JOURNAL OF Evolutionary Biology

doi: 10.1111/jeb.12169

# Investigating the production of sexual resting structures in a plant pathogen reveals unexpected self-fertility and genotype-by-environment effects

C. TOLLENAERE & A.-L. LAINE

*Metapopulation Research Group, Department of Biosciences, University of Helsinki, Helsinki, Finland*

*Keywords:*

adaptation;
chasmothecia;
genotype-by-environment interactions;
homothallism;
mating system;
mating-type genes;
powdery mildews;
sexual resting structures.

## Abstract

The sexual stage of pathogens governs recombination patterns and often also provides means of surviving the off-season. Despite its importance for evolutionary potential and between-season epidemiology, sexual systems have not been carefully investigated for many important pathogens, and what generates variation in successful sexual reproduction of pathogens remains unexplored. We surveyed the sexually produced resting structures (chasmothecia) across 86 natural populations of fungal pathogen *Podosphaera plantaginis* (Ascomycota) naturally infecting *Plantago lanceolata* in the Åland archipelago, southwestern Finland. For this pathosystem, these resting structures are a key life-history stage, as more than half of the local pathogen populations go extinct every winter. We uncovered substantial variation in the level of chasmothecia produced among populations, ranging from complete absence to presence on all infected leaves. We found that chasmothecia developed within clonal isolates (single-strain cultures). Additionally, these clonal isolates all contained both MAT1-1-1 and MAT1-2-1 genes that characterize mating types in Ascomycetes. Hence, contrary to expectations, we conclude that this species is capable of haploid selfing. In controlled inoculations, we discovered that pathogen genotypes varied in their tendency to produce chasmothecia. Production of chasmothecia was also affected by ambient temperature (E) and by the interaction between temperature and pathogen genotype (G × E). These G, E and G × E effects found both at a European scale and within the Åland archipelago may partly explain the high variability observed among populations in chasmothecia levels. Consequently, they may be key drivers of the evolutionary potential and epidemiology of this highly dynamic pathosystem.

## Introduction

Sexual reproduction may enhance genetic diversity by creating new allelic combinations. This reshuffling of allelic combinations is considered fundamental for evolution, as growing evidence suggests that increased recombination can speed up adaptation to environmental change (Goddard *et al.*, 2005; Cooper, 2007; Becks & Agrawal, 2012). Because parasites and their hosts are expected to be constantly co-evolving, pathogen species live in an ever-changing environment, as illustrated by the famous 'Red Queen' metaphor (van Valen, 1973; Morran *et al.*, 2011). This is particularly true for obligate pathogens, whose survival is inseparable from their host species. Hence, the capacity of pathogen populations to adapt to their host's changes may be affected by their reproductive strategy (McDonald & Linde, 2002; Barrett *et al.*, 2008a). The reproductive strategies of pathogen species are characterized by tremendous diversity. Many pathogens have the ability to complete their disease cycle asexually, yet sex is often conserved in pathogen life histories (Agrios, 2005;

*Correspondence:* Charlotte Tollenaere, Institut de Recherche pour le Développement (IRD), UMR Résistance des Plantes aux Bioagresseurs (IRD-CIRAD-UM2), 911, Avenue Agropolis, BP 64501 F-34394 Montpellier Cedex 5, France. Tel.: 00 33 (0) 4 67 41 62 61; fax: 00 33 (0)4 67 41 62 83; e-mail: charlotte.tollenaere@ird.fr

Heitman, 2006). In addition to the genetic consequences of sex in terms of recombination, sexual structures constitute a resting stage in many pathogen species, allowing survival during the absence of a suitable host or when the environmental conditions are unfavourable (Agrios, 2005). Understanding the factors affecting the sexual life-history stage of pathogen species is therefore critical from both evolutionary and epidemiological aspects.

Studies of pathogen sexual reproduction have been complicated by the obscurity of this life-history stage, making direct observations often impossible. Neutral molecular markers are increasingly becoming available, allowing inferences on the mode of reproduction and mating system from population genetics structure – evidencing recombination events (Varga & Toth, 2003; Saleh *et al.*, 2012), or on the opposite, assessing predominant clonality (Rougeron *et al.*, 2009). Molecular tools also allow the identification of mating-type loci (i.e. genomic regions regulating mating compatibilities) in many species (Heitman *et al.*, 2007). However, although we are beginning to grasp how ecological context may fundamentally impact on pathogen life histories (Barrett *et al.*, 2008a; Vale *et al.*, 2008; Wolinska & King, 2009), the study of sexual life history of pathogen populations within an ecological framework is lagging far behind investigations focusing on the clonal growth stage.

Fungi are characterized by extraordinary diversity of life cycles and reproductive strategies. In heterothallic fungi, sex occurs only after the mating of two strains of opposite mating types, whereas homothallic fungi are universally compatible, that is able to undergo sex even within the same clone (Billiard *et al.*, 2011, 2012). The fungal phylogenetic tree shows frequent transitions between homothallism and heterothallism (Billiard *et al.*, 2011), and within-species variation in reproductive system has also been reported (Marra *et al.*, 2004; Alby *et al.*, 2009; Klaassen *et al.*, 2012). Recent studies evidenced among-population variation in recombination rates that could be attributed to environmental variation (Barrett *et al.*, 2008b; Hosid *et al.*, 2010), indicating the importance of the ecological context to understand the role of specialized sexual cycles of pathogenic fungi. More generally, documenting the highly diverse mode of reproduction of fungal species will likely contribute to uncover the mystery of sex persistence (Billiard *et al.*, 2011, 2012).

Powdery mildews (Erysiphales, Leotiomycetes, Ascomycetes) are one of the most conspicuous and well-known groups of parasitic fungi (Spencer, 1978). Due to their diversity and abundance, powdery mildews are a significant component of natural plant communities (Glawe, 2008), and they cause damage worldwide on commercially important crops (Dean *et al.*, 2012). However, they remain poorly investigated in terms of sexual reproduction, which occurs via the production of particular sexual resting structures named chasmothecia (syn. cleistothecia), most likely because the obligate biotrophy of powdery mildews renders them impossible to culture on artificial media (Bélanger *et al.*, 2002). The mating system of powdery mildew fungi has been experimentally verified in only a few species. The first investigation of the mating-type genes in the powdery mildew group was recently published (Brewer *et al.*, 2011), extending the possibility to use molecular tools to investigate breeding systems (i.e. the proximate mechanisms controlling mating systems, named homo- vs. heterothallism in fungi, Billiard *et al.*, 2012) to the Erysiphales family. Studies published to date on the breeding system of 12 powdery mildew species are summarized in the Appendix S1. Heterothallism is by far the most common breeding system as it was reported for all the studied species, although four studies evidenced homothallism for some species also reported to be heterothallic. Moreover, although environmental cues may be important for triggering the production of sexual resting structures, detailed investigations on environmental factors are restricted to very few powdery mildew species: *Erysiphe necator* (Gadoury & Pearson, 1988; Legler *et al.*, 2012) and *E. pulchra* (Mmbaga, 2002). High sensitivity of powdery mildews to abiotic conditions is expected, as these fungi complete their life cycle on the surface of the host, rather than inside the host (Schnathorst, 1965).

