Size Variation of the Nonrecombining Region on the Mating-Type Chromosomes in the Fungal *Podospora anserina* Species Complex

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Abstract

Sex chromosomes often carry large nonrecombining regions that can extend progressively over time, generating evolutionary strata of sequence divergence. However, some sex chromosomes display an incomplete suppression of recombination. Large genomic regions without recombination and evolutionary strata have also been documented around fungal mating-type loci, but have been studied in only a few fungal systems. In the model fungus *Podospora anserina* (Ascomycota, Sordariomycetes), the reference S strain lacks recombination across a 0.8-Mb region around the mating-type locus. The lack of recombination in this region ensures that nuclei of opposite mating types are packaged into a single ascospore (pseudohomothallic lifecycle). We found evidence for a lack of recombination around the mating-type locus in the genomes of ten *P. anserina* strains and six closely related pseudohomothallic *Podospora* species. Importantly, the size of the nonrecombining region differed between strains and species, as indicated by the heterozygosity levels around the mating-type locus and experimental selfing. The nonrecombining region is probably labile and polymorphic, differing in size and precise location within and between species, resulting in occasional, but infrequent, recombination at a given base pair. This view is also supported by the low divergence between mating types, and the lack of strong linkage disequilibrium, chromosomal rearrangements, transspecific polymorphism and genomic degeneration. We found a pattern suggestive of evolutionary strata in *P. pseudocomata*. The observed heterozygosity levels indicate low but nonnull outcrossing rates in nature in these pseudohomothallic fungi. This study adds to our understanding of mating-type chromosome evolution and its relationship to mating systems.

Key words: convergence, mating-type chromosomes, fungi, fungal chromosomes, sex chromosomes, automixis, pseudohomothallism, evolutionary strata, transposable elements.

Introduction

The causes and consequences of recombination suppression raise fascinating questions in evolutionary biology. In the long term, the absence of recombination leads to the accumulation of deleterious mutations (Muller 1932; Bachtrog 2005). However, the suppression of recombination is sometimes favored by selection, for example when it allows the maintenance of beneficial combinations of alleles (Kirkpatrick and Barton 2006; Hoffmann and Rieseberg 2008; Schwander et al. 2014). The suppression of recombination has been widely studied on sex chromosomes and often leads to high levels of differentiation between sex chromosomes (Bergero and Charlesworth 2009) and degeneration of the nonrecombining sex chromosome (Bachtrog 2013). However, some sex chromosomes remain little differentiated due to rare

recombination events, undetectable in crosses but occurring frequently enough over evolutionary scales to prevent differentiation and degeneration (Guerrero et al. 2012). The suppression of recombination on sex chromosomes often extends in a progressive manner, generating patterns of evolutionary strata, for example, decreasing levels of divergence between sex chromosomes with increasing distance from the sex-determining genes (Bergero and Charlesworth 2009). Such patterns of evolutionary strata have been firmly established in many plants and animals (Bergero and Charlesworth 2009), and even for sex chromosomes with an incomplete suppression of recombination (Darolti et al. 2020).

Recombination is also suppressed around mating-type loci in some fungi (Bakkeren and Kronstad 1994; Fraser et al. 2004; Menkis et al. 2008; Branco et al. 2018; Hartmann et al. 2020),

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although this phenomenon has been much less studied than in plant and animal sex chromosomes. Evolutionary strata have also been reported around some fungal mating-type loci (Fraser et al. 2004; Menkis et al. 2008; Branco et al. 2017, 2018; Hartmann et al. 2020), indicating a stepwise extension of the recombination suppression away from the mating-type loci. Several of the fungi in which evolutionary strata have been detected are automictic (i.e., with mating occurring among products of a single meiosis), for example, in the anther-smut *Microbotryum* fungi and *Neurospora tetrasperma* (Menkis et al. 2008; Branco et al. 2017; Hartmann et al. 2020). In these fungi, the suppression of recombination and its stepwise extension around mating-type loci have occurred on multiple independent occasions in closely related species (Sun et al. 2017; Branco et al. 2018). Some evidence indicate that recombination suppression also occurs around the mating-type loci in nonautomictic fungi (Hartmann et al. 2020). Further studies on a wider range of fungi will be required to determine whether recombination suppression and evolutionary strata are the rule in automictic fungi and whether it is also frequent in other fungi.

The genome of the model fungus *Podospora anserina* includes a 0.8-Mb region, encompassing the mating-type locus, that displays suppressed recombination (Grognet et al. 2014). In this fungus, the occurrence of a single crossing-over event between the centromere and the mating-type locus ensures the copackaging of two nuclei of opposite mating types in dispersing spores and, thus, self-fertility through automixis (Grognet and Silar 2015). An inhibition of recombination around the mating-type locus has probably been selected to ensure the packaging of sexually compatible nuclei within the same ascospore. The ability to reproduce sexually from a single spore, due to the presence of two nuclei of different mating types, is called pseudohomothallism in fungi, by analogy to homothallism, that is, when a single haploid clonal lineage can perform the whole sexual cycle. By contrast, heterothallism is the situation in which spores or mycelia are haploid and have to be of different mating types to be compatible for mating.

Podospora anserina is a suitable model for studying the evolution of recombination suppression around mating-type loci in fungi, as it belongs to a complex of closely related pseudohomothallic species (Boucher et al. 2017; Ament-Velásquez 2020), referred to hereafter as the *P. anserina* complex. The percentage of nucleotide sequence difference within the species complex lies in the range of 1–3% (Boucher et al. 2017; Vogan et al. 2019; Ament-Velásquez 2020). These fungi carry a single mating-type locus with two alleles, *mat+* and *mat−* (Debuchy and Coppin 1992; Debuchy et al. 1993; Debuchy and Turgeon 2006). The S strain of *P. anserina* has been extensively studied and the exact limits of the region without crossing-over events have been identified based on segregation analyses and comparative genomics (Grognet et al. 2014). However, the suppression of recombination around the mating-type locus has not been studied in other strains or species of the complex.

The actual mating system of pseudohomothallic species of the *P. anserina* complex in natural populations has not been

studied either. It is thus unclear whether these species mostly self or outcross, and knowledge of the mating system is important for understanding the evolution of patterns of recombination around mating-type loci. It has long been assumed that pseudohomothallic fungi reproduce exclusively by selfing in nature. However, pseudohomothallism does not prevent outcrossing (Billiard et al. 2011; Billiard et al. 2012). Indeed, the anther-smut *Microbotryum* fungi, despite being mostly automictic, do outcross at low but nonnegligible frequencies when suitable mates are available (Giraud et al. 2005; Vercken et al. 2010; Abbate et al. 2018). In *P. anserina*, spores with a single nucleus—and thus carrying a single mating type—are regularly produced at low frequency in asci (Beckett and Wilson 1968), which could promote some level of outcrossing (van der Gaag 2005). In vitro experiments of vegetative and sexual compatibility among *P. anserina* natural isolates yielded estimates of potential outcrossing rates between 1% and 5% (van der Gaag 2005), but actual outcrossing rates in nature are unknown.

In lineages with high rates of selfing, the regions of suppressed recombination around the mating-type locus can be inferred directly from plots of heterozygosity over the genome in a heterokaryotic individual. Indeed, selfing leads to genome-wide homozygosity, except in regions linked to the mating-type locus. As mating can occur only between gametes carrying different alleles at the mating-type locus, it restores heterozygosity at mating-type locus and at all genes linked to the mating-type locus (Branco et al. 2017, 2018). In a population that generally selfs, outcrossing events typically lead to genome-wide heterozygosity, and each subsequent selfing event increases the size of the homozygous regions until only genomic regions linked to the mating-type locus remain heterozygous. The *P. anserina* S strain has been selfed in laboratories for many generations, and the only region that remains heterozygous is the one without recombination around the mating-type locus (Grognet et al. 2014). Furthermore, the divergence between alleles in a nonrecombining region can be used to estimate the time since linkage to the mating-type locus. Indeed, for all genes linked to mating-type locus, alleles remain associated with one mating type or the other, such that alleles associated with alternative mating types gradually diverge with time since recombination suppression, by independently accumulating substitutions. The divergence between alleles at genes around the mating-type locus between genomes of opposite mating types thus provides a good proxy for the time since these genes became linked to the mating-type locus, and can be used to detect patterns of evolutionary strata (i.e., decreasing divergence with increasing distance from the mating-type locus). Synonymous divergence (dS) is often used as a proxy for evolutionary time in this context (Bergero and Charlesworth 2009).

We analyze here the mating-type chromosomes of the seven pseudohomothallic species of the *P. anserina* species complex (Boucher et al. 2017; Ament-Velásquez 2020). Mycelia of *Podospora* fungi are heterokaryotic in natural conditions, that is, each mycelium carries nuclei of opposite mating types, with karyogamy occurring immediately before

meiosis and diploidy thus being a very brief stage. Homokaryons (i.e., with only one nucleus type) can however be obtained from the rare haploid spores produced in asci. We sequenced the two homokaryotic genomes of opposite mating types separately for each heterokaryotic *Podospora* strain, using the closest known species, *Cercophora samala*, as an outgroup. *Cercophora samala* is heterothallic, producing eight haploid spores following meiosis (Udagawa and Muroi 1979; P. Silar, pers. obs.); its rate of outcrossing in natural conditions is however unknown, as for *Podospora* species. We addressed the following questions: 1) Are there regions lacking recombination in the pseudohomothallic species of the *P. anserina* complex and the closely related heterothallic species *C. samala*? 2) If yes, has recombination ceased completely since before species radiation? 3) Do the size and location of the region lacking recombination vary between strains and/or species? 4) Can we detect evidence of evolutionary strata, for example, a pattern of decreasing divergence between alleles associated with alternative mating types with increasing distance from the mating-type locus? 5) Can we detect footprints of outcrossing in these pseudohomothallic species, in the form of stretches of heterozygosity in the genome? 6) Can we find evidence of degeneration in nonrecombining regions, such as the accumulation of transposable elements and nonsynonymous substitutions?

Results

Variability in the Size of the Region Lacking Recombination around the Mating-Type Locus between *Podospora* Species

We investigated whether there were regions without recombination around the mating-type locus in seven pseudohomothallic species of the *P. anserina* complex, and if so, whether their size and location differed between species. We studied the *mat+* and *mat−* haploid genomes of one heterokaryotic strain of each *Podospora* species, using available high-quality genome assemblies and performing additional Illumina genome sequencing (supplementary table S1, Supplementary Material online). A high degree of genome synteny has been reported among these species, that is, with no major rearrangements among species (Silar et al. 2019; Vogan et al. 2019; Ament-Velásquez 2020). We therefore used a read mapping approach to call SNPs in the *mat+* genome of the S reference strain (hereafter referred to as S+, as in Vogan et al. 2019, corresponding to S *mat+* in Grognet et al. 2014) and we built pseudosequences of genes. Using autosomal SNPs, we retrieved the same species relationships as described previously (fig. 1; supplementary fig. S1A, Supplementary Material online; Boucher et al. 2017; Ament-Velásquez 2020). We computed per-gene dS between the *mat+* and *mat−* homokaryotic genomes of each heterokaryotic strain, using only the genes present in the S+ genome for which orthologs were present in the two homokaryotic corresponding genomes and filtering out repetitive elements. We used the gene order in the *P. anserina* S strain for the plotting of dS, all genomes being collinear to the *P. anserina* S strain genome around the mating-type locus

(Silar et al. 2019; Vogan et al. 2019; Ament-Velásquez 2020). For all species, dS values were high around the mating-type locus, which is located on chromosome 1 (fig. 1), strongly suggesting a lack of recombination around the mating-type region.

