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The anther smut disease on *Gypsophila repens*: a case of parasite sub-optimal performance following a recent host shift?

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Abstract

The study of how parasites adapt to new hosts is of great importance for understanding the emergence of new diseases. Here, we report a study of the anther smut disease on *Gypsophila repens* (Caryophyllaceae). In contrast to what is usually found on other host species, infected natural populations of *G. repens* are extremely rare. Moreover, symptoms of diseased plants are incomplete and highly variable over the time. These results suggest that the fungus infecting *G. repens* is a case of a parasite not capable of exploiting its host optimally. Molecular analyses of *Microbotryum violaceum* strains infecting this and other Caryophyllaceae revealed that this sub-optimal behaviour probably resulted from a recent host shift from the morphologically similar plant *Petrorhagia saxifraga*. With its exceptionally low virulence and prevalence, but apparent self-sustainability, the disease on *G. repens* may thus represent an interesting case study for investigating the conditions leading to adaptation of parasites on new hosts.

Introduction

Studies of host shifts and inter-species disease transmission are important to understanding the emergence of new diseases in humans or crops (Antonovics *et al.*, 2002). Indeed, nearly 75% of emergent human diseases have their origins in animal populations (Cleaveland *et al.*, 2001; Taylor *et al.*, 2001). Examples include the SARS-agent (Tobler *et al.*, 2003), malaria (Waters *et al.*, 1991), HIV (Paraskevis *et al.*, 2003) and several viral human diseases in Southeast Asia (Mackenzie *et al.*, 2001), like avian influenza. In an agricultural context, host shifts of phytophagous insects (Thomas *et al.*, 2003a) and of fungal, viral and bacterial pathogens (Anderson *et al.*, 2004) from wild plant species are recognized to cause severe damage to crops. Moreover, studying the conditions allowing host shifts that lead to sustainable and virulent disease is also important to our understanding of host specificity and species richness in parasites (Thompson, 1994). Indeed there are many examples of parasites that exhibit rapid specialization and host-specific differentiation following the emergence of a disease on a new host (Drès & Mallet, 2002). In the present study, we investigated the possibility of a host shift of the anther smut fungus *Microbotryum violaceum* (= *Ustilago violacea*), onto the Caryophyllaceous *Gypsophila repens*. We also studied the characteristics and the dynamics of the disease in one population in order to analyse the performance of the pathogen to this plant host.

Natural populations of *G. repens*, as is the case for many other members of the Caryophyllaceae (Thrall *et al.*, 1993a), harbour the fungus *M. violaceum* that causes anther-smut disease. This heterobasidiomycete fungus induces the production of anthers containing only a mass of spores and destroys the ovary, thereby completely sterilizing the plant. This effect is also observed in pistillate flowers that normally do not produce anthers (Uchida *et al.*, 2003). Inoculum is thus transmitted by pollinators that visit diseased plants and transport spores to healthy plants (Baker, 1947). *Microbotryum violaceum* is an obligate parasite on a wide range of species in the

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Caryophyllaceae. It has been recorded on 92 species in Europe and on 21 in North America (Thrall *et al.*, 1993a). Isolates from the different plant species are genetically differentiated (Perlin, 1996; Garr *et al.*, 1997; Shykoff *et al.*, 1999; Bucheli *et al.*, 2000; Giraud *et al.*, 2002; Van Putten *et al.*, 2004), exhibit some morphological differences (Vanky, 1998) and a certain degree of specialization (Baker, 1947; Biere & Honders, 1996a). Therefore, *M. violaceum* is divided into host races, i.e. sibling fungal species or subspecies that are specialized on a restricted number of plant species.

The anther smut disease on *G. repens* appears to be quite peculiar for two reasons. Although *G. repens* is widespread and common in South and Central Europe (Blamey & Grey-Wilson, 1989), infected populations are very rare. This is in contrast to patterns observed in many other host species infected with this fungus, where infected populations are quite easy to find (J. A. Shykoff & T. Giraud, personal observations). Over 5 years of survey in the Alps, only one of more than 100 populations was found to contain diseased plants. This population was found in 1995 in Grosio, in the Italian Alps. Our intensive survey throughout the Swiss Alps in 1994 and in 2001 revealed no other infected populations, though one isolated infected individual was found growing near a large infected population of *Silene latifolia*, *Silene dioica* and their hybrids in the Münstertal in eastern Switzerland in 1994 (Bucheli *et al.*, 2000). In 2002, an intensive survey of the French part of the Pyrenees Mountains was unsuccessful in detecting the disease on *G. repens*. In addition to the rarity of diseased populations of *G. repens*, infection of this host leads to very atypical symptoms. While anther-smut typically leads to complete sterilization since all flowers are converted into spore-bearing structures, in *G. repens* plants are often partially diseased with a fraction of the flowers remaining healthy. Though partially diseased plants with distinct healthy and diseased branches can be found in other hosts, for example in *S. latifolia*, this pattern of disease expression is assumed to result from the plant becoming diseased earlier in the current period of flowering and disease is not yet systemic (Biere & Honders, 1996b). However, in *G. repens* partially diseased plants can be found at first flowering of the season, and healthy and diseased flowers can be found on the same branch. Moreover, whereas diseased flowers typically have greatly reduced or aborted stigmas in most host species (e.g. Shykoff & Kaltz, 1997), some infected *G. repens* flowers have functional stigmas and are able to produce seeds (López-Villavicencio *et al.*, 2003). Theoretically, partial sterilization has been considered to be a nonoptimal parasitic strategy for castrating pathogens (Obrebski, 1975; Jaenike, 1996; Antonovics *et al.*, 2002). Host reproduction demands resources that will be unavailable for parasite reproduction. Reproduction will furthermore draw energy away from host survival, leading to a reduction in parasite's transmission time (Baudoin, 1975; Obrebski, 1975).

