**Understanding Adaptation, Coevolution, Host Specialization and Mating System by
combining Population and Comparative Genomics in Castrating Anther-Smut Fungi**

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Abstract

Anther-smut fungi constitute a powerful system to study host-pathogen specialization and coevolution, with hundreds of *Microbotryum* species specialized on diverse Caryophyllaceae plants, castrating their hosts through particular manipulation of hosts' reproductive organs that facilitates disease transmission. *Microbotryum* fungi also have exceptional genomic traits, including dimorphic mating-type chromosomes, that make this genus also an excellent model for the evolution of mating systems and their influence on population-genetic structure and adaptive potential. Important insights into the adaptation, coevolution, host specialization and mating system evolution have been gained using anther-smut fungi, in particular with the recent advent of genomic approaches. We argue and illustrate based on the *Microbotryum* case studies that using a combination of genomic analyses is a powerful approach, where comparative genomics, population genomics and transcriptomics data allow the integration of different evolutionary perspectives and across timescales. We also highlight current challenges and future studies that will contribute to advance our understanding of mechanisms involved in adaptive processes in fungal pathogen populations.

Keywords : comparative genomics, population genomics, transcriptomics, adaptation, positive selection, selective sweeps, divergence, gene flow, rearrangements, suppressed recombination

Introduction

Pathogens thrive using living organisms as nutritional resources, which reduces their host fitness. This leads to coevolutionary arms races, in which pathogens are selected for increased abilities of host infection and exploitation, while hosts are selected for mechanisms of resistance to particular diseases. Such coevolution occurs on short evolutionary scales, as a never-ending process of adaptation and counter-adaptation (113). Across macro-evolutionary scales, some pathogens also may undergo host shifts, forming new species by specialization in combination with new hosts (42). Coevolution is a very different evolutionary process from host specialization, despite the terms often being used interchangeably, and may involve different genomic mechanisms and/or molecular interactions that have yet to be well resolved (42).

An integrated understanding of the ecological and genetic/genomic mechanisms underlying both coevolution and host specialization by pathogens is of fundamental importance. These phenomena indeed represent cases of rapid adaptation, diversification and long-term species interactions, shedding light on the processes generating and maintaining biodiversity and ecosystem dynamics. Furthermore, knowledge on the genomic mechanisms involved in coevolution and host specialization in fungal pathogens is important for controlling crop and animal diseases and preventing emerging diseases that are a rising threat in domestic and wild populations (52, 70). Fungi are the most important plant pathogens, causing dramatic crop diseases, including many devastating diseases that are newly emergent following host shifts (9, 45, 51).

Fungal pathogens also have to cope with their abiotic environment, such as temperature and humidity (2, 35, 44, 47, 129). Understanding the mechanisms of adaptation to climatic variables is thus similarly of fundamental and applied interest. Adaptation ability is however impacted by genetic diversity and gene flow, which are themselves influenced by dispersal rates and mating systems, that are therefore important life history traits to study for an integrated understanding of evolution, adaptation, population subdivision and speciation (18, 60, 63).

From the advent of modern genetics a century ago, anther-smut fungi (*Microbotryum violaceum* species complex, previously *Ustilago violacea*) have served as useful models for the molecular controls of mating and adaptations to abiotic conditions (1, 17, 23, 29, 61, 62, 73, 93, 123, 124). With advances in population genetics and genomics, emphasis has grown with regard to the natural diversity within this pathogen group, the dynamics of diseases, the mating systems and genetic differentiation in relation to host plants in natural ecosystems (2, 27, 32, 38, 39, 58, 65, 67, 97, 105, 111, 131, 132). The anther-smut fungi belong to the *Microbotryum* genus (basidiomycetes), which castrate plants of the Caryophyllaceae family, replacing the pollen with their spores and aborting ovaries (Figure 1B). They constitute an excellent model pathosystem, with hundreds of closely related fungal species specialized on different host plants, resulting from numerous host shifts, with conspicuous symptoms, a rich scientific history and occurring in natural ecosystems (Figure 1A) (83, 92, 97, 111, 118). Furthermore, they are phylogenetically close to the rust fungi as damaging crop pathogens (127). Most *Microbotryum* species are highly host-specific, but a few are more generalist, parasitizing closely related host species (Figure 1A) (98, 105). Other *Microbotryum* species, while distantly related, co-occur on the same host species, representing cases of convergence (Figure 1A) (2, 97).

87

88 Host-pathogen coevolution in the *Microbotryum* model systems has been suggested based on
89 patterns of plant local adaptation (43, 49) and congruent plant-pathogen genetic structure (49).
90 *Microbotryum* species show little pre-zygotic isolation and increasing post-zygotic isolation
91 strength with phylogenetic distance (28, 41, 98), which may allow gene flow among closely
92 related species. Moreover, abiotic factors have been shown to play a role with the disease
93 interactions in important ways (2), and *Microbotryum* fungi display an interesting mating
94 system, with predominant automixis (i.e., intra-tetrad selfing), which has fostered multiple
95 chromosomal rearrangements across the genus linking the mating-type loci controlling
96 gamete compatibility (25, 26). A consideration of these features altogether allows the
97 studying adaptation, coevolution, host specialization, differentiation and mating systems with
98 unique power.

99

100 For tackling this complex suite of questions, comparative genomics and population genomics
101 constitute highly relevant and complementary approaches, addressing different time scales of
102 evolution. In contrast to life history traits, ecology and population structure, which have been
103 extensively studied (7, 10, 22, 90), the genetic basis of interactions between *Microbotryum*
104 fungi and their hosts is still little known; genomic approaches can elucidate the mechanisms
105 and the functions involved in adaptation, coevolution, host specialization and speciation in
106 this pathosystem. Analyses of gene expression between different stages of the life cycle can
107 also inform on these processes. In particular, the pathogen's mating systems also influence
108 adaptation, coevolution and host specialization (63, 66), especially in anther-smut fungi that
109 are obligately completing the sexual cycle upon every disease transmission. Genomics can
110 further help to understand the evolution of mating systems by studying the changes at the
111 mating-type loci.