In the *P. anserina* S strain, the high dS values around the mating-type locus corresponded precisely to the genomic region previously reported to lack recombination on the basis of progeny segregation analyses (in blue in fig. 1; Grognet et al. 2014). The dS values thus delimit the same nonrecombining region as segregation analyses in the S strain, which confirms that it is a suitable indicator of the lack of recombination as previously shown in other automictic fungi. In the *Podospora pauciseta* CBS237.71 strain, the region in which dS was high was smaller than the nonrecombining region of the S strain, indicating that the region without recombination was much smaller in this strain (fig. 1; table 1). Conversely, in other strains, dS was high in a larger region around the mating-type locus than in the *P. anserina* S strain (fig. 1; table 1). We investigated whether this pattern of high dS values was due to the presence of a larger region lacking recombination in these species or to recent outcrossing events leading to heterozygosity, by checking the genome-wide distribution of dS values. Isolated genes with high dS values were considered to be probably due to errors in sequencing, variant calling, gene models or unidentified repeats, and were not taken into account. We found that dS values were equal or close to zero for most genes in regions of chromosome 1 other than that around the mating-type locus, hereafter referred to as pseudoautosomal regions (PARs), as well as for genes on other chromosomes hereafter referred to as autosomes, in the *Podospora pseudoanserina* CBS124.78, *Podospora bellaemahoneyi* CBS112042, and *Podospora comata* T strains, as expected for recombining regions under a predominant selfing regime (see genome-wide patterns of heterozygosity in supplementary fig. S2, Supplementary Material online). The heterozygous regions around the mating-type locus in these strains thus more likely resulted from a lack of recombination rather than outcrossing events. Using this approach, we were able to infer the existence of larger regions lacking recombination around the mating-type locus in *P. pseudoanserina*, *P. bellae-mahoneyi*, and *P. comata* than in the *P. anserina* S strain (fig. 1).

By contrast, we observed large clusters of heterozygous genes, that is, genomic regions of at least 15 genes with nonzero dS values and with a maximum distance between two consecutive genes with nonzero dS values of 150 kb, on chromosomes 2, 4, 5, and 7 in the *Podospora pseudopauciseta* CBS411.78 strain, and on chromosome 7 in the *P. pseudocomata* CBS415.72 strain (supplementary fig. S2B and table S2, Supplementary Material online). These clusters of high dS in several autosomal genomic regions probably resulted from recent outcrossing events in nature; the large sizes, ranging from 422 kb to 1.5 Mb, and the contrast with completely homozygous chromosomes, indeed render it highly unlikely that the clusters of high dS only result from errors in sequencing or variant calling (supplementary fig. S2B and table S2, Supplementary Material online). The sequenced
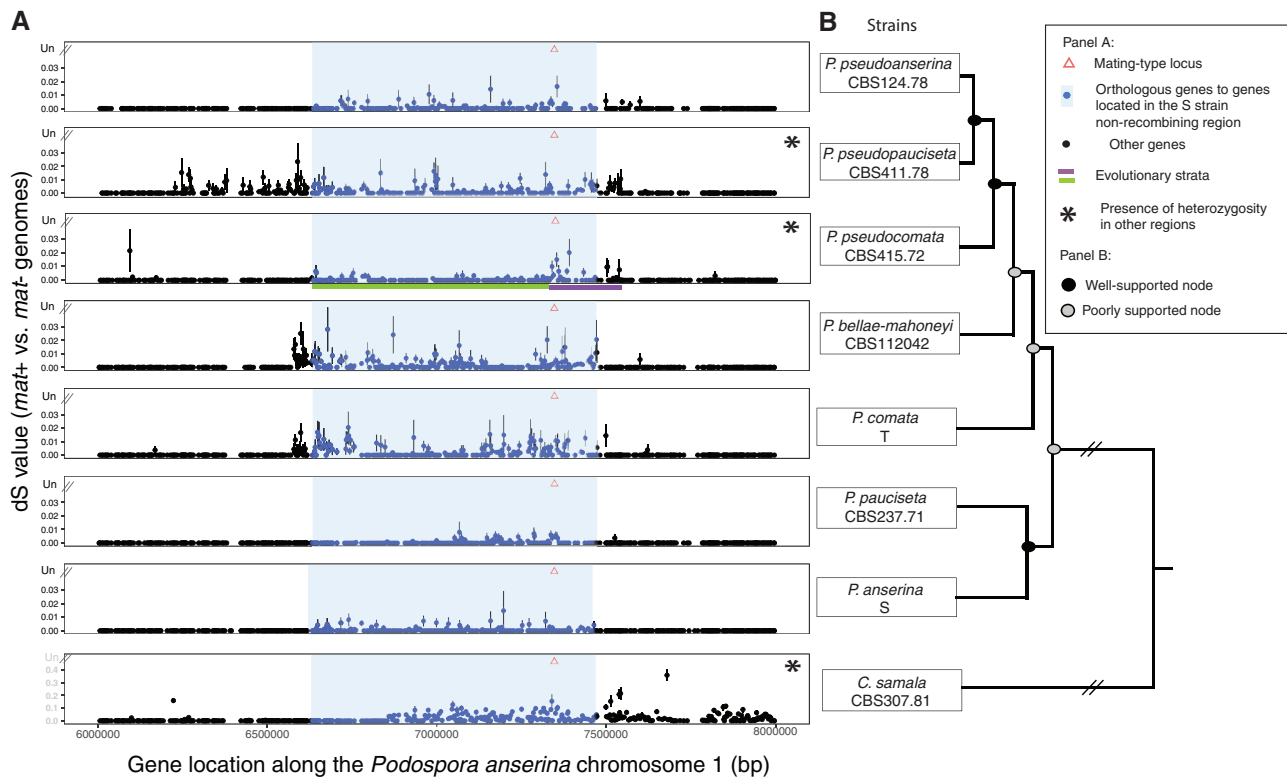
Fig. 1. Heterozygosity in the mating-type locus region in seven closely related pseudohomothallic *Podospora* species and the heterothallic *Cercophora samala* outgroup. (*A*) Per-gene synonymous divergence (d*S*) between the *mat*+ and *mat*− homokaryotic genomes of a heterokaryotic strain for each species, plotted against location on *Podospora anserina* chromosome 1. For each species, only nontransposable element genes with an ortholog in the *P. anserina* S+ genome were analyzed. Genes located in the nonrecombining region of the S+ genome, as inferred from segregation analyses performed by Grognet et al. (2014), are shown in blue. Dots indicate mean values and bars indicate the standard deviation of d*S* per gene. No d*S* values were computed for mating-type genes, due to the high sequence divergence between idiomorphs, indicated as unalignable (Un) and by empty red triangles. Different scales were used on the *y*-axis for the *Podospora* spp. and the *C. samala* strain, due to differences in d*S* ranges. High d*S* values indicate recombination suppression or outcrossing. Colored bars below the plots indicate putative evolutionary strata, where identified. Strains with other highly heterozygous regions within their genomes are indicated by an asterisk (see supplementary fig. S2, Supplementary Material online). (*B*) Cladogram showing the most likely evolutionary relationships between the seven pseudohomothallic *Podospora* species, with *C. samala* as an outgroup. Node support is based on the phylogenomic analyses performed by Ament-Velásquez (2020).

genomes of opposite mating types were isolated from heterokaryotic strains collected in natural populations, which underwent an unknown number of selfing events in the laboratory before sequencing. Patterns of heterozygosity can thus indicate the occurrence of outcrossing in nature, but not its frequency. The larger heterozygous regions around the mating-type locus than that in the *P. anserina* S strain (fig. 1) may, thus, be due to outcrossing events in the *P. pseudopauciseta* CBS411.78 and *P. pseudocomata* CBS415.72 strains. We also found high d*S* values in the heterothallic outgroup *C. samala* CBS307.81 strain around the mating-type locus, encompassing 104 additional orthologous genes (fig. 1; supplementary fig. S2*A*, Supplementary Material online), and for most of the orthologous genes on chromosomes 4 and 6, suggesting relatively recent outcrossing (supplementary fig. S2*B*, Supplementary Material online). Following an outcrossing event, one expects that circa 30 generations of selfing are required to obtain genome-wide homozygosity in *P. anserina* (supplementary note 1, Supplementary Material online). The homozygosity observed

along entire autosomes and in the PARs in these three strains constitutes a footprint of the multiple rounds of selfing events occurring in the laboratory before the isolation and sequencing of opposite mating types, and possibly also occurring in nature. Due to the heterozygosity resulting from outcrossing, it was not possible to determine the exact size of the region without recombination in these three lineages.

Variable Size of the Regions Lacking Recombination around the Mating-Type Locus in *P. anserina* and *P. comata*

We also investigated whether the size of the region displaying heterozygosity around the mating-type locus varied between strains within species. We studied the *mat*+ and *mat*− homokaryotic genomes of nine additional heterokaryotic strains of *P. anserina* and of one additional heterokaryotic strain of *P. comata*, using available high-quality genome assemblies and performing additional Illumina whole-genome sequencing (supplementary table S1 and fig. S1,

Supplementary Material online). In all nine additional *P. anserina* strains, dS was high around the mating-type locus, but the heterozygous region differed in size and precise location between strains (fig. 2; table 1). In three strains (10 b_P1_A1, Wa21 and Wa28), the heterozygous region was smaller than that in the S strain, ranging from 550 to 821 kb. This finding demonstrates that the genomic region lacking recombination differs not only between species, but also between strains within species, possibly due to the lability of the region lacking recombination and frequent changes in location.

For the other six strains (PSN240, Wa46, Wa53, Wa58, Wa63, and Wa87), the region of high dS values extended farther from the mating-type locus than that in the S strain. Remarkably, the heterozygous region in the Wa63 strain extended to genes located at ~565 kb away in the left flanking region and 316 kb away in the right flanking region of the region lacking recombination in the S strain. Four of these strains (PSN240, Wa46, Wa58, and Wa87) had completely homozygous autosomes (supplementary fig. S3, Supplementary Material online), indicating that the large heterozygous regions around the mating-type locus were due to a lack of recombination. The other two strains (Wa53 and Wa63) had clusters of heterozygosity in the PARs or autosomes (supplementary fig. S3 and table S2, Supplementary Material online). The large size of the heterozygous region around the mating-type locus in these two strains may, therefore, be due to relatively recent outcrossing events. We therefore performed ten generations of selfing, to assess the sizes of the nonrecombining regions (see below). In *P. comata*, the heterozygous region was larger in the T strain than in the Wa139 strain (supplementary fig. S4, Supplementary Material online; table 1). We found no clusters of heterozygosity in other genomic regions in either strain (supplementary figs. S2 and S4, Supplementary Material online), providing evidence for genuine variation in the size of the nonrecombining region also in *P. comata*.

