A hyperparasite affects the population dynamics of a wild plant pathogen

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Abstract

Assessing the impact of natural enemies of plant and animal pathogens on their host's population dynamics is needed to determine the role of hyperparasites in affecting disease dynamics, and their potential for use in efficient control strategies of pathogens. Here, we focus on the long-term study describing metapopulation dynamics of an obligate pathogen, the powdery mildew (*Podosphaera plantaginis*) naturally infecting its wild host plant (*Plantago lanceolata*) in the fragmented landscape of the Åland archipelago (southwest Finland). Regionally, the pathogen persists through a balance of extinctions and colonizations, yet factors affecting extinction rates remain poorly understood. Mycoparasites of the genus *Ampelomyces* appear as good candidates for testing the role of a hyperparasite, i.e. a parasite of other parasites, in the regulation of their fungal hosts' population dynamics. For this purpose, we first designed a quantitative PCR assay for detection of *Ampelomyces* spp. in field-collected samples. This newly developed molecular test was then applied to a large-scale sampling within the Åland archipelago, revealing that *Ampelomyces* is a widespread hyperparasite in this system, with high variability in prevalence among populations. We found that the hyperparasite was more common on leaves where multiple powdery mildew strains coexist, a pattern that may be attributed to differential exposure. Moreover, the prevalence of *Ampelomyces* at the plant level negatively affected the overwinter survival of its fungal host. We conclude that this hyperparasite may likely impact on its host population dynamics and argue for increased focus on the role of hyperparasites in disease dynamics.

Keywords: disease, hyperparasite, metapopulation, molecular detection, plant pathogen, regulation

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Introduction

Pathogens are frequently cited as important drivers of population dynamics of their hosts (Anderson & May 1991; Tompkins *et al.* 2002). Well-known examples include demographic cycles driven, at least partly, by parasites in the red grouse (Hudson *et al.* 1998; New *et al.* 2009) and the Soay sheep (Gulland *et al.* 1993). Highly virulent parasites may lead to a dramatic decrease of their host population (Harding *et al.* 2002), ultimately driving some populations to extinction (Vredenburg *et al.* 2010). Such disease-induced extinctions are not predicted by simple models of parasite dynamics where parasites should always go extinct before their hosts (Anderson & May 1991). However, small

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pre-epidemic host population size and the presence of alternative hosts (reservoirs) may lead to the extinction of a species by its parasites (de Castro & Bolker 2005). Hence, while the effect of pathogens on their host population dynamics appears highly plausible, empirical knowledge of whether and how parasites regulate host population size in natural populations remains limited, with most evidence being indirect (Schmid-Hempel 2011). Moreover, to date research on the effect of pathogens on host population dynamics has mostly focused on vertebrate hosts (Schmid-Hempel 2011).

Many species inhabit fragmented landscapes and may persist regionally as a metapopulation, that is an assemblage of spatially delimited local populations interconnected by migration and experiencing limited lifespan. The regional persistence of the metapopulation depends on a balance between local extinctions and colonizations (Levins 1969; Hanski 1999). The development of the metapopulation theory has been accelerated in the face of habitat fragmentation and the need for conservation applications for species inhabiting spatially fragmented landscapes. In this context, diseases are frequently mentioned as factors increasing extinction risk when enhancing migration levels through establishment of corridors (Simberloff & Cox 1987; Hess 1994). A modelling study confirmed that increasing migration within a host-pathogen metapopulation may lead to extinction of the host population under certain conditions (Harding *et al.* 2012). The impact of pathogens on their host's metapopulation dynamics was also highlighted in a recent study focusing on infectious disease in coral reefs (Sokolow 2009). In spite of these two recent studies, theoretical and empirical assessment of the relevance of diseases as drivers of metapopulation dynamics remains surprisingly rare in the ecological literature.

The fungal pathogen *Podosphaera plantaginis*, a powdery mildew (Erysiphales, Ascomycota), persists as a metapopulation in the fragmented dry meadows of its host plant *Plantago lanceolata* in the Åland archipelago (Laine & Hanski 2006; Jousimo *et al.* 2014). Approximately 4000 patches of the host plant have been surveyed annually since 2001, representing one of the few long-term studies of plant pathogens (see also Antonovics 2004; Smith *et al.* 2011). This pathosystem is characterized by a very low prevalence at the metapopulation scale (proportion of infected meadows remained generally lower than 7%) and very high local turnover (Jousimo *et al.* 2014). Approximately 40% of the local pathogen populations go extinct every winter (Tack & Laine 2014), rendering it critical that we delineate the factors affecting off-season survival of the pathogen population to understand regional dynamics. To this purpose, Tack & Laine (2014) used reciprocal transplant experiments to explore the interplay between different