Because *M. violaceum* is rare in natural populations of *G. repens* and since infected individuals express atypical symptoms and are able to reproduce, we attempted to determine whether the disease in the Italian population at Grosio may represent a case nonoptimal behaviour of *M. violaceum* resulting from a recent host shift. Incomplete symptoms have been documented previously for anther smut disease on *Silene vulgaris*, as the result of an incipient host shift from *S. latifolia* in North America (Antonovics *et al.*, 2002; Hood *et al.*, 2003). We analysed the characteristics and the dynamics of the anther smut disease on the plant *G. repens* by following symptoms on marked individuals in the Italian population over three consecutive years and by performing artificial inoculations in the greenhouse. Additionally, we attempted to identify a potential origin for the host shift by comparing the genetic similarity of the population of *M. violaceum* from *G. repens* found in Italy to samples from other hosts found in the same regions.

Material and methods

Populations survey and monitoring

Gypsophila repens is a perennial species distributed in the mountains of South and Central Europe (Blamey & Grey-Wilson, 1989). It is common in sunny and periodically dry places in rocky and grassy habitats. This species is gynodioecious, with populations composed of female and hermaphrodite individuals (having pistillate and perfect flowers, respectively), although some gynomonoecious individuals (bearing both types of flowers) may be present. Plants flower from early June until late October and flowers are pollinated by small solitary bees and syrphid flies (J. A. Shykoff & M. López-Villavicencio, personal observation). Pollinators visit many flowers on the same plant and typically visit neighbouring plants before moving over longer distances.

In order to study the characteristics and the dynamics of the disease on *G. repens*, we followed the fate of approximately 700 marked plants that represent almost all the individuals growing on a *ca.* 1500 m^2 southern exposed steep rocky slope of the Italian population (46°17′24″N and 10°15′11″E). Other plants, some of them infected, also grow on rock walls and rock outcrops within the pasture nearby. For three consecutive years (2000–2002) the size (i.e. number of branches), sex and infection status of each plant were recorded during the flowering season. To estimate the parasite-induced host mortality, we compared mortality rate for each year between healthy and diseased individuals. In this species, contrary to what is typically observed in other Caryophyllaceae, the percentage of flowers in infected individuals that show symptoms of the disease seems to follow a continuous distribution, and there are differences in the degree of sterility among symptomatic flowers. While some flowers present an aborted ovary

and spore-filled anthers, others produce spore-filled anthers but carry functional ovaries and stigmas that make them able to produce seeds, although in smaller numbers than healthy individuals (López-Villavicencio *et al.*, 2003). For the purpose of this study, we categorized the individuals in the population in three classes: (1) completely sterile: 'CS' i.e. plants presenting only flowers with aborted ovary and spore filled anthers, (2) plants with spore-bearing anthers and functional stigmas: 'FS'; these plants have all their flowers smutted, with at least some flowers presenting healthy stigmas and potentially able to produce seeds and (3) partially infected: 'PI', i.e. plants bearing both healthy and diseased flowers; as these plants have completely healthy flowers, they are able to produce pollen and seeds.

Fungal strain collection

We collected fungal teliospores from the 60 diseased *G. repens* individuals of the Italian population during July 2001 and we mapped the plants to the nearest 1 m. This infected population of *G. repens* is in sympatry with the Caryophyllaceae *Dianthus sylvestris* and *Dianthus carthusianorum*, both of which are also infected by *M. violaceum* and share with *G. repens* some of its pollinators (M. López-Villavicencio, personal observation). Isolates collected from *D. sylvestris* and *D. carthusianorum* are phylogenetically close to those collected from *G. repens* (phylogenies based on ITS, alpha-tubulin and beta-tubulin genes, T. Giraud, M. Le Gac, M. E. Hood, A. Widmer & J. A. Shykoff, unpublished data). Since we attempted to identify a potential origin for the host shift we collected fungal teliospores from 30 diseased plants of *D. sylvestris* and from 10 diseased plants of *D. carthusianorum* from the same location in order to compare their genetic similarity with *M. violaceum* from *G. repens*.

In July and August 2003, we collected fungal teliospores from 12 diseased individuals on the Caryophyllaceae *Petrorhagia saxifraga* in a population located in St. Johann im Walde in the Austrian Alps (46°55′21″N and 12°36′01″E). We also collected samples from five diseased individuals from the species *Dianthus neglectus*, in Valle de Pesio, Italy (44°11′48″N and 7°40′44″E) and from five diseased *D. neglectus* in St. Anna, Italy (44°15′37″N and 7°07′17″E). These other host species are usually found in sympatry with *G. repens*, the two plants share pollinators and are phylogenetically close (T. Giraud, M. Le Gac, M. E. Hood, A. Widmer & J. A. Shykoff, unpublished data). However, we did not find diseased populations of these plant species close to the studied Italian population of *G. repens*.