112

113 In this review, we discuss the recent insights into our understanding of adaptation,
114 coevolution and host specialization in anther-smut fungi gained from gene expression data
115 and comparative genomics (part 1) and from population genomics (parts 2 & 3). We then
116 discuss insights gained from genomics on mating system evolution (part 4). We illustrate that
117 the combination of multiple genomic approaches is needed for a full understanding of
118 evolution, as comparative genomics, population genomics and transcriptomics address
119 different timescales and have power for detecting different footprints of adaptive events.
120 Finally, future challenges to be addressed using genomics tools are discussed (part 5).

121

122 **1- COMPARATIVE GENOMICS AND TRANSCRIPTOMICS APPROACHES TO** 123 **UNDERSTAND ADAPTATION AND HOST SPECIALIZATION IN ANTHER-SMUT** 124 **FUNGI**

125 The sequencing of genomes and transcriptomes of *Microbotryum* species sheds light on
126 pathogenicity, adaptation and specialization mechanisms across long evolutionary timescales;
127 speciation events in castrating *Microbotryum* fungi have been dated from 0.4 to 11 MYA (26,
128 72) (Figure 2A). Phylogenomics enables obtaining an accurate understanding of the lineage
129 histories, and comparative genomics is highly suitable to identify genetic changes associated
130 with diversification at such large evolutionary scales.

131

132 **Genome architecture and identification of candidate genes involved in pathogenicity** 133 **using expression data**

134 One of the best studied anther-smut species is *M. lychnidis-dioicae*, parasitizing the white
135 campion *Silene latifolia* (Figure 1B). The diploid genomes of the Lamole *M. lychnidis-dioicae*
136 strain was the first eukaryote genome to be assembled with new sequencing technologies (16).

Comparative analysis of the Lamole strain of *M. lychnidis-dioicae* with other basidiomycetes genomes revealed specific gene content features such as the absence of plant cell wall degrading enzymes and expanded repertoires of major facilitator superfamily transporters, secretory lipases, glycosyltransferases and enzymes that could manipulate host development (104). Such features are likely related to the castrating and biotrophic lifestyle of anther-smut fungi (104), where the fungus takes up a largely symptomless residence between the host cells in the plant's growing points/meristems until the host initiates flower development. Additionally, this pathogen has a remarkable ability to developmentally transform female host plants to take on a male-like floral structure, with the growth of stamens that then bare spores in place of pollen and the abortion of the ovary early in its development (13). Apart from the accumulation of transposable elements (TEs) in the non-recombining regions of the mating-type chromosomes, there was no genome compartmentalization into more or less repeat-rich regions on autosomes (16, 104), in contrast to some other fungal pathogens with isochore genomic architecture and localization of effector genes in repeat-rich regions (74). Nevertheless, transposable elements were locally associated across the Lamole *M. lychnidis-dioicae* genome with gene clusters of small secreted proteins and genes affected by within species presence-absence polymorphism, suggesting a role of transposable elements in genome rearrangements and duplications of genes putatively involved in host adaptation (80, 104). Although footprints typical of genome defense mechanisms against TEs, similar to repeat-induced point mutation (RIP), were identified in anther-smut genomes, a massive burst-like expansion of Gypsy-like retrotransposons in a *Microbotryum* strain suggested that persistent transposable elements activity and expansion can occur (86, 87).

Transcriptomics conducted at several *in vitro* stages allow detecting genes upregulated in certain conditions and thus likely involved in important functions at a given life stage.

Transcriptomic analyses using the Lamole *M. lychnidis-dioicae* strain enabled identifying genes likely associated with nutrient uptake, the mating program and the dikaryotic switch (54, 104, 125, 126, 136). *In silico* effector gene prediction combining *in planta* expression data, sequence conservation and predicted localization, allowed identifying small secreted proteins genes as candidate effectors, i.e. involved in pathogenicity, in *M. lychnidis-dioicae*, *M. silenes-dioicae* and *M. violaceum var paradoxa* (20, 96). Eight genes in *M. silenes-dioicae* and three genes in *M. violaceum var paradoxa* predicted to encode secreted proteins were further confirmed to be secreted using yeast secretion trap (20, 96). Compared expression data in male and female *S. latifolia* individuals during fungal infection revealed pathogen-mediated changes in sex-biased gene expression and altered sexual dimorphism in the host (137). Another transcriptome analysis of the early development stages of infected flowers detailed changes in gene expression in *M. lychnidis-dioicae*, identifying gene categories likely to manipulate the host development and reproductive system, such as potential effectors and virulence factors (125). Further coupling experiments of host and pathogen gene expression changes, and in further paired host-*Microbotryum* fungi, should help deciphering the major components of the tight host-pathogen interactions described in the system.

Comparative genomics studies within the *Microbotryum* genus

Comparative genomics among *Microbotryum* fungi, and with other plant pathogens, has provided insights into the specificity of castrating biotrophic pathogens growing intracellularly (115) relative to other forms of parasitic nutritional ecology. Comparative genomics among anther-smut fungi specialized on different hosts can help unravel the genomic determinants of host specificity as well as the shared pathogenicity mechanisms. Indeed, while substantial insights has been gained by the study of individual genomes of *Microbotryum* species, whether features such as the conspicuous lack of cell-wall degrading

enzymes in the *M. lychnidis-dioicae* Lamole genome are common to the genus cannot be known without a comparative genomics analysis that addresses both distantly and closely-related species (77).

Early comparative studies focused on orthologous genes across single pass Sanger-sequenced cDNA libraries, i.e. expressed sequence tags, from four *Microbotryum* species. The primary focus was looking for signals of positive selection in terms of frequent amino-acid changes (4). A subset of the genes evolving under positive selection between species was further shown to be under strong purifying selection within two closely-related *Microbotryum* species, *M. lychnidis-dioicae* and *M. silenae-dioicae*, suggesting that adaptive changes concomitant with host shifts can be later fixed due to strong functional constraints within species (69). Although the inferred function of some of the orthologous groups with signals of positive selection could be associated with aspects of virulence or speciation, none of these displayed features of effectors (such as secretory signals), likely because the expressed sequence tags did not exhaustively cover the genomes. Indeed, only 53 clusters of orthologs shared by at least three species and at least 300 nucleotides long could be retrieved (4). Therefore, even though these analyses demonstrated the utility of comparative genomics to identify candidate genes for diversifying selection in non-model organisms, the lack of whole genome sequences prevented any insight about presence-absence polymorphisms or substitutions both known to be important for adaptation to new hosts.