This study aims at investigating variation – and its underlying causes – in the production of sexual resting structures in *Podosphaera plantaginis*, a powdery mildew specialized on *Plantago lanceolata*. In the Åland archipelago (south-west Finland), *Pl. lanceolata* occurs in network of approximately 4000 meadows, and *Po. plantaginis* has been demonstrated to occupy this network as a highly dynamic metapopulation, that is, an assemblage of spatially delimited local populations interconnected by migration and experiencing extinction/colonization cycles (Levins, 1969; Hanski, 1999). There is considerable variation in the probability of overwinter survival of local pathogen populations (Soubeyrand *et al.*, 2009) and variation in the ability of pathogen populations to adapt to their host population resistance structure (Laine, 2005, 2008), both processes being likely affected by the production of sexual resting structures. Given the literature on powdery mildews described above, we expected that the production of chasmothecia would require the meeting of the two different mating types (heterothallism hypothesis). Interestingly, recent genetic investigation revealed that approximately half of the pathogen populations did not contain any genetic diversity (monomorphic pathogen populations with only one multilocus genotype sampled, Tollenaere *et al.*, 2012). Availability of compatible mating type could thus limit sexual reproduction in some populations and impede overwintering survival; this mechanism would contribute to explain why local

pathogen populations typically persist only for 1–2 years (Laine & Hanski, 2006).

To measure the extent of variation in the production of chasmothecia in natural populations of *Po. plantaginis* in the Åland archipelago, we first recorded their frequency across 86 natural populations. Uncovering striking variation at the population level, from 0% to 100% of infected leaves bearing chasmothecia, we then tested what could generate this variation with the following aims. First, we tested the breeding system by (1) carrying out single- and two-strain inoculations and measured chasmothecia production and (2) amplifying the two mating-type genes in purified strains of *Po. plantaginis*. We discovered that, contrary to most powdery mildew species, *Po. plantaginis* from Åland is homothallic, that is, able to produce chasmothecia through haploid selfing. The Åland archipelago represents the extreme northern distribution range of *Po. plantaginis* species, and haploid selfing ability could be restricted to such peripheral populations. Indeed, smaller population size at the edge of geographical distribution could lead to the selection of universal compatibility (Lloyd, 1992), as described in various plant species (see for example Runions & Geber, 2000). We thus also assessed haploid selfing ability in *Po. plantaginis* sampled from other European locations and found them to be homothallic as well. Discarding the mate availability hypothesis, we then investigated whether pure haploid clones differed in their ability to produce sexual resting structures, and we found that the production of chasmothecia varied between pathogen genotypes, both at the European geographical scale and also within the small area of the Åland archipelago (tens of kilometres). We also assessed whether the abiotic environment could generate variation in chasmothecia production by testing strains originating from various localities across Europe under different temperature and daylight conditions. This experiment revealed an effect of the environmental conditions (temperature), of the origin of the strain and of their interaction on the production of resting sexual structures.

## Materials and methods

### Pathosystem

*Podosphaera plantaginis* (Castagne; U. Braun & S. Takamatsu) is an obligate pathogen specific to its host plant *Plantago lanceolata* L. (Plantaginaceae). During summer, the pathogen experiences repeated cycles of clonal reproduction, with the haploid mycelium producing haploid asexual wind-dispersed spores named conidia. The mating of two mycelia consists in a brief diploid phase, allowing the production of sexual resting structures (chasmothecia), in which meiosis followed by mitosis leads to the production of eight haploid ascospores. The obligate pathogen survives the period of host dormancy (plant die back to rootstock in winter) as chasmothecia, and ascospores re-initiate the epidemics in the following spring. Chasmothecia are conspicuous, dark, spherical structures approximately 90 $\mu$m in diameter, on the leaf surface. They are visible with the naked eye (see Fig. 1a), especially as they are generally densely aggregated.

Previous studies revealed the strain-specific resistance (Laine, 2004) and small-scale local adaptation for infectivity (Laine, 2005) in this pathosystem. Pathogen genotypes collected within the Åland archipelago were shown to differ for their infectivity profile (Laine, 2004) and for fitness estimates integrating various components of the clonal summer growth (Laine, 2008). The ability of the chasmothecia to survive winter and re-initiate epidemics of the disease through production of ascosporic inoculum was recently demonstrated for this pathosystem (A. J. M. Tack & A.-L. Laine, unpublished). However, what generates variation in the production of sexual resting structures allowing off-season survival is not known.

### Field estimation of chasmothecia levels in the Åland archipelago

Since 2001, every year in September, the Åland archipelago has been surveyed by 30–40 students systematically recording *Po. plantaginis* presence/absence in about 4000 populations of *Pl. lanceolata* (Laine & Hanski, 2006). In September 2010, a total of 175 *Plantago* populations were infected by the powdery mildew and we selected 86 of them for more detailed investigations (See Fig. 2 for a map of the studied localities). In each subpopulation, we also estimated the proportion of infected leaves bearing chasmothecia by randomly choosing five infected plants and recording the presence/absence of chasmothecia on 10 randomly chosen leaves. As prevalence of the disease was generally low, fewer data were available in some cases. We only considered plants recorded for at least five leaves and populations analysed for at least three plants.

Genetic diversity of *Po. plantaginis* had already been characterized in 80 of these populations by collecting four or five infected leaves and subsequently genotyping these samples with a set of 27 SNP (Single Nucleotide Polymorphism) markers (Tollenaere *et al.*, 2012). Both samples for genetic characterization and chasmothecia level estimates were collected at the same time (September 2010). Half of the studied populations presented no diversity at all (only one multilocus genotype found in all the analysed samples, Tollenaere *et al.*, 2012), and we assessed in the current study the effect of genetic diversity (binary variable: some diversity or no) on the proportion of leaves bearing chasmothecia in each population using a logistic regression in JMP version 8 (SAS Institute Inc., Cary, NC, USA).