For each sampled diseased plant, several unopened flower buds were collected. As plants are usually infected by a single fungal strain of *M. violaceum* (Baird & Garber, 1979; but see Hood, 2003) all the buds belonging to the same individual were stored together into a 1.5 or 2 mL Eppendorf tube filled with silica gel. The tubes were then stored at room temperature until later use.

Artificial inoculation and detection of the fungus *in planta* by PCR

In order to investigate whether the symptom category was a trait of the pathogen strains, we inoculated seeds from the species *G. repens* with fungal isolates from the three categories of symptoms. Seeds from *G. repens* were collected from the Italian population during August 2001. Because each plant may receive pollen from different plants, seed collections constituted mixtures of half and full siblings (i.e. maternal families). We use seeds from 10 different plants that belonged to the three sexual categories. Fungal isolates used for inoculation were from 21 different diseased individuals: eight individuals completely sterile (CS), five individuals presenting functional stigmas (FS) and eight partially infected (PI). In October 2001, seeds were inoculated with different strains from each category of infection (CS, FS and PI). For each of these treatment combinations, teliospores from two infected buds of a same plant were suspended in 6 mL sterile water, vortexed and then spread on 1% water agar in a Petri dish, where about 20 seeds of *G. repens* were put to germinate at 25 °C. Viability of the teliospores was checked by growing them on standard medium (Cummins & Day, 1977) plus hazelnut extract (10%). Fungal isolates from *G. repens* do not grow on normal standard medium, in contrast to all other host races of *M. violaceum*. Moreover, even on this supplemented medium they grew much slower than isolates from other plants species. Fifteen days later, seedlings were transferred to the greenhouse into plastic multi-pot containers (0.1 L volume per plant). To avoid possible contamination between different treatments, each multi-pot received only seedlings inoculated with the same fungal strain. When rosettes had begun to form, plants were transferred in larger pots (0.9 L volume) and completely randomized with regard to position in the grid of plants kept in the greenhouse.

Plants started to flower in March 2002. Because none of the plants showed smutted flowers we tested using PCR whether the fungus had infected the plants despite the absence of symptoms. The DNA was extracted from meristem regions and roots of 10 plants using the Chelex (Biorad) protocol (Bucheli *et al.*, 2001). The PCR was performed using the GR26 primers described below. The PCR products were separated in 1% agarose gels. Control PCR without any DNA was systematically run.

Microsatellite isolation and genotyping

Microbotryum violaceum DNA was extracted using the Chelex (Biorad) protocol (Bucheli *et al.*, 2001), using one infected bud from diseased plants of *G. repens* and of *P. saxifraga* and one anther full of spores from diseased

plants of *D. sylvestris*, *D. neglectus* and *D. carthusianorum*. All the 49 microsatellite markers developed for *M. violaceum* (Bucheli *et al.*, 1998; Giraud *et al.*, 2002) were tested for amplification and polymorphism on 30 strains from *G. repens*.

Because none of the previously developed microsatellite markers was polymorphic within Italian isolates from *G. repens*, five additional positive clones of the microsatellite enriched-library of *M. violaceum* from *G. repens* constructed by Giraud *et al.* (2002) were sequenced. The PCR primers were designed for one locus, GR26, having 19 (GA) repeats (Genbank accession AY754871) using the software PRIMER3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Using the primers (5′-3′) TCGACCGCTCGCTAATACCA and TGAGGAACACATCG GGTCCT successful amplifications of expected size (160 bp) were obtained and the locus was polymorphic on Italian isolates from *G. repens*. The 60 fungal isolates from the Italian population of *G. repens* were genotyped with this GR26 marker.

A total of 88 fungal strains were then genotyped using the microsatellite markers GR11, GR15, GR18, GR19 (Giraud *et al.*, 2002), L17 and L18 (Bucheli *et al.*, 1998): 16 strains collected on *G. repens*, 12 strains on *P. saxifraga*, 40 strains on *D. sylvestris*, 10 strains on *D. carthusianorum* and 10 strains on *D. neglectus* (five from each population sampled). The marker GR26 could not be used in this analysis because it did not amplify in *Dianthus* host races. The PCR amplifications were performed and visualized as described by Giraud (2004).

AFLP genotyping

A total of 35 fungal strains were genotyped using AFLPs: 10 strains collected on *G. repens*, four strains on *P. saxifraga*, five strains on *D. sylvestris*, seven strains on *D. carthusianorum* and nine strains on *D. neglectus* (five from Valle de Pesio and four from St. Anna). This technique requires the use of purified DNA extracted from cultured teliospores (see below). Because some of the strains failed to grow under controlled conditions, the number of strains genotyped by AFLPs was smaller than the number of strains genotyped using the microsatellite markers. Diploid teliospores from single buds were plated on standard (Cummins & Day, 1977) or enriched medium (with hazelnut extract). Haploid sporidia, produced after germination and meiosis, were grown for 2–4 weeks at 25 °C. The DNA was extracted from total sporidial culture using the Qiagen Kit for plants. The DNA isolated from such pooled cultures of sporidia only represents the genetic material present in the original diploid teliospores if all meiotic products are able to proliferate on the plate. Strains having mating type-linked haplo-lethal alleles have been described in *M. violaceum* (e.g. Thomas *et al.*, 2003b), for which half of the meiotic products die after a few mitotic divisions (Hood & Antonovics, 2000). Some alleles may therefore be missing in some individuals, but this problem appears minimal in our study, since the objective was to compare alleles among different host races.

