathogenicity and virulence phenotypes (Abdennadher and Mills 2000; Russell and Mills 1994). EKs and the resulting image of chromosome number and chromosome length polymorphisms has been used to support a conspecific status for *T. controversa* and *T. caries* (Russell and Mills 1994). Such analyses in combination with telomere-specific RFLPs also revealed relationships among lineages of race-specific strains of *U. hordei* (Abdennadher and Mills 2000).

Stripe smut of grasses, *Ustilago striiformis* s.l., is a complex of smut fungi widely distributed over temperate and subtropical parts of the globe. Infection leads to the shredding and death of leaf tissue following the rupture of elongated sori. Stripe smut infects nearly 100 different grass species. Over 30 smut taxa have been described from members of this complex in the last 200 years. Analysis of ITS and large subunit ribosomal DNA (rDNA) regions permitted the re-evaluation of phylogenies for this group, one of the most difficult to decipher in smut systematics (Savchenko et al. 2014).

Re-evaluation of less-studied smut fungi in the genomics age. With the advent of the genomics age has come additional important resources that have recently been utilized at the molecular genetic level to investigate previously forgotten or neglected smut fungi (Toome et al. 2014; and for comparison of smuts with rusts (Duplessis et al. 2013). In one example, for *T. horrida*, among the more destructive fungal pathogens of rice (*Oryza sativa* L.), inspection of the draft genome sequence (Wang et al. 2015) identified potential genes encoding proteins associated with key virulence factors. Another example in which molecular genetic tools have been applied to understand both phylogenetic relationships and aspects of pathogenicity is the species *Tilletiara anomala* (Toome et al. 2014), which was included in an early study with six species of *Tilletiopsis* by comparison of small subunit rDNA sequences (Takashima and Nakase 1996). Such analyses suggested its clustering with *U. maydis* and *U. hordei*, but *Tilletiara anomala* is now placed under Georgerfimeriales (Exobasidiomycetes). The sole representative of this species was from culture and, thus, its role in nature is unknown. Environmental sequencing projects detected it in the intracellular fluid of rice plants in Japan, hence, it is now believed to be a potential plant pathogen or endophyte; also, microbial community sequences from the soil of deciduous forests in Estonia (Toome et al. 2014) implicated an association with plants, as is true for the majority of Ustilaginomycotina.

Recently, the genome sequence *Melanopsichium pennsylvanicum* was obtained, and *Melanopsichium pennsylvanicum* is closely related to *Ustilago maydis* and other sweet grass (*Poaceae*)-infecting smuts but is parasitic to a dicot plant (Sharma et al. 2014). *Melanopsichium pennsylvanicum* is a nonobligate biotrophic pathogen. As an exception among Ustilaginaceae, *Melanopsichium pennsylvanicum* colonizes the dicot genus *Persicaria* (Begerow et al. 2006) and is responsible for gall smut. To explore the evolutionary patterns resulting from host adaptation after this huge host jump, the sequenced genome of *Melanopsichium pennsylvanicum* was compared with the genomes of *U. maydis*, *Sporisorium reilianum*, and *U. hordei*. Although all four genomes had a similar completeness in the set of core genes, gene absence in *Melanopsichium pennsylvanicum* was most pronounced in putative secreted proteins, considered to be possible effector candidates (Sharma et al. 2014). Positive selection studies revealed that *Melanopsichium pennsylvanicum* had the highest number and proportion of genes under positive selection. Despite its reduced repertoire of predicted secreted proteins, putative effectors showed a higher proportion of positively selected genes than noneffector candidates.

Considering a “core” of secreted effectors required for pathogenicity, the 248 putative secreted effectors found in all four smut genomes might constitute such a set. On the other hand, the 92 found in all grass-parasitic smuts but with no ortholog in *Melanopsichium pennsylvanicum* might constitute a set of effectors important for successful colonization of grass hosts (Sharma et al. 2014).