Patterns of Evolutionary Strata

Despite the variability in size of the heterozygous regions around the mating-type locus between strains and species, we found no pattern suggestive of evolutionary strata in most of the strains studied. Only in *P. pseudocomata* CBS415.72, there seemed to be two different strata with different dS levels (fig. 1). We designed a method to systematically test whether evolutionary strata were present, by successively dividing the heterozygous region around the mating-type locus into two segments at every gene location and plotting differences of dS between segments for each segment limit (supplementary fig. S5, Supplementary Material online). In the presence of evolutionary strata, one expects a clear peak of differences in dS at the limit between two strata, as evolutionary strata are by definition segments with different differentiation levels. For most of the strains, we did not find any clear peak of dS differences (supplementary fig. S5, Supplementary Material online), as expected by visual inspection of the dS plots (figs. 1 and 2). Only in *P. pseudocomata* CBS415.72, we observed one clear peak of dS difference

(supplementary fig. S5, Supplementary Material online) and we divided the heterozygous region at this limit into green and purple regions (fig. 1 and supplementary fig. S5, Supplementary Material online). The dS level was in fact significantly higher in the purple region (mean dS = 0.00645) than in the green region (mean dS = 6.75e-05; multiple comparison Wilcoxon tests of dS differences: $P = 0.016$ between the purple and green regions, $P < 2e\text{-}16$ between recombining and nonrecombining regions). This pattern in *P. pseudocomata*, together with the high degree of homozygosity of its autosomes (except on chromosome 7; supplementary fig. S2, Supplementary Material online), strongly suggests the presence of two evolutionary strata, with the purple region lacking recombination for significantly longer than the green region. This finding further reinforces the view that the precise location of the genomic region without recombination changes over time, here extending in two successive steps.

Signatures of Recent Recombination Suppression or Frequent Gene Conversion Events

For the species for which multiple genome sequences were available—*P. anserina* and *P. comata*—divergence around the mating-type locus between the *mat+* and *mat−* homokaryotic genomes within heterokaryotic strains was significantly higher than that between recombining regions, that is, PARs and the autosomes, of two strains of the same species (pairwise multiple comparison Wilcoxon tests, *P*-values ranging from 7.3e-09 to 0.01062; supplementary fig. S6A and *B* and table S3B, S3C, S4B, and S4C, Supplementary Material online). This pattern reinforces the view that heterozygosity was due to a lack of recombination in these regions, and not just due to outcrossing: The dS increased around the mating-type locus following the independent accumulation of mutations in the opposite mating types, because of the lack of recombination. It does not simply reflect the within-species diversity. Nevertheless, the divergence between alleles around the mating-type locus within heterokaryotic strains was lower than that in recombining regions between species, indicating that recombination or gene conversion has occurred around the mating-type locus since the last speciation events (pairwise multiple comparison Wilcoxon tests, *P*-values <2e-16; fig. 3; supplementary tables S3A and S4A, Supplementary Material online), again supporting the view that the region without recombination is labile and/or incomplete across long evolutionary times.

The divergence between alleles around the mating-type locus within heterokaryotic strains differed significantly between several pairs of species (fig. 3; supplementary tables S3A and S4A, Supplementary Material online). The divergence between mating types was, for example, significantly higher within the *P. comata* T strain than in other *Podospora* species except the *P. bellae-mahoneyi* CBS112042 and *P. pseudopauciseta* CBS411.78 strains (pairwise multiple comparison Wilcoxon tests, *P*-values ranging from <2e-16 to 2.0e-06; fig. 3; supplementary tables S3A and S4A, Supplementary Material online). By contrast, we found no significant differences in within-strain dS levels around the mating-type locus

Table 1. Description of the Highly Heterozygous Region around the Mating-Type Locus in Seven Closely Related Pseudohomothallic *Podospora* Species.

Heterokaryotic Strain ID	Species	No. of Genes Orthologous to the *Podospora anserina* S Strain	No. of Additional Genes Compared with the *Podospora anserina* S Strain	Length in Long-Read Assembly (bp) (except for *)	Mean of dS Value for All Genes	Standard Deviation of dS Value for All Genes	Mean of dS Value for Genes with dS > 0	Standard Deviation of dS Value for Genes with dS > 0	No. of Genes with dS Value Equal to 0
CBS124.78	*Podospora pseudoanserina*	207	19	968,466	0.00101	0.00350	0.00486	0.00641	164
CBS411.78	*Podospora pseudopauciseta*	295	115	1,368,016	0.00216	0.00416	0.00541	0.00507	177
CBS415.72	*Podospora pseudocomata*	207	15	923,725	0.00102	0.00366	0.00501	0.00684	165
CBS112042	*Podospora bellae-mahoneyi*	231	20	914,193	0.00303	0.00529	0.00630	0.00615	120
T	*Podospora comata*	212	21	922,753	0.00307	0.00504	0.00674	0.00558	116
Wa139	*Podospora comata*	193	0	758,611	0.00304	0.00505	0.00683	0.00561	106
CBS237.71	*Podospora pauciseta*	151	0	691,375	0.00087	0.00354	0.00438	0.00698	121
S	*Podospora anserina*	229	0	838,139	0.00069	0.00180	0.00327	0.00265	169
Wa21	*Podospora anserina*	195	0	821,286	0.00076	0.00194	0.00302	0.00286	146
Wa28	*Podospora anserina*	177	0	706,611	0.00092	0.00238	0.00354	0.00356	131
Wa46	*Podospora anserina*	242	30	1,018,946	0.00085	0.00188	0.00315	0.00242	177
Wa53	*Podospora anserina*	258	44	1,030,818	0.00102	0.00216	0.00342	0.00272	181
Wa58	*Podospora anserina*	191	7	819,132	0.00079	0.00167	0.00284	0.00205	138
Wa63	*Podospora anserina*	423	209	1,749,834	0.00082	0.00207	0.00353	0.00299	325
Wa87	*Podospora anserina*	254	41	986,242	0.00079	0.00157	0.00287	0.00172	184
PSN240	*Podospora anserina*	223	28	841,584*	0.00075	0.00189	0.00324	0.00272	171
10b_P1_A1	*Podospora anserina*	155	0	550,662*	0.00087	0.00179	0.00315	0.00211	112

Note.—For each heterokaryotic strain, the following features are indicated: The total number of genes and the number of additional genes present that are orthologous to genes from the *P. anserina* S strain (229 genes were present in the nonrecombining region around the mating-type locus in the S strain, as reported by Grognet et al. 2014); the length of the region as inferred from orthologous gene location in the long-read assembly of one homokaryotic genome of each strain (except for the strains PSN240 and 10 b_P1_A1, for which no long-read assembly was available; the length of the region was based on the *P. anserina* S+ genome assembly for these two strains); the mean and standard deviation of dS values for all genes of the regions and for genes with a dS > 0 only; and the number of genes with a dS equal to 0.
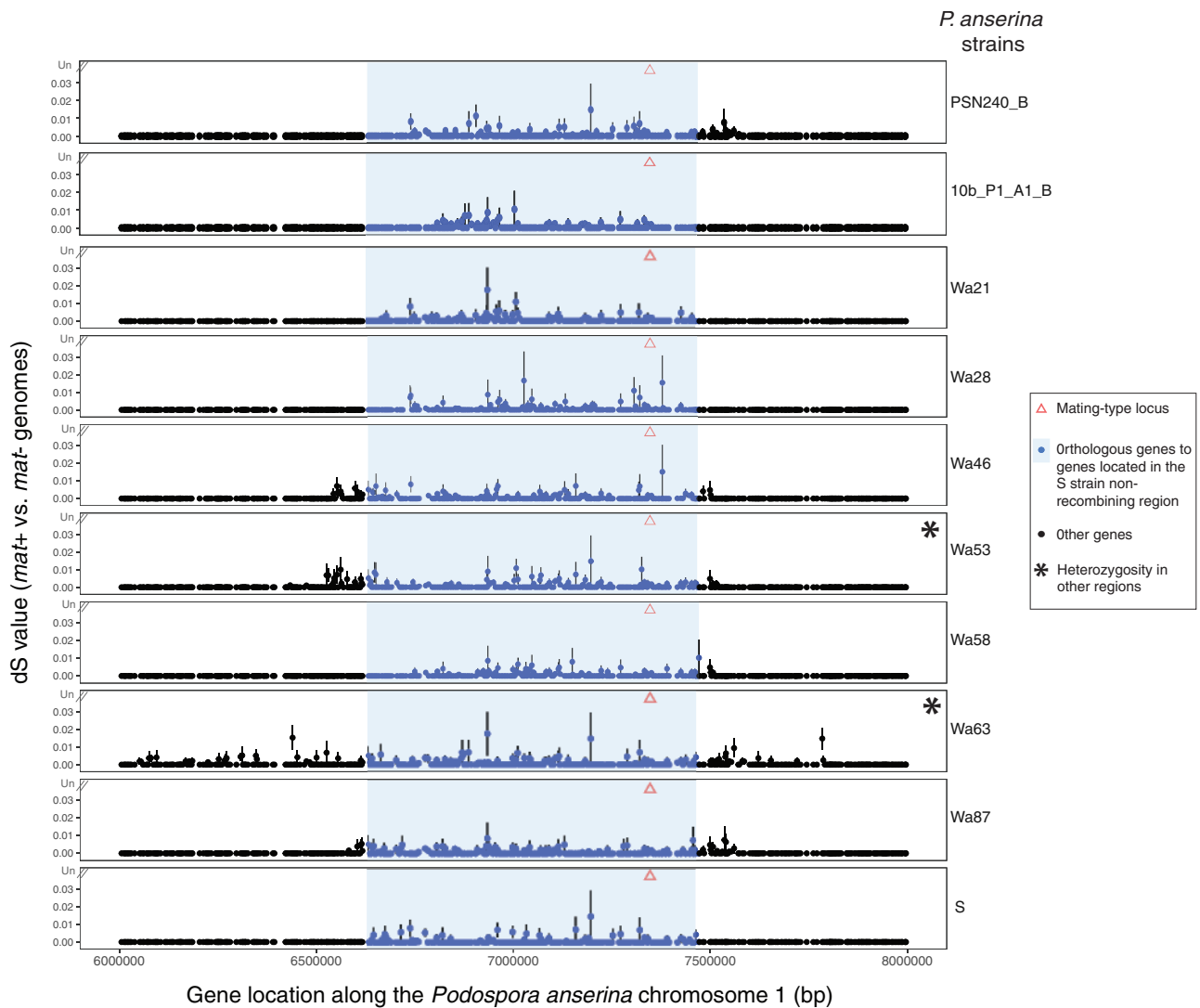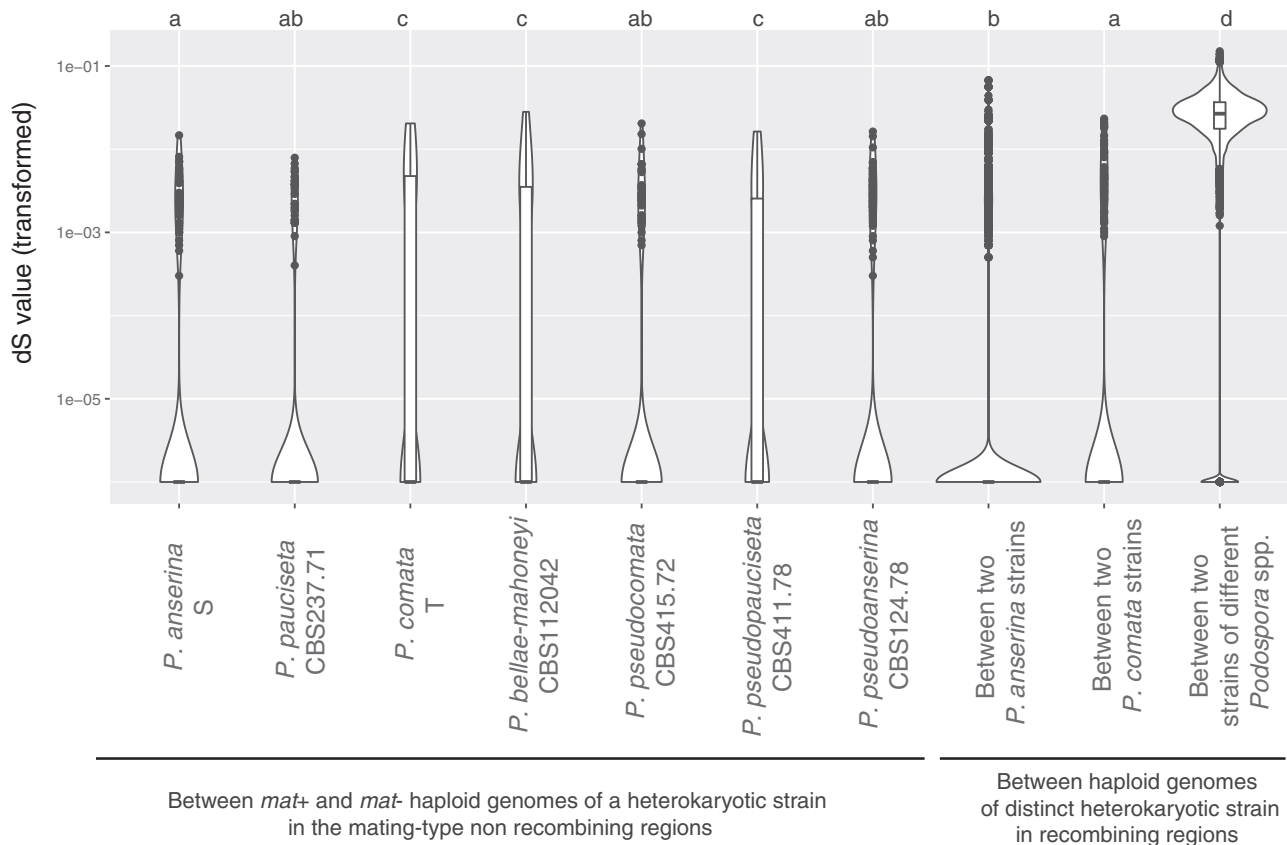
Fig. 2. Within-species variation in heterozygosity in the mating-type locus region of *Podospora anserina*. Per-gene synonymous divergence (dS) between the *mat+* and *mat−* homokaryotic genomes of the S strain and nine additional *P. anserina* heterokaryotic strains (PSN240, 10b_P1_A1, Wa87, Wa63, Wa58, Wa53, Wa46, Wa28, Wa21) plotted against location on *P. anserina* chromosome 1. Only nontransposable element orthologs common to the eight *P. anserina* strains studied were analyzed. Genes located in the nonrecombining region of the S genome, as described by Grognet et al. (2014) are shown in blue. Dots indicate mean values and bars indicate the standard deviation of dS per gene. No dS values were computed for mating-type genes due to high sequence divergence between idiomorphs, indicated as unalignable (Un) and with empty red triangles. High dS values indicate recombination suppression or outcrossing. Strains with other highly heterozygous regions are indicated by an asterisk (see supplementary fig. S3, Supplementary Material online).