AFLP protocol was adapted from Justesen *et al.* (2002) as follows: 50–100 ng of DNA was digested with five units of each restriction enzyme *Pst*I and *Rsa*I (isoschizomere of *Mse*I Biofermentas) for 2 h, followed by 3 h of ligation (0.1 μM of *Pst*I adapter, 1 μM of *Mse*I adapter, 0.2 mM ATP, one unit of T4 DNA Ligase, Biofermentas), without heat inactivation, in a 50 μL final volume of 1 × RL Buffer (Tris–HAc PH 7.5 10 mM, MgAc 10 mM, Kac 50 mM, DTT 5 mM, BSA 50 ng μL^{-1}). After a 1/20th dilution in ultra pure water, ligation products were used directly (no streptavidine purification) for the first amplification, using *Pst* and *Mse* primers (2 ngμL^{-1}) with no selective nucleotides, then diluted 1/20th in water and followed by a specific amplification (two selective nucleotides on both *Mse* and *Pst* primers). Table 1 lists the selective primers used, which were adopted from Hovmoller *et al.* (2002). Digestions, ligations and DNA amplifications were performed in a ICycler (Biorad, USA) and PCR products were visualized on a Sequigen apparatus (Biorad), using 6% polyacrilamide silver stained gels. Polymorphic AFLP fragments of strong intensity were scored as binary characters for each isolate.

Table 1 Primer combinations used for AFLPs, their polymorphism per population and Nei's diversity index for each host race of *Microbotryum violaceum* analysed in this study, using AFLP data.

	Number of polymorphic bands						
						D. neglectus	
Primer combinations	Total	*G. repens* ($n = 10$)	*P. saxifraga* ($n = 4$)	*D. sylvestris* ($n = 5$)	*D. carthusianorum* ($n = 7$)	Valle de Pesio ($n = 5$)	St. Anna ($n = 4$)
P(AA)M(AG)–P12M12	51	1	29	6	7	19	23
P(AA)M(CA)–P12M25	53	1	39	6	9	4	24
P(GC)M(AC)–P20M12	36	0	25	5	5	9	20
P(AA)M(CC)–P20M25	52	1	42	13	18	14	25
Nei's diversity index		0.55 ± 0.074	1.00 ± 1.17	1.00 ± 0.12	1.00 ± 0.07	1.00 ± 0.04	

Data analyses

Differences in mortality rates between infected and healthy individuals between 2000–2001 and 2001–2002 were determined by χ^2-test using JMP, Version 4.0 (SAS-Institute). Statistical analyses on population structure of *M. violaceum* on *G. repens* and on genic differentiation among host races were performed on microsatellite data using the updated Version 3.2a of the GENEPOP software available free at http://www.cefe.cnrs-mop.fr/. Isolation by distance was tested by computing a Mantel test between the two half-matrices of pair-wise genetic distances and pair-wise geographical distances (raw distances or log-transformed). Genetic distances between individuals were estimated as described in Rousset (2000). Nei's diversity index (Nei, 1987) was calculated using the package ARLEQUIN 2.000 (Schneider *et al.*, 2000).

Trees were constructed independently from AFLP and microsatellite data using the software POPULATIONS (http://www.pge.cnrs-gif.fr/bioinfo) and were visualized using TREEVIEW software (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html). The Neighbour-Joining method was used from a matrix of chord distances (Cavalli-Sforza & Edwards, 1967) and 1000 bootstraps were were run. Nei's distances and the UPGMA method gave similar topologies for each data set, as did genetic distances based on allelic sizes for microsatellites.

Results

Populations survey and monitoring

Over the 2000–2002 period, the total percentage of diseased plants in the Italian population of *G. repens* fluctuated from 19 to 32% (Table 2). The percentage of healthy individuals that became smutted was 8% from 2000 to 2001 and 5% from 2001 to 2002. Recovery was however also relatively high: 7% of the plants that were diseased in 2000 no longer presented symptoms in 2001 and 4% of the plants that were diseased in 2001 no longer presented symptoms in 2002. Symptoms of diseased plants in this population were themselves highly variable over the time. Some of the plants completely sterilized 1 year were able to produce a mixture of healthy and diseased flowers or flowers with healthy stigmas the next year and the other way around (Fig. 1).

Table 2 Results of the monitoring of the diseased population of *Gypsophila repens* in Grosio, Italy: number of individuals in the population in 2000–2002, percentage of infected individuals (total and by symptom category).

Year	Number of individuals in the population	Total percentage of diseased individuals	Percentage of diseased individual by symptom category		
			CS	FS	PI
2000	703	19	78	10	12
2001	619	29	48	17	35
2002	646	32	43	24	33

Plants were grouped in three symptom categories: (1) completely sterile: 'CS' (2) plants with spore-bearing anthers and functional stigmas: 'FS' and (3) partially infected: 'PI' i.e. plants bearing both healthy and diseased flowers (see Material and methods for further details).