factors (namely the pathogen population of origin and overwintering site) in determining infection intensity in the following season, but the precise mechanisms underlying these effects have not yet been elucidated. Pathogens may themselves be the target of parasitism, a phenomenon known as hyperparasitism. The striking impact that hyperparasites can have upon plant pathogens has been previously demonstrated by extensive studies of dsRNA viruses infecting *Cryphonectria parasitica*, the fungus responsible for chestnut blight (Milgroom & Cortesi 2004). The hyperparasite was shown to modulate the pathogen virulence and consequently the host plant population size (Davelos & Jarosz 2004) and to shape the pathogen diversity (Brusini *et al.* 2011). Between-population variability in hyperparasite prevalence was also evidenced, as well as between-strain differences in the hyperparasite effect on its mycohost (Robin *et al.* 2010). However, the potential impact of hyperparasites on the natural metapopulation dynamics of any plant pathogen has never been investigated.

The most widespread and oldest known natural enemies of powdery mildews are *Ampelomyces* spp. (Kiss 2008). *Ampelomyces* spp. are intracellular fungal mycoparasites, that is fungi parasitizing other fungi. Their hyphae grow inside the mycelia of their hosts, killing the powdery mildew hyphae by degeneration of the cell content. Asexual fruiting bodies of *Ampelomyces*, called pycnidia, are produced in the hyphae, conidiophores and immature chasmothecia (i.e. sexual resting structures, syn: cleistothecia) of their fungal hosts (see Fig. S1, Supporting information, and Kiss 2008). In other systems, it has been shown that a fraction of the powdery mildew overwintering structures are destroyed by *Ampelomyces* every year in the field (Kiss 1998; Füzi 2003). As the overwintering success in *P. plantaginis* depends on the abundance of these resting structures (Tack & Laine 2014), parasitism by *Ampelomyces* may have critical consequences on the dynamics of this pathosystem.

The impact of hyperparasites on the dynamics of natural pathogen populations is poorly understood. The effect of *Ampelomyces* mycoparasites on natural populations of powdery mildews has been scarcely documented, despite the commercialization of *Ampelomyces* as a biocontrol agent of powdery mildews (AQ10™ Biofungicide; Ecogen Inc., Langhorne, PA, USA; Kiss *et al.* 2004). The aim of this study was to document the prevalence of *Ampelomyces* within the metapopulation of *P. plantaginis* and to test whether the hyperparasite has an impact on extinction rates of its host.

For this purpose, we developed a molecular screening test to detect *Ampelomyces* spp. in field-collected mixed samples containing both the powdery mildew and the host plant DNA. We chose to amplify the ITS region as it,

the reference DNA region for fungal species identification (Schoch *et al.* 2012), by quantitative polymerase chain reaction (qPCR) method. We then applied this detection method to a global sampling within the Åland metapopulation. We use these data to assess whether the level of multiple genotype infection (i.e. when multiple powdery mildew strains infect the same host leaf) affects the probability that the sample contains an *Ampelomyces* infection. Assuming that each powdery mildew strain has a certain probability of carrying *Ampelomyces*, leaves infected by multiple powdery mildew strains would be more likely to harbour *Ampelomyces* than leaves infected by a single powdery mildew strain. Secondly, we tested for a negative impact of the prevalence of *Ampelomyces* on the key life history stage of *P. plantaginis* – overwintering. Our results revealed that taking the hyperparasite into account might be important for understanding its phytopathogenic hosts' population dynamics.

Materials and methods

Study system

Podosphaera plantaginis is an obligate pathogen specific to the ribwort plantain *Plantago lanceolata* (Plantaginaceae). This powdery mildew species has repeated cycles of asexual reproduction throughout the summer with the production of wind-dispersed conidia. It survives host dormancy in winter as sexual resting structures called chasmothecia (Glawe 2008).

Within the Åland archipelago (southwest Finland), populations of *P. lanceolata* are typically small (<1 ha) and fragmented, occurring mainly on dry meadows. Since 2001, the occurrence of *P. plantaginis* has been systematically surveyed in approximately 4000 populations of *P. lanceolata* throughout the Åland archipelago. The presence/absence of this pathogen in each *P. lanceolata* meadow is recorded by a group of students every year in early September, when the clonal summer spread of the pathogen has terminated. *Podosphaera plantaginis* is the only powdery mildew species known to infect *P. lanceolata* within the Åland islands (A.-L. Laine, unpublished data). Infection of leaves by powdery mildew is easily visible due to the white-greyish mycelial growth on the leaf surface. Upon discovery, an infected leaf has always been collected and dried, and the presence of the powdery mildew has been confirmed by subsequent microscopy of these samples (see also Laine & Hanski 2006).

Molecular characterization of the first field observations of Ampelomyces

In September 2011, potentially infected leaves were collected in 292 meadows during the September survey,

of which 282 were confirmed to support *P. plantaginis*. Obvious signs of pycnidia typical of *Ampelomyces* sp. were found in four of the 282 infected leaves collected. The location of these four observations is indicated in Fig. 1.