The number of high-quality genomes assemblies or shotgun sequencing from *Microbotryum* species/strains has exploded recently, reaching nearly a hundred as by late 2018 (Table 1; Figure 1A; (15, 16, 25, 26, 30, 56, 112, 134)). In comparative genomics, near-complete gene lists can be clustered to obtain groups of homologous sequences that can be then used to build

phylogenetic profiles of gene content. Such comparisons allow the identification of gene families that are species-specific and those that have been expanded or reduced in particular lineages (8, 76). Species- or population-specific genes are either derived from within-group innovation, a rather uncommon phenomenon (31), the result of differential losses or gene duplications (78), or due to the non-vertical acquisition of gene-coding genome fragments, for instance horizontal gene transfer (50). Expanded gene families require the escape from the rampant pseudogenization (non-functionalization) of duplicated genes (99), whereas reduced or complete losses of gene families is often related to ecological shifts (117), rendering the product of those genes no longer needed for survival (5). Understanding these processes is fundamental to the study of evolutionary ecology as they help to explain the genomic architecture underlying the phenomenon of adaptive divergence. *In silico* annotations and comparative analyses have identified hundreds of candidate effectors across multiple *Microbotryum* species, enriched in gene families showing presence-absence polymorphism across species (Figure 2B) (112), along with orthologous genes with landmarks of positive selection between species and purifying selection within species (20), thus generalizing and expanding previous findings. High-quality genome assemblies revealed little genomic rearrangements in autosomes (26).

Studies of positive selection based on the comparisons of non-synonymous and synonymous substitution rates (dN/dS ; (135)) and on the comparisons of the proportions of non-synonymous and synonymous polymorphisms within species and differences between species (McDonald and Kreitman test; (100)) revealed no signature of diversifying selection between sister *Microbotryum* species specialized on two closely related host species (15), but detected a dozen of genes encoding secreted proteins with signs of positive selection between more distantly related *Microbotryum* species specialized on more distant host species (Figure 2C)

(20). Future comparative genomics studies encompassing all currently sequenced genomes will likely have high power to detect genes involved in host specialization by allowing further disentangling the effects of pathogen and host phylogenetic distances. In particular, combining population and comparative analyses should be very powerful to identify genes under diversifying selection between species and purifying selection within species as well as species-specific gene gains and losses. Building gene genealogies based on whole genomes also allowed to resolve previously ambiguous relationships among some anther-smut species (Figure 1A). The comparison of repeat contents and genomic rearrangements between genomes will be a further key step to understand the role of genome dynamics in adaptive processes in anther-smut fungi.

2-POPULATION GENOMICS TO IDENTIFY ADAPTIVE GENETIC VARIATION IN NATURAL PATHOGEN POPULATIONS

Population genomics is a complementary approach to comparative genomics for understanding adaptation in pathogen populations. Population genomics indeed address more recent adaptive events, and on a broader range of evolutionary genetic phenomena, not only gene gains/losses and recurrent changes in amino-acids. Selective sweeps can be detected using population genomics, which can reveal positive selection on a single amino-acid change or basepair substitutions in non-coding regions. Furthermore, population genomics can address the questions of the genomic bases of host-pathogen coevolution and local adaptation, that constitute more recent selection compared to the long-term selection underlying host specialization, and possibly differential selection among geographically distant populations (Figure 2A) (36, 75, 107). In contrast to major fungal-plant pathosystems, no gene-for-gene relationship has been reported for anther-smut fungi. Instead, the probability of infection shows quantitative variation (6, 7, 33), which suggests a rather complex genetic basis of co-

evolution and host local adaptation. Genome-wide population genomics approaches in anther-smut fungi allowed identification of the complex genetic basis of recent adaptive events through genome scans of selective sweeps and gene-presence absence polymorphism (3, 15, 80).

Selective sweep analyses allow one to identify loci that have recently been under positive selection within populations and thus likely underlying coevolution and local adaptation, whereas genes involved in host specialization are likely under purifying selection within species after the initial adaptive events following host shifts. Analyses of whole genome sequences of 53 genomes of the anther-smut sister species *M. lychnidis-dioicae* and *M. silenes-dioicae* identified selective sweeps (Figure 2D) (15), likely resulting from dynamic co-evolutionary arm race of the fungus with its hosts. The overlap between genes differentially expressed *in planta* and *in vitro* and those lying within selective sweeps, together with functional annotations, provided clues to genes and functions involved in plant-pathogen interaction in the *Microbotryum-Silene* system. Candidate genes included glycoside hydrolases, pectin lyases and an extracellular membrane protein with CFEM domain (15). The pectin lyase function seems relevant in that *Microbotryum* fungi grow between cells of the meristem (115), which is a pectin-occupied space. Extracellular membrane proteins with a cysteine-rich CFEM domain are present in effectors in several fungal pathogens (95). This study was also an opportunity to test for differences in intensity of coevolution between anther smut fungi on different hosts. Interestingly, differences in the number and the location of the selective sweeps were found between sister species. Footprints of positive selection affected 17 % of the genome in *M. lychnidis-dioicae* and 1 % of the genome in *M. silenes-dioicae* (15). Selective sweeps were scattered throughout the genomes. Linkage disequilibrium was found to decay relatively slowly with physical distance along chromosomes, as expected for selfing species, but still indicated effective recombination.

Polymorphism in each fungal species was negatively correlated with the recombination rates along chromosomes, consistent with recurrent positive and/or background selection erasing diversity on larger genomic regions when recombination is less frequent (15).

Population genomics can also contribute to our understanding of the impact of recent anthropogenic factors on the genome and subsequent adaptation. Analyses of *M. lychnidis-dioicae* genomes along a gradient of ionizing radiation levels around Chernobyl showed no evidence of deleterious mutation accumulation in the form of non-synonymous substitutions (3). Lower mean values of dN/dS were even found in Chernobyl compared to other areas of the same eastern genetic cluster (3), which may be due to stronger selection in contaminated areas against individuals bearing mildly deleterious mutations, i.e. stronger purifying selection.