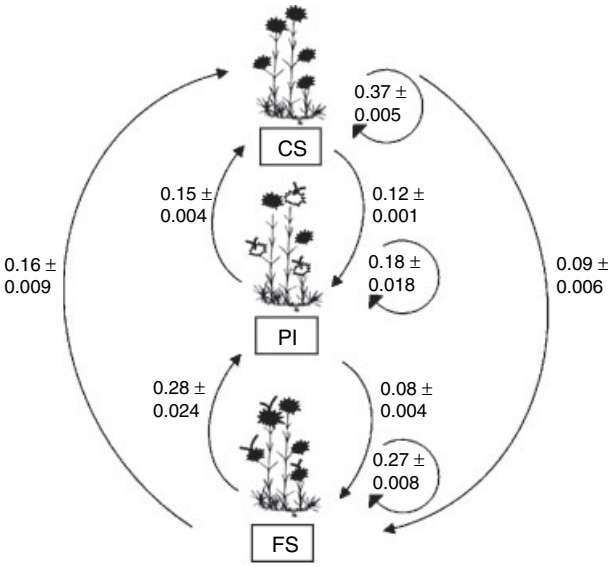

Fig. 1 Mean and SE of transition frequencies between the different classes of symptom categories of diseased *Gypsophila repens* individuals from 2000 to 2002 in Grosio, Italy. The three symptom categories are: (1) completely sterile: 'CS', (2) plants with spore-bearing anthers and functional stigmas: 'FS' and (3) partially infected: 'PI' i.e. plants bearing both healthy and diseased flowers. Only transitions between symptoms categories and probabilities of remaining in the same category are presented. Probabilities of recovery, transitions into nonreproductive categories and mortality are not represented but are taken into account in the values indicated on the figure.

No differences were found in mortality rate between healthy and diseased individuals either from 2000 to 2001 or from 2001 to 2002, suggesting that the parasite has a low impact on the mortality of the host (mortality 2000–2001: Pearson's $\chi^2_1 = 0.098$, $P = 0.7539$; mortality 2001–2002: Pearson's $\chi^2_1 = 1.460$, $P = 0.2269$).

Artificial inoculations and detection of the fungus *in planta* by PCR

None of the 302 plants of *G. repens* inoculated with *M. violaceum* presented teliospore-filled anthers once they flowered. The PCR run on whole DNA extracted from meristem regions and roots of growing plants using the marker GR26 detected no signal in any of the 10 plants tested, although infection can be detected using this protocol on other plant species (T. Giraud, unpublished data).

Genetic relationships among host races of *Microbotryum violaceum*

Fungal strains present on the four plant species *D. neglectus*, *D. carthusianorum*, *D. sylvestris* and *P. saxifraga* had highly different allelic frequencies both for AFLP and microsatellite data and there were private alleles for all host races, at least for some markers (Fig. 2). Pair-wise genic differentiation tests among the fungal populations present on these four plant species were highly significant, using either AFLP ($P < 0.00001$) or microsatellite data ($P < 0.00001$). In contrast, the population from *G. repens* was not significantly differentiated from the one on *P. saxifraga* and its alleles were a subset of the ones sampled on *P. saxifraga* (Fig. 2). The population on *G. repens* was indeed much less polymorphic than the one on *P. saxifraga* and on other host races (see Nei's diversity index for AFLP data in Table 1 and Figs 3 and 4).

Trees were reconstructed independently from AFLP (Fig. 3) and microsatellite data (Fig. 4). All bootstrap values for both trees were below 70%, but identical topologies were obtained for both AFLP and microsatellite data sets with all distances and branching methods used. Both trees confirmed clear differentiation among the host races *D. neglectus*, *D. carthusianorum*, *D. sylvestris* and *P. saxifrage*, and the strong genetic similarity between the strains collected on *G. repens* and the ones on *P. saxifraga*. The population on *G. repens* was nested within the one on *P. saxifraga* on both trees.

Within-population structure of *Microbotryum violaceum* on *Gypsophila repens*

Within-population structure of *M. violaceum* on *G. repens* was analysed by genotyping all the 60 fungal samples using the polymorphic microsatellite marker GR26. Four alleles and six genotypes were detected. Deviation from Hardy–Weinberg expectations was highly significant, with a deficiency of heterozygotes ($F_{IS} = +0.556$; $P < 0.00001$). There was no significant pattern of isolation by distance either when using raw geographical distances or log-transformed distances.

Discussion

A possible recent host shift of *Microbotryum violaceum* from *Petrorhagia saxifraga* onto *Gypsophila repens*

The failure of *M. violaceum* to produce stable and complete symptoms on *G. repens*, together with the rarity of the disease in natural populations of this plant, suggested that a recent host shift may have occurred on *G. repens* from another diseased Caryophyllaceae, as had been reported from *S. latifolia* to *S. vulgaris* (Antonovics *et al.*, 2002; Hood *et al.*, 2003). We therefore attempted to identify a potential origin for the host shift by comparing the genetic similarity of the population of *M. violaceum* from *G. repens* found in Italy to samples from other hosts usually found in sympatry: *Dianthus neglectus*, *D. carthusianorum*, *D. sylvestris* and *P. saxifraga*. The populations present on the four plant species *D. neglectus*, *D. carthusianorum*, *D. sylvestris* and *P. saxifraga* were strongly differentiated, representing true host races or even sibling species. The population on *G. repens* was in contrast genetically similar to the one on *P. saxifraga*. The fungus from the isolated *G. repens* individual found diseased near a large infected population of *S. latifolia*, *S. dioica* and their hybrids also had a genotype similar to our Italian isolates from *G. repens* and not to isolates from *Silene* spp. (Bucheli *et al.*, 2000). This, together with several gene sequences (T. Giraud, M. Le Gac, A. Widmer, M. E. Hood & J. A. Shykoff, unpublished