Another interesting case is that of *Pseudozyma flocculosa*. A natural antagonist of powdery mildews, this smut species is not considered a phytopathogen. Comparative genomics with its close relatives, *U. maydis*, *U. hordei*, and *S. reilianum*, revealed that, in addition to the presence of pivotal genes known to trigger the morphological and physiological changes linked to infection by smut fungi of their respective hosts, conservation of all other typical plant pathogenicity traits found in smuts was observed in *P. flocculosa* (Lefebvre et al. 2013). This finding indicates that *P. flocculosa* likely has evolved from a plant pathogenic ancestor common to all four species. Further analysis revealed that it has lost a specific subset of secreted effector proteins that influence virulence in *U. maydis* and *S. reilianum*, which could explain its nonpathogenic lifestyle (Lefebvre et al. 2013). Thus, *P. flocculosa* may have once been a virulent smut fungus that lost the specific effectors necessary for host compatibility. Interestingly, *P. flocculosa* appears to have acquired genes encoding secreted proteins not found in the compared Ustilaginales, including proteins hypothesized to have direct relevance to its ability to cause collapse of powdery mildew colonies (Lefebvre et al. 2013).

A model system in a nonagricultural setting: *Microbotryum lychnidis-dioicae*/Silene latifolia. Although members of the genus *Microbotryum* are most commonly found infecting the anthers of host plants in the family Caryophyllaceae, they can also be found on the anthers of members of families Dipsacaceae, Lamiaceae, Lentibulariaceae, and Portulacaceae (Kemler et al. 2006). Additionally, some members of *Microbotryum* can infect other organs, mainly of hosts in Polygonaceae. Studies using ITS nuclear rDNA sequences of members of almost all genera in Microbotryaceae (Kemler et al. 2006) confirmed anther smuts on Caryophyllaceae as a monophyletic group, with a native North American group that diverged from the European clade before the European species radiated on that continent. Also, a second group of anther smuts was found and contains parasites on Dipsacaceae, Lamiaceae, and Lentibulariaceae. At least the majority of the parasites of Asteraceae were monophyletic, while the ability to parasitize Polygonaceae was determined to be the ancestral state for the Microbotryaceae on Eudicot hosts. (Kemler et al. 2006).

The anther smut disease caused by the *Microbotryum violaceum* sensu lato (formerly known as *Ustilago violacea*), which evolved within the Microbotryales (Bauer et al. 2006; Begerow et al. 1997; Weiss et al. 2004), infects flowering species of Caryophyllaceae, and has been characterized as a sexually transmitted disease of the plant hosts (Antonovics 2005). It is a biotrophic disease that causes sterility in the host (Baker 1947), which some may consider as a better strategy for a pathogen than causing mortality (Sloan et al. 2008). In the male, the fungus disguises itself as the pollen so well that Linnaeus mistook the infected flower as a different species (Linné; 1737; Liro 1924) and that mistake was perpetuated into modern times (Oxelmann et al. 2001; Zhou et al. 2008), until the mistake was discovered in 2003 (Hood and Antonovics 2003). In fact, most herbarium collectors did not notice that their specimens were diseased (Hood et al. 2010). Since the teliospores occupy the space of the pollen in the anthers, infection in the female then seems futile, as there are no stamens (Kaltz and Shykoff 2001). To overcome this, the fungus alters the female reproductive organs by suppressing the gynoeceum and makes the rudimentary stamens continue development into anthers that can house the teliospores, effecting a sex change in the female host (Ruddat et al. 1991; Uchida et al. 2003, 2005).

Over an extended period of investigations, *M. violaceum* (originally called *Ustilago violacea*) was part of an extensive

series of mutant studies and provided classical genetic maps for pathogenic fungi prior to the development of modern molecular genetic approaches for such characterization (Garber and Ruddat 2002). For this dimorphic fungus, a variety of auxotrophic, dominant selective–fungicide resistance, and color mutants provided a versatile system for such classical genetics, and haploid isolates from extensive field collections were also maintained and available (Antonovics 2005; Antonovics et al. 2002). Additionally, while successful mating of haploid strains could only be observed microscopically, the lifecycle could be advanced artificially in the lab setting by providing extracts from suitable host species, thus allowing stable dikaryon formation in vitro (Castle and Day 1984; Kokontis and Ruddat 1986). Fundamentally different types of host compounds were found to trigger this switch in vitro, including lipid-like compounds such as tocopherols and phytols as well as compounds soluble in aqueous extracts, termed hyphal growth factors (Ruddat et al. 1991).