In addition to genome scans looking at signatures of positive selection, other population genomic approaches make use of the genetic variation in pathogen populations to identify the genomic architecture of local adaptation (19, 36, 107, 114). Population genomics enables the unravelling the genomic bases of adaptation to abiotic conditions by searching for correlations between local population allele frequencies and local environments (genetic-environment association methods) (82). Such approaches can be used in anther-smut fungi along altitudinal clines in Alpine populations on *Dianthus* or *Silene* hosts. Studies on the three species parasitizing *S. vulgaris* in particular could be interesting as elevation and climate has been shown to impact these anther-smut fungi (1, 2). Strong population structure as found in many *Microbotryum* species at European scale (2, 15, 27, 55, 105) might be a challenge to the use of such methods in particular, but these methods can be utilized at small geographical scales and/or in species with less population subdivision.

Gene copy number variation segregating within species is also a widespread and an important source of genetic variation and several examples of adaptive evolution through gene loss or gene gain have been identified in agricultural fungal plant pathogens (57). Population genomics allow to explore the extent and adaptive potential of such within-species variation. Gene presence/absence polymorphism was found to contribute to the genetic variation in populations of the two closely related species of castrating anther-smut fungi, *M. lychnidis-dioicae* and *M. silenes-dioicae* (80). Genes displaying presence/absence polymorphism were mostly recently acquired, in a single species, through duplications in multiple-gene families and few genes predicted to encode secreted proteins were affected, suggesting defense against host recognition by other genetic changes than gene loss or gain. Although most gene presence/absence polymorphisms were likely neutral, the putative functions of some genes affected by presence-absence polymorphism (e.g., secreted proteins) or their localization within previously identified selective sweeps suggested that some gene loss or gain events may be adaptive (80).

3-INSIGHTS INTO THE DYNAMICS OF DIVERGENCE AND GENE FLOW FROM POPULATION GENOMICS

By providing a glimpse into intra-specific genetic diversity and its variation across the genome, population genomics analyses are also highly useful to understand processes underlying species divergence and phylogeography, quantifying rates of gene flow and its heterogeneity along genomes, and providing accurate estimation of population size variations. The occurrence of multiple *Microbotryum* sister species pairs in sympatry makes the system a perfect model to study the dynamics of divergence and gene flow in fungal pathogen populations.

Contrasted patterns of interspecific gene flow in the *Microbotryum* genus: a speciation continuum?

The two pathogens *M. lychnidis-dioicae* and *M. silenes-dioicae* and their respective sister host plants, *Silene latifolia* and *S. dioica*, are ubiquitous in Europe and their geographic distributions are largely overlapping, providing an ideal system for research on the formation and maintenance of species in sympatry. Microsatellite data from samples across Europe revealed rare disease transmission events between the host species and rare pathogen hybrids (72, 132). However, these approaches using a dozen microsatellite markers may lack power. Analyses of whole genome sequences of many pathogen samples that appeared of pure ancestry based upon the microsatellite data then revealed no evidence for admixture, indicating that introgression does not persist beyond one or two generations (15). In the laboratory, both fungal species can infect both host plants (40, 64, 98). Experimental crosses showed little premating isolation by assortative mating between the two pathogen species (28, 98, 132), even at sympatric sites (110), and a lack of post-mating barriers (41, 98). Hybrids were viable and fertile at least through the F2 generation in the greenhouse (40, 98, 131). F2 hybrids produced by selfed F1s had mostly returned to homozygosity, suggesting that genomic content derived from one of the two parental species had already begun to be purged (28, 40). This latter finding, combined with the fact that introgression does not appear to persist in nature, is consistent with strong genome-wide selection by the host plant and the scattering of genes involved in host specialization across the genome, as revealed in genome scans of selective sweeps (15). F_{ST} values were found near their maximum all along their genomes (Figure 3A).

Whereas strict host specialization is often the rule on *Silene* species (15, 97, 133), on *Dianthus* hosts in contrast population genetics approaches revealed four *Microbotryum*

364 lineages with broader and overlapping host specificities (Figure 1A) (97, 105). One
365 *Microbotryum* lineage was found only on *D. pavonius* while the others occurred spread across
366 several host species, some of them being shared among *Microbotryum* lineages. The sympatry
367 of *Microbotryum* lineages within populations, in particular in the Alps, led to hybridization
368 (105). The individuals with mixed ancestry based on clustering analyses of microsatellite data
369 suffered from significant meiotic sterility, which confirmed they were hybrids between
370 species (105). The larger host ranges of *Microbotryum* lineages on *Dianthus* hosts may be
371 explained by the recent divergence of their host plants. The *Dianthus* genus has indeed
372 undergone a recent radiation in Europe with morphologically diverse European *Dianthus*
373 species restricted to small geographically restricted ranges (130). The full extent and
374 evolutionary consequences of the hybridization on pathogen dynamics and evolution remains
375 to be explored. Along this line, the *Dianthus-Microbotryum* system may become, in the
376 coming years, a tractable model to investigate the impact of gene flow during divergence, and
377 whether selection due to local/host adaptation can make some genomic regions more or less
378 permeable to gene flow, which represents a current debate in evolutionary biology (37). These
379 questions could not be addressed so far based on the population genomics analyses of *M.*
380 *lychnidis-dioicae* and *M. silenae-dioicae* as no genomic introgression could be detected in
381 natural populations (15). In contrast, the hybrids detected in natural populations on *Dianthus*
382 hosts with significant sterility suggest the occurrence of introgressions (105). Other pairs of
383 *Microbotryum* species might also be suitable to address these questions of the impact and
384 heterogeneity of gene flow along the genome. For example, anther-smut fungi on the closely
385 related and sympatric native American species *S. virginica* and *S. caroliniana* (11, 12) could
386 not be separated into host-specialized species based on a few gene genealogies (58, 92, 111).
387 In this system, population genomics should allow elucidating whether anther-smut fungi on
388 these American *Silene* species show host differentiation or genome-wide gene flow, or

introgression only in genomic regions not involved in host specialization. Based on the few genomes available so far (26), we find F_{ST} values between *Microbotryum* populations on the two hosts, *S. virginica* and *S. caroliniana*, that are lower and more heterogeneous along the genomes than between *M. lychnidis-dioicae* and *M. silenae-dioicae* referenced above (Figure 3B). These initial results suggests the occurrence of gene flow in some genomic regions. The situation of anther-smut fungi on *S. vulgaris*, with three distant lineages with convergent specialization on this same host species (2), would also be worth exploring using population genomics to determine the extent of introgression and its genomic localization, and whether the interspecific exchange of alleles has been deleterious or adaptive.

