doi:10.1111/j.1558-5646.2007.00144.x

EVOLUTION OF REPRODUCTIVE ISOLATION WITHIN A PARASITIC FUNGAL SPECIES COMPLEX

Mickael Le Gac,[1,2,3] Michael E. Hood,[4,5] and Tatiana Giraud[1,6]

[1]*Ecologie, Systématique et Evolution, UMR 8079 CNRS-UPS-ENGREF, Bâtiment 360, Université Paris-Sud, F-91405 Orsay, France*

[2]*E-mail: LeGac@zoology.ubc. ca*

[4]*Department of Biology, Amherst College, Amherst, Massachusetts 01002*

[5]*E-mail: MHood@amherst.edu*

[6]*E-mail: Tatiana.Giraud@u-psud. fr*

Received March 27, 2006
Accepted March 12, 2007

Despite important advances in the last few years, the evolution of reproductive isolation (RI) remains an unresolved and critical gap in our understanding of speciation processes. In this study, we investigated the evolution of RI among species of the parasitic fungal species complex *Microbotryum violaceum*, which is responsible for anther smut disease of the Caryophyllaceae. We found no evidence for significant positive assortative mating by *M. violaceum* even over substantial degrees of genetic divergence, suggesting a lack of prezygotic isolation. In contrast, postzygotic isolation increased with the genetic distance between mating partners when measured as hyphal growth. Total RI, measured as the ability of the pathogen to infect and produce a diploid progeny in the host plant, was significantly and positively correlated with genetic distance, remaining below complete isolation for most of the species pairs. The results of this study, the first one on the time course of speciation in a fungus, are therefore consistent with previous works showing that RI generally evolves gradually with genetic distance, and thus presumably with time. Interestingly, prezygotic RI due to gamete recognition did not increase with genetic distance, in contrast to the pattern found in plants and animals.

KEY WORDS: **Assortative mating, ecological isolation, extrinsic isolation, hybrid inviability, hybrid sterility, life cycle, postmating, speciation.**

Speciation remains a central topic of evolutionary biology, but definitive answers on how reproductive isolation (RI) evolves have proven difficult to obtain, mostly because speciation is a slow process and is frequently unobservable in an experimental setting. The best evidence for a time course of speciation comes from the relationship between the strength of RI between pairs of taxa and their divergence times (i.e., using the surrogate measure of genetic distance) (Coyne and Orr 2004, p 72). Such comparative studies were first performed in *Drosophila* (Coyne and Orr 1989, 1997), showing that both prezygotic behavioral isolation and intrinsic postzygotic isolation, that is, hybrid inviability and sterility, evolved gradually with time. A positive relationship between RI and genetic distance was later reported in diverse taxa, such as other insects (Presgraves 2002; Christianson et al. 2005), frogs (Sasa et al. 1998), birds (Price and Bouvier 2002; Tubaro and Litjamer 2002; Lijtmaer et al. 2003), fishes (Mendelson 2003; Russell 2003; Bolnick and Near 2005), and angiosperms (Moyle

[3]Present address: Department of Zoology, University of British Columbia, Vancouver, BC V6T1Z4, Canada

et al. 2004). Surprisingly few empirical studies have compared the evolutionary rates of pre- and postzygotic barriers, three of them dealing with animals (Coyne and Orr 1989, 1997; Mendelson 2003; Christianson et al. 2005) and one with plants (Moyle et al. 2004). In all these studies, prezygotic RI was found to increase with genetic distance either at the same rate or faster than postzygotic RI.

Comparative studies have thus provided valuable insights into the evolution of RI. However, such studies remain too scarce, and virtually all of them deal with animal species. Investigating a greater diversity of biological models will help unravel the general mechanisms governing the evolution of RI. Here, we investigated the evolution of RI among species of the parasitic fungal species complex *Microbotryum violaceum*, as the first comparison for rates of evolution of both pre- and postzygotic barriers in any fungus. This basidiomycete morphospecies includes several cryptic species that are highly specialized on different host species (Le Gac et al. 2007). We analyzed (1) the prezygotic trait of positive assortative mating (gamete recognition) between different sibling species, (2) the postzygotic trait of hybrid inviability linked to the production of infectious hyphae after mating, and (3) total RI, measured as the ability to produce a diploid progeny in host plants. We aimed to investigate the strength of these different reproductive barriers and their evolution. The goals were to assess (1) whether the pattern of increasing RI with genetic distance, found to be general in plants and animals, would also be observed in a fungus, and (2) whether the rate of evolution of prezygotic isolation would also be similar or higher than the rate of evolution of postzygotic evolution, as found in other organisms.