among the seven *P. anserina* Wa strains or between the two *P. comata* strains (pairwise multiple comparison Wilcoxon tests, P-values ranging from 0.91 to 1; supplementary fig. S6A and B and table S3B, S3C, S4B, and S4C, Supplementary Material online). The species-specific dS levels around the mating-type locus suggest that recombination has been absent for significantly longer time periods in some species than in others and/or that mutation rates and effective population sizes differ.

The dS values around the mating-type locus of the *C. samala* CBS307.81 strain (mean dS value of 0.035) were about seven times higher than those in the seven *Podospora* pseudohomothallic species (mean value of 0.0049; supplementary fig. S6C, Supplementary Material online and note the different scales on fig. 1 and supplementary fig. S2 and

tables S3D and S4D, Supplementary Material online). Such very high dS values around the mating-type locus in the *C. samala* CBS307.81 strain may be due to ancient recombination suppression, but the similarly high dS values on some autosomes (supplementary fig. S2, Supplementary Material online) instead suggest a recent cross between two very distant strains followed by a few generations of selfing. The *C. samala* heterokaryotic strain sequenced may thus be of hybrid origin, resulting from a cross between different species, or between individuals from differentiated populations, but this latter hypothesis would mean that *C. samala* has a level of diversity ten times higher than that of *P. anserina*.

We studied the tree topologies for alleles present in the *mat+* and *mat−* genomes of the seven species in the *P. anserina* species complex, for genes in the mating-type

FIG. 3. Between-species comparison of heterozygosity levels in the nonrecombining region around the mating-type locus. The per-gene synonymous divergence (dS) between the *mat+* and *mat−* homokaryotic genomes of a heterokaryotic strain, in the nonrecombining region around the mating-type locus, is presented for each species on the left. We retained only genes with orthologs in the S strain nonrecombining region, as identified by Grognet et al. (2014), and filtered out transposable elements. For comparison, per-gene dS was computed between homokaryotic genomes of two strains belonging to the same species (*Podospora anserina* or *Podospora comata*) or to different species, for 300 randomly sampled genes on chromosomes 2–7 (i.e., autosomes). The *y*-axis was log10 transformed to enhance visualization. Before log10-transformation of the *y*-axis, 0.000001 was added to all dS values so that dS values equal to zero have a $1.e^{-6}$ value on the log10-transformed *y* axis. Different letters indicate significantly different mean values in pairwise multiple comparisons performed with Wilcoxon tests with Bonferroni correction (see supplementary table S3A, Supplementary Material online). The number of genes used for each gene sequence comparison and the mean dS values are presented in supplementary table S4A, Supplementary Material online.

locus region with nonzero dS values for at least two *Podospora* species. For all but seven genes, we found that alleles clustered by species rather than by mating type (fig. 4A), and with strong bootstrap support. However, the nodes separating species were poorly resolved and were not always congruent between genes and with the genome-wide species phylogeny, as expected given the rapid and recent divergence of *Podospora* species (Boucher et al. 2017; Ament-Velásquez 2020). The lack of evidence for transspecific polymorphism in the heterozygous region around the mating-type locus provided supports for the view that recombination occurred around the mating-type locus after the last speciation events in the *Podospora* complex or that gene conversion or occasional recombination restored species topology. Only seven genes, distributed across the region of high heterozygosity without genomic clustering, showed no clustering per species of the *mat+* and *mat−* alleles, for the *P. pseudoanserina*, *P. pseudopauciseta*, and *P. pseudocomata*

strains, which formed a monophyletic clade (supplementary fig. S7, Supplementary Material online). This unresolved clustering may reflect very recent divergence of these three species, especially as the alleles did not cluster by mating type either (supplementary fig. S7, Supplementary Material online).

The gene tree topologies for the eight *P. anserina* Wa strains showed that *mat+* and *mat−* associated alleles clustered by mating type (but for one strain) only for the *APN2* (*Pa_1_20580*) gene, the gene physically closest to the mating-type locus (fig. 4). The other genes did not cluster by mating type (fig. 4B), indicating recent recombination or gene conversion events. The difference in pattern between the *APN2* and other genes indicates an older recombination suppression event at the *APN2* gene, close to the mating-type gene, than at other genes farther away, potentially constituting an older evolutionary stratum. This putative DNA-(apurinic or apyrimidinic site) lyase 2 (*APN2*) gene is close to the mating-type locus in almost all Pezizomycotina fungi (Butler 2007),
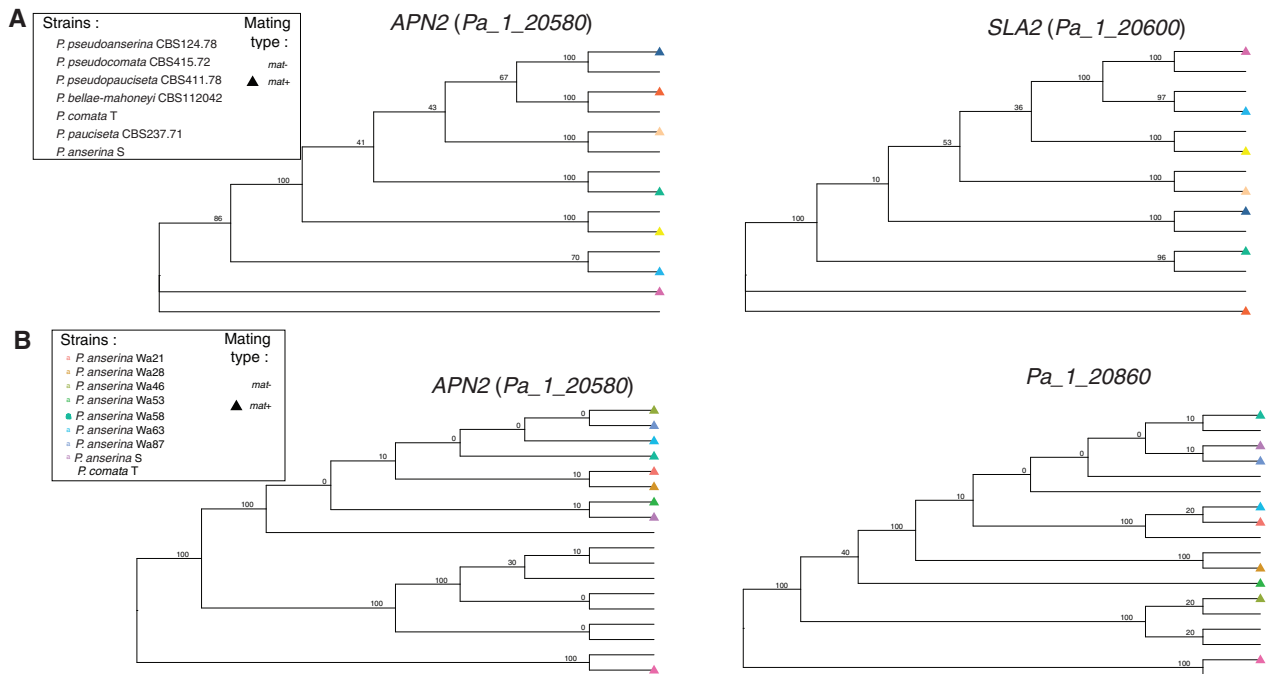
FIG. 4. Absence of transspecific polymorphism in the seven *Podospora* species studied (*A*) and allele clustering in the eight *Podospora anserina* strains (*B*) in the highly heterozygous region around the mating-type locus. Examples of single gene-based genealogies of alleles in *mat*+ and *mat*− genomes. The mating type and strain ID associated with alleles are indicated on cladograms by tip symbol and tip color, respectively. (*A*) Allele genealogies for the genes *APN2* (*Pa_1_20580*) and *SLA2* (*Pa_1_20600*), the genes physically closest on either side of the mating-type locus. Alleles cluster by species for the two genes. No outgroup was used for the analysis and the cladograms were not rooted. (*B*) Allele genealogies for the genes *APN2* and *Pa_1_20860*. Alleles of the *APN2* gene, which is physically close to the mating-type locus, cluster by mating type. Alleles of the *Pa_1_20860* gene, located further away from the mating-type locus, display poorly resolved clustering, regardless of mating type. The *Podospora comata* T strain was used as an outgroup for the analysis and the cladograms were rooted.

and must, indeed, have been closely linked to mating-type genes for a very long period. However, the *APN2* gene displays no transspecific polymorphism (fig. 4*A*), suggesting that recombination or gene conversion events likely occur, even if infrequently.