Another promising approach in anther-smut fungi for identifying genomic regions involved in host adaptation will be to perform genome scans of differentiation between closely related species or host races, if possible to avoid the potential pitfalls of such approaches (37). This could contribute to our understanding of the role of gene flow in the early stages of divergence and to identifying genomic regions less permeable to gene flow because of selection for host adaptation and/or genetic incompatibilities between lineages (24, 37). More generally, such population genomics approaches would be valuable to use in plant pathogen fungi.

Phylogeography and demographic history inferences

Microbotryum lychnidis-dioicae and *M. silenae-dioicae* also constituted case studies in providing one of the most clear-cut examples of phylogeographic structure in pathogens, thanks to a collection of samples whose density and geographical scale was unprecedented for a disease association in natural populations. In *M. lychnidis-dioicae*, clustering analyses based on microsatellite markers (133), as well as nuclear gene sequences (69, 72), revealed the

existence of three genetically distinct clusters, reflecting recolonization from well-recognized southern refugia after glaciation. Little admixture has been found between clusters based on microsatellites (49, 133), and this has later been confirmed by whole genome sequences (15). Indeed, SNPs (single nucleotide polymorphisms) revealed few shared polymorphisms and many fixed differences among the clusters, and pairwise F_{ST} values between them were high (0.56–0.74; Figures 3C and D), supporting low levels of inter-cluster gene flow (15). Whole genome sequences provided further insights into the age of divergence between the three *M. lychnidis-dioicae* lineages (Southern, Western and Eastern clusters), sequential size changes in the population size of derived lineages and also supported low levels of gene flow (15). Most notably, the pathogen genetic structure closely matched with the genetic structure of the host species *S. latifolia* with the same regionally defined Southern, Western and Eastern clusters, indicating that the anther-smut pathogen remained during the last glaciation in the same three distinct refugia as its host (i.e. in the Iberian, Italian and Balkan peninsulas) (49). The congruence of population structures between *M. lychnidis-dioicae* and its host appeared even stronger than what could be expected because of isolation by distance alone, suggesting that coevolution has played a significant role in the congruence of the population structures (49). Genome-wide gene presence-absence polymorphism recovered the same population structure (80). Inoculation experiments, indicating plant local adaptation for resistance to pathogens (49, 89, 91), were consistent with a contribution of adaptive factors to the observed congruence between pathogen and host population structures.

Microsatellite markers and genome-wide SNPs indicated that *M. silenes-dioicae* also exhibited a genetic structure, albeit with biogeographic patterns more difficult to interpret (15, 133) and very low F_{ST} values genome-wide (Figure 3E). Genome-wide gene presence/absence polymorphism revealed two different clusters with a more obvious east/west separation (80),

that may correspond to local adaptation of *S. dioica* clusters (81, 109), although this remains to be assessed. This case study shows the power of various kinds of population genomic studies to unravel weak and/or adaptive population subdivision.

4-UNRAVELLING MATING SYSTEM AND GAMETE COMPATIBILITY SYSTEMS USING BOTH COMPARATIVE GENOMICS AND POPULATION GENOMICS

The combination of comparative genomics and population genomics also can reveal remarkable transitions in mating systems by elucidating the changes in genomic mechanisms controlling mating compatibility. For the broad group of basidiomycete fungi, gamete compatibility is controlled by two loci acting at the haploid stage, mating being successful only between haploid cells carrying different alleles at both mating-type loci (34). The two mating-type loci are i) the PR locus which encodes pheromone genes and a pheromone receptor gene implicated in gamete recognition and fusion, and ii) the HD locus which encodes homeodomain protein-coding genes allowing, after fusion, for the maintenance of the dikaryon and hyphal growth (48, 94). Most basidiomycetes are outcrossing and have these two loci unlinked, although some fungi in this group have linked mating-type loci (103). Linkage of the two mating type loci is considered to be favored due to increased odds of gamete compatibility under selfing when mating-type loci are linked (103). Interestingly, most *Microbotryum* species are highly selfing and were long known to segregate only two mating type phenotypes, but it remained uncertain whether this was due to mating-type loci linkage or to the loss of role in mating-type determinism for one of the two mating-type loci, as both cases occurred in basidiomycetes (85, 103). Comparative genomics of well-assembled genomes allowed to resolve the complex genome architecture and long-term evolutionary history of the repeat-rich and rearranged mating type chromosomes in anther-smut fungi, and

population genomics datasets were essential for identifying young events of recombination suppression.

Population genomics confirmed high rates of selfing in all studied *Microbotryum* species, by showing high levels of genome-wide homozygosity (15, 25, 26) and confirmed massive recombination suppression on mating-type chromosomes (84, 134). High-quality genomes assemblies allowed reconstructing the history of genomic events underlying the shift in gamete compatibility system (25, 26). The long-read sequencing technology allowed assembling the two repeat-rich mating-type chromosomes of the Lamole *M. lychnidis-dioicae* strain, which confirmed linkage between the two mating-type loci HD and PR (Figure 4A) (16). Genome comparisons between multiple *Microbotryum* species showed that the ancestral state had unlinked mating-type loci on two distinct chromosomes, and that independent rearrangements and chromosome fusions occurred in multiple species, convergently linking the two mating-type loci by large regions without recombination (Figure 4B) (25, 26). This shows that natural selection can repeatedly lead to similar phenotypes through multiple different evolutionary pathways.