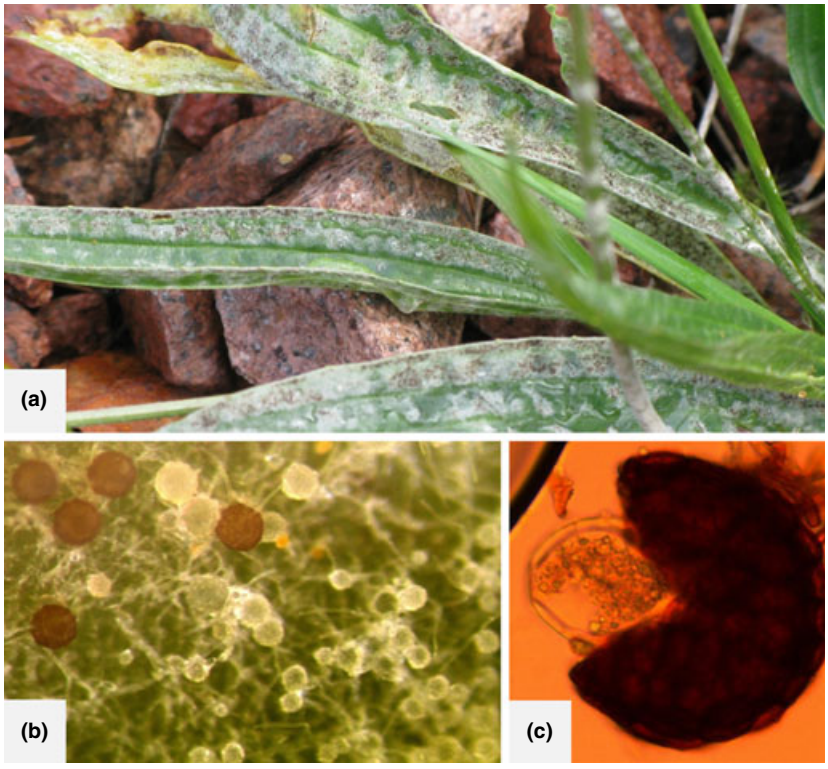


**Fig. 1** Pictures of the sexual resting structures (chasmothecia) of *Podosphaera plantaginis*. (a) Infected *Plantago lanceolata* leaves bearing chasmothecia (black dots) observed with the naked eye. (b) Chasmothecia at different maturation stages, observed under a stereomicroscope. (c) Crushed chasmothecium releasing a (still immature) ascus.

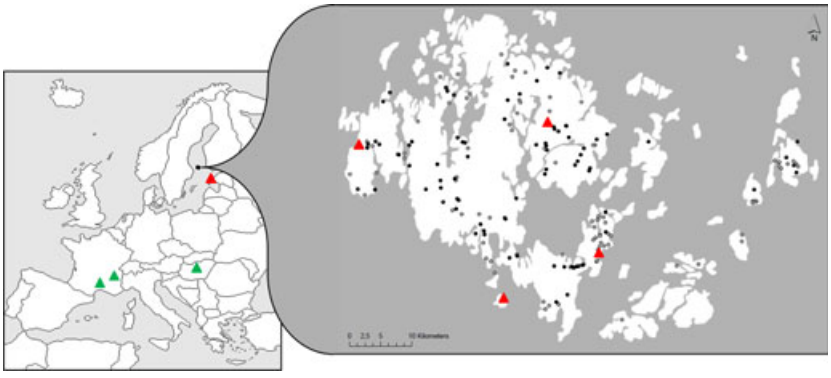

**Fig. 2** Map of the studied sites within Europe (left) and the Åland archipelago (right). The sampling localities are indicated with triangles: green triangles where southern strains were sampled and red triangles for northern strains. Within the Åland archipelago, all the 175 *Plantago* meadows infected by *Podosphaera plantaginis* are represented by a circle, with black circles indicating the 86 populations surveyed to estimate levels of chasmothecia.

### Sampling and isolation of *Po. plantaginis* strains

Infected leaves of *Pl. lanceolata* were sampled from different locations across Europe (see map in Fig. 2): the Åland archipelago in Finland, Saaremaa island in Estonia, Budapest in Hungary and Saint Jean du Gard and Saint Nazaire les Eymes, both being in France. Conidia from collected samples were transferred using a fine paintbrush onto new *Plantago* leaves (collected from greenhouse grown plant genotypes known to be widely susceptible). The inoculated leaves were placed in 9-cm Petri dishes with moist filter paper in a growth chamber at 20 °C with a 16L/8D photoperiod. Strains can be maintained in the laboratory by repeating this procedure every 2 weeks approximately (see also Laine, 2004, 2007). After at least one transfer, strains were purified by transferring an individual chain of conidia into a new leaf, using one hair from a paintbrush and laying the hair on the inoculated leaf.

### Experiment comparing chasmothecia production between single- and two-strain cultures

We compared the production of chasmothecia between purified fungal cultures and join cultures of two different strains. For this purpose, we used five strains originating from four populations in Åland (IDs 325, 1413, 2220 and 9031) and leaves from 11 different plants

grown in the laboratory from seeds collected in Åland. These 11 plant genotypes have been previously characterized as susceptible to the strains used (C. Tollenaere, unpublished results). Two colonies of approximately the same size were simultaneously brushed onto the same part of new leaves on 17 February 2011. In the single-strain inoculations (80 infected leaves in total), we used two colonies from the same strain, whereas the colonies came from different strains in the two-strain inoculations (120 infected leaves in total).

The experiment was continued until 25 days after inoculation (4 March 2011), and inoculated leaves were observed under a stereomicroscope (SMZ800; Nikon, Tokyo, Japan), and the number of chasmothecia was recorded for each leaf. To make sure the formed chasmothecia were fertile, we crushed some of them between two glass slides and observed their content using a microscope (DM LB; Leica Microsystems GmbH, Wetzlar, Germany). In the *Podosphaera* group, chasmothecia contain one ascus whose contents typically differentiate into eight ascospores when mature (Mori *et al.*, 2000). We compared the leaves inoculated with a single strain or a mix of two strains for the binary variable 'bearing chasmothecia or not' using logistic regression in JMP software and for the count variable 'total number of chasmothecia on the leaf' using a generalized linear model with quasi-Poisson distribution using R software (R Foundation for Statistical Computing, Vienna, Austria, http://www.R-project.org).

### Amplification of mating-type genes

Each of the five MAT genes described in *Eysiphe necator* (Brewer *et al.*, 2011) were found to have orthologs in the transcriptome data of *Po. plantaginis* (Tollenaere *et al.*, 2012). We were particularly interested in the genes that are specific of one of the two mating types in other powdery mildew species and consequently decided to focus on MAT1-1-1 and MAT1-2-1. We designed the following specific primers from the transcriptome library sequences: Pp_MAT1_1_F (AGA CCA CTT AGC CTT GAA CG) and Pp_MAT1_1_R (TAG AAG TGG AGG AGA CGG AA) for MAT1-1-1 gene and Pp_MAT1_2B_F (TTG ACG TCG CTT GAC TTC AG) and Pp_MAT1_2_R (CAG TCA ATG CTC CAG ATT CC) for MAT1-2-1 gene.

