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# Convergent recombination cessation between mating-type genes and centromeres in selfing anther-smut fungi

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**Key words:** young sex chromosomes, selfing mating system, automixis, centromere, fungi, recombination suppression, bipolar, tetrapolar, convergence, breeding system transition, central fusion automixis, supergenes, *Microbotryum saponariae*, *Microbotryum lagerheimii*

# Abstract

The degree of selfing has major impacts on adaptability and is often controlled by molecular mechanisms determining mating compatibility. Changes in compatibility systems are therefore important evolutionary events but their underlying genomic mechanisms are often poorly understood. Fungi display frequent shifts in compatibility systems and their small genomes facilitate elucidation of the mechanisms involved. In particular, linkage between the pre- and post-mating compatibility loci has evolved repeatedly, increasing the odds of gamete compatibility under selfing. Here, we studied the mating-type chromosomes of two anther-smut fungi with unlinked mating-type loci despite a selfing mating system. Segregation analyses and comparisons of high-quality genome assemblies revealed that these two species displayed linkage between mating-type loci and their respective centromeres. This arrangement renders the same improved odds of gamete compatibility as direct linkage of the two mating-type loci under the automictic mating (intra-tetrad selfing) of anther-smut fungi. Recombination cessation was found associated with a large inversion in only one of the four linkage events. The lack of trans-specific polymorphism at genes located in non-recombining regions and linkage date estimates indicated that the events of recombination cessation occurred independently in the two sister species. Our study shows that natural selection can repeatedly lead to similar genomic patterns and phenotypes, and that different evolutionary paths can lead to distinct yet equally beneficial responses to selection. Our study further highlights that automixis and gene linkage to centromeres have important genetic and evolutionary consequences, while being poorly recognized despite being present in a broad range of taxa.

# Introduction

Mating systems reflect the degree of selfing/outcrossing in natural populations and impact gene flow, the accumulation of deleterious alleles and adaptability (Lande and Schemske 1985; Igic et al. 2008; Charlesworth and Charlesworth 1987; Charlesworth et al. 1990; Charlesworth 2002; Hereford 2010; Lande 2015). Outcrossing can promote gene flow and therefore the rapid spread of beneficial alleles as well as the purge of deleterious alleles, while selfing is often associated with reproductive assurance and can help maintain favorable combinations of alleles at different loci. There is a wide diversity of mating systems in nature that strongly impact the evolution of organisms. Automixis with mating among products of a given meiosis that separated in the first meiosis division ("fusion of non-sister second division products" ; Lewis and John 1963), for example, is a little-known form of self-fertilization (Mogie 1986); such automixis is often called central fusion in animals, the term "fusion" referring to the union of gametes and the term "central" referring to the placement of the fusing gametes in an ordered tetrad (Suomalainen 1950; Goudie and Oldroyd 2014). This kind of automixis maintains heterozygosity at all loci for which there has been no recombination with the centromere (Engelstädter 2017; Zakharov 2005; Hood and Antonovics 2000; Lenormand et al. 2016; Hood and Antonovics 2004). This effect can extend over large portions of the genome when there is low levels of crossing-over (Hood and Antonovics 2000, 2004). Automixis with central fusion can thus maintain long-term heterozygosity, which can lead to the sheltering deleterious alleles or may be beneficial in cases of advantageous overdominance (i.e., heterozygote advantage; Engelstädter 2017). Automixis and its genetic and evolutionary consequences are poorly studied despite being relatively frequent (Mogie 1986), across a variety of taxa such as in fungi (Hood and Antonovics 2000; Zakharov 2005; Grognet et al. 2014; Menkis et al. 2008), plants (Asker 1980; Schön et al. 2009; Walker 1985;

Antonius and Nybom 1995; Cruden and Lloyd 1995), reptiles (Watts et al. 2006; Booth et al. 2010; Booth and Schuett 2015), fishes (Chapman et al. 2007; Dudgeon et al. 2017; Feldheim et al. 2017), birds (Schut et al. 2008), crustaceans (Nougué et al. 2015), nematodes (Van der Beek et al. 1998) and insects (Normark 2003; Suomalainen et al. 1976; Oldroyd et al. 2008).

Evolutionary transitions between mating systems are known to be relatively frequent (Goldberg and Igić 2012; Goldberg et al. 2010; Nieuwenhuis et al. 2013; Chantha et al. 2013; Hanschen et al. 2018). Changes in the genetic determination of gamete production or compatibility often underlie transitions in mating systems, such as the evolution of a self-incompatibility system. For example, in many species mating can only occur between males and females, which enforces outcrossing, and sexes are often determined by sex chromosomes (Beukeboom and Perrin 2014). In angiosperms, mating can also be restricted by a self-incompatibility locus, which promotes outcrossing in hermaphroditic species by preventing mating between genotypes carrying identical alleles (Vekemans et al. 2014). In most fungi, gamete compatibility is controlled at the haploid stage and only cells carrying different alleles at the mating-type loci can successfully mate (Billiard et al. 2011, 2012).

Fungi provide excellent eukaryotic models for studying the genomic changes involved in gamete compatibility transition, as they display highly diverse and labile mate-recognition systems (Nieuwenhuis et al. 2013; Billiard et al. 2012, 2011), as well as relatively small and compact genomes that allow for high-quality genome assemblies (Badouin et al. 2015; Gladieux et al. 2014; Branco et al. 2017; Faino et al. 2015; Sonnenberg et al. 2016; Sun et al. 2017a; Branco et al. 2018; Sun et al. 2017b). In basidiomycete fungi (e.g., rusts, smuts and mushrooms), mating type is most often controlled by two loci: (i) the PR locus, determining gamete fusion compatibility with a pheromone receptor and neighbouring pheromone genes, and (ii) the HD locus, determining compatibility for post-mating development with two homeodomain genes (Coelho et al. 2017). To successfully mate and produce offspring, two gametes must carry different alleles at both loci. In most basidiomycetes, the PR and HD loci segregate independently (Raper 1966; Nieuwenhuis et al. 2013). Multiple independent events of linkage of the two mating-type loci have been documented in several fungal species (Branco et al. 2017; Bakkeren and Kronstad 1994; Nieuwenhuis et al. 2013; Sun et al. 2017b). Such control of gamete compatibility inherited as a single locus is advantageous under selfing as it increases the odds of gamete compatibility among the gametes of a given diploid individual (Fig. 1; Coelho et al. 2017).

The plant-castrating anther-smut fungi belonging to the highly-selfing basidiomycete genus *Microbotryum* are particularly good systems for studying the genomic changes underlying shifts in gamete compatibility systems. Before the radiation of this genus, recombination suppression extended around each of the PR and HD loci (Branco et al. 2017). Several *Microbotryum* species underwent independent transitions to complete linkage between the mating-type loci through various chromosomal rearrangements that brought the HD and PR loci onto the same chromosome (Branco et al. 2017, 2018). In some of these species, the cessation of recombination subsequently expanded far beyond the mating-type loci in several successive steps to include the majority of the mating type chromosomes (Branco et al. 2017, 2018). Recombining pseudo-autosomal regions (PARs) remained at both edges of the mating-type chromosomes in many lineages (Branco et al. 2018).