Material and Methods

BIOLOGICAL MODEL

The basidiomycete fungal species complex *M. violaceum* is responsible for anther smut, a naturally occurring venereal disease of many insect-pollinated perennial plant species in the Caryophyllaceae (Thrall et al. 1993). The fungal life cycle is illustrated in Figure 1 (see also Day and Garber 1988). Diploid teliospores of *M. violaceum* are produced in the anthers of infected plants, thereby replacing the pollen. Deposited on a new host, teliospores undergo meiosis that leads to the production of haploid yeast-like sporidia of opposite mating types, named a1 and a2. These sporidia can be considered as the gametes of the fungus. Conjugation between sporidia of opposite mating types is required to initiate growth of dikaryotic infectious hyphae. It has recently been shown that *M. violaceum* is composed of several specialized cryptic species, that is, lineages evolving independently without nuclear gene exchange (Le Gac et al. 2007). The species within the *M. violaceum* complex will be referred to here as in Le Gac et al. (2007), based

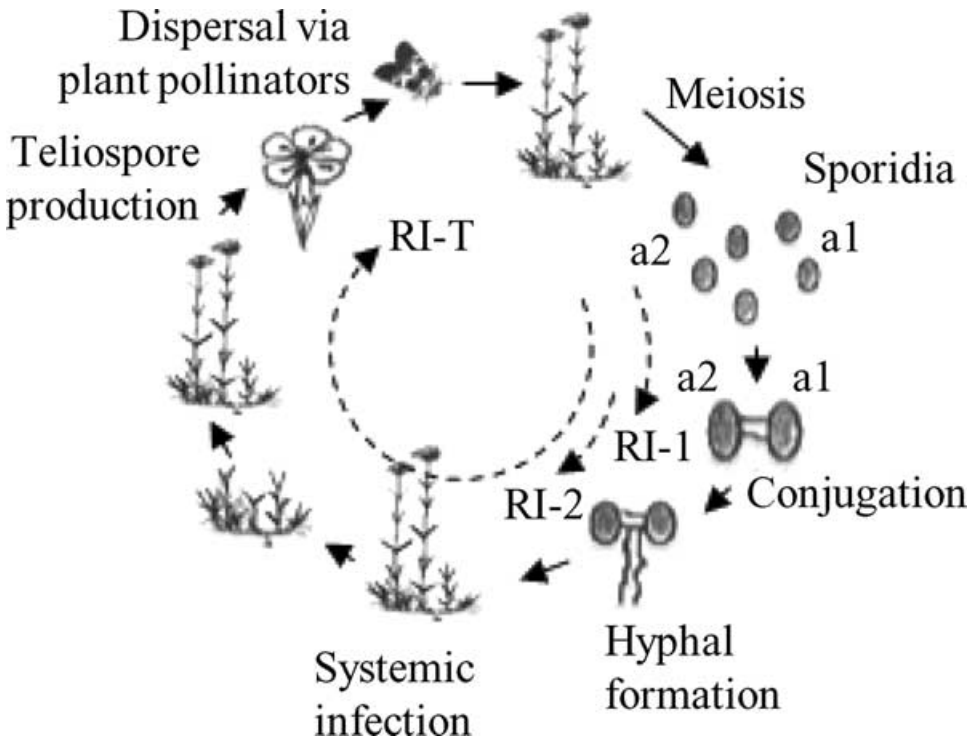

Figure 1. Life cycle of *Microbotryum violaceum*. Dotted arrows indicate the parts of the life cycle in which we investigated reproductive isolation. RI-1: inability to perform sporidial conjugation, RI-2: inability to produce of infectious hyphae, and RI-T: inability to infect the plant and produce a diploid progeny, corresponding to the total reproductive isolation measured in this study.