We sequenced the ITS regions of these four samples exhibiting pycnidia to confirm our visual identification of *Ampelomyces* and to identify their *Ampelomyces* lineage. For this purpose, we extracted DNA of 1 cm² samples of the dried infected *Plantago* leaves bearing pycnidia using E.Z.N.A. plant DNA kit (Omega Bio-Tek, Doraville, GA, USA). We amplified the ITS region according to the nested PCR protocol described in Ito & Takamatsu (2010), using primers P3 (5′-GCCGCTTCACTCGCCGT TAC-3′) and ITS5 (5′-GGAAGTAAAAGTCGTAA CAAGG-3′) in a first amplification, followed by a second PCR using ITS5 and ITS4 (5′-TCCTCCGCTTATTGA TATGC-3′). The PCR products were purified using Exosap-IT (GE Healthcare, Buckinghamshire, UK). Sequencing of the purified PCR products was performed by the Institute for Molecular Medicine Finland (FIMM, Helsinki, Finland) laboratory. We compared the obtained sequences to the GenBank nucleotide database (http://www.ncbi.nlm.nih.gov/genbank/).

In September 2013, a small-scale field survey was conducted in Åland to detect again the presence of *Ampelomyces* mycoparasites in powdery mildew colonies. Mycoparasites were collected from four sample sites (Fig. 1), and seven strains were isolated and maintained in culture as described in Liang *et al.* (2007). The ITS sequences were determined in these seven newly isolated Åland strains as described above.

Design of a molecular detection test for Ampelomyces spp.

To screen our field samples that consisted of a mix of plant, powdery mildew and possibly *Ampelomyces* DNA, we aimed at designing a primer pair specific for *Ampelomyces*, and amplifying different *Ampelomyces* lineages (see below), that would not amplify the fungal host *P. plantaginis*, nor the host plant *P. lanceolata*. The forward primer (AQ-F264, 5′-GATGAAGAACGCAGC GAAAT-3′) is located in a conserved region of the ITS sequence: 100% identity with *P. plantaginis* and 95% with *P. lanceolata*. The reverse primer (AQ-R462, 5′-GCT GCCAATTGCTTTGAGAT-3′) is located in a hypervariable region; however, it presents 60% identity with ITS sequence of the host plant and the powdery mildew fungal host. This partial overlap of the *Ampelomyces*-specific AQ-F264/AQ-R462 primers and the ITS sequence of *P. plantaginis* could not be avoided due to the diversity of ITS sequences determined in different *Ampelomyces* lineages (Pintye *et al.* 2012). However, the
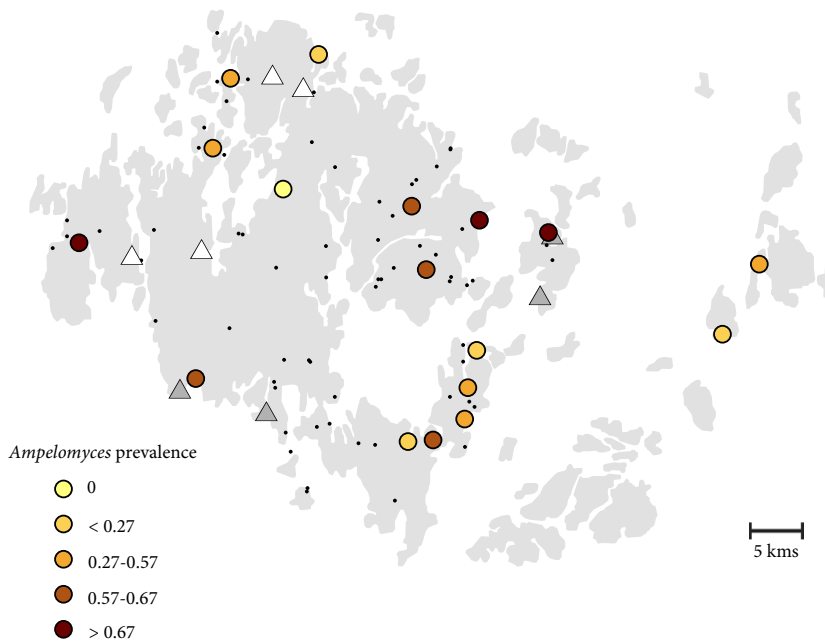
Fig. 1 Map of the study sites, indicating the location of (i) the initial observation of pycnidia in 2011 (four populations indicated with white triangles), (ii) the populations sampled in 2013 (four sites indicated with grey triangles), (iii) the populations having sufficient sample size (>22 samples) to estimate *Ampelomyces* prevalence (the colour gradient indicates the prevalence level) and (iv) all the sampled populations (black dots).