Throughout the modern work on *M. violaceum*, ecological studies have also dominated the investigations (Antonovics et al. 2002; Bucheli et al. 1998; Hood et al. 2010; Kaltz et al. 1999; Thrall et al. 1993) and the system has proved an excellent model for emerging infectious disease through host-shifts (Antonovics et al. 2002; Refrégier et al. 2008). This is especially important since *M. violaceum* has, historically, been used as an aggregate term for anther smut species that together infect almost 200 species in the Caryophyllaceae in North America and over 650 species in Europe (Thrall et al. 1993), and additional species in this complex expand the host range further. At the same time, it was realized that individual strains of the fungus appeared to be severely limited to infection of the host species from which they were originally isolated, although there were a few cryptic generalist species (Refrégier et al. 2008). As mentioned earlier, molecular genetic tools that were adapted for phylogenetic analysis (e.g., sequencing ITS regions of nuclear rDNAs) re-evaluated the taxonomic position of the anther smuts, removing many species from the Ustilaginales and placing them together in the Microbotryales, specifically in the genus *Microbotryum*. This repositioning placed them closer to the rust fungi than to the “true” smuts. Then, such techniques were applied to further delineate anther smuts isolated from different host species. Studies using RAPD (Perlin et al. 1997), AFLPs (J. Antonovics personal communication; Hughes and Perlin 2005), gene phylogenies (Denchev 2007; Garr et al. 1997; Lutz et al. 2005, 2008; Perlin et al. 1997; Piatek et al. 2012, 2013), microsatellites (Bucheli et al. 1998), and EKs (Hood 2005; Perlin 1996) led to the realization that host-limited strains of the fungus called *M. violaceum* were most likely different species or incipient new species of the fungus. This conclusion has continued to be investigated and supported by additional gene sequencing and electrophoretic karyotyping (Hood 2005) that, along with extensive ultrastructural and morphological studies, helped provide the current understanding of the phylogenies and taxonomy of this diverse fungal species complex (Bauer et al. 2006; Begerow et al. 2014; Vánky 2012).

As a model of a smut fungus that infects a much more genetically diverse group of host species compared with the better-characterized smuts that infect agriculturally important but genetically more homogenous hosts, we focus here on the anther smut species *Microbotryum lychnidis-dioicae*, isolated from *Silene latifolia*. The host *Silene latifolia*, a wild flower, is one of the most studied species among vascular plants that display sexual dimorphism and is also a model system of increasing scientific importance for the study of evolution of plant sex chromosomes (Mrackova et al. 2008). While it is known that steroids do not affect sex determination in this species (Ruddat et al. 1991), it is still unclear what does. Microscopic investigations of the infection process have provided important insights (Uchida et al. 2003, 2005) and, in some cases, breathtaking images of the interplay between fungus and host (Schäfer et al. 2010).

Recently, the genome of a previously well-characterized a_1 mating-type strain (Lamole p1A1) of *M. lychnidis-dioicae* was sequenced using both 454 pyrosequencing and Illumina technologies (Perlin et al. 2015), while another group sequenced both mating types and 12 more pairs of strains of the fungus (Whittle et al. 2015), and yet another study yielded an almost complete assembly of both mating types of the lineage previously sequenced, this time using the PacBio P5/C3 Single Molecular Real Time technology (Badouin et al. 2015). In addition, carefully chosen transcriptome data pertaining to multiple stages of the lifestyle of *M. lychnidis-dioicae*, both in vitro and in planta, are now available (Fontanillas et al. 2015; Toh 2015; Toh and Perlin 2015).

In the remainder of this review, we will attempt to summarize the many aspects of the research that have been done on *M. lychnidis-dioicae* and draw parallels with findings in other less-studied smut fungi.