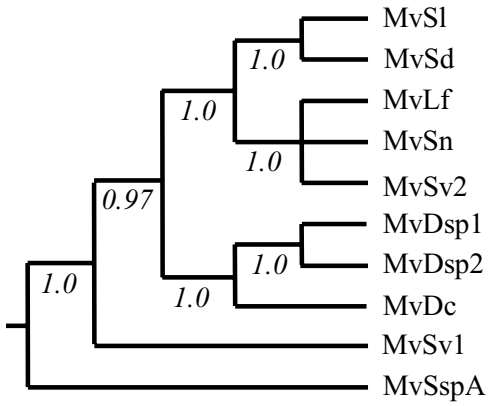

Figure 2. Phylogenetic relationships among the sibling species of the complex *Microbotryum violaceum* that are used in this study. Numbers in italic indicate the Bayesian posterior probabilities obtained using a 1746 bp concatenated dataset of three genes (Le Gac et al. 2007).

on their host species (Fig. 2). In particular, the species parasitizing the plant *Silene latifolia* is called MvSl and the one parasitizing *S. dioica* is called MvSd (see online Supplementary Appendix 1 or Le Gac et al. 2007).

We investigated the evolution of RI as a function of divergence time within the *M. violaceum* species complex, using the genetic distances among the species as an estimation of the relative times since divergence. The genetic distances were obtained using a 1746 bp concatenated dataset of three genes (Le Gac et al. 2007) and were calculated as the average distance between two taxa, as implemented in the Mega V2.1 software (Kumar et al. 2001). Following ModelTest v3.5 (Posada and Crandall 1998) results, genetic distances among species were calculated using a TRN+G model (gamma = 0.25).

Fungal strains for this study were collected on 11 host plants from nine localities in several regions of Western Europe and America between 2000 and 2004 (see online Supplementary Appendix 1) and stored at 4°C with silica gel. For the strains that had not been analyzed in Le Gac et al. (2007), at least one of the three genes was sequenced to ensure that they belonged to the fungal species corresponding to their host of collection. Fungal diploid teliospores were germinated and grown for one week at 23°C on GMB2 media (Le Gac et al. 2007). The resulting single-teliospore colonies were harvested individually, diluted, and then replated, yielding haploid single-sporidial colonies. Separate sporidial cultures were tested for conjugation with stock a1 and a2 sporidia (isolated from *M. violaceum* infecting *S. latifolia*) in 50 μL of sterile water. After 10 days at 4°C, sporidia suspensions were checked for conjugation under 400× magnification. Conjugations with either a1 or a2 sporidia were observed for all sporidia colonies, allowing their assignment to the a1 or a2 mating type.

Using the experiments described in the following sections, we quantified various components of RI according to the index proposed by Coyne and Orr (1989):

$$\mathrm{RI} = 1 - (\text{proportionofsuccessfulinterspecific crosses}/\text{proportionofsuccessfulintraspecificcrosses})$$

This index usually varies between 0 (no RI) and 1 (complete RI), but in some cases may be < 0 if interspecific crosses are more successful than the intraspecific crosses.

The reproductive barriers RI-1 (sporidial conjugation) and RI-2 (production of infectious hyphae) are independent. RI-T (ability to produce a diploid progeny in host plants) includes both RI-1 and RI-2 (Fig. 1) and may be considered as the total RI with regard to hybrid viability.