Ampelomyces prevalence

- 0
- < 0.27
- 0.27–0.57
- 0.57–0.67
- > 0.67

5 kms

qPCR method developed in this work did reliably distinguish the *Ampelomyces*-specific amplifications from false-positive results by setting up a proper cut-off value (see below).

Each qPCR reaction was performed in a final volume of 10 μL containing 5 μL SYBRGreen PCR Master Mix (Applied Biosystems, Madrid, Spain), 0.5 μL of each primer at 5 μM and 1 μL DNA sample. Assays were carried out using Bio-Rad CFX-496 (Hercules, CA, USA) qPCR machine with the following conditions: initial denaturation of 10 min at 95 °C, followed by 40 cycles of denaturation 30 s at 95 °C, annealing 30 s at 60 °C and extension 30 s at 72 °C; final extension lasted 5 min at 72 °C and the melting curve analysis from 45 °C to 95 °C by 0.5 °C increment every 5-s. The software Bio-Rad CFX Manager was used to visualize the results.

As the within-species diversity of *Ampelomyces* within Åland has not yet been studied, we assessed the generality of our detection method by testing six different *Ampelomyces* strains (A1, AQ10, B124-a, GYER, TP1 and Vitis32, see details in Table S1, Supporting information) belonging to four different lineages of the five described to date (Pintye *et al.* 2012). These strains were grown on Czapek-Dox medium supplemented with 2% malt. DNA was extracted using the same method as previously described. Ten-fold serial dilutions of the DNA isolated from these strains were used to establish standard curves.

To assess the reliability of the newly designed method and to determine a qPCR cycle threshold with which to score the binary response of infected or uninfected samples, we generated *Ampelomyces*-infected

P. plantaginis under laboratory conditions. Detached *P. lanceolata* leaves from a single maternal lineage were placed onto moist filter paper in 9-cm petri dishes and inoculated with single conidial chains of Åland-derived strains of *P. plantaginis* following the protocol described by Nicot *et al.* (2002). After 9 days of growth at 21 °C at 16 h:8 h, D:L mildew lesions were inoculated by spraying 0.069 ± 0.004 mL of either a suspension of *Ampelomyces* spores in water (5×10^5 spores/mL) or an equal volume of water directly onto the infected leaves. The *Ampelomyces* strains used in these mycoparasitic tests were isolated in Åland in September 2013 (see above). After 19 days, *Ampelomyces* treated lesions were observed under a dissecting microscope and scored on a four-category scale for presence of pycnidia: 'none' = no pycnidia observed, 'few' = one or two patches of very few pycnidia, 'moderate' = pycnidia covering <75% of *Podosphaera* infection, and 'many' = pycnidia covering more than 75% of *Podosphaera* infection. Control leaves infected with just *P. plantaginis* were also checked to confirm the absence of *Ampelomyces* contamination. Both treated and control leaves were prepared for DNA extraction following the same protocol as described for the large-scale field sampling (see below). The qPCR procedure was applied in triplicate for each treated sample ($n = 20$), for nine *P. plantaginis*-only controls and for five infection-free leaves. An additional 36 *P. plantaginis*-only controls were screened once. We tested for a correlation between the observed categories of pycnidia coverage and the result of the molecular test.

Finally, we used an in vitro method assessing the reliability of the newly designed molecular detection test through the use of plasmids containing the ITS sequence obtained from most *Ampelomyces* strains isolated in Aland (see the detailed methodology in Appendix S1, Supporting information).

Molecular procedures applied to a large-scale field sampling

In late September 2011, 282 *Plantago* populations were found to be infected by the powdery mildew during the survey of the entire meadow network. We chose 98 of these powdery mildew populations, located throughout the Åland archipelago (Fig. 1), for sample collection. In each population, five infected plants were randomly selected for sampling. Sampled plants were labelled using coloured wooden sticks, and plant positions were recorded using GPS. One leaf per plant was collected in most populations, whereas 17 populations were sampled more intensively with five infected leaves collected from four plants and ten infected leaves collected from one plant.

Powdery mildew-infected *Plantago* leaves collected in the field were first placed in a falcon tube. After being transported to the laboratory in a cool box, samples were prepared by collecting a 1-cm^2 piece of infected leaf as well as all the fungal material that could be scraped off the leaf with a scalpel into a 1.5-mL microcentrifuge tube kept at -80 °C. Both plant and fungal DNA were thus extracted jointly at the Institute of Biotechnology (University of Helsinki), using E.Z.N.A. plant DNA kit as described in Tollenaere *et al.* (2012). The molecular detection test described above was performed three times on each of these samples. We converted the cycle threshold (C_t) values of the qPCR assay into a binary response variable (positive vs. negative samples) using a cut-off value ($C_t = 24$) determined with the data obtained from experimentally infected samples (see the results section). For 25 randomly selected positive samples, we amplified the ITS region using conventional PCR with the primers AQ-F264 and AQ-R462, followed by one sequencing reaction (partial sequencing only with the same methodology described above) to ensure the amplicon was derived from *Ampelomyces* DNA.