The majority of studied anther-smut fungi undergo selfing by automixis (Hood and Antonovics 2000; Giraud et al. 2008; Vercken et al. 2010; Bueker et al. 2016; Gladieux et al. 2011) and have linked mating-type loci (Branco et al. 2018). Here we studied two closely related species, *Microbotryum lagerheimii* and *M. saponariae*, that have retained unlinked PR and HD mating-type loci located on different chromosomes (Fig.1; Hood et al. 2015), despite selfing mating systems (Fortuna et al. 2016; Abbate et al. 2018; Fortuna et al. 2018). However, *M. saponariae* displays the PR and HD mating-type loci completely linked to centromere of their respective chromosomes, which induces central fusion automixis and ensures the same odds of compatibility under selfing by automixis as would linkage between the mating-type loci (Fig. 1; Hood et al. 2015). While the mating-type loci are also known to be located on different chromosomes in *M. lagerheimii* (Branco et al. 2017), it is unclear whether the HD and PR loci are linked to the centromeres. In case they are linked to centromeres and given that *M. lagerheimii* and *M. saponariae* are sister species in available phylogenies (Fig. 2), recombination cessation with the centromeres could potentially predate their speciation event. An alternative hypothesis would be independent linkage events, with convergence for complete centromere linkage occurring in the two species after their divergence. In this study, we used segregation analyses and high-quality genome assemblies to investigate: 1) whether HD and PR loci are linked to the centromeres in *M. lagerheimii*, 2) whether linkage predates speciation between *M. lagerheimii* and *M. saponariae* or constitutes independent events, and 3) whether the PR and HD loci became linked to centromeres at similar dates in each species.

## Results

### Linkage of mating-type loci to centromeres in *M. saponariae* and *M. lagerheimii*

To test whether recombination was suppressed between the mating-type loci and their respective centromere in *M. lagerheimii*, we analysed PR and HD mating-type loci segregation within ordered linear tetrads using allele-specific PCR markers for each mating-type locus. When there is complete centromere linkage, alleles at both mating types always segregate at the first meiotic division, leading to the ordered linear *Microbotryum* tetrad with cells derived from opposite poles of meiosis I carrying alternate alleles at both the PR and HD loci (Fig. 1; Hood et al. 2015; Hood and Antonovics 2000). Conversely, when there is no centromere linkage of the mating type loci, mating-type alleles segregating at the second meiotic division results in only half the tetrads carrying alternate alleles at both loci in the opposite cells of the ordered linear tetrad (Figure 1). We found evidence supporting centromere linkage of mating-type loci in *M. lagerheimii*, with isolated meiotic products derived from opposite poles of meiosis I showing alternate alleles at both the PR and HD loci in all of the 78 meioses analysed. Given the number of tetrads analysed, the 95% confidence interval for the occurrence of recombination between at least one mating-type locus and its centromere was 0-5%. This indicates that the *M. lagerheimii* PR and HD loci are completely or nearly completely linked to their respective centromere, as in its sister species *M. saponariae* (Hood et al. 2015).

We compared the sequences of the mating-type chromosomes to investigate whether inversions could have contributed to linkage between mating-type loci and their centromeres. While the alternate HD mating-type chromosomes were collinear within both *M. saponariae* and *M. lagerheimii* (Figs. 3a and c), we observed a 51.4 kbp inversion between HD and the centromere in the *M. saponariae* lineage compared to the ancestral state, shared by *M. intermedium* and *M. lagerheimii* (Supplemental Figs. S2a and S3a). In both *M. saponariae* and *M. lagerheimii* the HD locus was located close to the centromere (distant by 138 kbp in *M. saponariae* and by 162 kbp in *M. lagerheimii*; Figs. 3a and 3c). In *M. lagerheimii* the alternate PR chromosomes ($a_1$ and $a_2$) also showed nearly complete collinearity (Fig. 3b), with only rearrangements around the PR locus, as typical in *Microbotryum* due to very old recombination suppression in this region (Branco et al. 2017). In contrast, *M. saponariae* displayed a large pericentric inversion distinguishing the alternate PR chromosomes. This inversion involved 593 kbp in the $a_1$ and 701 kbp in the $a_2$ PR mating-type chromosomes, representing 50% and 52% of $a_1$ and $a_2$ PR chromosome lengths (Fig. 3d). The two edges of the inversion were very close the centromere and the PR locus (Fig. 3d; inversion boundaries were distant by 35 kbp from centromeres and by 0 kbp from the edge of the PR-proximate region with ancient recombination suppression). The inversion appeared derived in the $a_2$ *M. saponariae* PR chromosome, as the *M. saponariae* $a_1$ PR chromosome was highly collinear to the *M. lagerheimii* $a_1$ and $a_2$ PR chromosomes (Supplemental Fig. S1). No further rearrangements were present within the large inversion beyond those located in the PR-proximal region (Fig. 3d). A small additional inversion was observed towards the PAR in the short arm of the *M. saponariae* $a_2$ PR mating-type chromosome (green region in Fig. 3d, involving 11 kbp and 20 kbp on the $a_1$ and $a_2$ PR mating-type chromosomes). This inversion may correspond to an additional step extending further recombination cessation towards the PAR.

Synonymous divergence ($d_S$) between alleles associated to the alternative mating types at the genes located between the PR-proximal region and the centromere in *M. lagerheimii* and *M. saponariae* provided further evidence for complete centromere linkage of the PR mating-type loci. For all genes linked to a mating-type locus, the same allele remains associated with the same mating-type, so that alleles associated to the alternate mating types accumulate independent mutations, showing increasing divergence ($d_S$) with time since the complete recombination cessation. To recover the history of recombination cessation, we plotted allelic divergence ($d_S$) along the ancestral gene order, as subsequent rearrangements may blur historical steps (Branco et al. 2017). We used the mating-type chromosomes of an outgroup species with independently segregating mating types (*M. intermedium*) as a proxy for the ancestral gene order; however, using the *M. lagerheimii* gene order gave similar conclusions given the few rearrangements observed (Supplemental Figs. S2 and S3; Branco et al. 2017). We observed very high levels of synonymous divergence around the PR and HD mating-type loci both in *M. lagerheimii* and *M. saponariae* (purple and blue genomic regions, respectively, Fig. 4). This result was expected given ancient recombination cessation proximal to each of the PR and HD mating-type loci (Branco et al. 2017). The non-zero $d_S$ values between the PR-proximal region and the centromere in *M. saponariae* and *M. lagerheimii* supported complete linkage to the centromere. In highly selfing organisms such as anther-smut fungi, homozygosity is high at almost all genes (*i.e.* $d_S$=0 between alleles on autosomes in a diploid individual; Supplemental Fig. S4) except in regions linked to mating-types (Branco et al. 2018). The lower $d_S$ values closer to the centromere than closer to the purple region, in both *M. saponariae* and *M. lagerheimii* (Figs. 4a and c), suggest stepwise extension of recombination cessation farther from the PR locus and to eventually reach the centromere. The synonymous divergence between the HD-proximal blue region and its centromere in both species was almost zero, although some genes exhibited non-zero $d_S$ value (Figs. 4b and d).