GAMETE RECOGNITION AMONG EIGHT M. VIOLACEUM SPECIES

We first investigated the evolution of RI due to gamete recognition (i.e., assortative mating) among eight *M. violaceum* species (RI-1, Fig. 1). A total of 323 crosses were performed, corresponding to four kinds of intraspecific and 26 kinds of interspecific crosses (see online Supplementary Appendix 2a). The full matrix of crosses was not performed because we could not obtain sporidia of both mating types for some species, probably due to mating-type-linked deleterious recessive mutations that occur commonly in lineages of this fungus (Hood and Antonovics 2000). Previous studies have shown that the addition of host extracts had no influence on the relative proportion of conjugations in vitro between different crosses (Kaltz and Shykoff 1999; Van Putten et al. 2003), supporting a lack of this potential form of environmental influence. Sporidia were therefore suspended in 1 mL of sterile water. Sporidial concentrations were determined using a Neubauer™ haemocytometer under 400× magnification. Each suspension was adjusted to obtain ca. 2.8×10^{11} cells. L^{-1}. Crosses were performed by pooling 25 μL of a1 and of a2 suspensions in ELISA plates that were stored at 4°C for 12 days. The proportion of conjugating sporidia was then counted. Because we isolated colonies of sporidia of a single mating type to perform the crosses, the observed conjugations could not reflect selfing: they could only be crosses between the two strains involved in the experimental cross. Moreover, plants inoculated with sporidia of a single mating type did not become infected (see below). Each of the 323 crosses was performed twice and results were pooled (i.e., for each cross, the sum of the number of conjugating sporidia in the two samples was divided by the sum of the number of sporidia in the two samples) after the repeatability of crosses was checked using a one-way analysis of variance (Zar 1984). The ANOVA was highly significant ($F_{(321,322)} = 6.62$, $P < 0.0001$), showing that conjugation was more similar between the two repeats of the same cross than among crosses, with an intraclass correlation coefficient of $r_i = 0.74$.

GAMETE RECOGNITION BETWEEN MVSL AND NINE OTHER SPECIES AND HYBRID HYPHAL GROWTH

We also investigated the evolution of gamete recognition and hyphal growth ability (RI-1 and RI-2, Fig. 1) by performing the conjugation tests in vitro. This included crosses of MvSl with intraspecific strains as well as with nine other species from the *M. violaceum* complex (see online Supplementary Appendix 2b). A total of 152 crosses were performed, the cross of a given species pair being performed multiple times, using different strains from the same species, from different populations (see online Supplementary Appendices 1 and 2b). Crosses were performed as above, except that sporidia were suspended in 1 mL of sterile water with α-tocopherol (2 g. L^{-1}), known to induce in vitro the formation of infectious hyphae after conjugation (Day and Garber 1988). We counted (1) the proportion of sporidia that were conjugating and (2) the proportion of conjugating sporidia that produced hyphae of a greater than one cell in length. Each cross was performed twice and results were pooled after the repeatability of crosses was checked ($r_i = 0.67, F_{(163,164)} = 5.1073, P < 0.0001$ for the conjugation test, $r_i = 0.95, F_{(150,151)} = 35.2393, P < 0.0001$ for the hyphae growth test).

INFECTION ABILITY BY HYBRIDS BETWEEN MVSL/MVSD AND NINE OTHER SPECIES

To investigate the ability of hybrids to infect host plants and produce a diploid progeny (i.e., total RI, RI-T, Fig. 1), we performed intra- and interspecific crosses followed by artificial inoculations, yielding 48 and 42 crosses involving MvSl and MvSd as the focal species, respectively (see online Supplementary Appendix 2). Sporidial concentrations were adjusted as indicated above. For each cross, 1.3×10^7 a1 and a2 sporidia and between 50 and 75 seeds from *S. latifolia* or *S. dioica* obtained from three natural populations for each plant species (different from the population from which the fungus had been isolated) were spread on two petri dishes and grown at 23°C. To control for contamination between treatments, some seeds were grown on two petri dishes without fungal sporidia or with sporidia of a single mating type. Ten days later, the most vigorous seedlings per cross (35 for *S. latifolia*, 25 for *S. dioica*) were planted into plastic pot containers (0.9 liter volume per plant) in the greenhouse. Plant positions were randomized with regards to their inoculation treatment, and plants were kept in the greenhouse until flowering. Flowering plants were scored as either diseased (with the fungus sporulating in the flowers) or healthy (with symptom less, healthy flowers). All infected flower buds were collected and flowering plants were segregated to avoid secondary disease transmission. None of the control plants that were grown on media without sporidia or with a single sporidia type (a1 or a2) was found to be infected, indicating the lack of between-treatment contaminations and the necessity of mating between a1 and a2 sporidia prior to infection.

The ability of the diploid teliospores, recovered from these infected buds, to produce viable meiotic products, which constitute the first step of hybrid fertility, was checked by plating a sample of the spores on GMB2 media and growing them at 23°C for ca. one week.