The collected samples consisted of all the fungal material found on one *Plantago* leaf but whether powdery mildew infection on that leaf consists of a unique strain or a mix of various strains may affect the pattern of infection by *Ampelomyces*. We consequently carried out a SNP (*Single Nucleotide Polymorphism*) assay specific for *P. plantaginis* in these same samples, allowing us to infer whether infection of the leaf is due to a unique

strain or various strains of powdery mildew (Tollenaere *et al.* 2012). As *P. plantaginis* is haploid, we considered the samples to be coinfected by various mildew strains if two alleles were detected in at least one SNP locus (see details in Tollenaere *et al.* 2012).

Linking the prevalence of Ampelomyces with its fungal host population dynamics

Firstly, we tested whether *Ampelomyces* prevalence increased with the level of coinfection (as estimated from the SNP genotyping of *P. plantaginis* samples described above). This analysis was performed on the whole data set (808 samples from 424 plants from 98 populations). Specifically, we modelled for each plant the proportion of leaves with *Ampelomyces* as a function of the fraction of leaves showing multiple genotypes of the powdery mildew (i.e. whether a unique strain or a mix of various strains was detected using SNP markers). As the response variable is a proportion, we used a binomial distribution and logit link. To account for variation among populations, we included population identity as a random effect. We used the framework of generalized linear mixed models as implemented in procedure GLIMMIX in SAS 9.3 (Littell *et al.* 2006).

Secondly, we assessed the impact of the prevalence of *Ampelomyces* hyperparasite on the overwintering of its fungal host from September 2011 to July 2012 at two spatial scales: a 1-m^2 quadrat and the population level. For both analyses, we used *Ampelomyces* prevalence and the fraction of leaves with chasmothecia (resting structures) in autumn 2011 as the explanatory variables of overwintering success. We included the latter variable as previous work has shown that the overwintering success of *P. plantaginis* can be affected by the abundance of chasmothecia (Tack & Laine 2014).

To analyse the impact of *Ampelomyces* prevalence on the overwintering of its fungal host at a small spatial scale, we relocated the plants ($n = 78$ plants in 16 of the 17 more intensively sampled populations) that had been marked as infected in September 2011 in early July 2012 at the onset of within season transmission (Soubeyrand *et al.* 2014; Ovaskainen & Laine 2006; for details on the July survey, please see Tack & Laine 2014). Powdery mildew overwintering success was inferred by recording the fraction of infected plants within the 1-m^2 quadrat surrounding the marked locations of plants recorded to be infected in autumn 2011. In autumn 2011, we visually scored the presence–absence of chasmothecia on ten randomly selected leaves (or at least five if the plant had <10 leaves) of the same five plants that were selected for the *Ampelomyces* sampling. The fraction of leaves bearing chasmothecia was estimated out of the total number of infected leaves at the

plant level; an additional population-level estimate was derived by averaging the estimate across the five plants (see also Tollenaere & Laine 2013 and Tack & Laine 2014). We used the procedure GLIMMIX (described above) to model the fraction of infected plants in quadrats in July 2012 as a function of the level of *Ampelomyces* (fraction of leaves infected) and chasmothecia (fraction of infected leaves with chasmothecia) on the focal sampled plant in the previous autumn. To account for variation among populations, we included population identity as a random effect.

To analyse the impact of *Ampelomyces* on overwintering at the population scale, we recorded the abundance of *P. plantaginis* in July 2012 using a categorical scale (for details on the methods, please see Tack & Laine 2014) in all 98 studied populations: (i) no infected plants, (ii) 1–10 infected plants, (iii) 10–100 infected plants, and iv) more than 1000 infected plants. This categorical scale facilitates the logistics of surveying infection across a large number of populations (Tack & Laine 2014). Mildew abundance in July 2012 was then used as the response variable for a weighted generalized linear model (glm) performed using R software (R Core Team 2013). The explanatory variables were the prevalence of *Ampelomyces* and the proportion of chasmothecia in the previous autumn at the population level, both estimated as described above. We used the quasi-Poisson family in the final model because of overdispersion of the data.

Results

ITS sequencing of Ampelomyces strains from Åland

The ITS sequences determined in the four infected dried *Plantago* leaves bearing *Ampelomyces* pycnidia, collected in Åland in September 2011, as well as those determined in six of seven strains isolated in the archipelago in 2013, were all identical (GenBank Accession nos: KC494280 and KM066091–KM066097) and approximately 97–98% similar to the ITS sequences of the *Ampelomyces* strains belonging to the 'Podosphaera (sect. Sphaerotheca) clade' identified by Park *et al.* (2010). The seventh strain isolated in 2013 belonged to the same clade but differed by a single SNP from the other *Ampelomyces* strains isolated in Åland.