Genomic landscape of *M. lychnidis-dioicae*. The first time the anther smut community was able to get a glimpse at the genomic landscape of *M. lychnidis-dioicae* was when an expressed sequence tag (EST) library (Yockteng et al. 2007) of the mated fungus prepared in the presence of α -tocopherol (which stimulated dikaryon formation) was made available via an online database, MICROBASE (<http://www.microbase.com>). In this study, a high frequency of genes without significant BLAST hits was encountered. The dissimilarity of pheromone receptor compared with other Basidiomycetes explained the failure to clone the gene using degenerate primers explained with sequences from *Ustilago maydis* and *U. hordei* (Yockteng et al. 2007).

Yockteng et al. (2007) predicted and some evidence was provided (Perlin et al. 2015) that *M. lychnidis-dioicae* lacks cell wall-degrading enzymes commonly found in plant pathogens but has expanded carbohydrate-active enzyme and protein domains, such as the major facilitator superfamily (MFS) domains and secretory lipase domain (Perlin et al. 2015). These findings were attributed to the biotrophic lifestyle of the fungus.

We can compare the results discussed above to those of another plant pathogen causing smut-like symptoms. *Ustilaginoida virens* Takah (teleomorph: *Villosiclava virens*) is an ascomycete fungus that causes a false smut disease of rice and has been recognized to be the fastest spreading rice crop disease (Zhang et al. 2014). Analysis of the five genotypes suggests that there was a recent expansion of the population, based on the high percentage of nonsynonymous substitutions. Similar to *M. lychnidis-dioicae*, *U. virens* was found to have fewer cell wall-degrading enzymes, namely, pectinases and cutinases (Zhang et al. 2014). In contrast to *M. lychnidis-dioicae*, it has a reduced repertoire of proteins predicted to be in the MFS (compare 80 MFS representatives for *U. virens* versus 246 for *Fusarium graminearum*, 180 for *Magnaporthe oryzae*, 190 for *Botrytis cinerea*, 144 for *Sclerotinia sclerotiorum*, 207 for *Cordyceps militaris*, 215 for *Metarhizium anisopliae*, and 177 for *Metarhizium acridum*) (Zhang et al. 2014). This reduction suggests a narrower range of nutrient specificity (Perlin et al. 2014).

Since the discovery of the clusters of effector genes in *U. maydis* (Kämper et al. 2006), the search for such clusters has been conducted on other newly sequenced genomes and was successful for some (Schirawski et al. 2010; Zhang et al. 2014) but not all smut fungi, e.g., *Melanopsichium pennsylvanicum* (Sharma et al. 2014) and *M. lychnidis-dioicae* (Perlin et al. 2015), coincidentally both infecting dicotyledons. In the case of *M. lychnidis-dioicae*, though a large cluster of such genes is not observed, among the 279 predicted secreted proteins, a number are predicted to bear the hallmarks of effectors, such as being small, cysteine-rich, and lacking any similarity to other proteins currently in the available databases (Perlin et al. 2015).

Mating chromosomes. Basidiomycetes have the most complicated mating systems among the fungi. Heterothallism, i.e., individual cells possessing a specific sex (also known as mating types), and requiring a compatible partner to complete the sexual life cycle, is

much more common than homothallism (selfing) in Basidiomycetes. This is in contrast to Ascomycetes, where homothallism occurs more frequently; one hypothesis is that homothallic species evolved from heterothallic ancestors (Lin and Heitman 2007). About 65% of basidiomycetes possess a tetrapolar system, while 25% use a bipolar system. Only about 10% are homothallic (Bakkeren and Kronstad 1994). In tetrapolar mating, there are typically two loci, e.g., *a* and *b*. Successful mating requires that the cells engaging in mating differ at both loci. The *a* locus encodes two proteins, a pheromone (or its precursor) and a receptor for the opposite mating-type pheromone; the two different forms are not properly alleles, as they share limited homology, and are, thus, referred to as idiomorphs (Turgeon and Yoder 2000). The *b* mating loci contain genes that code for two types of homeodomain transcription factor proteins, usually tightly linked. The two types of homeodomain transcription factors are termed HD1 and HD2, often homologs of the *Saccharomyces cerevisiae* mating proteins MAT α 2 and MAT α 1. Heterodimer formation resulting from mating between cells differing in the alleles at this locus is required to produce a functional transcription factor to activate genes necessary for the completion of the lifecycle (Hsueh and Heitman 2008). It is believed that multiallelic systems favor outcrossing in Basidiomycota (Hsueh and Heitman 2008). On the other hand, in some basidiomycetous fungi, mating is controlled by a bipolar system, where mating is controlled by a single allelic locus (termed variously *A*, *a*, or *b*). Bipolarity may have evolved by the *B* or *a* locus having lost functionality in determining specificity, as has occurred in the mushroom *Coprinellus disseminatus* (James et al. 2006), or both mating loci have become physically linked so as to act as a single locus. The latter strategy has occurred in the smut plant pathogen *U. hordei* (Bakkeren and Kronstad 1994), likely in the anther smut *M. violaceum* (Giraud et al. 2008), and in the human pathogen *Cryptococcus neoformans* (Lengeler et al. 2002).

