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Hybrid sterility and inviability in the parasitic fungal species complex *Microbotryum*

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Abstract

Microbotryum violaceum, the anther-smut fungus, forms a complex of sibling species which specialize on different plants. Previous studies have shown the presence of partial ecological isolation and F1 inviability, but did not detect assortative mating apart from a high selfing rate. We investigated other post-mating barriers and show that F1 hybrid sterility, the inability of gametes to mate, increased gradually with the increasing genetic distance between the parents. F2 hybrids showed a reduced ability to infect the plants that was also correlated with the genetic distance. The host on which the F2 hybrids were passaged caused a selection for alleles derived from the pathogen species originally isolated from that host, but this effect was not detectable for the most closely related species. The post-mating barriers thus remain weak among the closest species pairs, suggesting that premating barriers are sufficient to initiate divergence in this system.

Introduction

How one species diverges into two species remains a central question in evolutionary biology. Two of the main difficulties in studying speciation are the slow rate of the process, generally not observable in a researcher's lifetime, and the multiplicity and inter-relation of the factors contributing to divergence. Studying multiple barriers to gene flow between recently derived species, and their evolution with genetic divergence, is thus an essential approach to gain insights into speciation processes (Coyne & Orr, 2004), allowing the elucidation of the first and most important barriers to initiate reproductive isolation.

Two main types of barriers to gene flow are commonly distinguished, depending upon when in the life cycle they are effective. Prezygotic barriers may be caused by mating avoidance because of ecological isolation or assortative mating, or the failure of gametes to unite. Post-zygotic barriers occur when hybrids are formed but are inviable or sterile (Coyne & Orr, 2004). Within these two main categories, more kinds of reproductive isolation have been distinguished, the importance of which varies across biological systems. For instance, ecological prezygotic isolation results from niche specialization and has been invoked as the strongest barrier to gene flow in walking sticks, sticklebacks and many parasites (Crespi & Sandoval, 2000; Nosil *et al.*, 2005; Giraud, 2006; Giraud *et al.*, 2006; Vines & Schluter, 2006). In plants, specialized pollinator guilds, non-overlapping flowering times and high rates of self-pollination can substantially limit gene flow in a variety of species (Schemske & Bradshaw, 1999; Martin & Willis, 2007; Widmer *et al.*, 2009). Behavioural assortative mating prevents gene flow in many animals (Butlin, 1987).

Mechanisms of post-zygotic isolation can be manifested at any developmental stage, from maturation of the hybrid individual to its ability to successfully mate and contribute functional gametes. F1 hybrid inviability can be due to intrinsic mechanisms, such as Dobzhansky–Muller genetic incompatibilities (Orr, 1995), as is well known, for instance, in *Drosophila* (Presgraves *et al.*, 2003). F1 hybrid inviability can alternatively be caused by extrinsic mechanisms, when the hybrids are ill-suited

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to available environments (Rundle & Nosil, 2005). Hybrid sterility has been identified as the main barrier to gene flow in several organisms (Coyne & Orr, 1997; Castiglia & Capanna, 2000; Van Der Velde & Bijlsma, 2004; Greig, 2007). In some other cases, F1 hybrids are fertile, but they produce unfit F2 hybrids (Wade *et al.*, 2003; Niehuis *et al.*, 2008). Effective reproductive isolation between close species often results from the cumulative effects of several of these barriers. In organisms as different as house mice, reef fishes or sunflowers, investigations have found partial isolation at multiple steps in the life cycle (Ramsey *et al.*, 2003; Britton-Davidian *et al.*, 2005; Lai *et al.*, 2005; Smadja & Ganem, 2005; Crow *et al.*, 2007; Mendelson *et al.*, 2007).

Investigating the rate at which these barriers to gene flow evolve is essential for understanding the time course of speciation. Current mechanisms of reproductive incompatibility may indeed evolve well after gene flow has ceased, thus obscuring the essential barriers that contributed to the speciation process. Isolating mechanisms occurring later in the life cycle are most likely to be hidden by traits that evolve after speciation has been accomplished, because those occurring early in the life cycle will appear to be most important at the time of study (Schemske, 2000). Yet, it is essential to elucidate the first barriers in the process of speciation, because they were those causing the initial divergence. By studying the evolution of reproductive barriers between species across a range of genetic distances, it has been shown for instance in *Drosophila* and mushrooms that the youngest species pairs show strong prezygotic isolation in sympatry, but not in allopatry (Coyne & Orr, 1997; Le Gac & Giraud, 2008). This suggests that prezygotic barriers are essential in the early stages of divergence in sympatry, but not in allopatry. Similar approaches have shown that hybrid sterility evolves faster than hybrid inviability in a wide range of organisms (Wu, 1992; Coyne & Orr, 1997; Sasa *et al.*, 1998; Presgraves, 2002; Price & Bouvier, 2002; Scopece *et al.*, 2008). The importance of hybrid sterility may, however, be obscured at later stages of divergence because it occurs later in the life cycle than hybrid inviability.

Fungi are particularly suitable models for studying speciation, partly because of the presence of many complexes of sibling species that are amenable to being cultured and crossed in the laboratory (Giraud *et al.*, 2008a). Here we study the evolution of reproductive isolation in *Microbotryum violaceum* sensu lato, a fungal basidiomycete species complex responsible for the anther smut disease on many plant species in the Caryophyllaceae (Thrall *et al.*, 1993). *Microbotryum violaceum* sensu lato includes several sibling species highly specialized on their host plant and evolving independently without detectable gene flow despite their large overlap in range (Lutz *et al.*, 2005; Kemler *et al.*, 2006; Le Gac *et al.*, 2007a,b; Refrégier *et al.*, 2008).

Previous studies investigated pre- and post-zygotic barriers between the *Microbotryum* sibling species. 'Pre- and post-mating' are more appropriate terms to use in basidiomycetes than 'pre- and post-zygotic' because of their extended dikaryotic phase after mating and before nuclear fusion, which is the usual stage when a 'zygote' is said to be formed. The dikaryotic phase often represents the main part of the life cycle in basidiomycetes, as in *Microbotryum* where it is the infectious form. Partial premating ecological isolation results from differences in the habitats and pollinators of the host plants, but this reproductive isolation is not complete (Goulson & Jerrim, 1997; Van Putten *et al.*, 2007). *In vitro* crosses showed no evidence of assortative mating among *Microbotryum* species (Le Gac *et al.*, 2007b), but the high rate of selfing in nature, because of intra-tetrad conjugations (Hood & Antonovics, 2000, 2004; Giraud, 2004; Giraud *et al.*, 2005), has been invoked as a particular form of premating isolation (Giraud *et al.*, 2008b). Artificial inoculations of hybrids on plants revealed that partial post-mating isolation increased with genetic distance between the fungal species: hybrids had low infective ability and caused incomplete symptoms (Le Gac *et al.*, 2007b; Sloan *et al.*, 2008). Hybrids between the closest species pairs were however quite capable of causing infections. Hybrids also had a low production of viable gametes (Sloan *et al.*, 2008) possibly caused by the high karyotypic divergence that has been described between *Microbotryum* species (Hood, 2002; Hood *et al.*, 2003, 2004).