Design of the molecular detection test

We obtained successful amplification with all the six *Ampelomyces* strains presented in the Table S1 using the newly designed primer pair AQ-F264/AQ-R462. However, limited growth on the plate and subsequent low DNA concentration prevented the use of the strain

B124-a for the calibration curve. The standard curves were similar for the five remaining strains with slopes varying between −2.58 and −3.31 (Fig. S2, Supporting information). Correlation coefficients were high ($0.966 < r < 0.990$) indicating low interassay variability. Although there may be some differences of efficacy between the different *Ampelomyces* strains, the designed molecular test is likely to be applicable to any *Ampelomyces* spp.

When applying the molecular detection test to experimentally infected samples (Fig S3, Supporting information), we found that lesions showing visible evidence of *Ampelomyces* infection (categories: 'few', 'moderate', 'lots') had significantly lower C_t values than either of the control groups ('none', 'control') (Tukey's HSD, $P < 0.006$ in all cases). There was no significant difference in C_t values between 'control' samples and those that were exposed to, but did not grow, *Ampelomyces* ('none') (Tukey's HSD: $P = 0.95$). Based on these data, we set the C_t cut-off value for *Ampelomyces* presence/absence to 24 cycles, which is between the highest C_t obtained for samples bearing pycnidia and the lowest C_t found in samples not showing infection. Furthermore, 24 cycles is significantly lower than the amplification threshold of either control group ('control': one-sample *t*-test, $t = 5.7101$, d.f. = 4, $P = 0.004$, 'none': one-Sample *t*-test; $t = 2.848$, d.f. = 4, $P = 0.046$) and thus serves a suitable cut-off for determining the presence of established infections despite the potential for nontarget amplification mentioned above (Fig S3, Supporting information).

When the plasmid construction containing the ITS region of an *Ampelomyces* strain isolated in Åland was used in a dilution series prepared in DNA extracted from powdery mildew-infected *P. lanceolata* leaves, the standard curves showed that the lowest number of ITS copies leading to reliable amplifications was $10^4/\mu L$ (Average $C_t = 24.08$). The qPCR cut-off, $C_t = 24$, used for field samples, is very close to the cycle threshold for $10^4/\mu L$. Detection of 10^3 or less ITS copies was not reliable due to the effect of the DNA extracted from *Ampelomyces*-free powdery mildew-infected *P. lanceolata* leaves. Therefore, this experiment also confirmed $C_t = 24$ to be a robust threshold for detecting *Ampelomyces* in field-collected samples.

Ampelomyces detection within the Åland archipelago

When applying the molecular test to the large-scale field sampling (951 samples), a straight shape instead of the expected sigmoid for the qPCR assay (evolution of the fluorescence through time) was obtained for 126 samples (13.2% of the data set). This suggests possible inhibition of the reaction, and these samples were

removed before further analyses. The powdery mildew SNP assay was applied to all the samples. SNP genotyping assay worked for all the samples except 12 (1.3% of the data set), which were consequently removed from all the analyses as a negative result for such samples may be due to poor DNA quality. A total of 813 samples were finally used in this study.

We performed three replicates on each sample and found repeatability of 88.1%. We considered as positive the samples having C_t lower cycle threshold than 24 for all the amplifications performed.

Among the 813 analysed samples, 362 (44.5%) were positive for *Ampelomyces*. Between populations, prevalences varied between 0 and 89% in the 17 powdery mildew populations having sufficient sample size (minimum 22 samples, see Table S2, Supporting information). No spatial structure in *Ampelomyces* prevalence was evident at the metapopulation scale (Fig. 1).

Relationship between Ampelomyces prevalence and the fungal host population dynamics

There was a strong positive relationship between *Ampelomyces* prevalence and the proportion of powdery mildew infections that were attributed to multiple strains ($F_{1,325} = 5.68$ and $P = 0.02$; Fig. 2). We also detected significant variation in *Ampelomyces* prevalence among populations (log-likelihood ratio test for the random effect 'population': $\chi_1^2 = 81.43$ and $P < 0.001$).

At the population scale (96 analysed populations), *Ampelomyces* prevalence in autumn did not significantly affect powdery mildew overwintering success measured as the abundance of *P. plantaginis* in the following spring ($t = -0.462$, $P = 0.645$). At the small spatial scale (1-m² quadrat), we detected a significant negative impact of *Ampelomyces* prevalence in autumn 2011 on the overwintering success of the powdery mildew ($F_{1,60} = 4.22$ and $P = 0.04$; Fig. 3). We did not detect an effect of chasmothecia on mildew overwintering ($F_{1,60} = 0.47$ and $P = 0.50$).