The mating system is tightly linked to pathogenicity, especially in pathogens that require a host to complete their sexual life cycle. The mating chromosomes of *M. lychnidis-dioicae* provide one of the best-studied models. Even before a whole genome sequence was available, the mating-type chromosomes of *M. lychnidis-dioicae* have served as a model for investigating the evolution of sex chromosomes (Fontanillas et al. 2015; Hood et al. 2004, 2013; Petit et al. 2012; Whittle et al. 2015). This was the first fungus found, via electrophoretic karyotyping of products isolated from single meiotic events, to have heteromorphic mating-type chromosomes (a_1 , about 2.8 to 3.1 Mb, and a_2 , 3.4 to 4.2 Mb) (Hood 2002); moreover, the a_1 mating-type chromosome is more gene-dense than the a_2 mating-type chromosome (Hood et al. 2004). There is also an agreement that the autosomes are generally homogeneous among mating types, while a large nonrecombining region (NRR) is found on the mating-type chromosomes (Fontanillas et al. 2015; Hood et al. 2013). Comparative analysis of the mating chromosomes stems from optical mapping (Hood et al. 2013), and yet, there are genes in the NRR that are very similar in terms of genomic sequence (Fontanillas et al. 2015). At the same time, comparison of the a_2 pheromone of different *Microbotryum* species (Badouin et al. 2015; Xu et al. 2016) revealed high sequence variation.

By way of comparison, a more extensive analysis of the mating genetics and the specificity of mate recognition in the plant biotrophic basidiomycete family Ustilaginaceae (Kellner et al. 2011) was able to dissect a conserved triallelic pheromone-receptor system that preserved interspecific sexual compatibility for more than 100 million years. While the mating-systems are more optimized for intraspecific mating, the extensive capability for successful interspecies couplings has important implications. This study suggests that biallelic mating-type systems occurred by convergent evolution. Importantly, the analysis by Kellner et al. (2011) demonstrates that grass smuts are a valuable model group to study the hybrid-based genesis of novel genotypes and their evolutionary impact on speciation.

Even among the closely related smuts that affect corn (*U. maydis*, *S. reilianum*) or barley (*U. hordei*), in which the gene functions and sequences are very similar, the mating-type loci differ significantly in terms of structure (Bakkeren et al. 2008). Although they all have the genes encoding the two homeodomains (HD1 and HD2) next to each other, analysis of synteny among the corn smuts shows that the *U. hordei* *MAT-2* locus is inverted compared with *MAT-1* relative to what is found in the other two fungi. It was noted in *M. lychnidis-dioicae* that there are extensive rearrangements of genes (inversions and translocations) among fungal mating chromosomes; this could both be a cause and result of suppressed recombination (Badouin et al. 2015). The respective mating-type loci of *M. lychnidis-dioicae* were determined to be 0.6 Mb apart, but they are tightly linked, hence resulting in a bipolar system (Badouin et al. 2015). This is in contrast to *Ustilaginoides virens* that only possesses a single mating-type locus (Zhang et al. 2014); it is thus more similar to the case in *U. hordei* than to that in *U. maydis*.