We amplified mating-type genes both in 10 randomly chosen field samples from the Åland archipelago and in the eight purified strains (four from the Åland archipelago, one from Estonia, two from France and one from Hungary) used in the experiment described below. Spores were collected from infected leaves, and DNA was extracted using E.Z.N.A. plant DNA kit (Omega Biotek, Norcross, GA, USA), as described in Tollenaere *et al.* (2012). Amplification was performed in a final volume of 20 μL containing 0.4 μM of each primer, 0.3 mM deoxyribonucleotides (dNTPs), 1U Taq polymerase (DyNAzyme II; Finnzymes, Vantaa, Finland) and 2 μL DNA extract in the appropriate 1X buffer. Samples were subjected to an initial denaturation at 94 °C for 2 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 56 °C for 30 s and extension at 72 °C for 30 s, with a final extension step of 10 min at 72 °C. The PCR products were visualized on a 1% agarose electrophoresis gel with SYBR® Safe DNA Gel Stain (Invitrogen, Carlsbad, CA, USA). Some of the PCR products were then purified using ExoSAP-IT (GE Healthcare, Little Chalfont, UK) and sequenced at the Finnish Institute for Molecular Medicine (FIMM, Helsinki, Finland).

### Experiment assessing the effect of pathogen genotype, environmental conditions and their interaction on chasmothecia production

We inoculated full plants to assess whether the production of chasmothecia was affected by the origin of the pathogen strain and by environmental conditions, namely temperature (15 or 20 °C) and daylight duration (8 or 16 h light a day). Eight strains were used for this experiment (see map in Fig. 2): five originating from northern Europe (four collected from populations IDs 187, 325, 490 and 609 within the Åland archipelago and one from Saaremaa, Estonia) and three originating from southern Europe (Saint Jean du Gard and Saint Nazaire les Eymes in France, and Budapest in Hungary). Two replicates were performed for each pathogen strain*treatment (8 strains*4 treatments*2 replicates + 4 uninfected controls = 68 plants in total). We chose to inoculate plants originating from Netherlands because they have no history of co-evolution with any of the strains used. All the seeds derived from a cross between two widely divergent ecotypes (they are full-sibs). They were planted at the same time and kept in the greenhouse before the experiment.

Each plant was inoculated on 18 May 2012, by brushing one colony of the pathogen onto one leaf of the plant (this inoculated leaf was labelled with a black marker). Four plants were not inoculated and used as negative controls. Plants were then sealed inside two-window pollination bags (International Plant Breeding Supplies, PBS10-2) to contain infection on those plants and placed into four growth chambers. All the growth chambers were set to the optimal conditions for the pathogen's growth, namely 20 °C and 16 h light per day (Laine, 2008), during the first 14 days (until 1 June). Then, each of the four growth chambers was set to one temperature-by-light treatment, the temperature being 15 or 20 °C and daylight duration either 8 or 16 h. Plants were watered every 2–3 days. They were rigorously shaken to ensure within-plant propagation of the disease and rearranged within chambers every week. The experiment was stopped 37 days post-inoculation (24 June 2012). The total number of leaves, the

number of infected leaves ($N_I$) and the number of leaves bearing chasmothecia ($N_C$) were then scored. The two more severely infected leaves were sampled individually onto moist filter paper in Petri dishes. The following day (25 June 2012), these leaves were inspected under a stereomicroscope, and both the total number of dark (see Fig. 1b) chasmothecia ($N_{Cl}$) and the total area infected (A) were estimated. The product of the proportion of leaves bearing chasmothecia ($N_C/N_I$) and the density of chasmothecia ($N_{Cl}/A$) averaged over the two observed leaves was used as an estimate for chasmothecia production (CP). For the analysis of the whole data set, we used the MIXED procedure in SAS software (SAS Institute Inc., Cary, NC, USA) to test the effect of the origin of the pathogen strain (northern or southern Europe), light, temperature and their interactions on log-transformed CPx100; the exact strain nested in the origin was set as a random effect. To analyse the data set restricted to the Åland strain, we used the GLIMMIX procedure in SAS to test the effect of the pathogen strain, light, temperature and their interactions on CPx100 with a Poisson distribution.

## Results

### Field data from the Åland archipelago

The proportion of infected leaves bearing chasmothecia in the studied populations (Fig. 3) was on average 0.582, but revealed considerable variability, with values ranging between 0 (no chasmothecia observed) and 1 (all the leaves bearing chasmothecia). There is no difference ($\chi^2 = 0.016$, $P = 0.899$) in the proportion of leaves bearing chasmothecia between the populations exhibiting polymorphism (chasmothecia level: 0.598 ± 0.276) and the populations where no genetic diversity was found in the powdery mildew population (chasmothecia level: 0.568 ± 0.276).

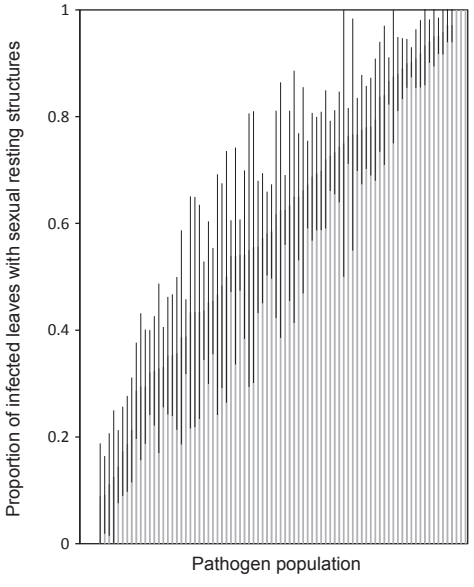


**Fig. 3** Distribution of the proportion of leaves bearing sexual resting structures in the 86 populations of *Podosphaera plantaginis* in the Åland archipelago.

### Experiment comparing chasmothecia production between single- and two-strain infections

Chasmothecia were produced both on the leaves infected by two strains and on the leaves infected by only one purified strain. The binary variable 'producing chasmothecia or not' was not affected by the number of inoculated strains (one single strain or a mix of two strains, $\chi^2 = 1.750$, $P = 0.186$, Fig. 4a). When chasmothecia were produced, their total number per leaf did not differ between single-strain or mixed inoculations ($t39 = 0.186$, $P = 0.853$, Fig. 4b). When observed under the microscope, the formed chasmothecia in case of single-strain infection were similar to the ones formed under mixed infection: they were still immature, but both contained an undifferentiated ascus (Fig. 1c) and consequently would likely produce mature ascospores.

### Mating-type genes amplification

All the samples (wild samples as well as purified laboratory strains) were shown to contain both mating-type genes (Appendix S2). The gene sequences were very similar to the sequences obtained at the nucleotide level for the closely related species *Po. xanthii* described by Brewer *et al.* (2011) (2.38% divergence at the nucleotide level for MAT1-1-1 and 2.25% for MAT1-1-2, Appendix S3).