Following recombination suppression, a chaos of rearrangements occurred on mating-type chromosomes (16), as well as TE and non-synonymous substitution accumulation (16, 54), as is typical in non-recombining regions, and in particular on sex chromosomes (14). The high-quality assemblies allowed the detailed characterization of extensive rearrangements and repeat accumulations on the two mating-type chromosomes (16, 25, 26). Another important characteristic feature of sex chromosomes was observed on the mating-type chromosomes of multiple *Microbotryum* species, i.e., the stepwise extension of the regions with recombination suppression. The progressive extension of the regions without recombination revealed a

pattern of clear “evolutionary strata”, i.e., decreasing divergence between alleles on the alternative mating-type chromosomes farther from the mating-type loci (Figure 4C). Population genomics was essential for providing evidence of early events of recombination suppression in several species, by showing the segregation of alleles according to their associated mating-type, decreased levels of diversity as expected under lower population effective sizes and that high divergence between alleles associated with the alternative mating types was due to balancing selection on mating types rather than elevated substitution rates (25, 26). Indeed, as soon as recombination ceased, alleles on the two mating-type chromosomes diverged gradually with time (Figure 4D). Finding such evolutionary strata in fungi, which lack male and female roles, challenged the classical view for the evolution of sex chromosomes. Indeed stepwise recombination suppression in sex chromosomes was thought to be due primarily to sexual antagonism, i.e., the selection to link genes with alleles beneficial in one sex, and deleterious in the other, to the sex determining gene (21). The finding of evolutionary strata in fungi without sexual antagonism indicates that alternative hypotheses should be explored to explain the progressive spread of recombination suppression, such as overdominance, epigenetic modifications associated with transposable elements or neutral rearrangements (88, 108).

5-CONCLUDING REMARKS AND PERSPECTIVES

A thorough understanding of the major roles played by pathogens requires the integrative study of both ongoing processes of coevolution and dynamics of specialization that impact the emergence new diseases. Investigations of the anther-smut fungi utilizing comparative approaches to genomics and gene expression profiles, combined with population-level studies, illustrate the strength of combining different genomic approaches addressing different

scales of evolution (Figure 2). The availability of genomic data for multiple sister species and multiple populations within species makes the anther-smut system quite exceptional for identifying the genetic mechanisms involved in adaptation, coevolution, host specialization and mating system at different evolutionary times (Table 1; Figure 1). Comparative genomics has long been the predominant approach for studying adaptation in plant fungal pathogens (46, 53, 59, 102, 107) and has provided important insights into the mechanisms of adaptation, e.g., through horizontal gene transfers, gene gains/losses, hybridization or recurrent amino-acid changes (71). Comparative genomics by definition does not consider population-level variation, such that population genomics is a complementary approach for insights into evolutionary processes acting at the local and regional scales. For example, several recent studies have revealed gene presence/absence polymorphism within species (78, 80, 119, 120). In addition, comparative genomics can only detect a specific type of positive selection, involving frequent changes of amino-acids. Positive selection of a single amino-acid change or of regulatory regions can only be detected by looking for selective sweeps using population genomics. Some recent studies based on population genomics have in fact revealed important aspects of adaptation in fungal plant pathogens, showing footprints of introgression, selective sweeps and amino acid-changes (68, 79, 101, 116, 121, 122).

Furthermore, cross referencing candidate genes that are highlighted by multiple indications of being subject to natural selection during parasitism as outlined here (e.g. genes found within a selective sweep, upregulated in the plant and having experienced gene family expansion compared to other fungal pathogens) can strengthen their putative roles as pathogen effector that are central in the specificity of fungal-plant combinations. Functional studies can help understanding the role of the candidate genes. Promising transformation protocol have been developed in *M. lychnidis-dioicae* (128) and will likely facilitate the characterization of key

genes involved in the interaction between the anther-smut fungi and their Caryophyllaceae host plants, an important challenge for the coming years. Transcriptomes and epigenomes of multiple *Microbotryum* species and multiple strains within species will likely be complementary to the current available genomic resources to identify the role of regulatory and epigenetic mechanisms in the adaptation of anther-smut fungi to their hosts and environment, their divergence and their mating-type chromosome organisation, contributing to further understanding the mechanisms involved in adaptive processes in fungal pathogen populations. It will also be interesting to investigate similar levels of among and within species sampling and genome sequencing using pathogens of different levels of obligate parasitism, including facultative and opportunistic pathogens, as well as hemibiotrophy and necrotrophy.

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930 **Tables and Figures**

931 **Table 1: Whole genome public resources in anther-smut fungi (*Microbotryum* genus).**

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Figure 1: Specialization and co-phylogenies of anther-smut fungi and their corresponding host plants. (A) Cladograms representing relationships between species of anther-smut fungi (left, *Microbotryum* genus) and species of host plants (right, mainly Caryophyllaceae), that are a consensus from previous phylogenetic analyses (26, 30, 111). Availability of short reads or long reads-based genome assemblies or population genomic data for the species of anther-smut fungi as presented in Table 1 is indicated with a black square near the fungal cladogram leaves. Dashed lines indicate specialization of a fungal species on a host species, with pink lines for fungal species infecting different hosts, and orange links for fungal species infecting the same host. The sequenced strain *M. intermedium* was sampled on *Salvia pratensis*, although this fungal species is usually found on *Scabiosa* hosts. (B) Infected host plants by anther-smut fungi. Numbers refer to host species as in panel A. Spores of anther-smut fungi are visible in the anthers of the flowers (Photo credits: Michael E. Hood, Tatiana Giraud, Maarten Strack van Schijndel).

Figure 2: Evolutionary processes in anther-smut fungi studied by comparative genomics and population genomics methods. (A) Schema highlighting differences in time scales between host specialization, species divergence, coevolution and local adaptation events in four host-specialized *Microbotryum* species. (B) Type of genomic variation investigated according to the evolutionary event time scales. (C) Examples of methods recently used to investigate various evolutionary events in anther smut fungi, focusing on between-species variation (1), between- and within-species variation (2), or only within-species variation (3). Information on gene annotation, gene expression and gene presence-absence polymorphism may be coupled to narrow down the number of candidate genes to be involved in host specialization, coevolution and local adaptation. (1) Study of gene content variation between whole genome shotgun assemblies of 19 *Microbotryum* species. Core and complementary

(species-specific) genomes were computed by sampling groups of 1 to 18 *Microbotryum* species (112). Increase in size of the complementary genome with the number of sampled genomes highlights the dynamic gene gain and loss within the *Microbotryum* genus. Genes contained in the complementary genome are putative candidate genes for host specialization.