Haplo-lethality and mating-type bias. Incidences of mating-type bias, in which only one mating type could be retrieved from germinated teliospores isolated in a particular wild population, were often observed. The phenomenon was formally reported (Oudemans et al. 1998) as a “consistent and widespread feature of the biology of the natural population” of the anther smut in *Silene latifolia*. Haplo-lethality (Antonovics et al. 1998) appeared to be the most suitable mechanism to explain the phenomenon. Such haplo-lethal alleles, often linked to mating type, would prevent survival of these haplotypes after meiosis; this problem was circumvented by early formation of a conjugation tube immediately after meiosis of the germinated teliospore, hence eliminating the need for both haplotypes to survive independently (Antonovics et al. 1998). Yet, mating-type bias had no effect on disease dynamics. Early fusion of opposite mating types (Nielsen 1972) to form two dikaryons that further divide into one dikaryon and eventually two monokaryons was also observed in *U. nuda*, which causes loose smut of barley. While this is the default mode of germination in *U. nuda*, it may be an alternate strategy in *M. lychnidis-dioicae* to survive haplo-lethality.

Transposable elements (TEs) and repeat-induced point (RIP) mutations. TEs are ubiquitous in eukaryotes and, though some have regarded them junk DNA (Kidwell and Lisch 2001), others have recognized these mobile genetic intracellular “parasites” as potential drivers of evolution, including, e.g., in the generation of exon structure (Dwyer 1998), founding of the sexual cycle in eukaryotic organisms (Bell 1993; Hickey 1982), and possibly, a force behind epigenetic mechanisms like chromatin formation and methylation (Bird 1995; McDonald 1990).

M. lychnidis-dioicae appears to be a rather unique Basidiomycete, with a hypermutation pattern among the TEs (Horns et al. 2012). Moreover, the density and diversity of TEs in this species is unusually high (Hood 2005; Hood et al. 2004). The most common TE found in the fungus is the *copia* element, which is very rare among fungi, even rarer in Basidiomycetes. One potential defense mechanism utilized by *M. lychnidis-dioicae* is a RIP machinery, although it does not appear to be as effective as those used in Ascomycetes (Hood et al. 2005).

By way of comparison, in *U. virens*, there appeared to be strong RIP activity, as evidenced by a high ratio of TpA/ApT (1.48) and a low value for (CpA+TpG)/(ApC+GpT) (< 1.0) (Zhang et al. 2014). Moreover, the high average ratio of di-nucleotide preference for *U. virens* also provided genome-level evidence for the efficiency of RIP, at a level similar to that in *Neurospora crassa* and higher than for other sequenced ascomycetes. Furthermore, alignment of homologous TEs revealed numerous point mutations representing C-to-T (G-to-A) transitions in the TE sequences, implying that RIP can also control TE activity in *U. virens* (Zhang et al. 2014).

Additional aspects of the interplay between pathogen and host. The above discussion of intracellular parasites (i.e., TEs) in an

organism that itself is considered a parasite or pathogen leads us now to a further discussion of host-pathogen interactions. Such relationships are often described as an “arms race” (Maor and Shirasu 2005). This competition can lead to coevolution of the pathogen with its host species (Refrégier et al. 2008). The pathogen is assumed to have an advantage due to faster generation time and larger population size (or progeny) and should do better on a host population with which they have evolved. However, in a study that examined sympatric and allopatric differences, indigenous *M. lychnidis-dioicae* did not infect the local host better than the “foreign” host (Kaltz et al. 1999). This suggests one or more innate defense mechanisms on the part of the host. Authors hypothesize that the dioecious hosts hold an advantage in this system by outcrossing, whereas the pathogen is likely to inbreed. In field studies of this system, diseased plants were found to flower earlier than healthy plants and neither the sex of the plant nor the plant and fungal population had measureable influence over this phenomenon (Shykoff and Kaltz 1998). However, it was concluded in the studies that the earlier flowering of a host population would correlate with the higher vulnerability to being infected. Various studies also found that the diseased plants appeared to produce a lower root/shoot ratio (Shykoff and Kaltz 1998), have larger asymmetry of floral petals (Shykoff and Kaltz 1998), and a reduction in inflorescence height, flower size, and nectar production of the flowers (Biere and Honders 1996). These papers suggested various explanations for the effects, either in favor of defense by the plants or offense by the fungus.