The sibling species of the *M. violaceum* complex thus have opportunities to mate in nature, do not show assortative mating and are capable of forming hybrids in the laboratory (Le Gac *et al.*, 2007b) but do not exchange genes in nature (Le Gac *et al.*, 2007a). High selfing rates, low F1 hybrid infection ability and low gamete production apparently contribute to genetic isolation, but may not be sufficient to completely prevent gene flow among the closest species pairs. Here, we wanted to assess whether other reproductive barriers could contribute to the lack of gene flow among *Microbotryum* species, and if these barriers increased with the genetic distance between the species, or were already strong between the closest species pairs. We therefore analysed several potential reproductive barriers in the progenies of 15 different crosses generated in a previous study (Le Gac *et al.*, 2007b) (three intraspecific, from two species, and 12 interspecific, from six species pairs). We assessed: (i) F1 hybrid sterility, in the form of inability of viable gametes to mate, and its relationship with genetic distances between the parents; (ii) some of the possible causes of F1 hybrid sterility by characterizing F1 gamete genotypes (number of alleles at the pheromone receptor gene and genome size); (iii) F2 hybrid inviability and its relationship with genetic distances between the parents by measuring F2 infection success; and (iv) the role of host environment as a factor in selecting for the fittest F2 hybrid genotypes.

Material and methods

Biological model

The life cycle of *M. violaceum* sensu lato is illustrated in Fig. 1. Diploid teliospores of the pathogen are produced in anthers of infected plants, thereby replacing the pollen. Deposited on a new host, teliospores undergo meiosis, leading to the production of haploid yeast-like sporidia of opposite mating types, called a_1 and a_2. They have sometimes been referred to as A1 and A2, but a_1 and a_2 should be preferred because of historical precedence. These sporidia and their mitotic descendants act as the gametes of the fungus. Conjugation between sporidia of opposite mating types is required to initiate growth of infectious dikaryotic hyphae. Mating in basidiomycete fungi is controlled by the mating type locus, which contains several genes, and in particular a pheromone receptor gene that has been characterized in *M. violaceum* (Yockteng *et al.*, 2007; Devier *et al.*, 2009). Sporidia from each mating type (a_1 and a_2) possess a specific allele of the pheromone receptor gene, the two alleles being highly divergent (Devier *et al.*, 2009).

The species within the *M. violaceum* complex will be referred to here as in the study by Le Gac *et al.* (2007a), based on their host species. Specifically, the species parasitizing the plant *Silene latifolia* is called MvSl, the one parasitizing *S. dioica* is called MvSd, the one parasitizing *S. nutans* is called MvSn, and the one parasitizing *Lychnis flos-cuculi* is called MvLfc. *Silene vulgaris* is parasitized by three distinct *Microbotryum* species, one of which will be used here, MvSv1. Some of the *Microbotryum* species have been given Latin names recently (Lutz *et al.*, 2005; Kemler *et al.*, 2006; Denchev, 2007a,b) but MvSl and MvSd are still referred to as the same species, *M. lychnidis-dioicae*, and MvLfc awaits taxonomic revision. MvSv1 has been described recently on *Lychnis viscaria* and given the name *M. lagerheimii* (Denchev, 2007a; Giraud *et al.*, 2008c). MvSn has retained the original name, *M. violaceum* sensu stricto (Lutz *et al.*, 2005; Kemler *et al.*, 2006).

Available F1 crosses

The successive steps of the hybrid life cycle analysed in this study are presented in Fig. 2. Diploid teliospores from F1 hybrids were available from Le Gac *et al.* (2007b). They had been dried under silica gel and stored at 4 °C. Two sets of F_1 hybrids were used: the one involving MvSl as one of the parents and obtained by inoculating the plant *S. latifolia* and the one involving MvSd as one of the parents and obtained by inoculating the plant *S. dioica*. Five F1 hybrid genotypes were available in the first set (two MvSl × MvSd and three MvSl × MvLfc) and seven F1 hybrids in the second set (two MvSd × MvSl, two MvSd × MvLfc, one MvSd × MvSn and two MvSd × MvSv1). We used, in addition, three non-hybrid F1 controls, i.e. strains resulting from crosses between two different strains of the same species: one MvSl × MvSl and two MvSd × MvSd crosses, so that a total of 15 F1 genotypes were analysed. For some F1 crosses, two infected plants had been obtained. As they corresponded to identical F1 genotypes, the results from these F1 strains were pooled. The different strains used for the experiments and the origins of the parental strains are described in Table 1 and in Le Gac *et al.* (2007a).

F1 sterility

We first tested the ability of viable sporidia produced by meiosis of F1 genotypes to engage in mating, i.e. conjugation among opposite haploid mating types (Fig. 2). To obtain gametes from each of the F1 progeny, we suspended their diploid teliospores in water and plated them on a nutrient agar medium (GMB2, Thomas *et al.*, 2003). Colonies were obtained, each corresponding

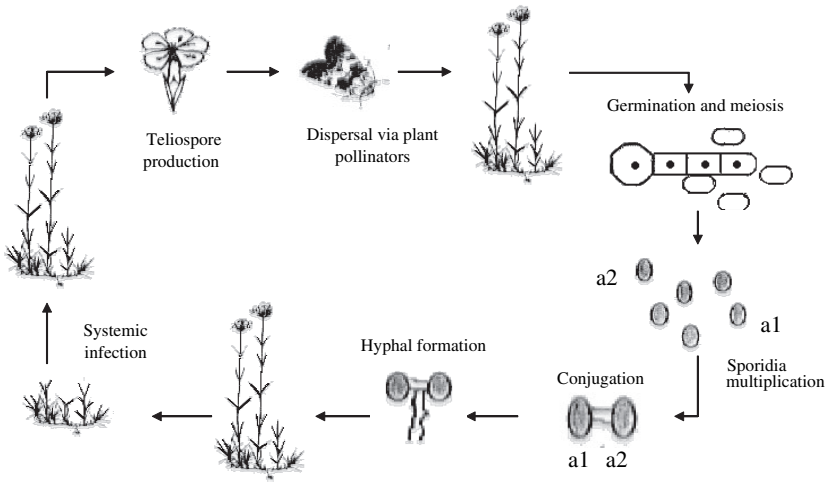

Fig. 1 *Microbotryum violaceum sensus lato* life cycle. The main phase is the infectious dikaryon. Sexual reproduction occurs just before infection of a host plant.

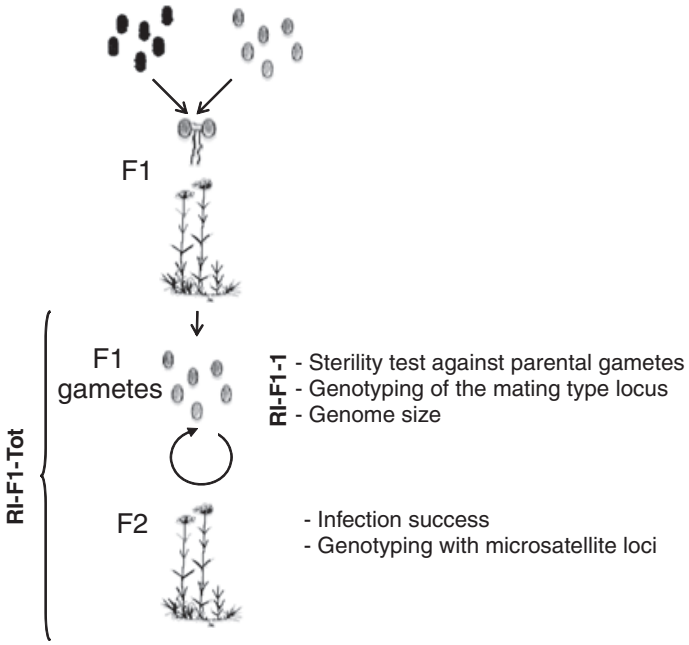

Fig. 2 The different barriers to gene flow analysed in this study. F1 hybrid sterility and F2 hybrid inviability were studied, as well as their underlying mechanisms. RI-F1-1 and RI-F1-Tot represent the reproductive barriers studied here. RI-F1-1: inability of F1-produced gametes to conjugate. RI-F1-Tot: inability to produce a diploid F2 progeny in host plants.

to the germination of a single teliospore, thus potentially including the haploid clones of up to four meiotic products of a single meiosis, two being of a_1 mating type and two of a_2 mating type (Fig. S1). We applied a second dilution step to 10 of these teliospore-derived colonies, plating them on GMB2, thus obtaining colonies corresponding to clones of a single haploid sporidia (Fig. S1). For each of the 10 teliospores per strain, one sporidial clone was randomly chosen for further analyses. The 10 single-sporidia-derived clonal lineages corresponded to 10 different meioses of the same diploid individual. The ability of sporidia (gametes) to bud by mitoses on medium allows the generation of millions of gametes of the same genotype.