Discussion

Although microscopic observation allows *Ampelomyces* detection, molecular tools can improve the feasibility and accuracy of large-scale screening of fungal pathogens. For this purpose, we aimed to design a molecular detection test of *Ampelomyces* spp. using a quantitative molecular assay based on species-specific ITS amplification. Although some lower-quality samples had to be removed from the data set, the molecular test was successful for the majority of our samples (86.8%), and repeatability over three replicates was fairly good (88.1%). The newly developed molecular test was
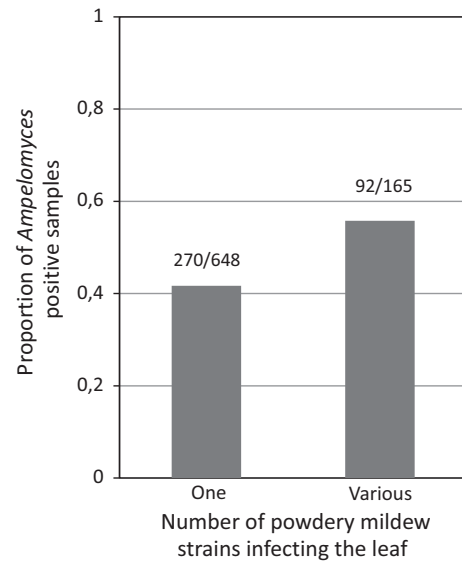

Fig. 2 Relationship between the powdery mildew genetic diversity (one vs. multiple strains) and the probability of infection by *Ampelomyces* at the leaf level.
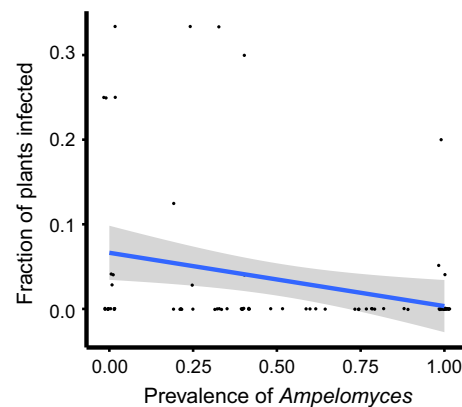

Fig. 3 The impact of the prevalence of the hyperparasite *Ampelomyces* in autumn 2011 on the overwintering success (fraction of infected plants in July 2012) of its powdery mildew host *Podosphaera plantaginis* at a small spatial scale (1-m² quadrat). Values are slightly jittered horizontally to prevent overplotting. The linear regression line and its 95% confidence interval (as implemented in *stat_smooth*, package *ggplot2* within R software) are for visual illustration.

applied to experimentally infected samples revealing a good correlation between the qPCR results and microscopic observations of pycnidia (presence and abundance), thus verifying its utility in screening samples collected from nature.

Furthermore, amplification worked well for the six selected strains tested, despite high genetic variability in their ITS sequences (Pintye *et al.* 2012), suggesting

that the test could be applied to any known lineage of *Ampelomyces*. This allows the test to be applied widely to assess *Ampelomyces* prevalence within any powdery mildew species, even without previous investigation of the genetic variability of the mycoparasite. Notably, different *Ampelomyces* lineages could coexist within the positive samples detected. Many more *Ampelomyces* strains from *P. plantaginis* samples within the Åland archipelago would have to be characterized genetically to get a comprehensive picture of the genetic diversity of this mycoparasite in the region. However, the data presented here (four pycnidial samples collected in 2011 and isolation of seven strains in 2013) suggest little genetic diversity as all the ITS sequences determined in these samples were highly similar (10 of 11 were identical) and closely related to those identified in the 'Podosphaera (sect. *Sphaerotheca*) clade' (Park *et al.* 2010). Preliminary data (partial sequencing of 25 randomly selected positive leaf samples, data not shown) are in accordance with this, as only one sample of 25 belonged to another *Ampelomyces* clade (Park *et al.* 2010). In contrast, Pintye *et al.* (2012) found high diversity of *Ampelomyces* strains in the grape powdery mildew and Kiss *et al.* (2011) showed the coexistence of different ITS haplotypes of *Ampelomyces* on the same powdery mildew-infected host plants. Further investigations on the genetic diversity of *Ampelomyces* from Åland would also require the use of rapidly evolving genetic markers (e.g. microsatellites, Kiss *et al.* 2011).