### Experiment assessing the factors affecting chasmothecia production

In the experiment assessing the effect of daylight and temperature on chasmothecia production, 49 of the 64 inoculated plants (76.6%) were infected at the end of the experiment, whereas the four control plants remained uninfected. Within infected plants, the proportion of infected leaves ranged between 3.3% and 68.7%, and the proportion of infected leaves bearing chasmothecia varied between 0% and 100%. Only 19 plants (29.7% of the total) had the originally infected leaf still alive (this leaf withered on the other plants). The factors affecting the production of chasmothecia are presented in the Table 1 and the Fig. 5. When analysing the whole data set (49 observations), both temperature and the interaction between origin and temperature were significant explanatory variables (Table 1). The production of chasmothecia was higher at 15 °C than at 20 °C for both southern and northern

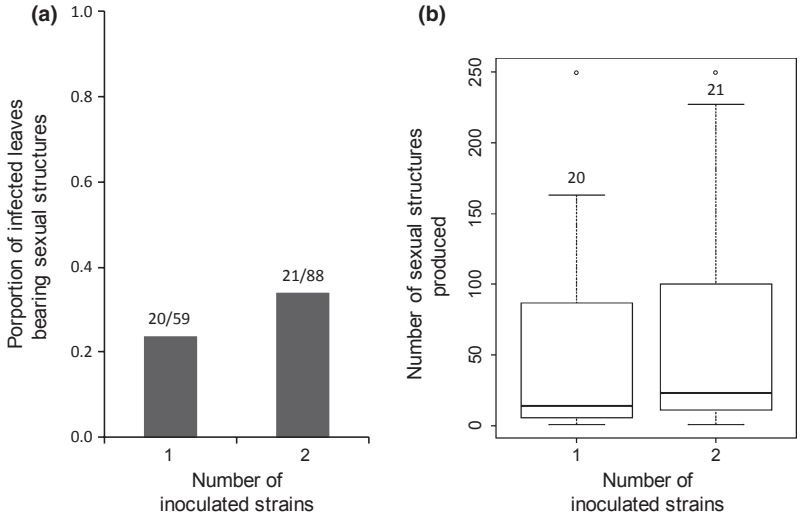


**Fig. 4** Absence of relationship between the amount of sexual resting structures produced and the number of *Podosphaera plantaginis* strains interacting under experimental conditions. (a) Comparison of the proportion of leaves bearing chasmothecia when a leaf is infected by one or two pathogen strains. (b) Comparison of the number of chasmothecia produced when a leaf is infected by one or two pathogen strains.

**Table 1** Factors affecting the estimate of experimental production of sexual resting structures (CPx100) in *Podosphaera plantaginis*.

| | Whole data set | | | Within Åland | | |
|---|---|---|---|---|---|---|
| Explanatory variable | d.f. | *F* | *P*-value | d.f. | *F* | *P*-value |
| Pathogen genotype (G) | 1 | 5.94 | 0.0507 | 3 | 5.05 | **0.0119** |
| Light treatment (L) | 1 | 0.30 | 0.5873 | 1 | 0.04 | 0.8387 |
| Temperature treatment (T) | 1 | 25.88 | **< 0.0001** | 1 | 6.29 | **0.0233** |
| G × T | 1 | 4.18 | **0.0489** | 3 | 3.69 | **0.0341** |

The variable 'Pathogen Genotype' corresponds to the origin (northern or southern European strain) in the case of the whole data set analysis and to the strain/population (four strains sampled from different populations) in the case of within-Åland analysis. Significant results (*P*-value < 0.05) are indicated in bold.

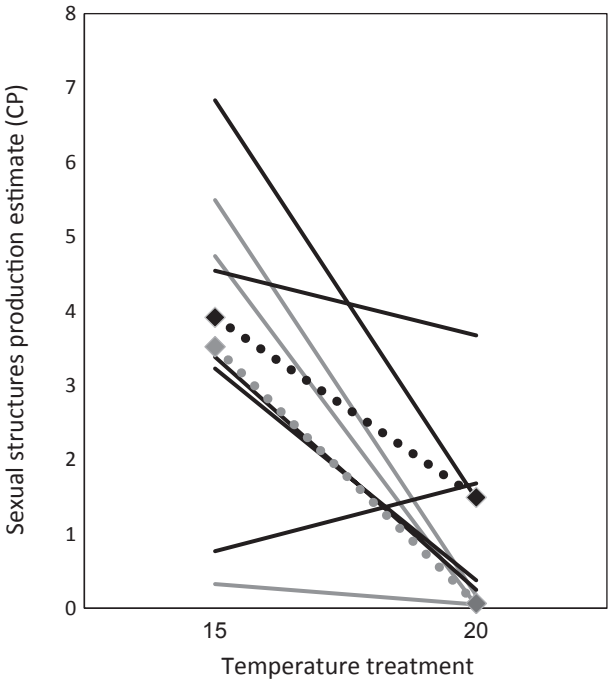


**Fig. 5** Effect of the temperature on the production of sexual resting structures in *Podosphaera plantaginis*. Average sexual resting structures (chasmothecia) production estimates (CP) are presented for each strain studied, as well as on average over the three southern European strains (France and Hungary, in grey) and the five northern strains (Finland and Estonia, in black).

European strains. But the strains also behaved differently depending on their origin (nonparallel reaction norm, Fig. 5) as northern strains produced significantly more chasmothecia at 20 °C than southern strains (*post hoc* comparison, $P = 0.0038$). The interaction between the strain and temperature was also a significant explanatory variable when analysing the data set comprising only the strains from the Åland archipelago (25 observations, Table 1). The effect of the light was non-significant in both analyses (Table 1).

## Discussion

In most pathosystems, the preponderance of studies has focused on the clonal pathogen transmission during the season of active host growth, whereas the production of sexual resting structures of the pathogen and inter-crop and overwinter survival has often remained comparatively neglected. However, this life-history stage is critical for both the evolutionary potential and multiple-year epidemiology of many pathogen species. This study aimed at a better understanding of the production of sexual resting structures in the *Plantago* powdery mildew and revealed that (1) this species is homothallic,

(2) pathogen strains differed in the extent to which they produced chasmothecia and (3) temperature conditions differently affected the production of chasmothecia depending on the origin of the pathogen. These genotype and genotype-by-environment effects may in part explain the highly variable levels of chasmothecia we observed in the natural pathogen populations.