Additional features suggest recent recombination or gene conversion events in the region around the mating-type locus. Some of the genes within nonrecombining regions had very low dS levels (even dS values equal to zero), possibly due to gene conversion events (fig. 1; table 1). Linkage disequilibrium patterns (supplementary fig. S8*B*, Supplementary Material online) further supported the existence of regular recombination or gene conversion events in the region around the mating-type locus. We indeed found no evidence of strong linkage disequilibrium around the mating-type locus in *P. anserina* (supplementary fig. S8*B*, Supplementary Material online) despite the lack of recombination events observed in progenies (this study; Grognet et al. 2014).

Further population genetic diversity indices also supported the occurrence of occasional recombination or gene conversion events in the region around the mating-type locus (supplementary fig. S8*A*, Supplementary Material online). Per-site nucleotide diversity (π) was lower in the mating type nonrecombining region of each mating type ($\pi_{mat-\ homokaryons} =$ 0.000378; $\pi_{mat+\ homokaryons} =$ 0.000373) compared with autosomes ($\pi_{mat-\ homokaryons} =$ 0.000663; $\pi_{mat-+homokaryons} =$

0.000670) and PARs ($\pi_{mat-\ homokaryons} =$ 0.000483; $\pi_{mat-\ homokaryons} =$ 0.000482). Lower diversity is expected in nonrecombining mating-type chromosomes due to half population effective sizes, but the difference was not significant (supplementary fig. S8*A*, Supplementary Material online; pairwise comparisons using Wilcoxon rank sum test, $P = 1$). Relative divergence index (F_{ST}) between the *mat*+ and *mat*− homokaryons was null in the PARs and autosomes (mean $F_{ST} = 0$) and was significantly higher in the mating type nonrecombining region, but the differentiation remained low (mean $F_{ST} = 0.0471$; pairwise comparisons using Wilcoxon rank sum test $P < 2e$-16). No strong differences were found in absolute divergence index (d_{XY}) between the mating types in the nonrecombining region (mean $d_{XY} =$ 0.000384) compared with the PARs (mean $d_{XY} = 0.000419$; pairwise comparison Wilcoxon rank sum test $P = 0.049$) and autosomes (mean $d_{XY} = 0.000575$; pairwise comparison Wilcoxon rank sum test $P = 0.098$).

Little Evidence for Rearrangement or Degeneration in Nonrecombining Regions

Nonrecombining regions would be expected to degenerate due to less efficient selection, and, in particular, due to the accumulation of genomic rearrangements and/or transposable elements. Previous comparisons of long-read assemblies
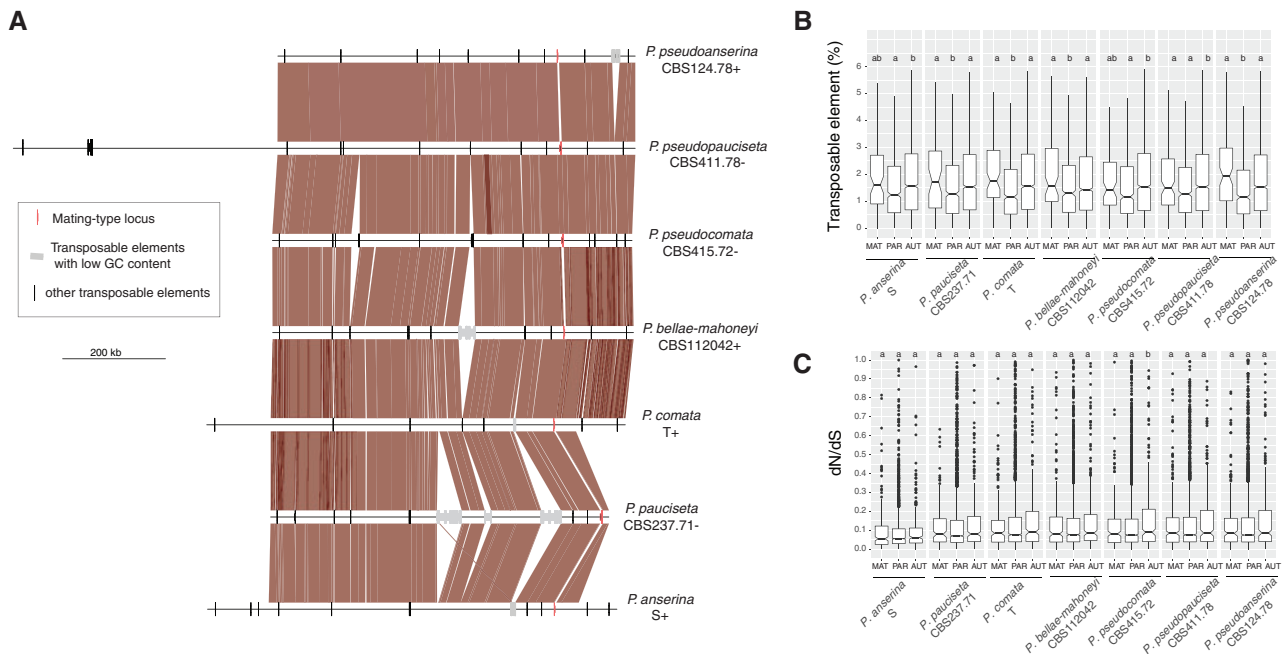
FIG. 5. Absence of signatures of sequence degeneration in the heterozygous region around the mating-type locus in the seven *Podospora* species studied. (A) Synteny between the genomes of the seven *Podospora* species studied, in the heterozygous regions around the mating-type locus, based on long-read genome assemblies. The location of the mating-type locus is shown in red, the islands of transposable elements with low GC content probably due to previous repeat-induced point mutation (RIP) events are shown in gray, and other transposable elements are shown in black. No inversions were detected. (B, C) Comparisons of genomic compartments between strains within species, and between species, for transposable element content (B) and dN/dS ratios relative to the *Cercophora samala* genome (C). We compared the following three genomic compartments: The heterozygous region around the mating-type locus (MAT), other regions on chromosome 1 (i.e., pseudoautosomal regions, PAR), and the autosomes (AUT), in the long read-based assembly of each species. Different letters indicate significantly different mean values according to pairwise multiple-comparison Wilcoxon tests (supplementary tables S6 and S7, Supplementary Material online). Transposable element content was calculated as the percentage of base pairs annotated as transposable elements in 10 kb nonoverlapping windows. Outliers were not plotted for (B). dN/dS ratios were computed between the *mat+* homokaryotic genomes of each *Podospora* species and the *C. samala mat+* genome, and only dN/dS values below 1 are plotted.

of a homokaryotic genome of each species suggested that synteny was conserved throughout the entire genome, including the mating-type region, both between species and between strains within species in the *P. anserina* complex (fig. 5A; Silar et al. 2019; Vogan et al. 2019; Ament-Velásquez 2020). Mapping against the S+ genome sequence revealed no evidence of large insertions/deletions, rearrangements or inversions in the mating-type locus region between homokaryotic genomes of opposite mating types in each heterokaryotic strain (supplementary table S5, Supplementary Material online). We also found no evidence of rearrangements in the mating-type locus region between the two homokaryotic genomes of the heterothallic *C. samala* CBS307.81 strain based on mapping against the S+ genome sequence (supplementary table S5, Supplementary Material online). Long-read sequencing of both mating-type chromosomes would however be needed to confirm the lack of rearrangements.

The PARs often display particular patterns, different from those of autosomes and nonrecombining regions on sex chromosomes or mating-type chromosomes (Otto et al. 2011; Corcoran et al. 2016; Hartmann et al. 2020). Here, we identified the PARs as regions of zero dS values on either side of the mating-type locus, as a dS value of zero indicates

homozygosity and, thus, recent selfing. In most strains, we found that there were significantly more transposable elements in the heterozygous regions around the mating-type locus than in the PARs (e.g., the recombining regions on chromosome 1), consistent with the suppression of recombination around the mating-type locus (fig. 5B; supplementary table S6, Supplementary Material online). The transposable element content of autosomes appeared to be intermediate between the PARs and the nonrecombining region, but was not significantly different from that of the nonrecombining region (fig. 5B; supplementary table S6, Supplementary Material online). All but two species had clusters of GC-poor transposable elements (TEs) in the heterozygous region around the mating-type locus (fig. 5A), as previously described in the S strain (Grognet et al. 2014). However, these clusters of GC-poor TEs probably originated from independent TE insertion events, as they are constituted by different types of transposable elements (data not shown). These GC-poor TE clusters were absent from *P. pseudocomata* and *P. pseudopauciseta*.

Nonrecombining regions are also expected to accumulate mildly deleterious nonsynonymous substitutions, which are typically investigated by calculating dN/dS (i.e., the ratio of nonsynonymous substitutions to synonymous substitutions)
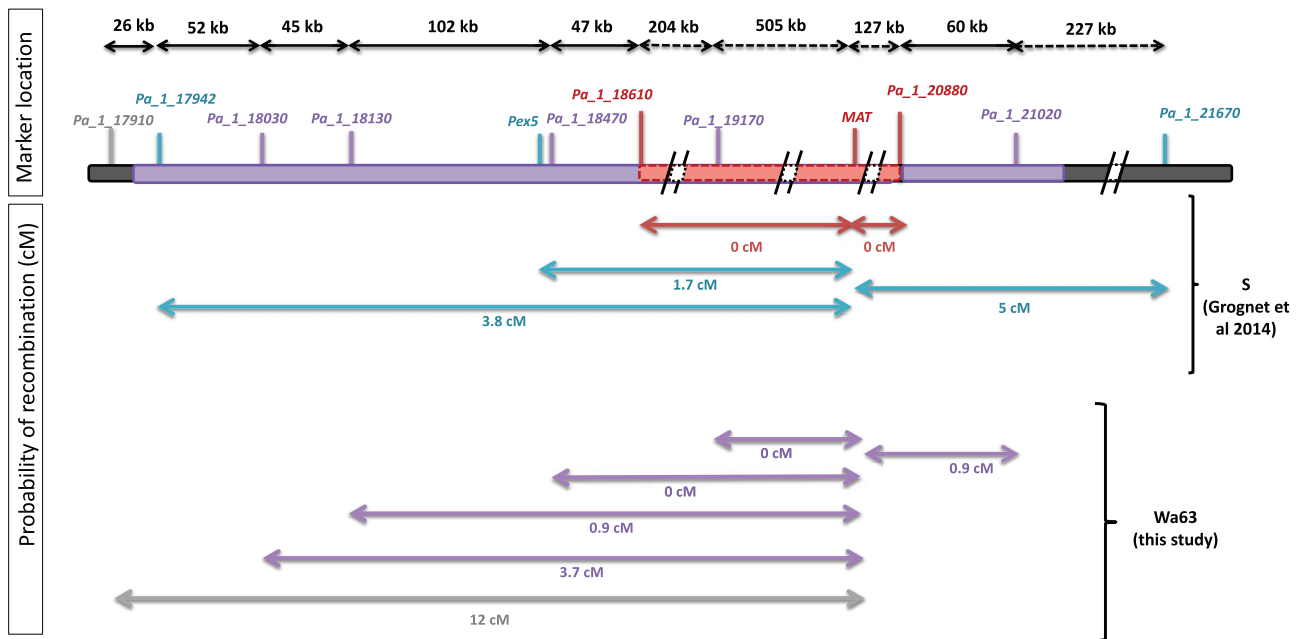
Fig. 6. Probability of recombination in centimorgans (cM) between markers and the mating-type locus in the *Podospora anserina* heterokaryotic strain Wa63 after several rounds of experimental selfing measured in 109 of the offspring compared with the *P. anserina* S strain. Relative marker locations compared with the mating-type locus and physical distance (kb) between adjacent markers are shown. Dashed lines indicate regions not represented to scale. The location of the mating-type locus is indicated as "MAT" in red, and the region shown to lack recombination in the *P. anserina* S strain is indicated as a light red rectangle, with markers located at the margin of the nonrecombining region (*Pa_1_18610* and *Pa_1_20880*) also indicated in red. Frequencies of recombination for the S strain, as inferred with the markers used by Grognet et al. (2014), are indicated in blue. The region with elevated dS in the Wa63 strain before experiment selfing but with zero dS in the S strain is indicated as a purple rectangle. Frequencies of recombination for the Wa63 strain, estimated from the genotypes at the five SNP markers according to the PCR-KASP assay for 84 of the F10 or F11 offspring and whole-genome sequencing data for 25 of the F10 offspring are shown in light purple. Frequencies of recombination with the *Pa_1_17910* marker, additional marker estimated from the genotypes obtained by the whole-genome sequencing of 25 of the F10 offspring, are shown in gray.