(2) Identification of genes under positive selection using polymorphism in one species and divergence with an outgroup using McDonald–Kreitman tests. An excess of the ratio between non-synonymous (D_N) and synonymous (D_S) substitutions between species compared to the ratio between synonymous (P_S) and non-synonymous (P_N) polymorphisms within species is indicative of positive selection within the focal species indicated by an asterisk. Examples are shown for the orthologous group ORTHAg06728 and ORTHAg05587 (20).

(3) Genome scan to identify selective sweeps based on allele frequency spectrum in *M. lychnidis-dioicae*. A selective sweep is characterized by an excess of rare variants. Composite likelihood ratio (CLR) tests estimate the probability of the presence of a selective sweep taking into account demographic history and genome-wide allele frequency spectrum (15).

Figure 3: Distribution of divergence along genomes between species of host-specialized anther-smut fungi (*Microbotryum* genus) based on F_{ST} genome scans. F_{ST} distributions are based on the genomes of five strains in each group for comparisons, except for strains on *S. caroliniana* for which only three genomes were available. (A) Divergence distribution between *M. silenes-dioicae* and *M. lychnidis-dioicae*. (B) Divergence distribution between *Microbotryum* fungi on *S. caroliniana* and *S. virginica*. (C) (resp. (D)) Divergence distribution between Southern and Northern (resp. Eastern) European genetic clusters of *M. lychnidis-dioicae* parasitizing *S. latifolia*. (E) Divergence distribution between Eastern and Western European genetic clusters of *M. silenes-dioicae* parasitizing *S. dioica*. In each panel, from top to bottom: density curve of genome-wide per-gene F_{ST} values; chromosomal

distribution of per-gene F_{ST} values on the species largest chromosome (on 2 Mb for each to ease comparisons); map showing the sampling location of sequenced strains (genomes used for F_{ST} distributions are shown as squares); pictures of infected hosts by each host-specialized species (Photo credits: Michael E. Hood). For each pairwise comparison, F_{ST} values were calculated per gene for all genes present on autosomal contigs (i.e. not belonging to mating-type chromosomes) and carrying at least 1 SNP using the PopGenome R package (106). Red dashed lines correspond to median F_{ST} values. Genomic data were described in (15, 26, 134). Mapping, SNP calling and F_{ST} calculations were described in (26, 80).

Figure 4: Genomic rearrangements and evolutionary strata on mating-type chromosomes of *Microbotryum lychnidis-dioicae* on *Silene latifolia*. (A) Circos plot allowing to retrieve the rearrangements events which occurred during the evolution of the *M. lychnidis-dioicae* mating-type chromosome. The two mating-type chromosomes (PR and HD mating-type chromosome) of *M. lagerheimii* are used as proxy for the ancestral state (25). The outer tracks represent contigs with scale in Mb. The blue and orange lines link orthologs, with inversions in orange. The blue and purple dots represent the HD and PR loci, respectively, and the yellow regions the centromeres. (B) Evolutionary scenario of the *M. lychnidis-dioicae* mating-type chromosome evolution from the two ancestral mating-type chromosomes through a centromere-to-telomere fusion event, which brought the PR and HD loci onto the same chromosome and allowed their linkage through a recombination suppression (dashed lines). (C) Demonstration of stepwise recombination suppression using per-gene synonymous divergence and their respective standard error ($dS \pm SE$) between alleles from *M. lychnidis-dioicae* associated to the a_1 versus a_2 mating types along the mating-type chromosome gene order of *M. lagerheimii*, as a proxy for ancestral gene order. Evolutionary strata of different divergence levels (colored differently) shows that

1009 recombination suppression extended stepwise from the HR and PR mating-type loci. (D)
1010 Examples of two gene genealogies showing contrasted clustering levels of alleles at non-
1011 mating-type genes associated with the a_1 versus a_2 mating types (dark grey and light grey
1012 squares, respectively, at the tips of the gene genealogy). The left panel shows the gene
1013 genealogy of a gene belonging to the pseudo-autosomal region (or PAR), with no trans-
1014 specific polymorphism, i.e., intermingled alleles associated with a_1 and a_2 mating types. The
1015 right panel shows the gene genealogy of a gene belonging to the non-recombining region,
1016 with completely separated alleles associated with a_1 versus a_2 mating types of both *M.*
1017 *lychnidis-dioicae* and *M. silenae-dioicae*, and thus trans-specific polymorphism. The branch
1018 length scale is indicated at the bottom of each gene genealogy.

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Table 1 : Whole genome public resources in anther-smut fungi (Microbotryum genus).