We found increased transposable element content in the HD and PR chromosomes in *M. saponariae* and *M. lagerheimii* compared to their autosomes. There were differences within each species between $a_1$ and $a_2$ mating-type chromosomes (Supplemental Fig. S5). Such transposable element accumulation and differences between homologous chromosomes further supported complete recombination cessation.

## Absence of trans-specific polymorphism between centromeres and the HD- and PR-proximal regions in *M. saponariae* and *M. lagerheimii*

We used genealogies of genes located between centromeres and the HD- and PR-proximal blue and purple regions, respectively, to assess whether linkage of mating-type loci to centromeres in *M. saponariae* and *M. lagerheimii* derived from a single event predating their speciation or from independent events in each lineage. If recombination cessation predates speciation, the alleles associated to the alternative mating-types will cluster by mating type rather than by species (which is called trans-specific polymorphism), as the alleles will have been linked to mating types since before the speciation. In contrast, if linkage is more recent than speciation, alleles will cluster by species as recombination will have broken any allelic association with mating-type within species after speciation. None of the orthologous groups corresponding to genes located between the PR- or the HD-proximal purple and blue regions and their centromeres displayed trans-specific polymorphism shared by *M. saponariae* and *M. lagerheimii*. We could use nine genes in the HD mating-type chromosome (Supplemental Fig. S6a) and ten genes in the PR mating-type chromosomes (Supplemental Fig. S6b), for which both alleles were available in all species with available genomes (the genes are indicated by red arrows on Fig. 4). These findings indicate independent events of complete mating-type-loci-centromere linkage in *M. saponariae* and *M. lagerheimii*.

We found further support for recombination suppression occurring after *M. saponariae* and *M. lagerheimii* speciation by dating the differentiation between alleles associated with $a_1$ versus $a_2$ mating types in gene genealogies. We computed a phylogenetic tree using the concatenated alignments of the nine and ten genes with both alleles available in all genomes and located between the HD-proximal region and the centromere, or the PR-proximal region and the centromere, respectively. We calibrated the tree nodes using the speciation date between *M. lychnidis-dioicae* and M. *silenes-dioicae*, previously estimated at 420 ky (Gladieux et al. 2011). Although these estimates are not robust absolute dates, they are useful to obtain relative dates of speciation and chromosome evolution events. Recombination cessation between the PR-proximal purple region and the centromere was younger in *M. saponariae* and *M. lagerheimii* (95% confidence interval 171 – 302 and 80 – 158 ky, respectively, Fig.5a) than their speciation event (95% confidence interval 2,997 – 4,386 ky, Fig. 5b). The date of recombination cessation between the HD-proximal blue region and the centromere was even younger in both species (95% confidence interval 0.1 - 18 and 3 - 26 ky, respectively, Fig.5a).

## Discussion

Here, we document convergent evolution of increased odds of gamete compatibility under automixis by independent linkage events of mating-type loci and centromeres in two closely related fungal species. Such linkage represents further convergence in gamete compatibility patterns with other congeneric lineages, that were previously shown to have achieved similar gamete compatibility odds through multiple independent direct linkage events between PR and HD mating-type loci (Branco et al. 2018). Linkage of the two mating-type loci, one to each other or to their centromere, are equally beneficial under automixis in terms of gamete compatibility odds (Fig. 1; Hood et al. 2015). We found here that the two mating-type loci in *M. lagerheimii*, while located on separate chromosomes, are completely linked to their respective centromeres, as in *M. saponariae*. Furthermore, we showed that in these sister species of anther-smut fungi such linkage occurred through independent recombination cessation events. Convergence of mating-type loci and centromere linkage seems to have also occurred at much larger phylogenetic scale within fungi. *Cryptococcus amylolentus*, a distant *Microbotryum* fungal relative, also displays both HD and PR genes linked to different centromeres (Sun et al. 2017a).  Our study thus shows that natural selection can lead repeatedly to similar genomic changes but also to distinct and equally beneficial solutions under a shared evolutionary pressure. These findings contribute to our understanding of evolution and the degree to which it is repeatable.

Segregation analyses showed that the HD and PR loci are linked to their respective centromere in *M. lagerheimii*, as previously shown in its sister species *M. saponariae* (Hood et al. 2015). While the inclusion of a finite number of analysed meiotic tetrads leaves the possibility that linkage to centromeres is only nearly complete, our genomic results support complete linkage in the PR chromosome. We found substantial differentiation between alleles associated to alternative mating-types at genes between the PR-proximal region and its centromere in both species, as well as a large inversion in *M. saponariae*. Additional evidence of complete recombination cessation is that the HD chromosome in *M. saponariae* and the PR chromosomes in both species are size dimorphic, with size co-segregating with mating type alleles (Hood et al. 2015). Chromosome size dimorphism is likely due to the differential transposable element amounts we found between alternate HD and PR chromosomes in each species.

The absence of trans-specific polymorphism and the more recent inferred linkage dates compared to the speciation event between *M. saponariae* and *M. lagerheimii* strongly support that recombination cessation was independent in the two species. The recent origins of complete linkage between the two mating-type loci and their centromeres in both species are corroborated by the low synonymous divergence values between $a_1$ and $a_2$-associated alleles and the lack of extensive rearrangements in the regions without recombination between the mating-type locus proximal regions and the centromeres.

For linkage of mating-type loci to centromeres to be beneficial under selfing by automixis, both HD and PR mating-type genes need to be linked to their respective centromere (Zakharov 1986, 2005). However, for both *M. lagerheimii* and *M. saponariae*, the PR linkage to centromere evolved long before the HD-centromere linkage. The PR locus-centromere linkage alone provides no advantage concerning gamete compatibility odds when mating occurs within a tetrad; however the PR-centromere linkage may have been generated in several steps extending the recombination cessation region beyond mating-type genes, as previously described (Branco et al. 2017). This would be consistent with the apparent heterogeneity in the $d_S$ values in the genes between the PR-proximal region and the centromere, with highest $d_S$ values for genes closer to PR than to the centromere (Fig. 4). Under this hypothesis, expansion of the regions of suppressed recombination would have occurred through processes unrelated to the mating system, such as the accumulation and methylation of transposable elements in non-recombining regions and in their margins (Ponnikas et al. 2018). Once the PR-centromere linkage was achieved, selection for HD-centromere linkage may have occurred and been selected for increasing odds of compatibility under automixis.