We first investigated the capacity of *Po. plantaginis* to reproduce via haploid selfing using three approaches. First, field survey showed that natural pathogen populations varied considerably in their level of chasmothecia, ranging from the absence of sexual resting structures to the presence of chasmothecia on all the observed infected leaves. However, the proportion of chasmothecia observed was not affected by the number of strains circulating within the population: both monomorphic (single-strain) pathogen populations and polymorphic (multistrain) populations had similar levels of resting structures allowing off-season survival. Second, chasmothecia were produced under experimental conditions, even when infecting leaves with a single purified strain. Chasmothecia production levels were similar in experimental conditions involving a unique strain or a mix of two different strains. Finally, all the tested samples, including purified haploid strains, contained both mating-type loci in their genomes, as found in most homothallic Ascomycete species (Lin & Heitman, 2007). Preliminary observations revealed that the number of ascospores varies up to eight in mature chasmothecia, even in experimental infections involving a unique strain (R. Alanen, unpublished data), which excludes the hypothesis of pseudo-homothallism – a case where sexual reproduction occurs without syngamy (Billiard *et al.*, 2012), which is reflected in the consistent production of four rather than eight ascospores per ascus. This study consequently proves that *Po. plantaginis* is a homothallic species and constitutes the first report combining molecular and experimental evidence of haploid selfing ability within the powdery mildew group. Confirmed reports of homothallism appear to be rare within the powdery mildews compared with confirmed reports of heterothallism (Appendix S1). This may be an artefact due to the low number of species for which the breeding system has been described. Alternatively, this family could indeed have experienced few transitions from heterothallism to homothallism and hence form an exception within Ascomycetes (Billiard *et al.*, 2011). The powdery mildew family is characterized by its obligate biotrophy and by a large and complex genome (Spanu *et al.*, 2010). Whether some constraints impede the transition between breeding systems, or whether preventing same-clone mating was selectively maintained in Erysiphales, remains an open question and certainly deserves further investigations. Based on the sequencing of ITS region (GenBank accession number: JX442063) and comparison with previously published data sets (Ito & Takamatsu, 2010; Takamatsu *et al.*, 2010), *Po. plantaginis* belongs to the basal paraphyletic assemblage of the subsection *Magnicellulatae* within the genus *Podosphaera* (C. Tollenaere, unpublished). Assessing the breeding system of related species would shed light on the evolutionary history of sexual reproduction in this fungal group.

In heterothallic fungi, increased disease prevalence enhances the probability of contact between the two mating types (Schnathorst, 1965; Gadoury *et al.*, 2012). The production of sexual resting structures is thus density dependent and maximal at the end of the growing season. This mechanism is absent in homothallic fungi, rendering critical the environmental signals triggering the production of the resting structures at the right moment, namely before the off-season begins. The daylight duration did not affect the production of sexual resting structures in our experiment. This result is congruent with a previous study documenting no effect of daylight duration treatment (10–12 h light per day) on chasmothecia production in *E. pulchra* from Tennessee (Mmbaga, 2002). On the other hand, a decrease in temperature from 20 °C to 15 °C increased the production of chasmothecia for both northern and southern European strains. The temperature decrease occurring at the end of the summer would thus induce the production of the resting structures in *Po. plantaginis*. Strong temperature effect on chasmothecia production had been evidenced in *E. pulchra* (Mmbaga, 2002) and in *E. necator*, with an optimal temperature of 15 °C (Gadoury & Pearson, 1988) or 20 °C (Legler *et al.*, 2012). However, potential differences between pathogen genotypes were not investigated in these previous studies. Similarly, the timing of ascospore release was strongly affected by environmental factors in the oak powdery mildew *E. alphitoides*, but no between-strain differentiation was evidenced across Europe (Marcais *et al.*, 2009). Genetic differentiation among populations, as a consequence of divergent selection, is, however, a key prediction of the Geographic Mosaic Theory of Coevolution (Thompson, 2005; Laine, 2009). This study demonstrates that the effect of the temperature on the production of chasmothecia differs depending on the origin of the strains (genotype-by-environment effect). Similarly high levels of chasmothecia were produced for both strains from the north and the south of Europe at 15 °C, whereas northern strains produced significantly more chasmothecia than southern strains at 20 °C. The production of sexual resting structures would thus be higher and encompasses most of the growing season in the north of Europe, reflecting their key role in the pathogen survival during cold northern winters. This is in accordance with the observation of chasmothecia already in July in the Åland islands. Milder winter conditions in Southern Europe would have allowed the pathogen to evolve reduced duration

of sexual resting structures production to the period predating winter (when temperatures drop below 15 °C).

This study also reveals the very small spatial scale for which variability in the experimental production of sexual resting structure can be evidenced. Indeed, our strains collected 17–42 km apart within the Åland archipelago all proved to be genetically different (data not shown) and to differ in their level of chasmothecia production. Moreover, the interaction between temperature and the strain genotype affected the production of sexual resting structures within the Åland archipelago as well. Hence, G × E effects can also be detected at small spatial scales. These effects of the pathogen genotype, the environment and their interaction (G, E and G × E effects) may in part explain the high variability observed in the sexual resting structures levels within the Åland metapopulation. Indeed, only asexual spores (conidia) were observed in some populations, whereas all the leaves were bearing sexual resting structures in others (chasmothecia production levels ranging between zero and one, see Fig. 3). This aspect is critical as the level of chasmothecia estimated in the field in autumn affects the overwintering success and is consequently thought to be a key driver of the metapopulation dynamics in this pathosystem (A. J. M. Tack & A.-L. Laine, unpublished). In addition to their importance as ecological factors, G × E interactions affecting fitness components are also a prerequisite for local adaptation (Kawecki & Ebert, 2004). Both G × E interaction and local adaptation have often been described for seed production in plants (see e.g. Nagy & Rice, 1997; McLeod *et al.*, 2012), but not previously to our knowledge on the production of sexual structures in a fungal plant pathogen. Local adaptation in terms of infectivity has also been evidenced at small spatial scales within the Åland metapopulation (Laine, 2005). Furthermore, G × E effects were also documented on the asexual stage of this species, with temperature differentially affecting the summer clonal spread of the different pathogen genotypes (Laine, 2007, 2008). Additional experiments with strains sampled from Åland could well reveal small-scale local adaptation for less explored life-history traits of the fungus such as the sexual stage investigated in this study.

The effect of the host plant genotype was not taken into account in this experiment as we used plants from similar genetic background (full-sibs) originated from a country where the pathogen was not sampled (and consequently not sharing a co-evolutionary history with the studied strains). However, the effect of the host plant on chasmothecia production would be very interesting to test, as the maturation of chasmothecia may be affected by the host cultivar (Gadoury & Pearson, 1988) and/or by the leaf physiological status (Jhooty & McKeen, 1962; Schnathorst, 1965). Taking the host plant variability into account would allow to encompass the whole complexity of potential pathogen genotype-by-host genotype-by-environment ($G_P \times G_H \times E$) interactions (Vale *et al.*, 2008; Wolinska & King, 2009; Bryner & Rigling, 2011) towards a better understanding of the spatiotemporal variation in selective pressure that parasite species may face in a mosaic of selective pressures.

In conclusion, our results show that *Po. plantaginis* is homothallic, but this does not imply that haploid selfing would be the prominent mating system in the wild (Billiard *et al.*, 2012). Haploid selfing (or same-clone mating, or intrahaploid mating, Billiard *et al.*, 2011) does not involve recombination, and its genetic consequences are identical to asexual reproduction (Billiard *et al.*, 2012). Outcrossing could be frequent in the populations comprising various pathogen genotypes, and in that case, natural selection may influence selfing vs. outcrossing rates. Monomorphic populations lacking compatible mating types may on the other hand exhibit exclusively haploid selfing. Estimating the frequency of haploid selfing *in natura* would provide a better understanding of how selective pressures shape mating patterns in the wild and how important is recombination for the co-evolution between host and pathogen species. More generally, investigating pathogen sexual reproduction through both ecological and genetic approaches offers an exciting future venue of research and is needed to uncover the drivers of parasite evolutionary potential and between-season epidemiology.