We tested the ability of these F1 gametes to mate with the parental gametes used to generate these hybrids. The parental gametes had been stored since the experiment of Le Gac *et al.* (2007b) at −20 °C in Eppendorf tubes filled with silica gel. We prepared cell suspensions of each hybrid gamete and each parental gamete by stirring a single colony in 500 μL of sterile tap water. Concentration was adjusted to achieve a dilution compatible with microscopic observation (i.e. between 10^6 and 10^8 cells/mL). For each F1 gamete, we tested three things: (i) its ability to mate with a_1s – by mixing 30 μL of its suspension with 30 μL of its a_1 parental gamete; (ii) its ability to mate with a_2 parental gametes – with the same protocol; and (iii) its ability to conjugate with its clones – by letting 60 μL of the suspension for conjugation (we suspected that this could occur for hybrid gametes carrying the two mating types after an aberrant meiotic segregation). We let conjugation proceed for 5 days at 10 °C in enzyme-linked immunosorbent assay (ELISA) plates wrapped with plastic film. We then looked for conjugation events in each well by examining 5 μL of the suspension under the microscope. The conjugation result was considered as positive when at least three conjugations were observed when going rapidly through the sample. We inferred the mating type of each gamete from the results of the conjugation tests: 'a_1' when conjugation succeeded with the a_2 parental gamete only, 'a_2' when conjugation occurred with the a_1 parental gamete only, 'a_1 a_2' in case of success with both a_1 and a_2 parental gametes, and '- -' when they were unable to mate and when no conjugation was observed at all. Each cross was repeated twice with fresh colonies. Results were mostly reproducible (77%), and only results consistent between the two repeats were taken into account in the analyses. Discrepancy between repeats for some samples seemed to be due to aneuploidy resolution between sequential sporidia plating. F1 sterility was calculated here as the number of gametes unable to mate divided by the sum of all gametes tested.

Genotype of F1 gametes at pheromone receptor genes

To investigate whether abnormal meiotic segregation occurred in the hybrids, and in particular for genes belonging to the mating-type locus, the presence of abnormal number of copies of pheromone receptor genes in the sporidia was assessed. Either two copies of the mating-type locus in the haploid sporidia or the complete absence of mating-type genes is indicative of incorrect meiosis, and might also directly cause sterility because the pheromone receptor is involved in the fusion

Table 1 Information on the origin for the strains used in the study and results of the sterility and viability experiments.

	a_1 parent			a_2 parent						Sterility					Pheromone receptor content								Percent of F1 homozygous loci	
Gen. dist.	Species	Strain	Origin	Species	Strain	Origin	Host plant	F1 code	F2 code	- -	a_1a_2	a_1	a_2	Prop. sterile	- -	a_1a_2	a_1	a_2	Prop. abn.	No. inf./ healthy plants	Prop. infected plants	Percent of F2 hetero-zygous loci	From adapted parent	From non-adapted parent
0.002	MvSl	140.01	The Pyrenees, France	MvSl	141.01	The Pyrenees, France	*S. latifolia*	C and G	MvSl × MvSl 1	0	0	7	3	0	2	3	12	3	0.25	6/24	0.2	100%	0%	0%
0.002	MvSd	72.04	The Alps, Switzerland	MvSd	72.50	The Alps, Switzerland	*S. dioica*	sd1	MvSd × MvSd 1	0	0	2	7	0	0	2	1	7	0.2	5/12	0.29			
0.002	MvSd	sdh2	Vosges, France	MvSd	72.37	The Alps, Switzerland	*S. dioica*	sd2	MvSd × MvSd 2	0	0	7	3	0	0	2	5	3	0.2	1/9	0.1			
0.01	MvSd	336	Brittany, France	MvSl	141.01	The Pyrenees, France	*S. latifolia*	F	MvSl × MvSd 2	0	0	0	9	0	3	0	0	7	0.3	3/23	0.12	63–87%	6–18%	7–24%
0.01	MvSl	100.06	The Alps, Switzerland	MvSd	72.37	The Alps, Switzerland	*S. latifolia*	M	MvSl × MvSd 3	0	0	1	0	n.c.	0	3	7	0	0.3	3/19	0.14	80–85%	5–15%	5–19%
0.01	MvSl	141.01	The Pyrenees, France	MvSd	72.37	The Alps, Switzerland	*S. dioica*	sd3	MvSd × MvSl 1	1	0	9	0	0.1	0	0	10	0	0	2/14	0.13			
0.01	MvSd	sdh1	Vosges, France	MvSl	100.07	The Alps, Switzerland	*S. dioica*	sd4	MvSd × MvSl 2	0	0	5	4	0	0	6	4	0	0.6	2/9	0.18			
0.027	MvLfc	109.34	The Alps, Switzerland	MvSl	141.01	The Pyrenees, France	*S. latifolia*	B	MvSl × MvLfc 3	6	0	0	4	0.6	0	4	1	5	0.4	0/26	0			
0.027	MvLfc	92.04	The Alps, Switzerland	MvSl	40.01	Paris region, France	*S. latifolia*	H	MvSl × MvLfc 1	8	0	0	2	0.8	0	8	0	2	0.8	2/24	0.08	0–13%	81–92%	6–8%
0.027	MvSl	140.01	The Pyrenees, France	MvLfc	92.03	The Alps, Switzerland	*S. latifolia*	I and J	MvSl × MvLfc 2	0	0	13	0	0	1	16	2	1	0.85	0/27	0			
0.028	MvSd	72.50	The Alps, Switzerland	MvSn	79.01	The Alps, Switzerland	*S. dioica*	–	MvSd × MvSn	–	–	–	–	–	–	–	–	–	–	0/19	0			
0.030	MvLfc	109.34	The Alps, Switzerland	MvSd	72.37	The Alps, Switzerland	*S. dioica*	sd5	MvSd × MvLfc 1	4	0	3	1	0.5	0	6	4	0	0.6	0/19	0			
0.030	MvSd	sdh1	Vosges, France	MvLfc	109.53	The Alps, Switzerland	*S. dioica*	sd6	MvSd × MvLfc 2	10	0	0	0	1	0	9	1	0	0.9	0/16	0			
0.059	MvSv1	sm3	Somerset, UK	MvSd	72.37	The Alps, Switzerland	*S. dioica*	sd7	MvSd × MvSv1 1	7	0	0	0	1	0	10	0	0	1	0/21	0			
0.059	MvSd	sdh1	Vosges, France	MvSv1	300.27	The Pyrenees, France	*S. dioica*	sd8	MvSd × MvSv1 2	1	1	0	5	0.143	0	7	1	2	0.7	0/17	0			

Gen. Dist. is the genetic distance between the parental species of the F1 strains, and name and origin of the parents are given. For sterility and pheromone receptor content, values correspond to the number of individual gametes having the corresponding phenotype or genotype. Prop. is used for proportion, Abn. means abnormal and refers to a number of allele of the pheromone receptor different to what is expected (either 0 or 2 instead of 1). - -, when used for sterility refers to the gametes unable to mate with any of the parental strains whereas a_1a_2 refers to those mating with both. When used regarding pheromone receptor content, - - concerns gametes with no allele of the pheromone receptor gene whereas a_1 a_2 concerns gametes carrying both alleles. n.c. indicates a proportion that was not calculated due to the too small number of data available.

between sporidia during mating in the basidiomycetes basal to the homobasidiomycetes (Bakkeren & Kronstad, 2007). The alleles of the pheromone receptor corresponding to the a_1 and a_2 mating types are highly divergent (Yockteng *et al.*, 2007; Devier *et al.*, 2009), so that they can be differentially amplified with specific polymerase chain reaction (PCR) primers. DNA was extracted from a single colony of F1-produced gametes using the Chelex (Bio-Rad Laboratories, Marne-la-Coquette, France) protocol (Bucheli *et al.*, 2001). We tested for the presence of each allele of the pheromone receptor gene by PCR amplification, using the primers Contig660-F and Contig660-R for the receptor specific to a_1, and Contig588 F1 and Contig588R2 for the receptor specific to a_2 (Yockteng *et al.*, 2007). The PCR ran for 12 cycles with a decrease in annealing temperatures from 62 to 50 °C, then for 30 cycles at 50 °C. Reference sporidia of known mating types were used as controls.