Alternatively, the close physical proximity of the HD locus to the centromere may be sufficient to render recombination events infrequent enough that recombination cessation between the PR locus and its centromere would be immediately beneficial for increasing the odds of gamete compatibility under automixis. This hypothesis of rare recombination between the centromere and the HD would explain the very low $d_S$ values in this region and the collinearity between $b_1$ and $b_2$ HD mating-type chromosomes in *M. saponariae* despite the inversion that occurred between HD and the centromere since its speciation from *M. lagerheimii*. This hypothesis is not incompatible with the PR-centromere linkage having evolved by successive evolutionary steps. Low recombination rates have been invoked in sex chromosomes to explain low differentiation between alleles on X and Y chromosomes in some animals (Stöck et al. 2013).

Although inversions are often thought to play a major role in suppressing recombination (Wright et al. 2016; Lemaitre et al. 2009; Wang et al. 2012), non-recombining regions with conserved collinearity have been reported in several fungi (Grognet et al. 2014; Branco et al. 2017; Jacobson 2005; Branco et al. 2018; Sun et al. 2017b). In this study, we only found inversions in the region with recent recombination cessation between the *M. saponariae* PR chromosomes. Finding that the limits of this inversion are precisely the centromere and the PR-proximal region is consistent with a role of inversions in recombination suppression, although we cannot exclude that the inversion occurred as a subsequent rearrangement after recombination cessation. The remaining mating-type loci and centromere linkage events occurred via different proximate mechanisms not involving rearrangements. Elucidating the proximal mechanisms suppressing recombination by exploring for example changes in DNA methylation and heterochromatin marks, as well as Spo11-dependent formation of double-strand breaks (Keeney 2008; Termolino et al. 2016), will be interesting in future studies.

Our results have general implications beyond mating systems in fungi and evolutionary convergence, providing an excellent illustration of the benefits of mating via central fusion automixis. Broader implication of central fusion automixis has been rarely considered, despite occurring in a wide range of taxa such as in fungi (Hood and Antonovics 2000; Zakharov 2005; Grognet et al. 2014; Menkis et al. 2008), plants (Asker 1980; Schön et al. 2009; Walker 1985; Antonius and Nybom 1995; Cruden and Lloyd 1995), and insects (Normark 2003; Suomalainen et al. 1976; Oldroyd et al. 2008). Theoretical models and reviews have highlighted the evolutionary and genetic consequences of central fusion automixis in maintaining heterozygosity (Zakharov 1986; Hood et al. 2005; Zakharov 2005; Engelstädter 2017; Antonovics and Abrams 2004), but few cases have been experimentally studied so far. Our findings illustrate how linkage to centromere under central fusion automixis can generate a sort of pseudo-linkage among genes on different chromosomes and preserve heterozygosity. Such maintenance of heterozygosity can be beneficial in a variety of cases (Ferreira and Amos 2006), such as the sheltering of deleterious alleles (Hood and Antonovics 2000) or overdominance in immune systems (Hraber et al. 2007) and other functions, as suggested at several loci in the case of the central fusion automictic Cape honeybees (Goudie et al. 2014).

## Materials and Methods

To conduct segregation analyses we isolated *M. lagerheimii* haploid cells from opposite poles of meiosis I across replicate meioses from the same diploid parent using micromanipulation (Hood et al. 2015). We investigated mating-type segregation by PCR amplification of allele-specific markers. The *M. lagerheimii* strain used for segregation analyses was collected on *Lychnis flos-jovis* in Valle Pesio, Italy (GPS 44.188400, 7.670650).

Genome analyses were conducted in the *M. saponariae* and *M. lagerheimii* assemblies. We used alternative mating types isolated from a single diploid spore of *M. saponariae* parasitizing *S. officinalis* (cell 1268, PRAT 47, $a_1$ $b_1$, and cell 1269, PRAT 48, $a_2$ $b_2$) collected near Chiusa di Pesio, (GPS coordinates 44.31713297, 7.622967437 on July 8th, 2012). We used the *M. lagerheimii* genome previously published ($a_1$ $b_1$ and $a_2$ $b_2$ assemblies GCA_900015505.1 and GCA_900013405.1, respectively; (Branco et al. 2017). DNA was extracted with the QIAGEN Genomic-tip 100/G (ref. 10243; Courtaboeuf, France) and Genomic DNA Buffer Set (ref. 19060) following manufacturer instructions and using a

Carver hydraulic press (reference 3968, Wabash, IN, USA). Haploid genomes were sequenced using the P6/C4 Pacific Biosciences SMRT technology (UCSD IGM Genomics Facility La Jolla, CA, USA). Assemblies of the genomes were generated with the wgs-8.2 version of the PBcR assembler (Koren et al. 2012). Contigs were aligned with optical maps of the two mating-type chromosomes obtained previously (Hood et al. 2015), with MapSolver software (OpGen), see statistics on assemblies in Supplemental Tables S1 and S2. We obtained orthologous groups with orthAgogue (Ekseth et al. 2014) based on BLASTP+ 2.2.30 followed by Markov clustering (Van Dongen 2000). We aligned the protein sequences of 780 fully conserved single-copy genes with MAFFT v7.388 (Katoh and Standley 2013) and obtained the codon-based CDS alignments with TranslatorX (Abascal et al. 2010). We used RAxML 8.2.7 (Stamatakis 2006) to obtain maximum likelihood gene trees for all 780 fully conserved single-copy genes and a species tree with the concatenated alignment. We estimated synonymous divergence ($d_S$) and its standard error with the yn00 program of the PAML package (Yang 2007).

We used nine orthologous groups (8,525 aligned codons) for dating the recombination cessation between the HD-proximal region and the centromere, and 10 orthologous (10,200 aligned codons) groups for dating recombination cessation between the PR-proximal region and the centromere. Divergence times were estimated using BEAST v2.4.0 (Drummond and Rambaut 2007), with the XLM inputs being generated using BEAUTi (Drummond et al. 2012).

Transposable elements were identified and annotated *de novo* in the high-quality genome assemblies, using both LTR-harvest (defaults parameters; Ellinghaus et al. 2008); and RepeatModeler (defaults parameters; Smit and Hubley 2015). We identified *de novo* centromeric-specific repeats (Melters et al. 2013) using Tandem-Repeat Finder (TRF v. 4.07b; Benson 1999), see Additional file 1.

### Data access

The PacBio (Pacific Biosciences) genome assemblies from this study have been submitted to the European Nucleotide Archive (ENA; https://www.ebi.ac.uk/ena) under accession number GCA_900015975 for the $a_1$ genome and GCA_900015475 for the $a_2$ genome of *Microbotryum saponariae* from *Saponaria officinalis*.

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## Author contributions

TG and MEH designed and supervised the study. TG, MEH and SB contributed to obtain funding. MEH, AS and TG obtained the genomes.  FC, RRDLV, SB and MAC performed the genomic analyses. MEH and AS performed the segregation analyses. FC, TG and MEH wrote the manuscript with contributions from all other authors.