for normalization according to the rate of neutral substitutions. We did not find, in any of the *Podospora* species, significantly higher dN/dS in the heterozygous regions around the mating-type locus than in other genomic regions (i.e., PARs and autosomes; fig. 5C; supplementary table S7, Supplementary Material online). Thus, overall, we found no evidence of degeneration in the mating-type nonrecombining regions relative to the autosomes, in terms of rearrangements, transposable element content and nonsynonymous substitutions, consistent with the inference that recombination has not been absent from these regions for very long or that the suppression of recombination is not complete.

Dynamics of Heterozygous Region Size following Multiple Generations of Selfing

The larger size of the heterozygous region in some strains may be due to recent outcrossing events, especially those with clusters of heterozygosity in autosomes. We therefore performed experimental selfing crosses to determine the limit of the nonrecombining region for three *P. anserina* strains (Wa63, Wa53, and Wa87). These three strains had large heterozygous regions around the mating-type locus (fig. 2) and the strains Wa63 and Wa53 had heterozygosity clusters in autosomes; dS between mating types in the autosomal heterozygous regions was significantly lower than in the mating-type nonrecombining region and the dS levels in autosomes

were similar to those between two *P. anserina* strains, supporting their origin from an outcrossing event (supplementary fig. S6D, Supplementary Material online). We performed 10 or 11 successive selfing crosses, in each generation crossing monokaryotic spores from different asci for each strain as intertetrad mating leads more rapidly to homozygosity than intratetrad mating (supplementary note 1, Supplementary Material online). We genotyped ~50 F10 or F11 progeny of each experimental selfing line by whole-genome sequencing (supplementary table S8, Supplementary Material online) or in kompetitive allele specific PCR (KASP-PCR) assays (supplementary tables S9–S12, Supplementary Material online). For the Wa63 cross, we found that the heterozygous region around the mating-type locus was smaller in the F10 and F11 progenies than in the parental strain, but it remained larger than that in the *P. anserina* S strain. The nonrecombining region in the Wa63 strain was, therefore, also larger than that in the S strain (fig. 6; supplementary fig. S9 and table S10, Supplementary Material online). For the Wa53 and Wa87 selfing progenies, the heterozygous region around the mating-type locus in the F10 progeny was of the same size as that in the parental strains as we did not identify any recombinant in the F10 progeny (supplementary figs. S10, S11 and tables S11, S12, Supplementary Material online). Conversely, as expected after multiple generations of selfing, the clusters of heterozygosity

in the other genomic regions of the Wa63 and Wa53 parental strains (supplementary fig. S3, Supplementary Material online) were not maintained. These results suggest that the region of suppressed recombination around the mating-type locus is also genuinely larger in these strains than in the *P. anserina* S strain.

We also crossed the Wa87 and Wa63 with each other, as they had heterozygous regions of different sizes around the mating-type locus, for investigation of the size of the heterozygous region in the offspring (supplementary fig. S12, Supplementary Material online). After outcrosses in both directions regarding the *mat+* and *mat–* mating types (Wa87+ × Wa63– and Wa87– × Wa63+), followed each by ten experimental intertetrad selfing crosses, the heterozygous region in the F10 offspring was different from that in the parental strains. The right-hand limit of the heterozygous region in the offspring corresponded to that of the parent with the smallest heterozygous region or to that of the S strain, whereas the left-hand limit appeared to be intermediate between that of the two parents (supplementary fig. S12*B*, *C*, Supplementary Material online).

Outcrossing in Pseudohomothallic Species

The clusters of heterozygosity in regions other than the mating-type locus for two *P. anserina* strains (Wa53 and Wa63; supplementary fig. S3 and table S2, Supplementary Material online), the CBS415.72 *P. pseudocomata* and the CBS411.78 *P. pseudopauciseta* strains (supplementary fig. S2, Supplementary Material online) suggest that outcrossing does occur in nature in *P. anserina* and its closely related pseudohomothallic species, contrary to the widespread assumptions that have long been held for pseudohomothallic fungi. We cannot infer the precise frequency of selfing versus outcrossing events in natural populations of *P. anserina* and its closely related pseudohomothallic species, because most strains underwent an unknown number of selfing events in the laboratory before genome sequencing. The PSN240, 10b_P1_A1, and Wa139 strains did not undergo selfing in the laboratory after collection from natural conditions and before genome sequencing. These three strains had no clusters of heterozygosity in regions other than the mating-type locus, indicating high rates of selfing in natural conditions; >30 generations of selfing are needed for a strain to become completely homozygous in these species (see details in supplementary note S1, Supplementary Material online).

Discussion

The evolution of recombination suppression has been studied in detail for sex chromosomes, but much less for fungal mating-type chromosomes. Recombination had previously been experimentally shown to be genuinely suppressed in the *P. anserina* S strain (Grognet et al. 2014). We found here evidence for a lack of recombination around the mating-type locus in all the six other pseudohomothallic *Podospora* species studied. Indeed, we found heterozygosity in the regions flanking the mating-type locus in several *Podospora* species, despite the homozygosity of all autosomes, thereby demonstrating a lack of recombination in

the mating-type region. The regions lacking recombination were of different sizes in different *P. anserina* strains, with some smaller and others larger than that in the reference S strain, as shown by the variable size of the heterozygous region around the mating-type locus, even in strains with completely homozygous autosomes. In *P. anserina* strains with heterozygous regions on autosomes, the size of the heterozygous region around the mating-type locus remained different from that in other strains after ten generations of selfing.

Not only did the regions lacking recombination differ in size between strains and species, but we also found that dS levels were species-specific and that there was no transspecific polymorphism. Overall, this suggests that the latest recombination events occurred later than the speciation events. One hypothesis to explain the lack of transspecific polymorphism is that recombination ceased completely in each species independently after the last speciation events. Such parallel evolution indicates that evolution can be repeatable under a given selective pressure, in this case, probably the need to compact nuclei of opposite mating types into single dispersing spores. This convergence of genomic organization under a shared selective pressure can be observed at a larger phylogenetic scale, as other fungi have evolved recombination suppression on mating-type chromosomes to favor automixis; this is the case, for example, in the anther-smut *Microbotryum* fungi and *N. tetrasperma* (Menkis et al. 2008; Branco et al. 2018), and also in multiple independent events in the anther-smut *Microbotryum* fungi and in human pathogen *Cryptococcus* fungi (Branco et al. 2018; Sun et al. 2019). The pattern suggestive of recombination suppression in *C. samala* may indicate that recombination suppression around mating-type loci is not restricted to automictic fungi, as previously suggested (Hartmann et al. 2020). However, the very high levels of divergence between mating types and on some autosomes suggest that the sequenced heterokaryotic *C. samala* strain originated from a recent outcrossing event, possibly between different species with different breeding systems. Analyses of additional *C. samala* strains are required to determine whether recombination suppression really does occur around the mating-type locus in this species.

An alternative explanation for the lack of transspecific polymorphism is that infrequent gene conversion events occur within the nonrecombining region. This hypothesis is supported by the existence of genes with no divergence between alleles in the middle of the heterozygous region and gene conversion has been reported in the nonrecombining regions of other fungal mating-type chromosomes (Menkis et al. 2010; Sun et al. 2012; Idnurm et al. 2015). Another, nonexclusive explanation for the overall pattern is that recombination is not entirely suppressed around the mating-type locus, but is instead inhibited in a region that can vary between strains and species. Across evolutionary time periods, the region lacking recombination would move around the mating-type locus, such that recombination still occurs, but highly infrequently. This hypothesis is also consistent with the lack of genomic rearrangements between mating types, the lack of linkage disequilibrium within populations and the

lack of genomic degeneration or TE accumulation around the mating-type locus relative to autosomes. Actually, very rare events of recombination could be detected in the *P. anserina* S strain near the mating-type locus by using a mutant screening (Silar and Picard 1994). If the lack of recombination has been selected to enforce the existence of a single crossing-over event around the mating-type locus through interference, the precise location of the region lacking recombination may not need to be fixed or precise. This situation is different from that in other fungi in which recombination suppression has been extensively studied around mating-type loci. In *Microbotryum* and *Cryptococcus* basidiomycetes, recombination suppression has evolved as a means of permanently linking the mating-type loci to each other and/or to the centromere, and in *N. tetrasperma*, it has evolved to link the mating-type locus to the centromere (Menkis et al. 2008; Branco et al. 2017; Carpentier et al. 2019). In these cases, complete suppression of recombination is beneficial, whereas in *Podospora*, interference could occur even if the precise region lacking recombination varies. Our findings support the emerging view that recombination suppression might be labile and incomplete on sex-related chromosomes, as recently reported in several sex chromosome systems, from frogs to guppies (Grossen et al. 2012; Guerrero et al. 2012; Darolti et al. 2020).

We found patterns suggestive of evolutionary strata in *P. pseudocomata* (i.e., distinct regions with different levels of divergence between mating types). Such a pattern of evolutionary strata indicates that the most recent recombination events occurred longer ago in the region immediately around the mating-type locus, with more recent recombination events occurring in more distant regions, consistent with a stepwise extension of the region lacking recombination. This is consistent with the interpretation of a region lacking recombination being labile in *Podospora* fungi, with possibly extension of this region over time. This finding provides additional support for the view that evolutionary strata do exist in fungal mating-type chromosomes (Hartmann et al. 2020). Here, in *Podospora*, the observed pattern of evolutionary strata may be best explained by recurrent changes in the exact limits of the recombination-free region, which does not need to be at a particular place to enforce a single crossing-over event between the mating-type locus and the centromere. It will be interesting to sequence further genomes to check whether the pattern is general within this species and to check that the pattern is not affected by outcrossing events.