The proportion of gametes with an abnormal genotype at the pheromone receptor gene was calculated for each F1 hybrid as the sum of sporidia with no receptor and with two receptor alleles, divided by the total number of sporidia. We investigated whether the proportion of gametes with an abnormal genotype was correlated with the genetic distance between the parental strains.

Genome size analysis of F1 gametes

We estimated the genome size of F1 gametes using flow cytometry (Kullman, 2000). The method has been validated for *Microbotryum*, which will be published elsewhere (M.E. Hood unpublished). This technique was performed using an Agilent Technology BioAnalyzer 2100 (Agilent Technologies Inc., Santa Clara, CA, USA) according to manufacturer's recommendations for on-chip staining. For each sample, a small colony was transferred to 50 mM Na citrate in 95% cold ethanol, and kept overnight at 4 °C. The cells were rinsed in 50 mM Na citrate and suspended in 50 mM Na citrate containing 0.1 mg mL^{-1} RNase A. Enzymatic action was allowed for 2 h at 37 °C. Cells were washed with 50 mM Na citrate, counted with a haemocytometer and suspended in Cell Buffer Solution (Agilent Technology) so as to obtain approximately 3000 cells per μL. An aliquot of 7 μL for each prepared sample was then loaded onto the BioAnalyzer Cell Chip with 7 μL of Sytox Green solution that stains nuclei according to DNA content, and incubated at room temperature for 2 h. Genome size was evaluated by using the Sytox Green fluorescence exhibited by the majority of the cells (see Fig. S2). A reference strain was used in each run for calibration. Relative excess of genome size was calculated according to the size exhibited by the control strain with the following formula:

$$\text{Relative excess of genome size} = \frac{\text{sample fluorescence pick} - \text{lowest reference fluorescence pick}}{\text{lowest reference fluorescence pick}}$$

As already shown by karyotype analyses (Hood, 2002; Hood *et al.*, 2003), genome size exhibited some variability even within species, so that even non-hybrid parental strains and intraspecific F1s could exhibit some excess in genome size compared with the reference strain.

Inability of F1s to produce infectious F2s

To investigate the ability of F1 hybrids to produce a viable diploid F2 progeny, we artificially inoculated plants with F1 teliospores, allowing their meiotic products to mate on the plants and give rise to infections. This experiment integrates all aspects of F1 hybrid sterility and F2 inviability (Fig. 2). Teliospores were suspended in 1 mL of sterile water, and concentration was adjusted to obtain approximately 2.8×10^{11} cells L^{-1}. For each hybrid, 1 mL of teliospore solution and between 50 and 70 seeds were spread on two Petri dishes and grown at room temperature. F1 hybrids obtained on *S. dioica* (with MvSd as one of the parents) were inoculated again on *S. dioica*, and F1 hybrids obtained on *S. latifolia* (with MvSl as one of the parents) were inoculated again on *S. latifolia*. To control for contamination between treatments, seeds were grown on two Petri dishes without teliospores for both plant species. Ten days later, 30 seedlings per treatment were planted in plastic pots (0.9 L volume per plant) and randomized in the greenhouse. Flowering plants were scored as either diseased (with the fungus sporulating in the flowers) or healthy (no symptoms, healthy flowers). All infected flower buds were collected and flowering plants were segregated to avoid secondary disease transmission. No control plants that were germinated on medium without sporidia became infected, indicating the lack of between-treatment contaminations in the greenhouse.

Genotype of F2 hybrids

DNA was extracted from F2 teliospores as described for F1-produced sporidia and genotyped using 29 microsatellite markers chosen for their ability to discriminate at least one pair of hybrid's parental species (Table S1). F1 hybrids should be heterozygous at all loci discriminating the parents, while recombination can take place before the production of F2s, leading to homozygosity for half of the loci on average under neutrality. However, as mating can occur only between sporidia of opposite mating types, heterozygosity will be preserved at all loci linked to

the mating type after a selfing event, and recombination is suppressed along the most part of the chromosome carrying the mating-type locus in *Microbotryum* (Hood, 2002). Moreover, because of the high prevalence of intra-tetrad mating and first division segregation of mating type, heterozygosity can also be preserved in *Microbotryum* at all loci linked to autosomal centromeres (Hood & Antonovics, 2004). A high proportion of heterozygous markers was therefore expected in the F2 generation, unless a strong selection favoured the rare inter-tetrad mating events that would produce homozygous individuals. Plants were incubated within a solution containing more than 2.8×10^{11} teliospores so that strong selection for the fittest F2 genotypes could indeed take place. We thus scored the percentage of heterozygous markers in the F2s and, when homozygosity was detected, the parental provenance of the allele was determined by comparison with parental genotypes. As the parents of the F1 were genotyped, the alleles found in the F2 generation produced in the greenhouse could easily be assigned to the parental species. We investigated whether F2s more strongly resembled just one of the parental types as the genetic distance between the parents increased. Such a selection for one of the parental genotype could indeed occur because of genetic or karyotypic incompatibilities between the genomes of the different species and/or because of selection imposed by the host environment on which the pathogen species had evolved. This analysis was only performed on F2s obtained on *S. latifolia*, because there was no successful F2 infection on *S. dioica* by crosses between distant parents.

Quantification of RI

We quantified several components of reproductive isolation (RI) according to the index proposed by Coyne & Orr (1989), as in Le Gac *et al.* (2007b):

$$\mathrm{RI} = 1 - \frac{\text{proportion of successful inter-specific crosses}}{\text{proportion of successful intra-specific crosses}}$$

This index usually varies between 0 (no RI) and 1 (complete RI), but in some cases may be negative if interspecific crosses are more successful than intraspecific crosses. When multiple intraspecific crosses were considered, the most successful one was used as the reference for the calculation of RI. The reproductive barriers studied here were named RI-F1-1 (inability of F1-produced gametes to conjugate) and RI-F1-Tot (inability to produce a diploid F2 progeny in host plants). Note that RI-F1-Tot contains all components of reproductive isolation, including RI-F1-1 (Fig. 2).

Genetic distance between species

The genetic distances were calculated using the 700-bp sequence of the γ-tubulin nuclear gene (Le Gac *et al.*, 2007a). A pairwise distance matrix was obtained using the Kimura two-parameter model (Kimura, 1980) and the genetic distance between species was calculated as the average distance among the different strains within species, using the software MEGA (Kumar *et al.*, 2001). We used the single gene for which we had sequences for all the strains involved in the present study. To check that the use of a single gene did not bias the genetic distance estimations, we compared the genetic distances obtained based on the sequences of the γ-tubulin, the β-tubulin and the EF1α elongation factor (Le Gac *et al.*, 2007a) for the species pairs with available sequences. The correlations between all three distances were highly significant and the correlation coefficients were close to one (between γ-tubulin and β-tubulin: $r = 0.987$, $P < 0.00001$; between γ-tubulin and EF1α: $r = 0.990$, $P < 0.00001$).