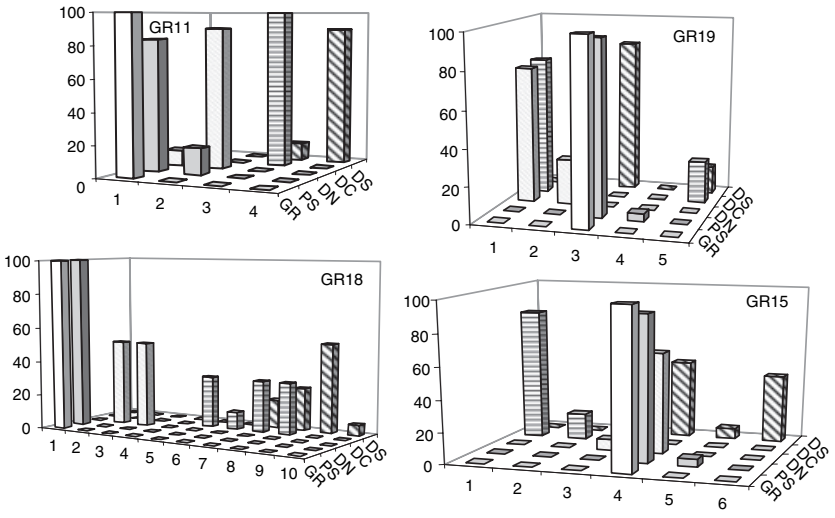

Fig. 2 Histograms of allelic frequencies for the microsatellite markers in fungal populations of *M. violaceum*. Alleles are represented in size order and by host plant: GR, *G. repens*; PS, *P. saxifraga*; DS, *D. sylvestris*; DC, *D. carthusianorum*; DN, *D. neglectus*.

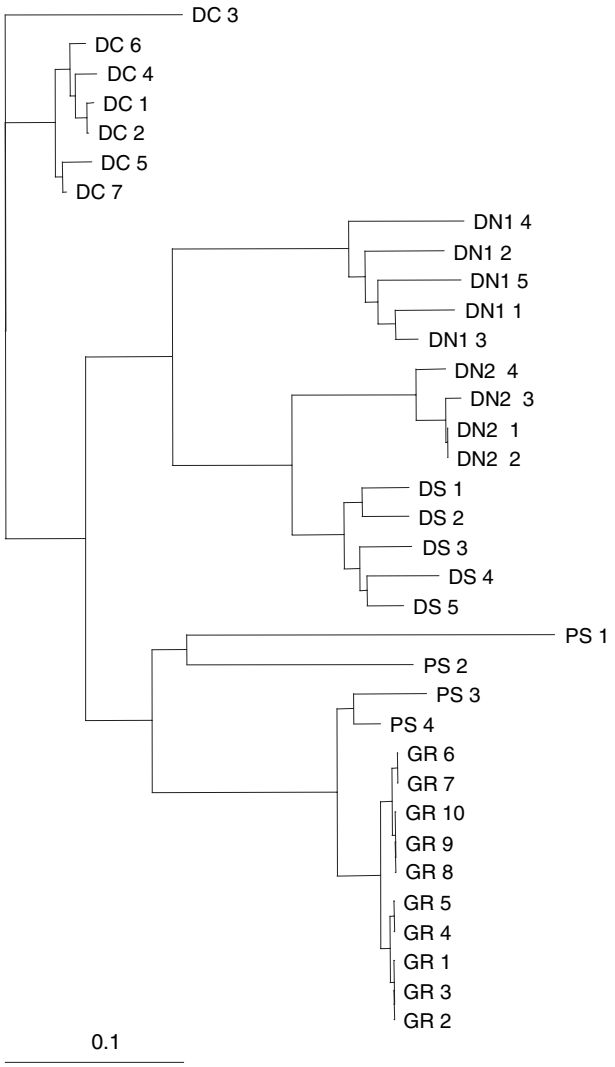

Fig. 3 Unrooted tree reconstructed from individual AFLP genotypes of *M. violaceum*. Bootstrap values were below 70% and were therefore not indicated on the figures. Strains are labelled by host plant: GR, *G. repens*; PS, *P. saxifraga*; DS, *D. sylvestris*; DC, *D. carthusianorum*; DN1, *Dianthus neglectus* from Valle de Pesio; DN2, *D. neglectus* from St. Anna.

data), indicates that there has been a recent host shift of the fungus onto *G. repens*, most likely from *P. saxifraga*.

Furthermore, our microsatellite and AFLP data revealed almost no genetic variability within the fungal population on *G. repens*. In pathogen populations, the absence of polymorphism is usually related to transmission bottlenecks in which the disease is founded in a population by only a few genotypes (Bergstrom *et al.*, 1999). Since the population on *G. repens* was genetically close and nested within the population on *P. saxifraga*, the lack of genetic diversity in the fungal population on *G. repens* strengthens the hypothesis that it may result from the transmission of a limited subset of isolates with a similar genotype to those found in the *P. saxifraga* population.