STATISTICAL ANALYSES

When testing for a relationship between RI and genetic distance by using the same species in multiple pairs, two problems must be faced (Fitzpatrick 2002): (1) phylogenetic nonindependence, due to common ancestry, and (2) statistical redundancy. To handle the phylogenetic nonindependence, phylogenetically independent contrasts are traditionally used (Felsenstein 1985). Following the method of nested average (Fitzpatrick 2002; Fitzpatrick and Turelli 2006), we used the phylogenetically independent comparisons to test for a correlation between RI and genetic distance (see online Supplementary Appendix 3). 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). Further, properly nested averaging makes the method somewhat robust to violation of this assumption (Omland 1997).

Statistical analyses were performed using JMP version 3.1.5 (SAS Institute). Data significantly deviated from normality (Shapiro-Wilk W test) and none of the transformations we tried improved the distribution. We therefore used nonparametric Spearman rank tests to investigate the existence of correlations between species divergence and RI.

Results

The correlations between the different RI measures and genetic divergence are shown in Figure 3. Both experiments evaluating the frequency of conjugation (i.e., involving the crossing matrix and the crosses involving MvSl with other species) yielded no evidence for significant increase in gamete recognition rates (RI-1, Fig. 1), even after substantial genetic divergence (Figs. 3A [i and ii], 3B [i and ii]). In contrast, the postzygotic trait of failure to produce infectious hyphae (RI-2, Fig. 1) increased with the genetic distance between mating partners (Figs. 3A [iii], 3B [iii]).

Regarding the inoculation experiments, 69.5% (n = 1680) of the *S. latifolia* plants inoculated had flowered within the eight months after their transfer to the greenhouse, and among them 4.9% were diseased. Regarding the inoculations of the host *S. dioica*, 37.5% (n = 1050) of the plants had flowered within the eight months after their transfer to the greenhouse, and among them 7.9% were diseased. On both hosts, total RI (RI-T, measured as the inability to produce a diploid progeny in the host plant) was significantly and positively correlated with genetic distance,