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# Figure legends

**Figure 1: Odds of compatibility among gametes of a diploid individual in basidiomycete fungi.** Gametes are fully compatible only if they carry different alleles at both mating-type loci, the PR (including pheromone receptor and pheromone genes, with $a_1$ and $a_2$ alleles) and HD (including homeodomain genes, with $b_1$ and $b_2$ alleles) loci. A) With PR and HD mating-type loci unlinked from each other and from the centromeres (shown here located in different chromosomes in blue and red), the percentage of compatibility of a given gamete among the other gametes produced by the same diploid individual is 25% across multiple meioses (a given gamete is compatible with one of every four gametes), and the percentage is 33% within tetrad (a given gamete is compatible with one of the other three gametes in the tetrad) or 67% (a given gamete is compatible with two of the three remaining gametes in the tetrad) depending on segregation of the mating type alleles. The different types of gametes produced are tetratypes (TT), parental ditypes (PD) or non-parental ditypes (NPD), which depends on allele segregation and on whether a crossing-over occurred between one of the two loci and the centromere. B) With PR and HD mating-type genes linked to the centromeres of different chromosomes (blue and red), the percentage of compatibility of a given gamete among the other gametes produced by the same diploid individual is 25% across multiple meioses but 67% within a tetrad (a given gamete is compatible with two of the three other gametes in the tetrad) due to the segregation of the variation occurring only at meiosis I for both mating type loci. The different types of gametes produced are parental ditypes (PD) or non-parental ditypes (NPD), which depends on segregation. C) With HD and PR loci fully linked to each other on the same chromosome, the percentage of compatibility of a given gamete among the other gametes produced by the same diploid individual is 50% across multiple meioses (a given gamete is compatible with one of every two gametes), and 67%

within a single meiotic tetrad (a given gamete is compatible with two of the three other gametes in the tetrad). The light blue background shows the opposite cells of ordered tetrads, both in a *Microbotryum* linear tetrad representation (bottom panel) and in the different types of possible tetrads depicted depending on mating-type locus linkage.

**Figure 2**: **Phylogenies of anther-smut fungi and their mating-type loci linkage.** A) *Microbotryomycete* phylogenetic tree based on 780 orthologous genes, including the studied *Microbotryum* species (shown in the anthers of their host plants) and the outgroup *Rhodosporidum babjevae*. The empty circles indicate full bootstrap support. Tree internode certainty with no conflict bipartitions (the normalized frequency of the most frequent bipartition across gene genealogies relative to the summed frequencies of the two most frequent bipartitions) is given above the branches, indicating good support for the bipartitions. Black bars at right indicate unlinked mating-type loci, dark grey linkage of mating-type loci to centromeres and light grey mating-type loci linkage.

**Figure 3**: **Intraspecific comparison of gene order between mating-type chromosomes.** Comparison of gene order between HD and PR chromosome pairs in *Microbotryum lagerheimii* (a and b) and *M. saponariae* (c and d). The outer tracks represent contigs, staggered every 200 kilobases. The HD, PR and pheromone genes are indicated by blue, dark purple and small light-purple circles, respectively. Blue and orange lines link alleles, the latter corresponding to inversions. The link width is proportional to the corresponding gene length. Yellow regions on the contig track indicate the centromeres (regions with low gene density, high TE density and enriched in tandem-repeats marked as pink marks). The black marks along the right contigs track indicate genes that have no synonymous substitutions between $a_1$ and $a_2$ alleles within species ($d_S$=0). Green marks indicate transposable elements (TEs) and grey marks non-TE genes. The ancient regions of recombination suppression are indicated on

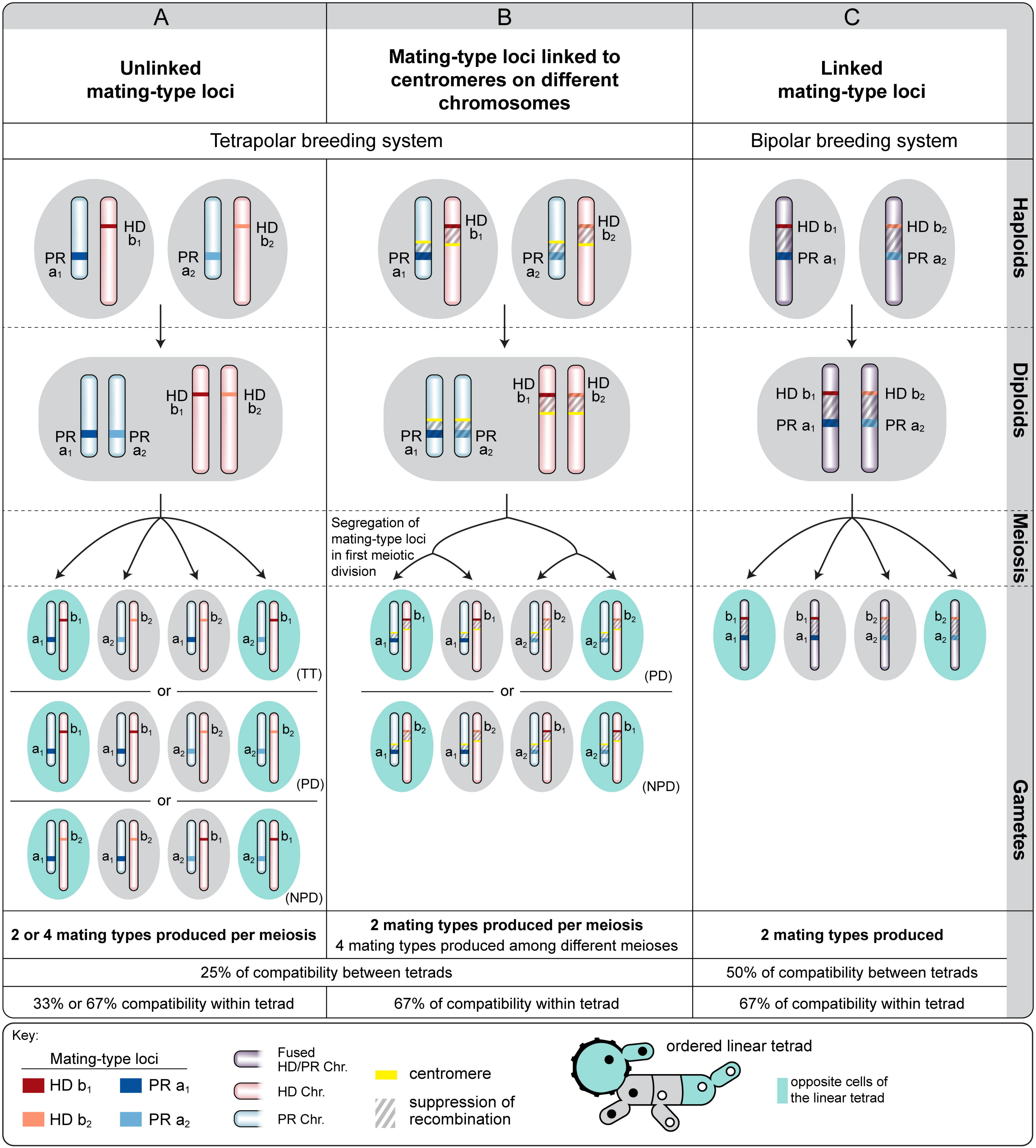
the outer track in blue for the HD locus and in purple for the PR locus. **a)** Comparison of the $b_1$ (left, orange) and $b_2$ (right, light orange) HD *M. lagerheimii* mating-type chromosomes. **b)** Comparison of the $a_1$ (left, orange) and $a_2$ (right, light orange) PR *M. lagerheimii* mating-type chromosomes. **c)** Comparison of the $b_1$ (left, red) and $b_2$ (right, light red) HD *M. saponariae* mating-type chromosomes. **d)** Comparison of the $a_1$ (left, red) and $a_2$ (right, light red) PR *M. saponariae* mating-type chromosomes. The large green arrow indicates the large inversion between the two mating-type chromosomes encompassing the mating-type locus and the centromere. The green regions on the contig track of each mating-type chromosome indicate the small inversion that likely occurred after the large inversion linking the PR locus to the centromere, extending the region of suppressed recombination.