Statistical analyses

When testing for a relationship between reproductive isolation and genetic distance by using the same species in multiple pairs, two problems must be faced (Fitzpatrick, 2002): (i) phylogenetic non-independence, because of common ancestry and (ii) statistical redundancy. To handle the phylogenetic non-independence, we used phylogenetically independent contrasts (Felsenstein, 1985) following the method of nested average (Fitzpatrick, 2002; Fitzpatrick & Turelli, 2006; see Le Gac *et al.*, 2007b, for more details on the method used and its application for the case of reproductive isolation in *Microbotryum*). The statistical redundancy in the matrix conjugation experiment (i.e. the same species are involved in multiple crosses) should not be a problem if it is safe to assume that within-clade divergence is independent of between-clade divergence (Fitzpatrick, 2002). Furthermore, properly nested averaging makes the method somewhat robust to violation of this assumption (Omland, 1997). We also performed analyses on the complete data sets because of the high karyotype variability in *Microbotryum*, both between and within species (Hood, 2002; Hood *et al.*, 2003). Indeed, at least part of the sterility in hybrid crosses probably results from karyotypic differences (see results). Independent chromosomal rearrangements that occurred in terminal branches of the phylogeny (not shared by multiple species pairs or even in different strains of the same species) should result in independent evolution of reproductive isolation between species pairs for different strains of the same two species, even if they are not phylogenetically independent.

As a result of the number of observations being relatively low because of weak hybrid viability, we could not test for normality. We therefore used the Spearman rank correlation test in R (R Development Core Team, 2006) to analyse the relationships between reproductive

isolation (inability to mate or to infect host plants) or genetic features of the hybrids (pheromone receptor content or genome size) and genetic distance between the parental strains. To test for homogeneity between repetitions of the conjugation experiment, we used χ^2 tests in Excel (Microsoft). To test for a deviation from a balanced sex ratio in strains from the F1 generation, we could not use χ^2 tests, because some expected values were below 5. We therefore used the Fisher exact tests, separately on hybrids and nonhybrids, using R (R Development Core Team, 2006).

Logistic regressions test whether a nominal variable can be explained by nominal, ordinal or and/or continuous explanatory variables. We used logistic regressions to test whether the inability of F1 gametes to mate (nominal variable) could be significantly explained by pheromone receptor content, genome size and/or genetic distance between parental species. We also wanted to test which of these three factors was a better predictor of the inability of F1 gametes to mate. To do so, we compared the percentage of variance that each of these factors was able to explain when included alone in the analysis, and we also included the factors sequentially in a forward analysis. Logistic regressions were performed using the software JMP (SAS Institute, 2004).

Results

F1 sterility

Inability of F1 gametes to mate

We obtained 10 to 20 (for crosses having yielded two infected plants) different gamete genotypes (=monosporidial colonies) from each of the fifteen available F1s and assessed their ability to mate by conjugation assay with their two parental gametes (a_1 and a_2). Most (69%) of the gametes conjugated with only one of their parental gametes (a_1 or a_2), and were therefore classified as being either a_1 or a_2. One gamete was able to conjugate with both mating types and with its clones. Interestingly, it was a gamete from an F1 obtained by crossing the most distant species, MvSd and MvSv1 (Table 1). Thirty-seven of the gametes (30%) were unable to conjugate, neither with the a_1 or a_2 parent nor with their clones, and all of them were the products of interspecific crosses. The proportion of F1 gametes unable to mate significantly increased with the genetic distance between the parental species for the complete data set (rho = 0. 77, P = 0. 003; Fig. 3a). The correlation on independent contrasts was not significant, but the Spearman rank correlation coefficient remained as high as on the complete data set (rho = 0.77, P = 0.222). The sterility of gametes

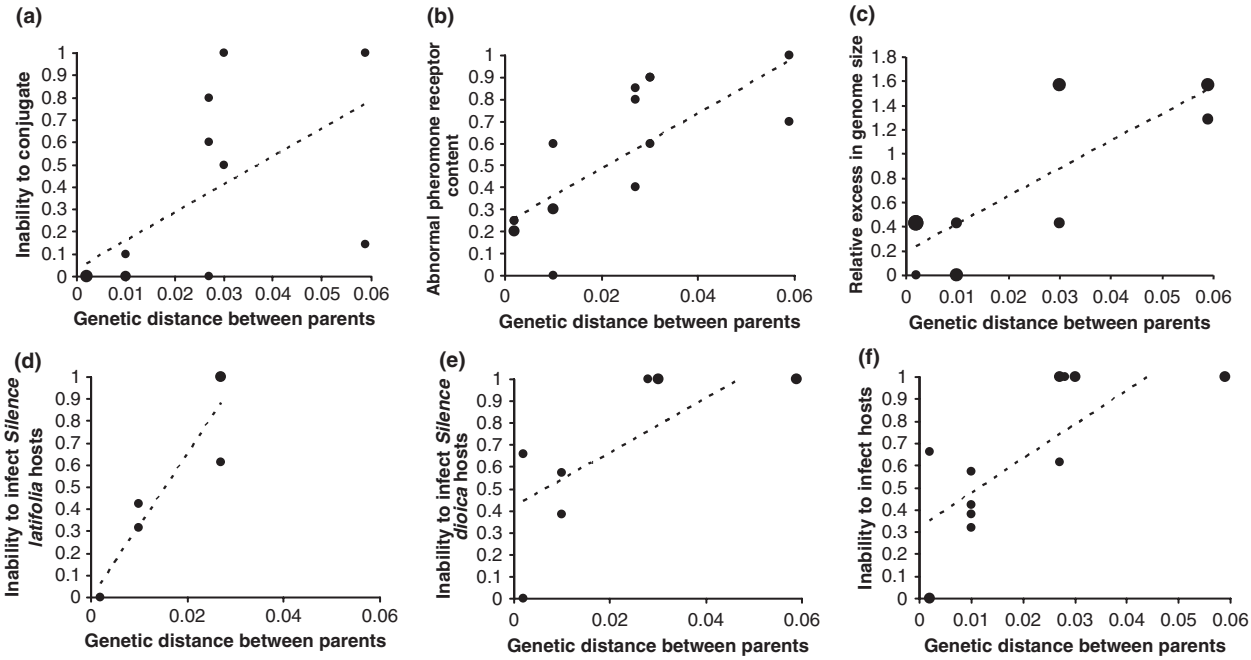

Fig. 3 Evolution of different reproductive barriers with increasing genetic distance between parents. (a) Evolution of F1 sterility, in the form of inability of viable F1-produced gametes to mate; (b) evolution of the abnormal pheromone receptor content of viable F1-produced gametes; (c) evolution of the relative excess of genome size of viable F1-produced gametes; (d) inability of F2 hybrids having MvSl as one of their parental strains to infect *Silene latifolia*; (e) inability of F2 hybrids having MvSd as one of their parental strains to infect *S. dioica*; (f) pooled data from (d) and (e). The size of the dots is proportional to the number of points that are superimposed.

deriving from crosses between closely related species (MvSl and MvSd) remained low (Fig. 3a).

Considering only the gametes that conjugated with a single one of their parental gametes (a_1 or a_2) and that were therefore classified as being either a_1 or a_2 (Table 1), we tested for a deviation from a balanced sex ratio in F1 strains. The deviation was marginally significant in intra-specific crosses (Fisher exact test, $P = 0.06$), while it was highly significant in hybrids (Fisher exact test, $P = 1.322e\text{-}10$). The sex ratio bias was not always in the same direction, either a_1 or a_2 gametes being over-represented. Interestingly, the over-represented mating type was the one from MvSl in hybrids involving this species as a parent.