A recent RNA-Seq analysis (Zemp et al. 2015) was able to further dissect the interaction of the pathogen and its host, presenting a strong link between the sex of the host and the gene expression changes. The gene expression response was complex and involved a higher percentage of the host genes than would normally be expected. There was a general pattern mirroring that of the opposite sex upon infection and the expression patterns were in line with the physiological changes observed in the infected plants.

By way of comparison with other fungi that infect anthers, gene expression was examined in the anthers of maize plants infected with *U. maydis* (Gao et al. 2013). This study suggested that the fungus turns on specific genes in the anthers; these changes in its transcriptome are correlated with dramatic changes to all host cell types in the anthers, both in numbers and size. In fact, there appear to be different gene sets responsible for disease in the sexual organs and the other parts of the plants (Gao et al. 2013). For example, *U. maydis* expressed 2,280 genes uniquely in tassels compared with 1,513 genes only in the anther samples. Such differences, including the expression of different subsets of its predicted secretome (i.e., 206 genes in anthers and 111 in tassels), may explain why *U. maydis* has such an extensive repertoire of effectors (Gao et al. 2013).

Although some members of genus *Microbotryum* can be found infecting other organs, mainly of hosts in the Polygonaceae (Kemler et al. 2006), this is in stark contrast to the *M. lychnidis-dioicae*/*Silene latifolia* system, in which, typically, only the anthers or pseudo-anthers house the teliospores and demonstrate obvious differences in macroscopic and microscopic morphologies. This may also help explain the smaller predicted secretome in *M. lychnidis-dioicae* (i.e., 279, compared with 554 in *U. maydis*; Gao et al. 2013) and the lower number of small secreted proteins (SSPs) (i.e., putative effectors) that were up-regulated in planta (i.e., 48). Nevertheless, the finding that these predicted SSPs were up-regulated late in infection further implicated them as candidate effectors in this system (Perlin et al. 2015). It will be interesting to compare the gene sets found in the maize pathosystem to the new RNA-Seq data of the infected floral buds vs. uninfected floral buds in the *M. lychnidis-dioicae*/*Silene latifolia* system (S. S. Toh, Z. Chen, T. Kalbfleisch, D. J. Schultz, C. A. Cuomo, and M. H. Perlin, unpublished data). Moreover, in the case of *M. lychnidis-dioicae*, there is also the suppression of the gynoceum and

proliferation of androecium in the female flower, phenomena for which additional insights will likely materialize from RNA-Seq analyses (S. S. Toh *unpublished*). Further, since a large number of studies (Carlsson-Granér 1997; Carlsson-Granér and Thrall 2015; Chung et al. 2012; Gibson et al. 2013) show variation in susceptibility of *Silene* spp. to infection by *Microbotryum* spp., such transcriptomics may inform analysis of changes in one or both host or pathogen gene expression that could, at least partially, explain such variation in host resistance.

Robust transformation system. A reliable and reproducible system for the introduction and maintenance of exogenously supplied DNA is a critical tool for the manipulation of a study organism (Alberts et al. 2002). As an example in which this tool has been applied to a lesser-studied smut, we find *U. esculenta*, mentioned previously. To investigate the outcome of the cultivation of infected wild rice as food and help determine whether the “vegetable” has any economic worth, molecular genetics methods were desired. Thus, a protoplast transformation method was developed recently that can perform both targeted gene knock-out and overexpression (Yu et al. 2015).

Several attempts at developing a transformation system for *Microbotryum* species that would allow investigation of genes of interest at a molecular genetics level have been made (Bej and Perlin 1989a and b, 1991; Perlin et al. 1990), mostly modeled after other transformation systems in tractable organisms like *Saccharomyces cerevisiae* and *U. maydis*. Disappointingly, these early successes were not reproducible by other researchers; even biolistic approaches proved ineffective (M. H. Perlin *unpublished*). Thus, none of these approaches were pursued further. Despite such disappointments, technological advances in other subdisciplines can still provide important clues for sustained progress where it was previously lacking. For example, *Agrobacterium*-mediated transformation has been used in plants since the 1980s (Caplan et al. 1983). The use of *Agrobacterium*-mediated transformation in fungi (Abbott et al. 2013; Ianiri et al. 2011), from the very tractable *Saccharomyces cerevisiae* to various recalcitrant Pucciniomycotina yeasts (Abbott et al. 2013; Bundock et al. 1995; Ianiri et al. 2011), eventually provided the basis for a usable, reproducible transformation system for *M. lychnidis-dioicae*. In fact, a system using *Agrobacterium*-mediated transformation had been reported for sugarcane smut *U. scitaminea* (Sun et al. 2014).