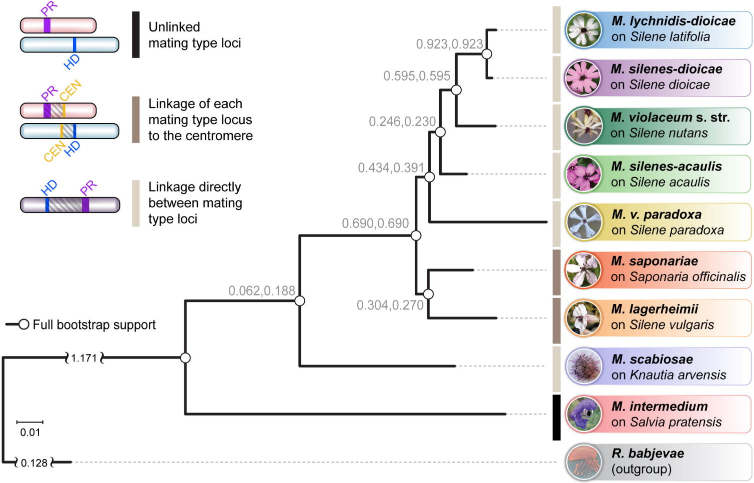
**Figure 4**: **Per-gene synonymous divergence and respective standard error ($d_S$ ± SE) between alleles associated to the $a_1b_1$-$a_2b_2$ mating types along the mating-type chromosomes within diploid *Microbotryum lagerheimii* and *M. saponariae* individuals.** Synonymous divergence is plotted against the genomic coordinates of the $a_1$ $b_1$ mating-type chromosomes of *M. intermedium* for all single-copy genes shared by the mating-type chromosomes, as a proxy for ancestral gene order. Divergence between the $a_1$ and $a_2$ pheromone receptor (PR) was too extensive (Devier et al. 2009) and could not be computed (noted as "unalignable"). The positions of the centromeres are indicated by yellow dots. Genes with $d_S>0$ between mating types around the PR and HD mating-type loci in *M. lagerheimii* are colored in purple and blue, respectively. The purple and blue regions correspond to the older PR- and HD-regions of suppressed recombination that evolved before and at the base of the radiation of the clade, respectively (Branco et al. 2017, 2018). Red arrows indicate the genes used for dating recombination cessation events. **a)** Per-gene synonymous divergence between mating types in *M. lagerheimii* along the gene order of the

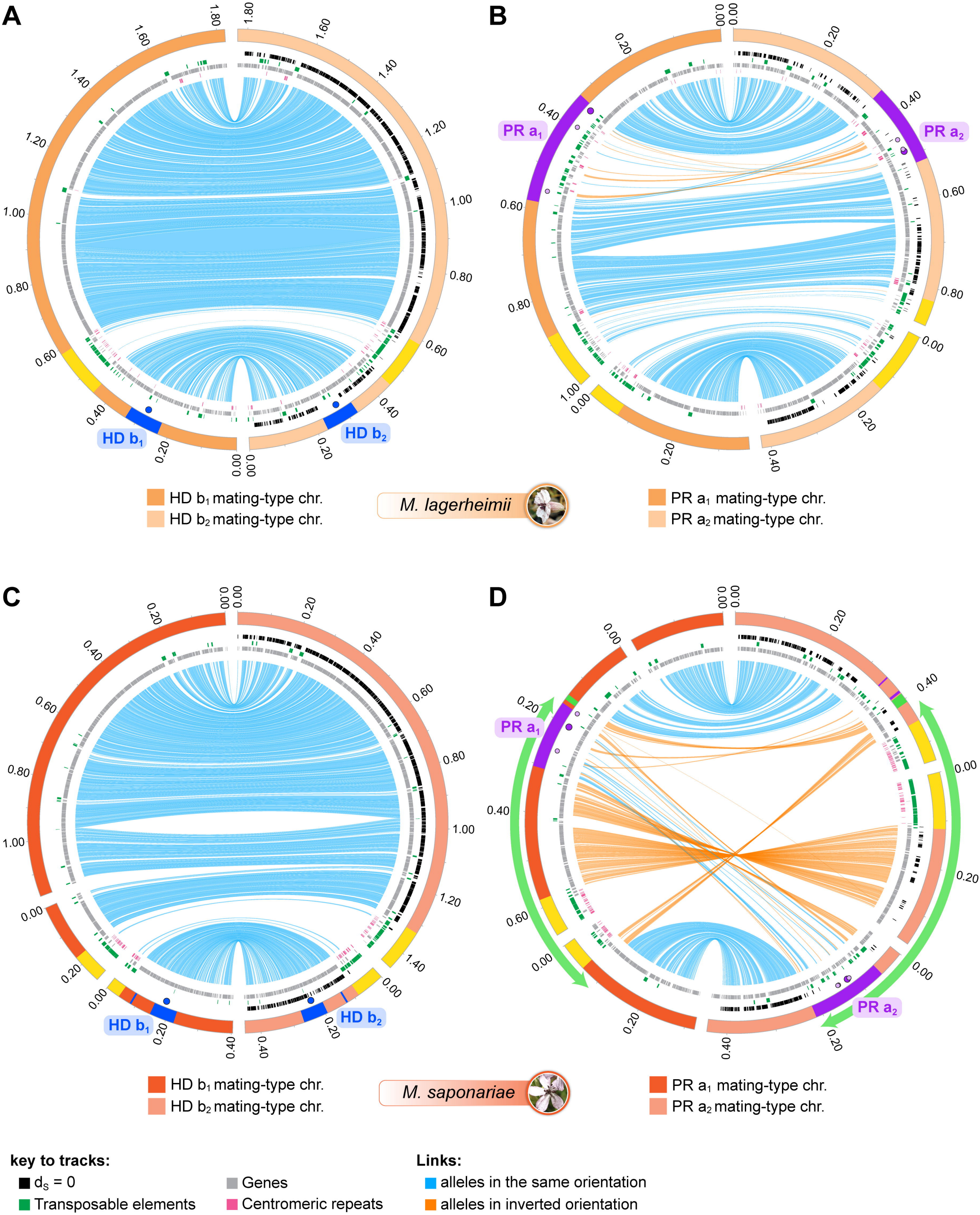
$a_2$ PR *M. intermedium* mating-type chromosome. **b)** Per-gene synonymous divergence between mating types in *M. lagerheimii* along the gene order of the $b_2$ HD *M. intermedium* mating-type chromosome. **c)** Per-gene synonymous divergence between mating types in *M. saponariae* along the gene order of the $a_2$ PR *M. intermedium* mating-type chromosome. **d)** Per-gene synonymous divergence between mating types in *M. saponariae* along the gene order of the $b_2$ HD *M. intermedium* mating-type chromosome.