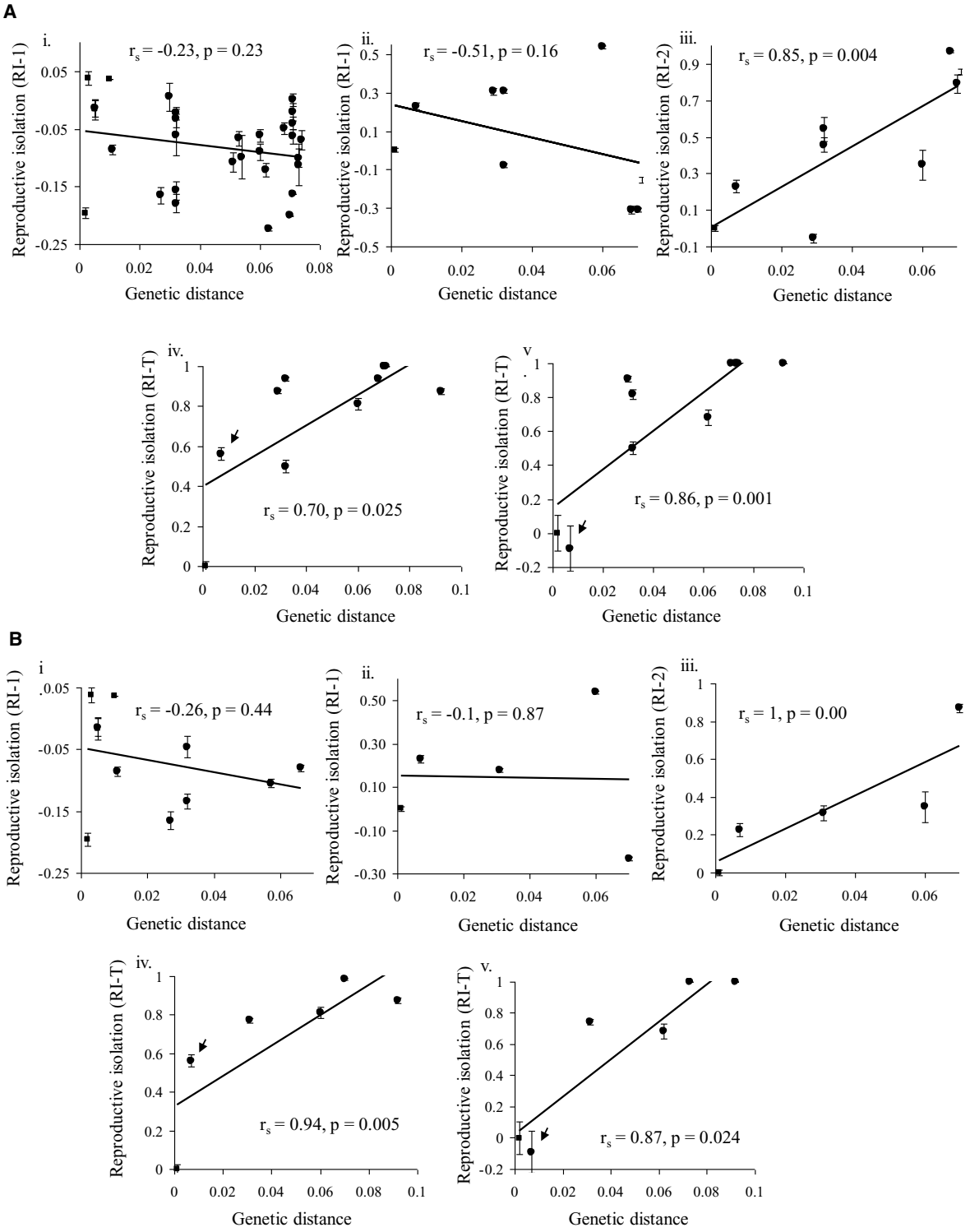

Figure 3. Reproductive isolation (RI) plotted against genetic distance among ten *Microbotryum* species, (A) considering the entire datasets, (B) considering the corrected datasets; (i) mean RI-1 (inability to perform sporidial conjugation) among the *Microbotryum* species according to the conjugation matrix experiment, standard errors indicated; (ii) mean RI-1 (inability to perform sporidial conjugation) within MvSl and between MvSl and the other *Microbotryum* species, standard errors indicated; (iii) mean RI-2 (inability to produce of infectious hyphae) within MvSl and between MvSl and the other *Microbotryum* species, standard errors indicated; (iv) RI-T (inability to produce a diploid progeny in host plants) within MvSl and between MvSl and the other *Microbotryum* species onto *S. latifolia*; (v) RI-T (inability to produce a diploid progeny in host plants) within MvSd and between MvSd and the other *Microbotryum* species onto *S. dioica*. Best-fit lines, Spearman rho, and *P*-values are indicated. Black squares correspond to intraspecific crosses. Arrows indicate the single interspecific cross inoculated on both hosts.

but remaining below complete isolation for most of the species pairs (Figs. 3A [iv and v], 3B [iv and v]).

Interestingly, the single type of interspecific cross that was inoculated on both host plants *S. latifolia* and *S. dioica*, MvSl × MvSd, appeared to display different infection success on the two host species. The ability of the MvSl × MvSd hybrid to infect *S. dioica* seemed as high as that of the intraspecific progeny MvSd × MvSd (Fisher's exact test, $P = 0.7$) whereas the infection proportion of the same MvSl × MvSd hybrid on *S. latifolia* was much lower than that of the intraspecific progeny MvSl × MvSl (Fisher's exact test, $P = 0.03$; see arrows on Figs. 3A [iv and v], 3B [iv and v]).

Samples of the diploid teliospores from each of the infected buds were plated on GMB2 media to investigate the first step of hybrid fertility. For all infected buds, numerous colonies of haploid sporidia grew, indicating viable meiotic products.

Discussion

The results of this study indicate that (1) prezygotic RI due to gamete recognition evolves much more slowly than postzygotic RI, as there was no evidence for a significant increase in assortative mating rates, even after substantial genetic divergence; (2) postzygotic RI involving the inability to produce infectious hyphae increased with genetic distance; (3) the total RI, measured here as the ability to cause disease and to produce a progeny, also increased with genetic distance; (4) at least some teliospores from each hybrid infection were able to produce viable meiotic products.