Proximal mechanisms preventing recombination do not appear to involve rearrangements in any of the strains analyzed here, although long-read sequencing would be needed to be certain. Recombination suppression without inversion has been reported in other fungi (Sun et al. 2017; Branco et al. 2018). The low-GC and TE-rich region in the nonrecombining region appeared as a good candidate for being involved in the recombination suppression but functional deletions have shown that it was not the case (Grognet et al. 2014). Another possible mechanism of recombination suppression is DNA methylation and/or chromatin compaction (Kent

et al. 2017; Furman et al. 2020). In *P. anserina*, DNA methylation has not been detected during vegetative growth (Grognet et al. 2019) but other developmental stages and other methylation marks remain to be investigated.

Despite *P. anserina* being considered a model organism (Silar 2013), few studies have been performed on natural populations and the nature of the actual mating system (selfing vs. outcrossing) of this species remains unknown. Pseudohomothallism facilitates automixis, but does not prevent outcrossing (Billiard et al. 2012). Our analysis of heterokaryotic strains revealed that outcrossing does occur, as a previous study suggested it was possible based on in vitro estimates of rates of vegetative and sexual compatibility among natural isolates (van der Gaag 2005). Other automictic fungi have also been found to outcross at low frequency, including the *Microbotryum* anther-smut fungi (Giraud et al. 2005; Vercken et al. 2010; Abbate et al. 2018).

Conclusion

In conclusion, our study adds to what is known of fungal mating-type chromosome evolution and its relationship to mating systems, and makes a more general contribution to our understanding of the evolution of recombination suppression on sex-related chromosomes. This is one of the first studies to document such high levels of polymorphism in the size and precise location of nonrecombining regions. This variability may be related to 1) the proximal mechanisms preventing recombination (i.e., not involving inversions), and 2) the evolutionary cause of the lack of recombination (i.e., the promotion of a single crossing-over event between the mating-type locus and the centromere rather than the linking of mating-type genes to each other or to centromeres). Further studies on a wider range of fungi are required to determine whether recombination suppression around mating-type genes is a general pattern, whether it is always associated with automixis, when evolutionary strata evolve and, more generally, why and how they evolve (Hartmann et al. 2020).

Materials and Methods

Fungal Strains, Sequencing Data, and Genome Assemblies

We studied seven pseudohomothallic *Podospora* species, using publicly available data for homokaryotic strains. We used both high-quality genome assemblies obtained by Sanger sequencing or long-read technology sequencing (P6/C4 Pacific Biosciences SMRT or MinION Oxford Nanopore) and genome sequencing data based on Illumina short reads (supplementary table S1, Supplementary Material online). Genome assemblies and Illumina sequencing data for homokaryotic strains (either *mat+* or *mat−*) were used for comparative genomics and read-mapping approaches, respectively. For *P. anserina*, we used available homokaryotic genome assemblies and Illumina sequencing data for the following strains: S (Espagne et al. 2008), Wa63, Wa87, Wa53, Wa58, Wa28, Wa21, and Wa46 (Vogan et al. 2019). For *P. comata*, we used homokaryotic genome assemblies and

Illumina sequencing data for the T strain (Silar et al. 2019), also sometimes referred to as the T_D strain (Vogan et al. 2019) and the Wa139 strain (Vogan et al. 2020). For *P. pauciseta*, we used homokaryotic genome assembly and Illumina sequencing data for the CBS237.71 strain (Vogan et al. 2019). For *P. pseudoanserina*, *P. pseudopauciseta*, *P. pseudocomata*, and *C. bellae-mahoneyi*, we used homokaryotic genome assemblies and Illumina sequencing data for the strains CBS124.78, CBS411.78, CBS415.72, and CBS112042 (Ament-Velásquez 2020). We also studied the homokaryotic Illumina sequencing whole genome data for the CBS307.81 strain of *C. samala*, a heterothallic species (Ament-Velásquez 2020).

In accordance with a previous study (Vogan et al. 2019), we refer to homokaryotic genomes using the ID of the corresponding heterokaryotic strain followed by the mating-type allele. For example, the assembled genome of the *mat+* homokaryon derived from strain Wa63 is referred to as Wa63+. We used the version of the S+ assembly available from the Joint Genome Institute MycoCosm website (https://mycocosm.jgi.doe.gov/mycocosm/home, last accessed November 15, 2018) as "Podan2" (Espagne et al. 2008) and annotations improved by Vogan et al. (2019) available from the GitHub repository https://github.com/johannessonlab/SpokBlockPaper (last accessed June 1, 2020). We downloaded assemblies of the Wa and CBS237.71– strains from the Dryad Digital Repository, 10.5061/dryad.vm1192g (Vogan et al. 2019). We downloaded the assembly of the Wa139– strain from the GitHub repository https://github.com/johannesson-lab/SpokBlockPaper (last accessed January 15, 2021) (Vogan et al. 2020) and assemblies of CBS124.78+, CBS411.78–, CBS415.72–, and CBS112042+ from GenBank under BioProject ID PRJNA685103 (Ament-Velásquez 2020). The T+ assembly was downloaded from the European Nucleotide Archive under BioProject ID PRJEB24890 and accession number GCA_900290415.1. We downloaded raw Illumina data from public NCBI Short Read Archive database using the BioProject IDs and BioSample numbers presented in supplementary table S1, Supplementary Material online.

For each homokaryotic strain for which a high-quality genome assembly was available, we sequenced the genome of the homokaryotic strain of the same heterokaryon but with the opposite mating type, except for the S– strain for which Illumina data were already available (Grognet et al. 2014). For the other strains, we generated whole-genome sequencing data with Illumina NextSeq500 or HiSeq 3000 150 bp paired-end sequencing technology, at a mean coverage of 20×, as previously described (Ament-Velásquez 2020; supplementary table S1, Supplementary Material online). Briefly, following their reception, all CBS *Podospora* pseudohomothallic strains were subjected to one selfing event in the laboratory before genome sequencing, to isolate homokaryons of opposite mating types and for strain rejuvenation. When the *P. anserina* Wa strains were first isolated in Wageningen, each strain was selfed to produce a stock of spores for the collection. The strains were often grown from these spores, and homokaryons were isolated after a second selfing event. In some cases, there was another selfing event to produce further stocks of spores. No records were available for the

number of selfing events per strain or indicating whether or not the homokaryons were tetrad siblings, so we were unable to use homozygosity levels to infer the rate of outcrossing in nature. We know only for the *P. comata* strain Wa139 that there was no selfing event before sequencing, because the two homokaryons (tetrad sisters) were isolated directly from horse dung (collected in Uiterwaarden Wolfswaard, the Netherlands in 2016). We also studied two additional *P. anserina* strains, PSN240 (collected from cow dung in the Cantabrian Mountains, Spain in 2017) and 10 b_P1_A1 (collected from horse dung in Orsay, France, in 2014; supplementary table S1, Supplementary Material online). As for *P. comata* strain Wa139, there was no selfing event before the sequencing of the *mat+* and *mat–* homokaryotic genomes of these strains. The two sequenced *C. samala* homokaryons were isolated from eight-spore asci in perithecia formed by the F0 CBS307.81 strain incubated on {M0 + miscanthus} medium, the F0 CBS307.81 strain thus likely being originally a heterokaryon. We grew the CBS307.81+ homokaryon. We grew each homokaryotic strain on cellophane sheets in Petri dishes of M2 medium for three to four days. We scraped the mycelium off the plates, to obtain ~100–200 mg of mycelium per strain, which was stored in a 1.5 ml Eppendorf tube at −80 °C for at least 24 h. The mycelium was then freeze-dried for ~20 h. We extracted DNA from the freeze-dried material with the commercial Nucleospin Soil kit from Macherey Nagel. Library preparation and genome sequencing were outsourced to the sequencing core facility of I2BC, Université Paris-Saclay (Centre de Recherche de Gif—http://www.i2bc.paris-saclay.fr/, last accessed June 1, 2020). Given the large numbers of read pairs with read mates mapping onto different chromosomes during the mapping procedure (~20% of mapping read pairs; analyses not shown), we used each newly sequenced paired-end sequencing run as two independent single-end sequencing runs for read mapping approaches and de novo assembly (see details below). All newly generated Illumina genomes were deposited in GenBank under BioProject IDs PRJNA523441 and PRJEB693675 (see supplementary table S1, Supplementary Material online for BioSample numbers).

Processing of Whole-Genome Sequencing Data

We first trimmed the Illumina raw reads with Cutadapt v2.1 software (Martin 2011). We used the software in a paired-end mode for the data already published, using the following options: –q 10,10; –minimumlength 50; –a AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC; -A AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGTAGAT CTCGGTGGTCGCCGTATT. We used the software in single-end mode for the newly sequenced genomes, with the following options: –q 10,10; –minimumlength 50; –a AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC –a GGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGG GGGGGGGGGGGGGGGG.

Illumina sequencing data were further processed with both a mapping-based approach and a de novo assembly-based approach. For the mapping-based approach, we mapped Illumina trimmed reads against the mat+ S genome assembly with the

short-read aligner bowtie2 v2.3.4.1 (Langmead et al. 2009), with the following software options: –very-sensitive-local –phred33 -X 1000. We removed PCR duplicates with the MarkDuplicates tool of Picard tools version 1.88 (http://broadinstitute.github.io/picard, last accessed October 30, 2019). We locally realigned the mapped reads, with the RealignerTargetCreator and IndelRealigner tools of the Genome Analysis Toolkit (GATK), to improve alignment accuracy in indel regions. The percentage of mapped reads ranged from 93.2% to 98.5% in the seven pseudohomothallic *Podospora* species and was 41.7% and 39.9% for the *C. samala mat+* and *mat–* genomes, respectively. We performed SNP calling with the HaplotypeCaller tool of GATK version 3.7 (McKenna et al. 2010). After running HaplotypeCaller in the haploid mode on each genome individually, we performed joint variant calls with GenotypeGVCFs on a merged gvcf variant file. SNP calls were filtered for quality with VariantFiltration, in accordance with GATK Good Practice for the hard filtering of variants (QUAL < 250; QD < 2; MQ < 30.0; −10.5 > MQRankSum > 10.5; −5 > ReadPosRankSum > 5; FS > 60; SOR > 3). We performed additional filtering steps with vcftools, to retain only biallelic SNPs with a high genotyping rate (>90%). In total, we obtained ∼2,230,000 biallelic SNPs in analyses including the seven pseudohomothallic *Podospora* species and the heterothallic *C. samala* strain, and ∼1,710,000 biallelic SNPs in analyses excluding *C. samala*. The variant call format files are available on request. We used the SNPs to reconstruct pseudogenome sequences, replacing the nucleotide bases of the S+ genome with the corresponding SNPs where necessary, and retrieving gene coding sequences based on S+ gene models (Espagne et al. 2008), with a customized script (available on request). We looked for deletions, inversions and duplications in the 17 genomes sequenced with Illumina technology, by mapping them against the S+ genome with the method implemented in Delly software, which uses paired ends, split reads and read depth to detect structural variation (Rausch et al. 2012). We ran the "germline SV calling" pipeline, retaining only homozygous variants, which were then filtered further with the following parameters: Number of supporting paired-end reads (PE) > 7 and mapping quality score (MAPQ) > 20. In parallel, all haploid genomes newly sequenced with Illumina in this study were assembled de novo with SPAdes v3.13.0 (Bankevich et al. 2012) and the following default options: –careful -k 21,29,37,45,53,61,79,87 –trusted-contigs. Assembly quality was assessed with Quast v 5.0.0rc1 (Gurevich et al. 2013). The resulting new assemblies consisted of between 300 and 1,056 contigs longer than 1,000 bp, with a mean N50 of ∼150 kb.