Our study revealed that *Ampelomyces* is a very common parasite within *P. plantaginis* populations, as 363 of the 813 (44.5%) samples analysed were found to be infected. Data from 17 intensively sampled populations (with at least 22 samples per population) indicated that *Ampelomyces* was present in the vast majority of the populations (only one of 17 had no positive sample). While the sampling was not optimal for detecting within-population variability (i.e. we measured multiple leaves on a few plants for each population), we did note striking variability in hyperparasite frequency between powdery mildew populations, which ranged between 0 and 89% (Table S2). Although the four initial samples of *Ampelomyces* were collected in the northern part of the Åland archipelago, subsequent analysis did not detect any spatial pattern in *Ampelomyces* prevalence (Fig. 1). Very few studies have documented the prevalence of *Ampelomyces* in field-collected samples of powdery mildew hosts. Kiss (1998) documented the prevalence of *Ampelomyces* in field-collected samples representing 27 powdery mildew species infecting 41 host plant genera in Hungary and Romania and found *Ampelomyces* spp. in 4.3–68.8% of the studied samples, depending on the fungal host genera considered. This study also reported that the intensity of mycoparasitism

(proportion of powdery mildew structures infected within sample) ranged from 0.15 to 65% in powdery mildew mycelia (Kiss 1998), while it remained lower than 4% in chasmothecia from grapevine powdery mildew populations from northern Italy (Angeli *et al.* 2009). The methodologies used in these studies were, however, very different from the one applied in the present study, and the results can therefore not easily be compared with each other.

We found that the dynamics of the powdery mildew host and the hyperparasite *Ampelomyces* were interrelated. First, the probability of an infected leaf sample to contain also *Ampelomyces* differed depending on whether the powdery mildew infection was caused by a single strain or multiple strains (i.e. coinfection). While disentangling the cause and effect may be difficult, this result supports the prediction that leaves infected by multiple powdery mildew genotypes have a higher probability to be infected by a powdery mildew strain bearing *Ampelomyces*, as a consequence of higher exposure. Secondly, we found an effect of *Ampelomyces* presence/absence on the overwintering success of the powdery mildew in the immediate surroundings (1-m² quadrat) of an infected plant. Off-season survival and re-initiation of the epidemics is a critical but poorly understood stage of many plant pathogens, and high stochasticity is often invoked (Soubeyrand *et al.* 2009). The factors classically involved in the seasonal disease spread (pathogen genotype, host genotype and the environment) were recently shown to affect the overwintering stage in the pathosystem studied here (Tollenaere & Laine 2013; Tack & Laine 2014). Our results show that a hyperparasite may significantly affect the overwintering success of its phytopathogenic host, as we found a significant effect of the presence/absence of *Ampelomyces* on overwinter survival of the powdery mildew at the local scale (immediate neighbours of focal plants). However, no effect of *Ampelomyces* was detected on the overwintering success at the population level. This suggests that occurrence of *Ampelomyces* is highly aggregated within the powdery mildew populations, having a highly localized effect on its host. Other variables, such as climate, are likely to be important determinants of extinction at the population scale. However, the aggregated sampling scheme may also have contributed to the lack of a significant effect at the population scale. More intensive sampling across multiple populations would help resolve whether *Ampelomyces* could be a driver of extinctions at the population level in the *P. plantaginis* metapopulation dynamics.

A significant effect of the hyperparasite on its host's overwintering would be a case of local disease-induced extinction, a phenomenon that could more easily be explained with the presence of alternative hosts

according to theoretical studies (de Castro & Bolker 2005). In our system, we frequently observed other plant species infected by powdery mildew in the *P. lanceolata* infected meadows (C. Tollenaere, pers. com.), and consequently, various powdery mildew species possibly hosting *Ampelomyces* are likely to co-occur with *P. plantaginis*. Some host specificity has been suggested to maintain *Ampelomyces* lineages (Park *et al.* 2010), but it has also been clearly shown that the same haplotypes of these hyperparasites can infect various powdery mildew species in the field (Kiss *et al.* 2011), and this may be the case in our system too. Testing other possible fungal hosts for presence/absence and diversity of *Ampelomyces* would determine the importance of other mycohosts for the interaction between *P. plantaginis* and *Ampelomyces* in the Åland metapopulation.

To conclude, we argue that the understanding of host-pathogen metapopulation dynamics may need to extend the scale of the study system into a tri-trophic spatial network and take into account the role of natural enemies of plant and animal pathogens. In addition to the fundamental knowledge gained on the regulation of natural population dynamics (see also Lafferty *et al.* 2008), studying such tri-(or more) trophic interactions in wild populations may lead to important applications such as the prevention of disease spread through biological control (for example Nuss 1992).

Acknowledgements

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Data accessibility

DNA sequences: GenBank Accession nos: KC494280 and KM066091–KM066097. Data sets used for analyses at the leaf, plant and population scale: Dryad doi:10.5061/dryad.mv332.

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1. Characteristics of the six *Ampelomyces* strains used to test the newly designed molecular detection method.

Table S2. Prevalence of *Ampelomyces* observed in the 17 powdery mildew populations presenting at least 22 samples.

Fig. S1. Illustrations on the study system.

Fig. S2. Calibration curves obtained for five different *Ampelomyces* strains (see Table S1 for details on the strains).

Fig. S3. Results of the molecular detection test applied on powdery mildew samples experimentally infected by *Ampelomyces*.

Appendix S1. Supplementary experiment assessing in vitro the reliability of C_t = 24 as a cut-off value.