As in all other comparative studies investigating the evolution of postzygotic RI, we found that hybrid inviability in *M. violaceum* increased with genetic distance, and thus presumably with time since lineage isolation. The significant correlation between genetic distance and hybrid inviability is consistent with most speciation theories in which RI is the by-product of gradual genetic divergence. The increase in RI due to inability of hybrids to produce infectious hyphae in vitro is probably due to an increase in intrinsic RI (genetic incompatibilities independent of the environment; e.g., Orr 2001; Coyne and Orr 2004; Bolnick and Near 2005). Extrinsic RI (RI due to genes performing more poorly in some environments than in others, Rundle and Whitlock 2001) is probably also involved in *M. violaceum*, as illustrated by the differences in success of the MvSl × MvSd hybrid on the two hosts tested, *S. latifolia* and *S. dioica*. The lack of a decrease in hybrid success of MvSl × MvSd on *S. dioica* hints that genetic incompatibilities may not be very important to the viability of hybrids at this small genetic distance. Further experiments involving backcrosses of F1 hybrids would allow the more rigorous documentation of extrinsic RI (Rundle and Whitlock 2001).

We examined the first step of hybrid fertility, by spreading hybrid teliospores onto petri dishes. Numerous fungal haploid colonies grew, indicating that at least some meioses occurred and that at least some of the meiotic products were viable in each kind of cross. However, the protocol used did not allow the determination of whether some hybrid teliospores did not germinate and for the germinating ones, whether all four meiotic products were viable and able to conjugate. Studies are under way to analyze hybrid sterility within the *M. violaceum* complex more thoroughly.

In conclusion, this study is the first to investigate the rates of pre- and postzygotic isolation in a fungus and one of the too few investigations into the time course of speciation. The results are consistent with previous works showing that RI generally evolves gradually with genetic distance, and thus presumably with time. Interestingly, prezygotic RI due to gamete recognition evolved much more slowly than postzygotic isolation, in contrast to the pattern found in other organisms. This may be due to some specificities of fungal life cycles (Giraud et al. 2006; Giraud 2006). However, more studies on a greater variety of models are required to unravel the general mechanisms governing the evolution of RI.

ACKNOWLEDGMENTS

We thank O. Jonot, L. Saunois, O. Cudelou, R. Yockteng, C. Raquin, B. Wenden and T. Eglin for their help with plant care, in vitro cultures and conjugation experiments. We thank A. Thomas, M. Lopez-Villavicencio, J. Enjalbert, C. Collin, A. Widmer, and M. Bartoli for help with teliospore collection. We thank J. A. Shykoff and O. Kaltz for helpful discussions and comments on previous versions of the manuscript and B. M. Fitzpatrick, J. A. Shykoff, E. Klein, F. Rousset, F. Austerlitz and O. Martin for help with statistical analyses. We thank D. Funk and three anonymous referees for constructive comments. This research was funded by an ACI JC from the French Ministère de la Recherche.

LITERATURE CITED

Bolnick, D. I., and T. Near. 2005. Tempo of hybrid inviability in centrarchid fishes (Teleostei: Centrarchidae). Evolution 59:1754–1767.

Christianson, S. J., J. G. Swallow, and G. S. Wilkinson. 2005. Rapid evolution of postzygotic reproductive isolation in stalk-eyed flies. Evolution 59:849–857.

Coyne, J. A., and H. A. Orr. 1989. Patterns of speciation in Drosophila. Evolution 43:362–381.

———. 1997. "Patterns of speciation in Drosophila" revisited. Evolution 51:295–303.

———. 2004. Speciation. Sinauer Associates, Sunderland, MA.

Day, A. W., and E. D. Garber. 1988. *Ustilago violacea*, anther smut of the caryophyllaceae. Adv. Plant Pathol. 6:457–482.

Felsenstein, J. 1985. Phylogenies and the comparative method. Am. Nat. 125:1–15.

Fitzpatrick, B. M. 2002. Molecular correlates of reproductive isolation. Evolution 56:191–198.

Fitzpatrick, B. M., and M. Turelli. 2006. The geography of mammalian speciation: mixed signals from phylogenies and range maps. Evolution 60:601–615.

Giraud, T., L. Villaréal, F. Austerlitz, M. Le Gac, and C. Lavigne. 2006. Importance of the life cycle in host race formation and sympatric speciation in parasites. Phytopathology 96:280–287.