## Acknowledgments

We are grateful to Ayco Tack for providing the Estonian strain and Levente Kiss for Hungarian samples. We also thank Jérome Gleyze and Ludivine Cabirou for their help in strain sampling, as well as Kalle Meller, Virpi Ahola, Otso Ovaskainen and Anne Galodé for their attempts to find *Plantago* powdery mildew in different locations. We are grateful to Arjen Biere for providing *Plantago lanceolata* seeds from Netherlands and to Krista Raveala for taking care of the plants and fungal strains. We thank Hanna Susi, Marin Brewer and David Gadoury for technical advises and helpful discussions throughout the work. The manuscript benefited from helpful comments from Ayco Tack, Tatiana Giraud, David Gadoury and two anonymous reviewers. This work was supported by funding from the Academy of Finland (Grant Nos 250444, 136393, 133499) and European Research Council (PATHEVOL; 281517) to A.-L. Laine.

## References

Agrios, G.N. 2005. *Plant Pathology*, 5th edn. Academic Press, California.

Alby, K., Schaefer, D. & Bennett, R.J. 2009. Homothallic and heterothallic mating in the opportunistic pathogen *Candida albicans*. *Nature* **460**: 890–893.

Barrett, L.G., Thrall, P.H., Burdon, J.J. & Linde, C.C. 2008a. Life history determines genetic structure and evolutionary potential of host-parasite interactions. *Trends Ecol. Evol.* **23**: 678–685.

Barrett, L.G., Thrall, P.H., Burdon, J.J., Nicotra, A.B. & Linde, C.C. 2008b. Population structure and diversity in sexual and asexual populations of the pathogenic fungus *Melampsora lini*. *Mol. Ecol.* **17**: 3401–3415.

Becks, L. & Agrawal, A.F. 2012. The evolution of sex is favoured during adaptation to new environments. *PLoS Biol.* **10**: e1001317.

Bélanger, R.R., Bushnell, W.R., Dik, A.J. & Carver, T.L.W. 2002. *The Powdery Mildews: A Comprehensive Treatise*. American Phytopathological Society Press, St. Paul.

Billiard, S., Lopez-Villavicencio, M., Devier, B., Hood, M.E., Fairhead, C. & Giraud, T. 2011. Having sex, yes, but with whom? Inferences from fungi on the evolution of anisogamy and mating types. *Biol. Rev.* **86**: 421–442.

Billiard, S., Lopez-Villavicencio, M., Hood, M.E. & Giraud, T. 2012. Sex, outcrossing and mating types: unsolved questions in fungi and beyond. *J. Evol. Biol.* **25**: 1020–1038.

Brewer, M.T., Lance, C.D.B., Paolo, C.C., Spanu, P.D. & Milgroom, M.G. 2011. Identification and structure of the mating-type locus and development of PCR-based markers for mating type in powdery mildew fungi. *Fungal Genet. Biol.* **48**: 704–713.

Bryner, S.F. & Rigling, D. 2011. Temperature-dependent genotype-by-genotype interaction between a pathogenic fungus and its hyperparasitic virus. *Am. Nat.* **177**: 65–74.

Cooper, T.F. 2007. Recombination speeds adaptation by reducing competition between beneficial mutations in populations of *Escherichia coli*. *PLoS Biol.* **5**: 1899–1905.

Dean, R., Van Kan, J.A.L., Pretorius, Z.A., Hammond-Kosack, K.E., Di Pietro, A., Spanu, P.D. *et al.* 2012. The top 10 fungal pathogens in molecular plant pathology. *Mol. Plant Pathol.* **13**: 414–430.

Gadoury, D.M. & Pearson, R.C. 1988. Initiation, development, dispersal, and survival of cleistothecia of Uncinula-Necator in New York vineyards. *Phytopathology* **78**: 1413–1421.

Gadoury, D.M., Cadle-Davidson, L., Wilcox, W.F., Dry, I.B., Seem, R.C. & Milgroom, M.G. 2012. Grapevine powdery mildew (*Erysiphe necator*): a fascinating system for the study of the biology, ecology and epidemiology of an obligate biotroph. *Mol. Plant Pathol.* **13**: 1–16.

Glawe, D.A. 2008. The powdery mildews: a review of the world's most familiar (yet poorly known) plant pathogens. *Annu. Rev. Phytopathol.* **46**: 27–51.

Goddard, M.R., Godfray, H.C. & Burt, A. 2005. Sex increases the efficacy of natural selection in experimental yeast populations. *Nature* **434**: 636–640.

Hanski, I. 1999. *Metapopulation Ecology*. Oxford University Press, New York.

Heitman, J. 2006. Sexual reproduction and the evolution of microbial pathogens. *Curr. Biol.* **16**: R711–R725.

Heitman, J., Kronstad, J.W., Taylor, J.W. & Casselton, L.A. 2007. *Sex in Fungi*. American Society for Microbiology, Washington.

Hosid, E., Grishkan, I., Yusim, E., Frenkel, Z., Wasser, S.P., Nevo, E. *et al.* 2010. The mode of reproduction in natural populations of ascomycetous fungus, *Emericella nidulans*, from Israel. *Genet. Res (Camb)* **92**: 83–90.

Ito, M. & Takamatsu, S. 2010. Molecular phylogeny and evolution of subsection Magnicellulatae (Erysiphaceae: Podosphaera) with special reference to host plants. *Mycoscience* **51**: 34–43.

Jhooty, J.S. & McKeen, W.E. 1962. The perfect stage of *Sphaerotheca macularis* on strawberry plants. *Plant Dis. Rep.* **46**: 218–219.

Kawecki, T.J. & Ebert, D. 2004. Conceptual issues in local adaptation. *Ecol. Lett.* **7**: 1225–1241.

Klaassen, C.H., Gibbons, J.G., Fedorova, N.D., Meis, J.F. & Rokas, A. 2012. Evidence for genetic differentiation and variable recombination rates among Dutch populations of the opportunistic human pathogen *Aspergillus fumigatus*. *Mol. Ecol.* **21**: 57–70.

Laine, A.L. 2004. Resistance variation within and among host populations in a plant-pathogen metapopulation: implications for regional pathogen dynamics. *J. Ecol.* **92**: 990–1000.

Laine, A.L. 2005. Spatial scale of local adaptation in a plant-pathogen metapopulation. *J. Evol. Biol.* **18**: 930–938.