	Fungal species name	Host plant of sampling	Number of distinct genotypes	References	Public database	Project ID/ Strain ID / Accession ID*
Long read based assemblies	<i>M. intermedium</i>	<i>Salvia pratensis</i> **	1	(25)	GenBank	PRJEB15277 : Microbotryum Intermedium Assembly (GCA_900096595)
	<i>M. lagerheimii</i>	<i>Silene vulgaris</i>	1	(25)	GenBank	PRJEB12080 : MvSv-1253-A1-R1 (GCA_900015505); MvSv-1253-A2-R1 (GCA_900013405)
	<i>M. lychnidis-dioicae</i>	<i>Silene latifolia</i>	2	(16) (80)	GenBank GenBank	PRJEB12080 : MvSi-1064-A1-R4 (GCA_900015465); MvSi-1064-A2-R4 (GCA_900015445) PRJNA437556 : MvSi-1318_A1 (GCA_003121365); MvSi-1318_A2 (GCA_003121355)
	<i>M. violaceum sensu lato (M.v. caroliniana)</i>	<i>Silene caroliniana</i>	1	(26)	GenBank	PRJEB12080: MvCa-1250-A1-R1 (GCA_900014965); MvCa-1250-A2-R1 (GCA_900014955)
	<i>M. violaceum sensu lato (M.v. paradoxa)</i>	<i>Silene paradoxa</i>	1	(26)	GenBank	PRJEB12080 : MvSp-1252-A1-R1 (GCA_900015495); MvSp-1252-A2-R1 (GCA_900015485)
	<i>M. violaceum sensu stricto</i>	<i>Silene nutans</i>	1	(25)	GenBank	PRJEB12080: MvSn-1249-A1-R1 (GCA_900015425); MvSn-1249-A2-R1 (GCA_900015455)
	<i>M. saponariae</i>	<i>Saponaria officinalis</i>	1	(30)	GenBank	PRJEB12080 : MvSof-1268-A1-R1 (GCA_900015975); MvSof-1269-A2-R1 (GCA_900015475)
	<i>M. scabiosae</i>	<i>Knautia arvensis</i>	1	(26)	GenBank	PRJEB12080 : MvKn-1118-A1-R1 (GCA_900008855); MvKn-1118-A2-R1 (GCA_900015415)
	<i>M. silenes-acaulis</i>	<i>Silene acaulis</i>	1	(26)	GenBank	PRJNA437556: ASM366583v1 (GCA_003665835); ASM366582v1 (GCA_003665825)
	<i>M. silenes-dioicae</i>	<i>Silene dioica</i>	1	(25)	GenBank	PRJEB16741 : MsdSd1 (GCA_900120095); PRJNA437556 : MsdSd12 (ID requested)
Whole genome shotgun assemblies	<i>M. carthusianorum</i>	<i>Dianthus superbus</i>	1	(112)	GenBank	PRJNA437556 : MvDC3-001-A2-G1 (ID requested)
	<i>M. coronariae</i>	<i>Lychnis flos-cuculi</i>	1	(112)	GenBank	PRJNA437556 : MvLf-1062-A1-G1 (ID requested)
	<i>M. lagerheimii</i>	<i>Silene vulgaris</i>	1	(112)	GenBank	PRJNA437556 : MvSv1-300-38-G1 (ID requested)
	<i>M. lychnidis-dioicae</i>	<i>Silene latifolia</i>	1	(104) (112)	GenBank GenBank	PRJNA41281 : p1A1 Lamole (GCA_000166175) PRJNA437556 : MvSiA1A2r2 (ID requested)
	<i>M. major</i>	<i>Silene otites</i>	1	(112)	GenBank	PRJNA437556 : MvSo-338-G1 (ID requested)
	<i>M. silenes-acaulis</i>	<i>Silene acaulis</i>	1	(112)	GenBank	PRJNA437556 : MvSa-10-04-A1-G1 (ID requested)
	<i>M. silenes-dioicae</i>	<i>Silene dioica</i>	1	(112)	GenBank	PRJNA437556 : MvSd-IT02-32-2-17A-A2-1141 (ID requested)
	<i>M. silenes-inflatae</i>	<i>Silene vulgaris</i>	1	(112)	GenBank	PRJNA437556 : Sv2-78-06-G1 (ID requested)
	<i>M. stellariae</i>	<i>Myosoton aquaticum</i>	1	(112)	GenBank	PRJNA437556 : MvMa-946-A1-G1 (ID requested)
	<i>M. superbum</i>	<i>Dianthus pavanus</i>	1	(112)	GenBank	PRJNA437556 : MvDp-1065-A2-G1 (ID requested)
	<i>M. superbum</i>	<i>Dianthus seguieri</i>	1	(112)	GenBank	PRJNA437556 : MvDC1-001-A2-G1 (ID requested)
	<i>M. violaceum sensu lato</i>	<i>Silene sp.</i>	1	(112)	GenBank	PRJNA437556 : MvSc-a-1127-A2-G1 (ID requested)
	<i>M. violaceum sensu lato (M.v. caroliniana)</i>	<i>Silene caroliniana</i>	1	(112)	GenBank	PRJNA437556 : MvCa-1131-A1-G1 (ID requested)
	<i>M. violaceum sensu lato (M.v. italica)</i>	<i>Silene italica</i>	1	(112)	GenBank	PRJNA437556 : MvSi-1128-A1-G1 (ID requested)
	<i>M. violaceum sensu lato (M.v. lemmonii)</i>	<i>Silene lemmonii</i>	1	(112)	GenBank	PRJNA437556 : MvSlm-001-A2-G1 (ID requested)
	<i>M. violaceum sensu lato (M.v. paradoxa)</i>	<i>Silene paradoxa</i>	1	(112)	GenBank	PRJNA437556 : MvSp-880-A1-G1 (ID requested)
	<i>M. violaceum sensu lato</i>	<i>Silene pusilla</i>	1	(112)	GenBank	PRJNA437556 : MvSpu-866-A1-G1 (ID requested)
	<i>M. violaceum sensu stricto</i>	<i>Silene nutans</i>	1	(112)	GenBank	PRJNA437556 : MvSn-1014-A1-G1 (ID requested)
	<i>M. violaceo-irregulare</i>	<i>Silene vulgaris</i>	1	(112)	GenBank	PRJNA437556 : MvSv3-001-G1 (ID requested)
Sequence archive (reads)	<i>M. lychnidis-dioicae</i>	<i>Silene latifolia</i>	39	(134) (15)	NCBI Short Read Archive NCBI Short Read Archive	PRJNA269361 PRJNA295022
	<i>M. silenes-dioicae</i>	<i>Silene dioica</i>	19	(15)	NCBI Short Read Archive	PRJNA295022
	<i>M. violaceum sensu lato (M.v. paradoxa)</i>	<i>Silene paradoxa</i>	4	(26)	NCBI Short Read Archive	PRJEB16741
	<i>M. violaceum sensu lato (M.v. caroliniana)</i>	<i>Silene caroliniana; Silene virginica</i>	11	(26)	NCBI Short Read Archive	PRJEB16741
	<i>M. saponariae</i>	<i>Saponaria officinalis</i>	1	(56)	GenBank	PRJEB11435

*Information were retrieved on public databases on 22.11.18. For long read based assemblies, assemblies of the two mating type a1 and a2 are indicated if available.
ID requested indicate that the genomic data were submitted to the public database and are currently processed
**the sequenced strain was sampled on *Salvia pratensis*, although the fungal species is usually found on *Scabiosa* hosts

A

Microbotryum

Hosts

B

Figure 1

Figure 2

Figure 3