Abnormal segregation as shown by gamete genotypes at the pheromone receptor gene

We tested whether F1 gametes had an abnormal genotype for the pheromone receptor of the mating-type locus, i.e. either two or zero alleles instead of one allele. For most of the gametes unable to conjugate (84%), both pheromone receptors were detected by PCR and none was identified as lacking a pheromone receptor. Among the hybrid gametes capable of conjugating, 34% carried both pheromone receptors, 63% carried a single one (which corresponded to the observed phenotype) and 3% carried none. The proportion of gametes exhibiting abnormal segregation pattern at the mating-type locus, i.e. having two or no pheromone receptor, was positively correlated with the genetic distance between the parents for the complete data set (rho = 0.83; $P = 0.0002$; Fig. 3b) and this correlation was marginally significant when using independent contrasts (rho = 0.95, $P = 0.0513$). The abnormal segregation of the pheromone receptor alleles was thus more frequent when parental strains were more distant, and remained low for parental strains from closely related species (MvSl and MvSd).

Genome size of F1 gametes

We carried out flow cytometry analyses on 20 randomly chosen F1 gametes from five different crosses (Table 2). All gametes from F1 crosses obtained with strains of the same or closely related species exhibited a genome size similar to that of parental sporidia. In contrast, gametes resulting from the most distant crosses all exhibited abnormally large genomes. The correlation between relative excess in genome size of the gametes and genetic distance between the parental strains was significant for the complete data set (rho = 0.71; $P < 0.001$; Fig. 3c). The test was not significant when conducted on independent contrasts but the Spearman rank correlation coefficient was even higher (rho = 0.80, $P = 0.333$). This tendency confirms that abnormal meiotic segregation in

Table 2 Genome size and corresponding phenotypes and genotypes of 20 randomly chosen gametes having MvSd as one of the parental strains.

F1 sporidia	Genetic distance between parental strains	Relative excess of genome size	Pheromone receptors (gene content)	Abnormal pheromone receptor content	Conjugation phenotype	Inability to mate
Sd1 1	0.002	0.43	a_1	n	a_1	n
Sd1 2	0.002	0.43	a_2	n	a_2	n
Sd2 1	0.002	0.43	a_1	n	a_1	n
Sd2 2	0.002	0.43	a_1	n	a_1	n
Sd2 9	0.002	0	a_1 a_2	y	a_1	n
Sd3 1	0.01	0.43	a_1	n	a_1	n
Sd3 2	0.01	0	a_1	n	a_1	n
Sd3 3	0.01	0	a_1	n	a_1	n
Sd3 4	0.01	0.43	a_1	n	–	y
Sd3 5	0.01	0	a_1	n	a_1	n
Sd5 1	0.03	1.57	a_1 a_2	n	–	y
Sd5 2	0.03	1.57	a_1 a_2	y	n.i.	n.i.
Sd5 3	0.03	0.43	a_1	n	a_1	n
Sd5 4	0.03	0.43	a_1	n	a_1	n
Sd5 5	0.03	1.57	a_1 a_2	y	a_1	n
Sd7 2	0.059	1.57	a_1 a_2	y	n.i.	n.i.
Sd7 3	0.059	1.29	a_1 a_2	y	–	y
Sd7 4	0.059	1.57	a_1 a_2	n	–	y
Sd7 5	0.059	1.57	a_1 a_2	y	–	y
Sd7 6	0.059	1.29	a_1 a_2	y	n.i.	n.i.

Relative excess of genome size was calculated according to the lowest fluorescence level obtained for control strains. n.i., indicates 'not identified'.

F1s increased with the genetic distance between the parental strains. Hybrids between moderately distant species generated both gametes with normal and abnormal genome sizes (see sd5 resulting from a cross between MvSd and MvLfc, in Table 2).

Relationship between inability of F1 gametes to mate, genome size and genotype at the pheromone receptor gene

To test which factor among pheromone receptor content – genome size or genetic distance – was a better predictor of the inability of F1 gametes to mate, a stepwise forward logistic regression, as well as three individual logistic regressions, were performed on the 17 gametes for which all data were available. In logistic regressions considering a single factor, genetic distance had a slightly better explanatory power ($R^2 = 0.444$; $P = 0.034$) than genome size ($R^2 = 0.429$; $P = 0.019$) and a much better explanatory power than pheromone receptor content ($R^2 = 0.049$; $P = 0.315$). The stepwise forward logistic regression including all three factors also indicated that genetic distance was the variable explaining best the ability to conjugate.

F2 inviability

Inability of F2s to infect host plants

Of the six F1s (inter- and intra-specific crosses) obtained on *S. latifolia* and inoculated again on *S. latifolia*, four yielded infected plants with F2 teliospores: the intraspecific MvSl × MvSl, the two hybrids resulting from the crosses between close species MvSl × MvSd and a single one of the three hybrids resulting from the cross between more distant species MvSl × MvLfc (Table 1). When infection was present, the highest rate was for the MvSl × MvSl intra-specific F1 (20%), the others ranging between 13.64% (MvSl × MvSd-3) and 7.69% (MvSl × MvLfc-1). We detected a significant correlation between the degree of reproductive isolation (RI-Tot-F1, i.e. proportion of infection failure by F2 inter-specific hybrids when compared with intra-specific F2s, Fig. 2) and the genetic distance between the parents (rho = 0.94, $P = 0.005$; Fig. 3d) for the complete data set. When using independent contrasts, the Spearman rank correlation coefficient remained high but the test was nonsignificant (rho = 1, $P = 0.333$).

From the seven F1 hybrids obtained on *S. dioica* and inoculated again on *S. dioica*, two yielded infected plants with F2 teliospores: MvSd × MvSl-1 (14% of infected plants) and MvSd × MvSl-2 (11% of infected plants). The two intra-specific F1 crosses MvSd × MvSd-1 and MvSd × MvSd-2 also yielded infections with 25% and 10% of diseased plants, respectively (Fig. 3e). The most distant crosses, MvSd × MvSn, MvSd × MvLfc and MvSd × MvSv1, yielded no F2 hybrids at all. As for the experiment on *S. latifolia*, a significant positive correlation was observed between total reproductive isolation and the genetic distance between the parents for the complete data set (rho = 0.84, $P = 0.005$), as well as for independent contrasts, at least marginally (rho = 0.950, $P = 0.0513$; Fig. 3e).

The proportions of plants infected by the intra-specific crosses MvSl × MvSl and MvSd × MvSd on the corresponding host plants were similar. We pooled the results of the two experiments (on *S. latifolia* and *S. dioica*, respectively; Fig. 3f). The correlation between total reproductive isolation and the genetic distance between the parents still held and was even more strongly supported (rho = 0.86, $P < 0.001$ for the complete data set; rho = 0.97, $P = 0.005$ when done on independent contrasts). As shown previously for F1 hybrids (Le Gac *et al.*, 2007b), post-mating reproductive isolation in F2 hybrids thus significantly increased with the genetic distance between the parents.

F2 genotypes

Twenty-nine microsatellite markers were found capable of discriminating at least one pair of hybrid parents in the set of hybrids on *S. latifolia*. The F2 genotypes were characterized as being either heterozygous or homozygous for these markers, and in the case of homozygosity, the parental *Microbotryum* species that was the contributor of the retained alleles was determined by comparing with the scored genotypes of the parents (Fig. 4). The intra-specific F2 cross MvSl × MvSl was heterozygous for all markers whereas all F2 hybrids had at least some homozygous markers. The proportion of homozygous markers increased with the genetic distance between the parents, with MvSl × MvSd F2 hybrids having between 36.4% and 13.3% of homozygous loci and MvSl × MvLfc F2 hybrids between 100% and 87.5% of homozygous loci. While selfing to produce the F2 generation should result, on average, in a 50% loss in heterozygous loci to homozygosity, Fisher's exact tests showed that the proportion of homozygous loci was significantly higher than 50% for the crosses MvSl × MvLf-1A and B ($P = 0.027$ and 0.002, respectively), and significantly lower than 50% for MvSl × MvSd-2C, 2B, 2A and 1C ($P = 0.020$, 0.026, 0.048 and 0.025, respectively). The test was not significant for MvSl × MvSd-1 A and B.