Interestingly, the plants *G. repens* and *P. saxifraga* are morphologically very similar, differing only on seed morphology and on the presence of involucral bracts at the base of the calyce in *P. saxifraga* (Douglas *et al.*, 1989). The two species have overlapping distribution ranges (Blamey & Grey-Wilson, 1989) and their morphological similarity suggests that they share most of their pollinators, what may have facilitated the host shift of *M. violaceum* from *P. saxifraga* to *G. repens*.

There was a strong heterozygote deficiency in the Italian population of *M. violaceum* on *G. repens*, as has been found for other host races of *M. violaceum* (Delmotte *et al.*, 1999; Bucheli *et al.*, 2001; Giraud, 2004). This suggests high selfing rates, particularly in *M. violaceum* since this fungus is able to perform intra-tetrad selfing (= intra-meiotic selfing), which maintains more heterozygosity at loci linked to centromeres and to the mating type locus than does classical, inter-tetrad selfing (Hood & Antonovics, 2000, 2004;). A high intra-tetrad selfing rate may facilitate establishment of a disease onto a new host from a limited number of strains because it allows avoiding the problems both of finding mates and of inbreeding depression.

The anther smut disease on the plant *Gypsophila repens:* a case of sub-optimal performance on a new host

In contrast to what is usually found on other Caryophyllaceae infected by *M. violaceum*, symptoms of the diseased plants of *G. repens* were both incomplete and highly variable between plants and over the time. Furthermore, we found high percentages of new infection and of recovery each year, much higher than has been reported for the anther smut disease on other host plants, where the incidence normally does not fluctuate greatly between years (Carlsson & Elmqvist, 1992; Roche *et al.*, 1995; but see Biere & Honders, 1996b). The percentage of recovery appeared each year slightly lower than the percentage of new infection, indicating that the disease may be spreading slowly within the population. However, recovery and new infection each year may be only apparent: because individual plants often changed between categories of infection from one year to the other, symptoms becoming more or less severe, the lack of smutted flowers may only be an extreme in a continuous distribution of symptom severity. Asymptomatic plants could actually remain infected but produce smutted flowers only some years.

Partial infection must bear a fitness cost for the parasite. Theoretically, it has been suggested that the optimal evolutionary strategy for castrating parasites should be to completely sterilize their hosts (Obrebski, 1975; Jaenike, 1996; O'Keefe & Antonovics, 2002).