Laine, A.L. 2007. Pathogen fitness components and genotypes differ in their sensitivity to nutrient and temperature variation in a wild plant-pathogen association. *J. Evol. Biol.* **20**: 2371–2378.

Laine, A.L. 2008. Temperature-mediated patterns of local adaptation in a natural plant-pathogen metapopulation. *Ecol. Lett.* **11**: 327–337.

Laine, A.L. 2009. Role of coevolution in generating biological diversity: spatially divergent selection trajectories. *J. Exp. Bot.* **60**: 2957–2970.

Laine, A.L. & Hanski, I. 2006. Large-scale spatial dynamics of a specialist plant pathogen in a fragmented landscape. *J. Ecol.* **94**: 217–226.

Legler, S.E., Caffi, T. & Rossi, V. 2012. A nonlinear model for temperature-dependent development of *Erysiphe necator* chasmothecia on grapevine leaves. *Plant. Pathol.* **61**: 96–105.

Levins, R. 1969. Some demographic and genetic consequences of environmental heterogeneity for biological control. *Bull. Entomol. Soc. Am.* **71**: 237–240.

Lin, X. & Heitman, J. 2007. Mechanisms of homothallism in fungi and transitions between heterothallism and homothallism. In: *Sex in Fungi: Molecular Determination and Evolutionary Implications* (J. Heitman, J.W. Kronstad, J.W. Taylor & L.A. Casselton, eds), pp. 35–57. ASM Press, Washington.

Lloyd, D.G. 1992. Self- and cross-fertilization in plants. II. The selection of self-fertilization. *Int. J. Plant Sci.* **153**: 370–380.

Marcais, B., Kavkova, M. & Desprez-Loustau, M.L. 2009. Phenotypic variation in the phenology of ascospore production between European populations of oak powdery mildew. *Ann. For. Sci.* **66**: 814.

Marra, R.E., Cortesi, P., Bissegger, M. & Milgroom, M.G. 2004. Mixed mating in natural populations of the chestnut blight fungus, *Cryphonectria parasitica*. *Heredity* **93**: 189–195.

McDonald, B.A. & Linde, C. 2002. Pathogen population genetics, evolutionary potential, and durable resistance. *Annu. Rev. Phytopathol.* **40**: 349–379.

McLeod, K.A., Scascitelli, M. & Vellend, M. 2012. Detecting small-scale genotype-environment interactions in apomictic dandelion (*Taraxacum officinale*) populations. *J. Evol. Biol.* **25**: 1667–1675.

Mmbaga, M.T. 2002. Ascocarp formation and survival and primary inoculum production in Erysiphe (sect. Microsphaera) pulchra in dogwood powdery mildew. *Ann. Appl. Biol.* **141**: 153–161.

Mori, Y., Sato, Y. & Takamatsu, S. 2000. Evolutionary analysis of the powdery mildew fungi using nucleotide sequences of the nuclear ribosomal DNA. *Mycologia* **92**: 74–93.

Morran, L.T., Schmidt, O.G., Gelarden, I.A., Parrish, R.C. II & Lively, C.M. 2011. Running with the Red Queen: host-parasite coevolution selects for biparental sex. *Science* **333**: 216–218.

Nagy, E.S. & Rice, K.J. 1997. Local adaptation in two subspecies of an annual plant: implications for migration and gene flow. *Evolution* **51**: 1079–1089.

Rougeron, V., De Meeus, T., Hide, M., Waleckx, E., Bermudez, H., Arevalo, J. *et al.* 2009. Extreme inbreeding in *Leishmania braziliensis*. *Proc. Natl. Acad. Sci. USA* **106**: 10224–10229.

Runions, C.J. & Geber, M.A. 2000. Evolution of the self-pollinating flower in *Clarkia xantiana* (Onagraceae). I. Size and development of floral organs. *Am. J. Bot.* **87**: 1439–1451.

Saleh, D., Xu, P., Shen, Y., Li, C.G., Adreit, H., Milazzo, J. *et al.* 2012. Sex at the origin: an Asian population of the rice blast fungus *Magnaporthe oryzae* reproduces sexually. *Mol. Ecol.* **21**: 1330–1344.

Schnathorst, W. 1965. Environmental relationships in powdery mildews. *Annu. Rev. Phytopathol.* **3**: 343–366.

Soubeyrand, S., Laine, A.L., Hanski, I. & Penttinen, A. 2009. Spatiotemporal structure of host-pathogen interactions in a metapopulation. *Am. Nat.* **174**: 308–320.

Spanu, P.D., Abbott, J.C., Amselem, J., Burgis, T.A., Soanes, D.M., Stuber, K. *et al.* 2010. Genome expansion and gene loss in powdery mildew fungi reveal tradeoffs in extreme parasitism. *Science* **330**: 1543–1546.

Spencer, D.M. 1978. *The Powdery Mildews*. Academic Press, London.

Takamatsu, S., Niinomi, S., Harada, M. & Havrylenko, M. 2010. Molecular phylogenetic analyses reveal a close evolutionary relationship between Podosphaera (Erysiphales: Erysiphaceae) and its rosaceous hosts. *Persoonia* **24**: 38–48.

Thompson, J.N. 2005. *The Geographic Mosaic of Coevolution*. Chicago University Press, Chicago.

Tollenaere, C., Susi, H., Nokso-Koivisto, J., Koskinen, P., Tack, A., Auvinen, P. *et al.* 2012. SNP design from 454 sequencing of Podosphaera plantaginis transcriptome reveals a genetically diverse pathogen metapopulation with high levels of mixed-genotype infection. *PLoS ONE* **7**: e52492.

Vale, P.F., Salvaudon, L., Kaltz, O. & Fellous, S. 2008. The role of the environment in the evolutionary ecology of host parasite interactions – meeting report, Paris, 5th December, 2007. *Infect. Genet. Evol.* **8**: 302–305.

van Valen, L. 1973. A new evolutionary law. *Evol. Theory* **1**: 1–30.

Varga, J. & Toth, B. 2003. Genetic variability and reproductive mode of *Aspergillus fumigatus*. *Infect. Genet. Evol.* **3**: 3–17.

Wolinska, J. & King, K.C. 2009. Environment can alter selection in host-parasite interactions. *Trends Parasitol.* **25**: 236–244.

## Supporting information

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

**Appendix S1** Literature review of the mating system in powdery mildew species.

**Appendix S2** Gel picture showing the amplification of both mating-type genes MAT1-1-1 and MAT1-2-1 in purified strains originating from various locations in Europe.

**Appendix S3** Alignment of the sequences of mating-type genes in *Podosphaera plantaginis* to the most related sequences described to date: the cucurbit powdery mildew *Po. xanthii* (from Brewer *et al.*, 2011, *Fungal Genetics and Biology*).

Data deposited at Dryad: doi:10.5061/dryad.r1k1f

*Received 8 January 2013; revised 11 March 2013; accepted 27 March 2013*