Increasing the genetic distance between parents of hybrids also seemed to influence the way parental loci were distributed in the F2 hybrids: among MvSl × MvSd F2 hybrids obtained on *S. latifolia* the per cent homozygous loci identical to the MvSl parent varied widely from 25% to 100%, showing no preferential representation of any of the parental genomes. In contrast, MvSl × MvLfc F2 hybrids had approximately 92% of their homozygous loci originating from the MvSl parent, suggesting that a selection occurred for retaining this parental genome. It therefore seems that F2 hybrids from more distant crosses retained more alleles from the fungal species naturally infecting the plant onto which they were passaged.

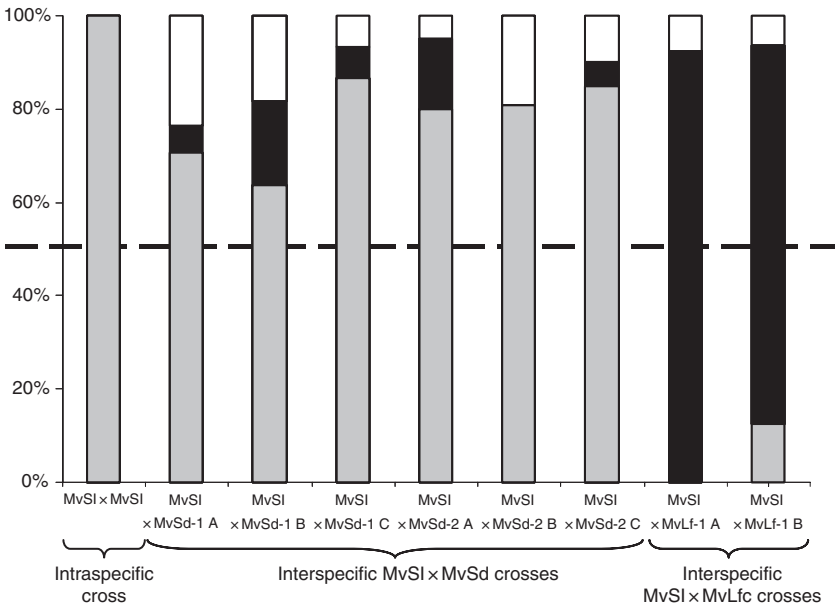

Fig. 4 Purge in F2 hybrids. Proportion of loci being either heterozygous (grey), or homozygous, with the allele from the parent adapted to the plant on which the inoculation was performed (black), or with the allele from the other parental strain (white). The dotted line show the expected average 50% loss in heterozygous loci to homozygosity by selfing to produce the F2 generation.

Discussion

Our aim in this study was to identify other reproductive barriers among *Microbotryum* species besides the high level of selfing by intratetrad mating (Giraud *et al.*, 2008b), partial ecological isolation (Van Putten *et al.*, 2007) and F1 hybrid inviability, and to elucidate which ones were the most important at early stages of divergence. We therefore investigated some traits linked to F1 sterility and F2 inviability, and some of the possible underlying mechanisms, as a function of the genetic distance between the parental species. Because few F1 hybrids and even fewer F2 hybrids were viable, sample sizes were low for some tests using independent contrasts. As a result, some correlations with genetic distance were nonsignificant even with high Spearman rank correlation coefficients; nevertheless, the tendencies appeared robust. Moreover, as we showed that karyotypic rearrangement (as suggested by abnormal genome sizes) was significantly correlated with sterility, and as there is a high karyotypic variability both between and within species (Hood, 2002; Hood *et al.*, 2003), analyses based upon the complete data sets may be appropriate: the sterility in crosses involving different strains from the same species, or involving phylogenetically nonindependent species pairs, may indeed still have an independent origin.

F1 sterility and underlying mechanisms

The proportion of F1 gametes unable to mate increased with the genetic distance between the parents, indicating that hybrid sterility increased with genetic distance between species. Here, we investigated only the mating behaviour of haploid genotypes that could grow in culture, but further sterility can arise from the inability to produce viable haploids. Previous studies have in fact shown that the proportion of teliospores capable of germinating and producing sporidia dramatically decreased in *Microbotryum* hybrids (Sloan *et al.*, 2008; M.E. Hood, unpublished data). Altogether, these results indicate that F1 sterility increases with genetic distance among *Microbotryum* species.

Cell fusion for mating in basidiomycete fungi basal to the homobasidiomycetes is regulated by interactions between pheromones and complementary receptors present at the mating-type locus (Bakkeren & Kronstad, 2007). Inability to conjugate in gametes produced by *Microbotryum* hybrids could therefore be due to a complete lack of the mating-type locus in the gametes as the result of abnormal segregation at meiosis. This would however be surprising because most of the examined *Microbotryum* species have dimorphic sex chromosomes, nonrecombining over much of their length and carrying the mating-type locus, in addition to other genes (Hood, 2002, 2005). Absence of the pheromone receptor gene in a gamete would therefore mean that the cell lacks sex chromosome and would probably be inviable. The sex chromosomes indeed harbour essential genes for haploid growth, as evidenced by the presence of deleterious alleles linked to the mating-type locus that prevent the growth of sporidia (Hood & Antonovics, 2000; Thomas *et al.*, 2003). In fact, here we identified few gametes with no pheromone receptor allele. Instead, the inability to conjugate seemed to correlate with the abnormal presence of two alleles in the haploid gametes. The sterility could therefore be due to a deregulation of the mating-type genes because of the aneupolyploidy of the gametes,

having two sex chromosomes instead of a single one. However, the inability to conjugate was much better explained by genetic distance or genome size than by pheromone receptor content, suggesting that factors other than the sole ploidy at the mating-type locus contributed to sterility.

Hybrid sterility has been shown to be caused by chromosomal differences between species (Delneri *et al.*, 2003), creating unbalanced gametes during F1 meioses, and/or by genetic incompatibilities (Fishman & Willis, 2001; Demuth & Wade, 2007), i.e. epistatic incompatibilities between alleles of the two species present at different loci (Orr, 1995; Barbash *et al.*, 2003). The two phenomena fuel each other as higher nucleotide diversity accumulates around rearranged chromosome areas, as shown in *Helianthus* and in *Sorex* (Lai *et al.*, 2005; Basset *et al.*, 2008), and as DNA sequence divergence decreases the propensity for chromosome pairing. High karyotypic variability among and within *Microbotryum* species has been described (Perlin, 1996; Hood *et al.*, 2003, 2004), as well as low hybrid gamete viability (Sloan *et al.*, 2008), suggesting that chromosome mispairing occurs in hybrid meioses. We support this idea, by showing that the viable F1 gametes resulting from distant crosses are unbalanced, having genome size close to twice the expected size. We cannot however exclude that genetic incompatibilities further contribute to hybrid sterility in *Microbotryum*.