Comparative Genomics

For comparative genomics analyses, we performed orthologous group reconstruction between the protein sequences of all homokaryotic genome assemblies with OrthoFinder software (Emms and Kelly 2019). For the S+ and T+ genome assemblies, we used published gene and repeat prediction models (Espagne et al. 2008; Silar et al. 2019; Vogan et al. 2019). For all other genome assemblies (both long read-based assemblies and de novo Illumina-based assemblies), we used the same gene prediction pipeline as previously

described (Vogan et al. 2020), adding, as evidence, the protein sequences of the *mat+* and *mat–* loci of the S strain (Espagne et al. 2008; Grognet et al. 2014). We filtered protein sequences based on OrthoFinder output, to restrict our investigations to one-to-one orthologs. We retrieved gene coding sequences for each protein with the gffread program (available from https://ccb.jhu.edu/software/stringtie/gff.shtml#gffread, last accessed April 15, 2020). We studied transposable element content, using the custom-built library "PodoTE-1.00" (available from https://github.com/johannessonlab/SpokBlockPaper/blob/master/Annotation/data/, last accessed April 15, 2020) and described in a previous study (Vogan et al. 2020) to annotate repeated elements in all species with RepeatMasker 4.0.9 (Smit et al. 2013). A per-window analysis of transposable element content was performed with a customized script (available on request).

Analyses of Heterozygosity and Allelic Divergence

We studied the heterozygosity of heterokaryotic strains, by calculating dS values per gene between the *mat+* and *mat–* homokaryotic genomes of the same heterokaryotic strain, as previously described (Branco et al. 2017, 2018). Briefly, we performed pairwise sequence comparisons and aligned orthologous gene sequences of the *mat+* and *mat–* homokaryotic genomes, using the codon-based approach implemented in translatorX, with default parameters (Abascal et al. 2010). We used the nucleotide alignment as input for the yn00 program in the PAML package, to calculate dS values (Yang and Nielsen 2000; Yang 2007). We used the same procedure to study divergence between genomes of the same species or different species. We calculated dN/dS ratios, by computing dS and dN values between allele of the *mat+* genome of each *Podospora* species and the allele of the *mat+* genome of the *C. samala* strain. Only genes orthologous to genes of the S+ *P. anserina* strain were used for these analyses. For all *Podospora* species, we used gene sequences obtained by pseudogenome reconstruction and checked the results with gene models obtained from de novo assemblies (data not shown). For *C. samala*, due to low rates of mapping onto the S+ genome (50%), we used only gene models obtained from de novo assemblies.

We compared dS values between *mat+* and *mat–* homokaryotic genomes of heterokaryotic strains in the nonrecombining regions around the mating-type loci to dS values between different strains in recombining regions. For the comparisons of the nonrecombining regions around the mating-type loci, we only kept genes with orthologs to genes of the S strain nonrecombining region defined in Grognet et al. (2014). For the comparisons in recombining regions, we randomly sampled 300 genes on chromosomes 2–7 (i.e., recombining regions; supplementary table S13, Supplementary Material online). Comparisons between different strains was performed using either the *mat+* or *mat–* homokaryotic genome of each strain. We performed 28 comparisons among *P. anserina* strains, one comparison between the two *P. comata* strains and 21 comparisons among the seven pseudohomothallic *Podospora* species (supplementary

table S14, Supplementary Material online). The significance of differences was assessed using pairwise multiple comparisons Wilcoxon tests and we applied the Bonferroni correction of *P*-values for multiple testing.

Gene Genealogies

We constructed gene genealogies, by generating codon-based alignments of orthologous gene coding sequences with translatorX (Abascal et al. 2010). We used the RAxML v. 8.2.11 program (Stamatakis 2014) to obtain maximum likelihood gene trees and obtained support for nodes by a bootstrapping procedure with 100 replicates. We plotted gene trees with cladograms, using the ggtree v.2.0.1 R package (Yu et al. 2017).

Genetic Diversity and Linkage Disequilibrium Analyses

To study divergence among strains, we built networks with the neighbornet method implemented in SplitsTree v4.16.2 (Huson 1998; Huson and Bryant 2006). We removed SNPs with missing data and used only autosomal SNPs. We computed linkage disequilibrium decay among the seven *P. anserina* Wa strains (i.e., 14 homokaryotic genomes) using the option –hap-r2 of the vcftools version 0.1.15 program (Danecek et al. 2011). We used SNPs without missing data and filtered out minor allele frequencies <0.2 and SNPs overlapping transposable elements. We drew a linkage disequilibrium heatmap with the LDheatmap v.1.0-4 R package (Shin et al. 2006). We computed the following genetic diversity statistics in nonoverlapping 10 kb windows: Nucleotide diversity per site (π), relative divergence index (F_{ST}) and the absolute divergence index (d_{XY}) between the *mat+* and *mat−* *P. anserina* Wa genomes using the PopGenome v.2.7.5 R package (Pfeifer et al. 2014). We excluded windows with fewer than three SNPs.

Statistical Analyses and Plots

All statistical analyses were performed in the R software v4.0. Plots were performed using the ggplot2 v3.2.1 R package (Wickham 2009).

Selfing Experiment

We performed ten generations of experimental self crosses for the heterokaryons Wa63, Wa53, and Wa87 (referred to as the F10 progeny), with an additional generation of experimental selfing for the heterokaryon Wa63 (referred to as the F11 progeny). Briefly, homokaryons of opposite mating types (*mat+/mat−*) of each heterokaryon were grown on M2 medium. For each generation of selfing, compatible homokaryotic ascospores from two different asci were used for crosses. The use of ascospores from different asci increased the probability of a return to homozygosity. We also performed two experimental outcrosses between homokaryons of the Wa63 and Wa87 strains. After crossing the Wa87+ and Wa63− homokaryons and the Wa87− and Wa63+ homokaryons, we performed ten generations of experimental selfing, as described above. At the last generation of experimental selfing (F10 or F11), ∼50 homokaryotic F10 and F11 offspring were grown for each cross (referred to as the F10Wa63, F11Wa63, F10Wa53,

and F10Wa87 offspring for self crosses of the Wa63, Wa53, and Wa87 heterokaryotic strains, respectively, and F10Wa87+×Wa63−, F10Wa87−×Wa63+ for the other two crosses). The mating types of the isolated F10 and F11 offspring were checked either by crossing them with S+ and S− tester strains or by polymerase chain reaction (PCR). Primers were designed to bind to the coding regions of the *FPR1* and *smr1* genes, which are specific to the *mat+* and *mat−* mating types, respectively, with Primer 3.0 (Rozen and Skaletsky 2000). For FPR1, we used 5′-GAAACTGATGTCGGCTCACC-3′ and 3′ TCGCGTATCATAGGAGGCAG 5′ as forward and reverse primers, respectively. For smr1, we used 5′-CTTTGATCCGCACTGCTCTC-3′ and 3′ GGTGTTTCCCTGGGTAGTCA 5′ as forward and reverse primers, respectively. PCR was performed in a volume of 25 µl containing 5–10 ng genomic DNA, 0.4 mM each of forward and backward primers, 0.025 mM dNTP, 0.75 U *Taq* polymerase (DreamTaq, Thermo Fisher, Inc.), in PCR buffer. PCR products were amplified over 30 cycles. The resulting amplicons were subjected to electrophoresis in 1.5% agarose gels.

We genotyped F10 or F11 offspring of the *mat+* and *mat−* mating types to determine whether recombination events had occurred around the mating-type locus. We first performed Illumina genome sequencing on a small number of strains, to obtain genotype information for both the mating-type locus region and the other chromosomes. In total, we sequenced with Illumina 25 genomes from F10Wa63 homokaryotic offspring (15 *mat+*/10 *mat−*), six genomes from F10Wa53 offspring (3 *mat+*/3 *mat−*), six genomes from F10Wa87 offspring (4 *mat+*/2 *mat−*), six genomes from F10Wa87+×Wa63− offspring (2 *mat+*/4 *mat−*), and six genomes from F10Wa87−×Wa63+ offspring (2 *mat+*/4 *mat−*) (supplementary table S8, Supplementary Material online). We mapped reads against the S+ genome assembly (Espagne et al. 2008) and performed SNP calling as described in the "Processing of whole-genome sequencing data" section. We retrieved information on genotype and allele frequency at SNPs segregating between the parental strains and located within coding sequences, using vcftools v0.1.15 (Danecek et al. 2011) and the VariantsToTable tool of GATK version 3.7 (McKenna et al. 2010).

We used the KASP (Kompetitive Allele-Specific) PCR assay (He et al. 2014) to obtain genotypic information for SNPs surrounding the mating-type locus region in a larger number of strains for the F10Wa63, F11Wa63, F10Wa53, and F10Wa87 crosses. We genotyped five SNPs segregating between the parental strains Wa63+ and Wa63− in 24 further of the F10Wa63 offspring (11 *mat+*/13 *mat−*) and 60 of the F11Wa63 offspring (32 *mat+*/28 *mat−*), two SNPs segregating between the parental strains Wa87+ and Wa87− in 38 further of the F10Wa87 offspring (17 *mat+*/21 *mat−*) and one SNP segregating between the parental strains Wa53+ and Wa53− in further 38 of the F10Wa53 offspring (19 *mat+*/19 *mat−*) with KASP-PCR technology (supplementary tables S10–S12, Supplementary Material online). SNPs were located in the regions flanking the mating-type nonrecombining region (supplementary figs. S9A, 10A, and 11A and table S9, Supplementary Material online). Primers

designed with BatchPrimer3 (You et al. 2008) and bought from Eurofins. We used the KASP Master Mix (LGC Biosearch Technologies) kit in accordance with the manufacturer's instructions. Briefly, for each separate sample, we set up 10 μl volume reactions containing 5 μl of diluted DNA (1/10), 5 μl of Master Mix KASP, and 0.1 μl of the primer mix. We used a qPCR program on a Biorad CFX96 thermocycler (supplementary table S15, Supplementary Material online). We read SNP alleles with Biorad CFX Manager software.

Supplementary Material

Supplementary data are available at *Molecular Biology and Evolution* online.

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Author Contributions

T.G. and P.S. conceptualized the study and acquired funding. T.G. supervised the study. S.L.A.V., A.A.V., and H.J. provided genome assemblies. F.E.H., S.L.P., M.B., A.S., V.G., P.G., and F.M. contributed to experiments. F.E.H. analyzed genomes. F.E.H. and P.S. analyzed progenies. F.E.H. and T.G. wrote the original draft. All authors edited the manuscript.

Data Availability

The data underlying this article are available either in the public NCBI Short Read Archive and GenBank databases under BioProject IDs PRJNA523441, PRJEB24890, PRJNA685103, PRJEB693675, from the Dryad Digital Repository 10.5061/dryad.vm1192g (last accessed January 15, 2021) or from the GitHub repository https://github.com/johannessonlab/SpokBlockPaper (last accessed January 15, 2021).

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