Thus, by adapting other *Agrobacterium*-mediated strategies and specifically exploiting the newly acquired genomic and transcriptomic data for *M. lychnidis-dioicae*, a robust transformation system has recently been developed (Toh et al. 2016). The method exploited recent genomic and transcriptomic data (Perlin et al. 2015) to provide endogenous promoters that would allow target genes to be preferentially expressed under different phases of the fungal lifecycle. Currently, overexpression of a heterologous marker using a native promoter as the driver has been successful (S. S. Toh *unpublished*) and development of a targeted knock-out system is underway (M. H. Perlin *unpublished*).

Conclusions. The smut fungi, though not a monophyletic group, form a very important focus for both basic biology and phytopathology. In fact, the diversity of this group is part of its charm. Smut fungi are well-suited to investigate the ecology and evolution of plant pathogens, as they are biotrophic yet various stages can be maintained on media in the laboratory. Along with the vast body of work already completed on smut fungi, the examples presented in this review should highlight the importance of these fungi, both as important pathogens but, also, as models for other basic and practical scientific areas of investigation. The focus of this review has been to emphasize that the advent of modern molecular approaches, especially genomics and transcriptomics, has benefited study of not only the already extensively studied smut pathogens of agricultural crops but, also, has provided the opportunity to explore less-studied representatives of smuts, many of which can provide insights due to their infection of

wild, uncultivated host species that are not genetically homogenous. These parasites of wild hosts can be critical as models to explore the emergence of infectious diseases through host shifts (Antonovics et al. 2002; Refrégier et al. 2008). As next-generation sequencing becomes ever more affordable, even little-known smut fungi (e.g., *T. anomala* [Toome et al. 2014]) or those whose understanding at the molecular level has lagged behind (e.g., *M. lychnidis-dioicae* [Perlin et al. 2015]) are able to receive attention and potentially advance as models of phytopathogenesis. As an example, *Testicularia* species were traditionally classified in Cintractiaceae with *Cintractia*, *Trichocintractia*, and related genera based on similar host plants and similarities in sorus development and basidial structure (Begerow et al. 2006; Vánky 2012). On the other hand, molecular phylogenetic analyses of several rDNA loci showed that family Cintractiaceae was nonmonophyletic (Begerow et al. 2006). Thus most members of Cintractiaceae were moved to other families (Begerow et al. 2006). However, due to the paucity of molecular data, the genus *Testicularia* was not included in that study (Begerow et al. 2014). The genome sequencing of *Testicularia cyperi*, underway at the Joint Genome Institute during the writing of this review, will provide the first whole genome reference sequence for a smut fungus in the Ustilaginales that is not in the Ustilaginaceae. These data will provide the ability for phylogenetic and phylogenomic reconstructions and, in comparative genomics studies, to uncover molecular bases governing production of sexual and anamorphic states and the evolution of phytopathogenicity in Ustilaginomycotina.

Comparative genomics has allowed identification of core sets of genes involved in pathogenicity but, also, subsets of genes that confer host preference (Sharma et al. 2014, 2015). Moreover, such comparisons, not possible in the pregenomics age, can provide likely paths in the evolution toward and away from pathogenic lifecycles (e.g., *P. flocculosa* [Lefebvre et al. 2013]). Modern technological advances can thereby propel previously forgotten fungi to new prominence as models for understanding the emergence of phytopathogenesis (e.g., *Melanopsichium pennsylvanicum* [Sharma et al. 2014]). The genome sequences combined with transcriptomics should contribute to new insights into the subtle genetic differences that can lead to drastic changes in fungal pathogen lifestyles, including possible transitions between pathogenicity and symbiosis or saprophobic growth as well as the emergence of infectious diseases through host shifts.

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