We observed a sex-ratio bias in the F1 hybrids, their diploid teliospores producing significantly more a_1 or a_2 than the balanced ratio expected due to the heterozygosity of teliospores for the mating type. High frequencies of strains with biased sex ratio have previously been reported in natural populations of *Microbotryum*, which has been shown to be caused by the presence of deleterious alleles linked to the mating-type locus that prevent the growth of haploid sporidia (Hood & Antonovics, 2000; Thomas *et al.*, 2003). These alleles can be maintained thanks to early intra-tetrad matings and possibly population-level selection (Hood & Antonovics, 2000; Tellier *et al.*, 2005). This phenomenon was however not expected here as the F1 generation analysed was generated using sporidia that had successfully been grown *in vitro*, and thus did not carry such deleterious alleles. The mating type ratio significantly deviated from 50 : 50 only in hybrids, suggesting that the sex-ratio bias may result from abnormal meioses caused by karyotytpic rearrangements that render meiotic products unbalanced. An alternative explanation may be that sporidia in some species grow faster than in other species and that the genetic determinism of growth rate is linked to the mating type. This would bias the representation of the mating type in colonies budding off teliospores (Fig. S1). In fact, sporidia from MvSl usually grow faster *in vitro* than those of other *Microbotryum* species (G. Refrégier, pers. obs.) and the over-represented gametes in our F1 hybrids were precisely those from the MvSl parent. Further experiments are however clearly required to understand the sex-ratio bias in hybrids.

F2 inviability and underlying mechanisms

In addition to F1 sterility, we found that F2 hybrids had a lower ability to infect plants than F2 resulting from intraspecific crosses, and that this inability increased with genetic distance between the parents. For hybrids between pathogen species adapted to different environments (such as to different hosts), poor hybrid viability can be due to extrinsic ecological isolation, i.e. the hybrids are ill-suited to parental environments (because the hybrids carry half a genome that is not adapted to the host on which it is inoculated; Rundle & Nosil, 2005), or to genetic incompatibilities between the genomes from the two different species (Orr, 1995). Sloan *et al.* (2008) observed that hybrids between different *Microbotryum* species had infection successes and proportions of incomplete symptoms on a given host plant that were intermediate between that of the parental species adapted to this host and that of the parental species not adapted to that host (from cross-inoculations). This suggested that extrinsic poor suitability accounted for the low infection capability of hybrids, although an additional role of intrinsic genetic incompatibilities cannot be excluded.

F2 hybrids could not be obtained from the most distant species for which we had F1 hybrids. Genotyping with microsatellites revealed that the F2 hybrids from the most distant species that we did obtain were highly homozygous, having preferentially retained the alleles from the *Microbotryum* species originating from the host on which experimental passaging was performed. This suggests that selection occurred among the pool of different meiotic products derived from the F1 teliospores, such that those gametes bearing a majority of alleles belonging to the parental home-type for that host plant were favoured. This calls to mind the selection arena hypothesis (Bruggeman *et al.*, 2004) with selection occurring among numerous progeny, even before competition with other congeners within the population as a whole. The fact that many teliospores of a diploid individual are usually deposited onto the same plant (Bucheli & Shykoff, 1999) allows such competition to occur among the progeny in *Microbotryum*. This selection arena weeds out the most unfit combinations of hybrids before they can participate in the next generation. This phenomenon should contribute to preventing introgression between moderately distant *Microbotryum* species, by allowing only the F2 hybrids with a purged genome (i.e. more homozygous) to occur. Because we can see this effect on numerous microsatellite markers, the selection seems to occur at many loci, which suggests that specialization loci and/or incompatible loci are widely spread across the whole genome.

Interestingly, the F2s resulting from intra-specific crosses or from crosses between close species (MvSl and MvSd) had retained significantly more heterozygous loci than the 50% expected. This is probably due to early intra-tetrad mating conjugations in *Microbotryum*, that are known to preserve heterozygosity at all loci linked to centromeres and to mating-type loci (Hood & Antonovics, 2004).

Evolution of the different types of reproductive barriers

Our results, together with previous studies, showed that different types of barriers acting at various stages of the life cycle can contribute to reproductive isolation. These include ecological isolation (Shykoff *et al.*, 1999; Van Putten *et al.*, 2007), high selfing rate (Giraud, 2004; Giraud *et al.*, 2005, 2008b), low infective ability of F1 hybrids (Le Gac *et al.*, 2007b), F1 sterility (this study, Sloan *et al.*, 2008), low infective ability and genomic purging of F2 hybrids (this study). The maintenance of species boundaries through the accumulation of different types of barriers to gene flow has been shown in other organisms such as monkeyflowers (Ramsey *et al.*, 2003), reef fishes (Crow *et al.*, 2007) or darters (Mendelson, 2003; Mendelson *et al.*, 2007).

To understand the process of speciation, it is however of paramount importance to elucidate which barriers arose first by looking at their evolution over time. Many barriers can indeed arise after the cessation of gene flow (Schemske, 2000). Our results, together with those obtained previously (Le Gac *et al.*, 2007b), showed that the different kinds of post-mating barriers (F1 inviability, F1 sterility and F2 inviability) in fact increase with genetic distance among *Microbotryum* species, which suggests the progressive accumulation of genetic incompatibilities, karyotypic differences and/or specialization. The apparent lack of hybrids in nature (Bucheli *et al.*, 2000) suggests that other barriers must act efficiently earlier in the life cycle. These early barriers would prevent gene flow among incipient species and allow the accumulation of genetic incompatibilities, karyotypic differences and/or specialization. We have not yet identified any premating barriers that would in themselves completely prevent gene flow, but the cumulative action of partial ecological isolation and high selfing rates may be sufficient, ensuring reproductive isolation among close species and thus the accumulation of genetic differences, leading to the appearance of post-mating barriers. Further work in contact zones between close *Microbotryum* species should help elucidate the most important barriers to gene flow in nature in the early stages of speciation. Van Putten *et al.* (2005) studied a zone of sympatry between MvSl and MvSd using only two microsatellite markers and it was not clear whether true hybrids were present. Only allelic diversity at each locus was considered and it is not clear whether hybrid genotypes were present. We are currently studying additional zones of sympatry, using numerous molecular markers, to determine if hybrids are formed in nature between close species and, in such a case, if we can find F1, F2 and/or subsequent hybrid generations.

The post-mating barriers appeared to be strong for moderately distant species, with high levels of inviability and sterility, high purging of one of the parental genomes, and with no F2 genotypes produced. It is also interesting to compare the evolutionary rates of the different kinds of post-mating barriers. Our results suggest that F2 inviability evolved more rapidly than F1 inviability, as F1 hybrids could be obtained for large genetic distances (Le Gac *et al.*, 2007b) for which F2 hybrids could not be obtained here (for the crosses MvSd × MvSn, MvSd × MvLfc, MvSd × MvSv1). This may be caused by recessive alleles that are masked in F1 hybrids but are revealed in F2 hybrids. Such lower performance of the F2 hybrids, already observed in *Mimulus* (Fishman & Willis, 2001), may therefore be due to a higher rate of accumulation of recessive than dominant genetic incompatibilities.

Conclusion

By documenting the evolution of hybrid inviability and sterility in the *Microbotryum* species complex, we showed that these barriers may rely at least partly on chromosomal rearrangements impairing meioses in hybrids, and probably also on recessive genetic incompatibilities. The post-mating barriers indeed appeared strong between moderately and highly distant species, with no F2 produced or with high levels of inviability and sterility. This should ensure definitive reproductive isolation, preventing gene flow even if premating barriers were to break down, for instance if higher levels of outcrossing evolved or if lower levels of ecological isolation occurred due to habitat disturbance. The post-mating barriers however remain weak among the closest species pairs, suggesting that premating barriers may be sufficient to initiate divergence among *Microbotryum* species.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Protocol for isolation of F1 gametes.

Figure S2 Flow cytometry analysis of one parental strain and five F1 gametes resulting from a MvSd × MvLfc cross. Corresponding phenotypes and genotypes of the gametes are specified for each gamete.

Table S1 Microsatellite markers used for F1 genotyping. Their ability to discriminate between parental strains for each type of hybrid is indicated by a '+'.

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