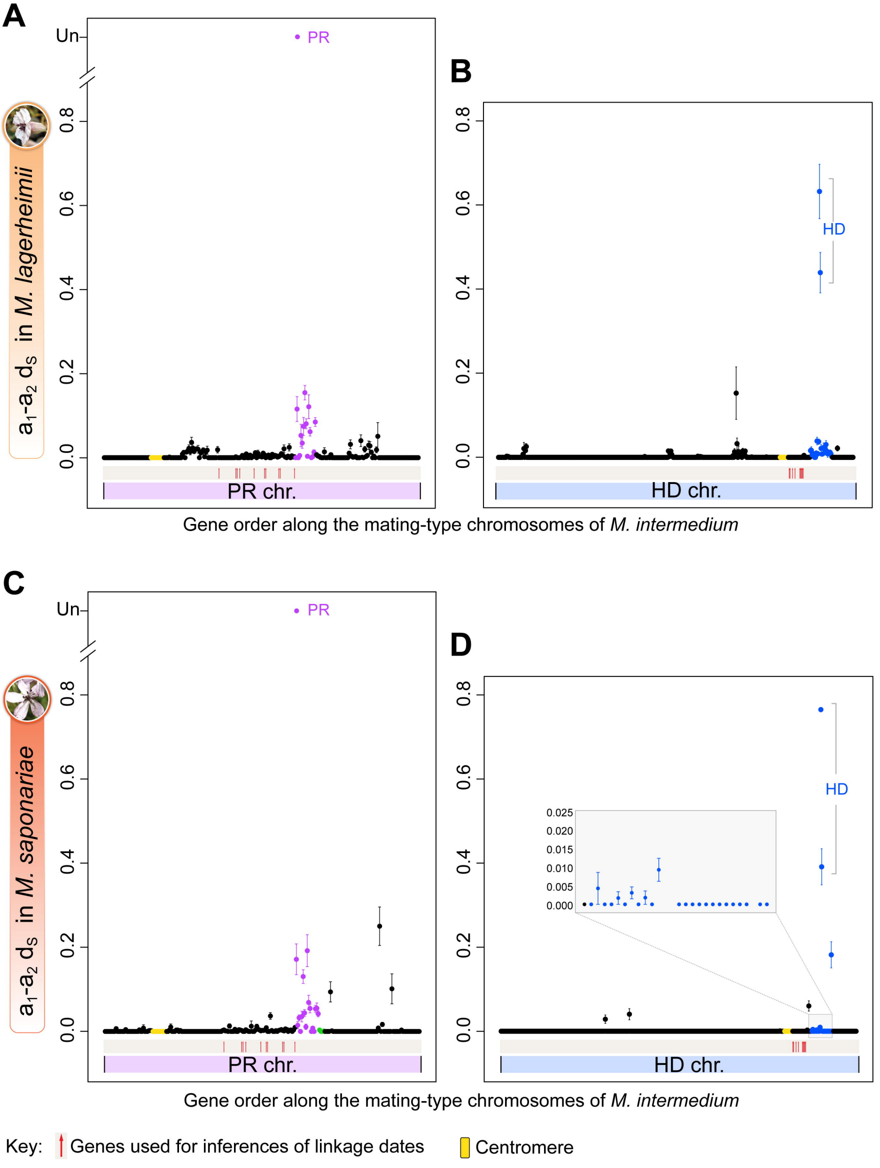
**Figure 5: Linkage date estimates across *Microbotryum*.** Linkage between mating-type loci (PR and HD) and centromeres, or between PR and HD loci was inferred from dates of divergence between alleles associated to the $a_1$ and $a_2$ mating types at genes linked to the mating-type loci. In gene genealogies, nodes separating alleles associated to the $a_1$ and $a_2$ mating types within species correspond to the date of linkage to the mating type loci (to the HD locus in blue and to the PR locus in purple). Genealogies of alleles associated to the alternative mating-types ($a_1$ and $a_2$) were reconstructed based on a concatenated alignment of both alleles at 9 genes ancestrally located between the centromere and the HD-proximal region (8,525 aligned codons) and of 10 genes ancestrally located between the centromere and the PR-proximal region (10,200 aligned codons). The genes used for this analysis, with both alleles in all species, are indicated by red arrows in the Figure 3. **a)** Marginal posterior densities for the most recent common ancestor date (MRCA time) estimated with BEAST v2.4.0 based on multiple sequence alignments of genes located between the HD-proximal (blue) or the PR-proximal (light purple) region and the corresponding centromeres in the ancestral gene order. **b)** Time-calibrated tree of $a_1$ and $a_2$ alleles with nodes drawn at the mean date (in million years, MY) for the genes on the HD chromosome. Inferred divergence dates for the genes located between the HD-proximal or the PR-proximal regions and the corresponding centromeres are shown in blue or purple fonts, respectively, to the right of the