Giraud, T. 2006. Selection against migrant pathogens: the immigrant inviability barrier in pathogens. Heredity 97:316–318.

Hood, M. E., and J. Antonovics. 2000. Intratetrad mating, heterozygosity, and the maintenance of deleterious alleles in *Microbotryum violaceum* (= *Ustilago violacea*). Heredity. 85:231–241.

Kaltz, O., and J. A. Shykoff. 1999. Selfing versus outcrossing propensity of the fungal pathogen *Microbotryum violaceum* across *Silene latifolia* host plants. J. Evol. Biol. 12:340–349.

Kumar, S., K. Tamura, I. B. Jakobsen, and M. Nei. 2001. MEGA2: molecular evolutionary genetics analysis software. Bioinformatics 17:1244–1245.

Le Gac, M., M. E. Hood, E. Fournier, and T. Giraud. 2007. Phylogenetic evidence of host-specific cryptic species in the anther smut fungus. Evolution 67:1526.

Lijtmaer, D. A., B. Mahler, and P. L. Tubaro. 2003. Hybridization and postzygotic isolation patterns in pigeons and doves. Evolution 57:1411–1418.

Mendelson, T. 2003. Sexual isolation evolves faster than hybrid inviability in a diverse and sexually dimorphic genus of fish (Percidae: Etheostoma). Evolution 57:317–327.

Moyle, L. C., M. S. Olson, and P. Tiffin. 2004. Patterns of reproductive isolation in three angiosperm genera. Evolution 58:1195–1208.

Omland, K. E. 1997 Correlated rates of molecular and morphological evolution. Evolution 51:1381–1393.

Orr, H. A. 2001. The genetics of species differences. Trends Ecol. Evol. 16:343–350.

Posada, D., and Crandall, K. A. 1998. MODELTEST: testing the model of DNA substitution. Bioinformatics. 14:817–818.

Presgraves, D. C. 2002. Patterns of postzygotic isolation in Lepidoptera. Evolution 56:1168–1183.

Price, T. D., and M. M. Bouvier. 2002. The evolution of F1 postzygotic incompatibilities in birds. Evolution 56:2083–2089.

Rundle, H. D., and M. C. Whitlock. 2001. A genetic interpretation of ecologically dependent isolation. Evolution 55:198–201.

Russell, S. T. 2003. Evolution of intrinsic post-zygotic reproductive isolation in fish. Ann. Zool. Fennici 40:321–329.

Sasa, M. M., P. T. Chippindale, and N. A. Johnson. 1998. Patterns of postzygotic isolation in frogs. Evolution 52:1811–1820.

Thrall, P. H., A. Biere, and J. Antonovics. 1993. Plant-life history and disease susceptibility—the occurrence of *Ustilago violacea* on different species within the Caryophyllaceae. J. Ecol. 81:489–498.

Tubaro, P. L., and D. A. Litjamer. 2002. Hybridization patterns and the evolution of reproductive isolation in ducks. Biol. J. Lin. Soc. 77:193–200.

Van Putten, W. F., A. Biere, and J. M. M. Van Damme. 2003. Intraspecific competition and mating between fungal strains of the anther smut *Microbotryum violaceum* from the host plants *Silene latifolia* and *S. dioica*. Evolution 57:766–776.

Zar, J. H. 1984. Biostatistical Analysis, 2 nd edition. Prentice-Hall, Englewood Cliffs, NJ.

Associate Editor: D. Funk

Supplementary Material

The following supplementary material is available for this article:

Appendix S1. Names of isolates, the mating type of the sopridia used in this study, the species they belong to within the *Microbotryum violaceum* complex, their host and location of collection, and the type of experiment for which they were used.

Appendix S2. Experimental design to investigate reproductive isolation within the *Microbotryum violaceum* species complex.

Appendix S3. Nested phylogenetic corrections used to test for a correlation between reproductive isolation and genetic distance.

This material is available as part of the online article from:
http://www.blackwell-synergy.com/doi/abs/10.1111/j.1558-5646.2007.00144.x
(This link will take you to the article abstract).

Please note: Blackwell Publishing is not responsible for the content or functionality of any supplementary materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.