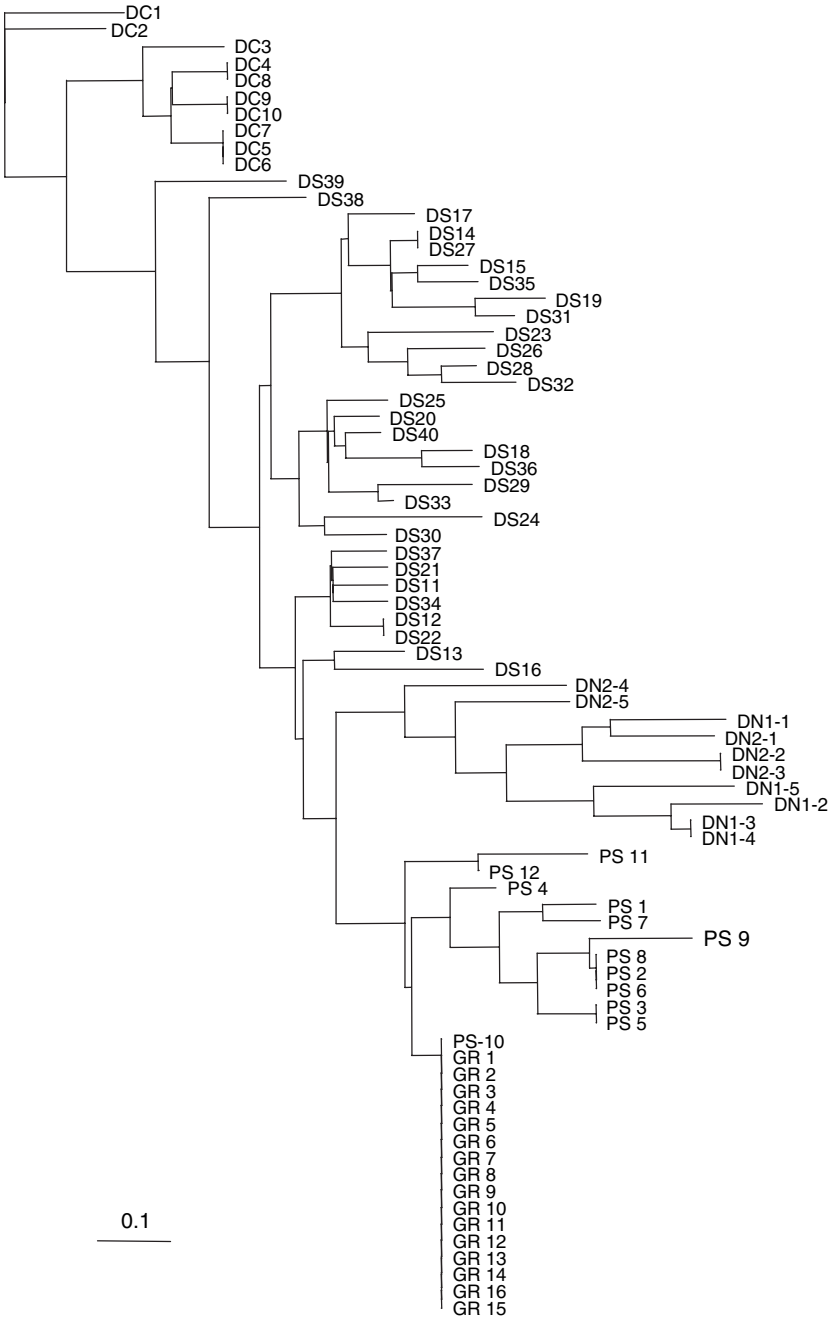

Fig. 4 Unrooted tree reconstructed from individual microsatellite genotypes of *M. violaceum*. Bootstrap values were below 80% and were therefore not indicated on the figures. Strains are labelled by host plant: GR, *G. repens*; PS, *P. saxifraga*; DS, *D. sylvestris*; DC, *D. carthusianorum*; DN1, *D. neglectus* from Valle de Pesio; DN2, *D. neglectus* from St. Anna. Labels of individuals are not the same as in Fig. 3. The single micro-satellite marker polymorphic on the fungal population from *G. repens*, GR26, could not be used in this analysis because it did not amplify in the *Dianthus* host races.

Nevertheless, reduced host sterilization in castrating parasites may evolve under particular kinds of population structure as in viscous populations with gene flow and colonization restricted to nearby sites for both host and parasite (O'Keefe & Antonovics, 2002). However, there are several lines of evidence suggesting that the atypical variability and low severity of the symptoms of the anther smut disease on *G. repens* is due to a sub-optimal performance of *M. violaceum* on this host species and not to alternative hypotheses, such as selection for reduced virulence of the fungus, variability in virulence among isolates of the fungus, or variability in resistance among plants. First, a lack of virulence variability has been reported in general in *M. violaceum* (Alexander & Antonovics, 1993; Kaltz & Shykoff, 2002) and the *M. violaceum* population from *G. repens* in Italy appears to have been subjected to a recent bottleneck, which may have reduced further variation in virulence. Second, symptoms changed over time for individual diseased plants, indicating that the

degree of disease severity was neither a characteristic of a particular fungal nor plant genotype, nor even of an interaction between the two. Third, artificial inoculations through a protocol that usually yields high percentages of infection in other host races (Alexander & Maltby, 1990; Kaltz *et al.*, 1999; Kaltz & Shykoff, 2002; Hood *et al.*, 2003) resulted here in none of the *G. repens* plants becoming infected. Finally, the chemical interactions between *G. repens* and its fungus seem less specific than in other plant species. In host species like *S. acaulis*, which exhibits more typical disease symptoms, spores of *M. violaceum* seem to chemically inhibit pollen germination on the stigmatic surface since both compete for germination space (Marr, 1998) and this has not been observed on *G. repens* (López-Villavicencio *et al.*, in press).

Microbotryum violaceum as a model system

In conclusion, the isolates on *G. repens* are likely to represent a recent host shift of *M. violaceum*. Contrary to the previous case of incipient host shift of *M. violaceum* reported from *S. latifolia* to *S. vulgaris*, where isolates present on *S. vulgaris* have just jumped from the close-by *S. latifolia* plants (Antonovics *et al.*, 2002; Hood *et al.*, 2003), the disease in *G. repens* seems to be self-sustaining. However, the symptoms are still not complete. It may then represent a further step in the process of adaptation to a new host than host shifts of *M. violaceum* reported from *S. latifolia* to *S. vulgaris*.

Microbotryum violaceum is already a model system in genetics (reviewed in Garber & Ruddat, 2002), disease transmission dynamics (e.g. Thrall *et al.*, 1993b; Antonovics *et al.*, 1995), host and pathogen coexistence (Antonovics & Thrall, 1994) and metapopulation dynamics (Antonovics *et al.*, 1994). This study, other recent works on several independent incipient host shifts of *M. violaceum* from *S. latifolia* to *S. vulgaris* (Antonovics *et al.*, 2002; Hood *et al.*, 2003) and studies on the history of host shifts in the association *Microbotryum*-Caryophyllaceae (Jackson, 2004), show that it can also be useful as a model to study mechanisms, sustainability, causes and consequences of parasite host shifts. In particular, it will be interesting to follow the fate of the disease on *G. repens*. Long-term observations of the host race on *G. repens* might reveal whether the disease will spread to other populations or become extinct. The evolution of the symptoms will also be interesting to follow, if rapid enough at a human scale, as there has been much debate in the recent literature about whether a newly established disease should evolve towards increased or decreased virulence (e.g. Lively, 1999; O'Keefe & Antonovics, 2002; Weiss, 2002) and which conditions allow evolution of optimal virulence from the point of view of the parasite. The anther smut disease on *G. repens* may thus represent an interesting case study of a self-sustainable host shift, yet unable to adapt completely to its new host.

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