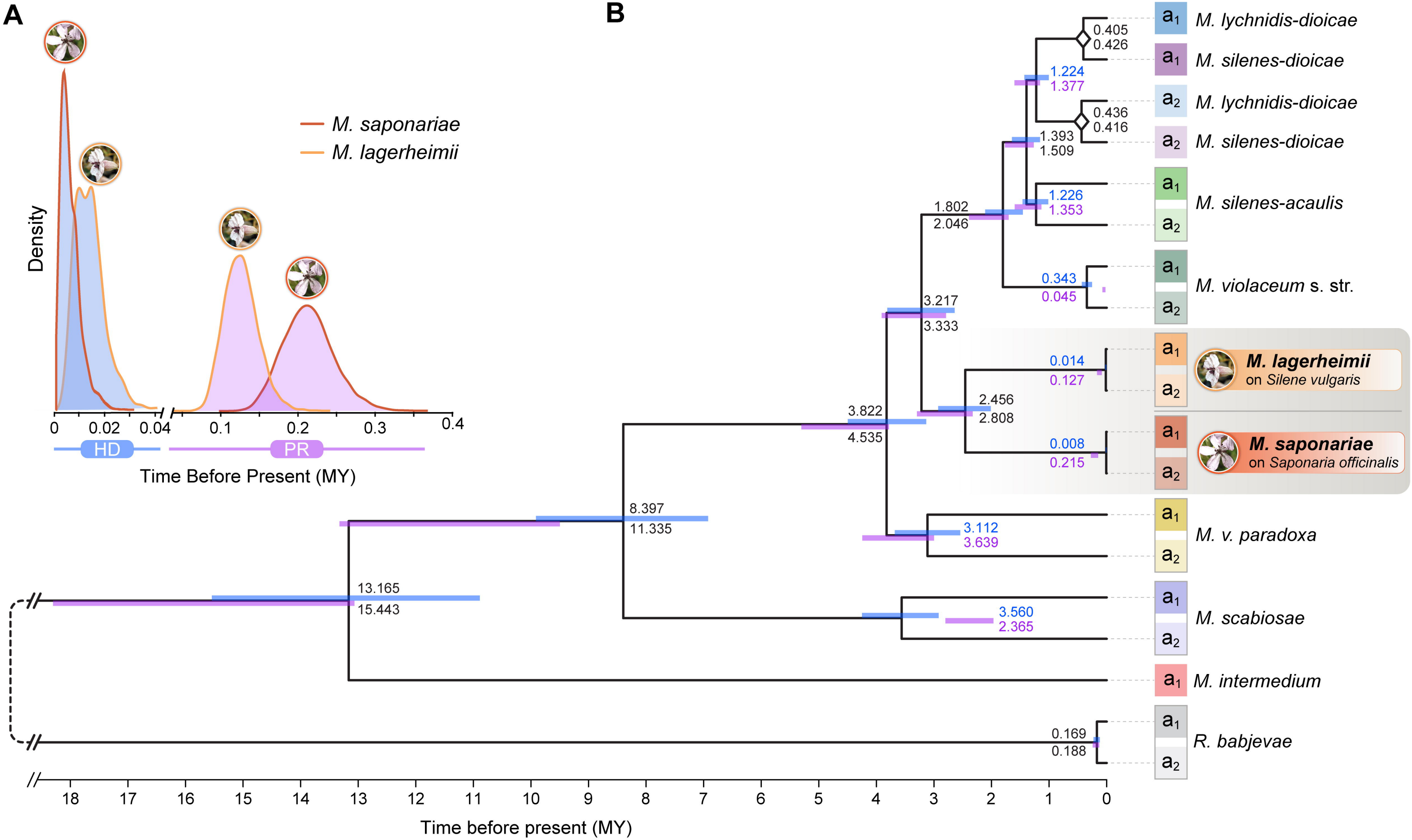
nodes. Light blue and light purple bars correspond to 95% confidence intervals. Speciation dates as inferred from each dataset are shown in black font on the right side of the nodes (bottom, PR set; up, HD set).


A
B
C
Unlinked mating-type loci
Mating-type loci linked to centromeres on different chromosomes
Linked mating-type loci
Tetrapolar breeding system
Bipolar breeding system
Haploids
Diploids
Meiosis
Gametes
PR $a_1$
HD $b_1$
PR $a_2$
HD $b_2$
Segregation of mating-type loci in first meiotic division
$a_1$
$a_2$
$b_1$
$b_2$
(TT)
or
(PD)
or
(NPD)
(PD)
or
(NPD)
2 or 4 mating types produced per meiosis
2 mating types produced per meiosis
4 mating types produced among different meioses
2 mating types produced
25% of compatibility between tetrads
50% of compatibility between tetrads
33% or 67% compatibility within tetrad
67% of compatibility within tetrad
67% of compatibility within tetrad
Key:
Mating-type loci
HD $b_1$
PR $a_1$
HD $b_2$
PR $a_2$
Fused HD/PR Chr.
HD Chr.
PR Chr.
centromere
suppression of recombination
ordered linear tetrad
opposite cells of the linear tetrad


PR
HD
Unlinked mating type loci
PR
CEN
CEN
HD
Linkage of each mating type locus to the centromere
HD
PR
Linkage directly between mating type loci
Full bootstrap support
1.171
0.128
0.01
0.062,0.188
0.690,0.690
0.434,0.391
0.246,0.230
0.595,0.595
0.923,0.923
0.304,0.270
M. lychnidis-dioicae on Silene latifolia
M. silenes-dioicae on Silene dioicae
M. violaceum s. str. on Silene nutans
M. silenes-acaulis on Silene acaulis
M. v. paradoxa on Silene paradoxa
M. saponariae on Saponaria officinalis
M. lagerheimii on Silene vulgaris
M. scabiosae on Knautia arvensis
M. intermedium on Salvia pratensis
R. babjevae (outgroup)


A
B
C
D
HD b₁
HD b₂
PR a₁
PR a₂
HD b₁ mating-type chr.
HD b₂ mating-type chr.
M. lagerheimii
PR a₁ mating-type chr.
PR a₂ mating-type chr.
HD b₁ mating-type chr.
HD b₂ mating-type chr.
M. saponariae
PR a₁ mating-type chr.
PR a₂ mating-type chr.
key to tracks:
■ dS = 0
■ Transposable elements
■ Genes
■ Centromeric repeats
Links:
■ alleles in the same orientation
■ alleles in inverted orientation


A
B
C
D
Un
PR
a1-a2 dS in M. lagerheimii
a1-a2 dS in M. saponariae
0.0
0.2
0.4
0.6
0.8
HD
0.025
0.020
0.015
0.010
0.005
0.000
PR chr.
HD chr.
Gene order along the mating-type chromosomes of M. intermedium
Key: Genes used for inferences of linkage dates
Centromere


A
Density
M. saponariae
M. lagerheimii
0
0.02
0.04
0.1
0.2
0.3
0.4
HD
PR
Time Before Present (MY)
B
0.405
0.426
1.224
1.377
0.436
0.416
1.393
1.509
1.226
1.353
1.802
2.046
0.343
0.045
3.217
3.333
0.014
0.127
2.456
2.808
3.822
4.535
0.008
0.215
8.397
11.335
3.112
3.639
3.560
2.365
13.165
15.443
0.169
0.188
a1
a2
M. lychnidis-dioicae
M. silenes-dioicae
M. lychnidis-dioicae
M. silenes-dioicae
M. silenes-acaulis
M. violaceum s. str.
M. lagerheimii
on Silene vulgaris
M. saponariae
on Saponaria officinalis
M. v. paradoxa
M. scabiosae
M. intermedium
R. babjevae
18
17
16
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
0
Time before present (MY)