From leaf to continent: the multi-scale distribution of an invasive cryptic pathogen complex on oak

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Abstract

The spatial distribution and niche differentiation of three closely related species (*Erysiphe alphitoides, E. quercicola* and *E. hypophylla*) causing oak powdery mildew was studied at scales ranging from the European continent, where they are invasive, to a single leaf. While *E. alphitoides* was dominant at all scales, *E. quercicola* and *E. hypophylla* had restricted geographic, stand and leaf distributions. The large-scale distributions were likely explained by climatic factors and species environmental tolerances, with *E. quercicola* being more frequent in warmer climates and *E. hypophylla* –in colder climates. The extensive sampling and molecular analyses revealed the cryptic invasion of *E. quercicola* in nine countries from which it was not yet recorded. The presence of the three species was also strongly affected by host factors, such as oak species and developmental stage. Segregation patterns between *Erysiphe* species were finally observed at the leaf scale, between and within leaf surfaces, suggesting competitive effects.

INTRODUCTION

Fungi have been increasingly recognized as an important group among invasive species (Desprez-Loustau *et al.* 2007, van der Putten *et al.* 2007, Mallon *et al.* 2015, Dickie *et al.* 2017), with some devastating consequences in the case of plant and animal pathogens (Fisher *et al.* 2012, Roy *et al.* 2017). Human-mediated transport has been identified as a major pathway for the introduction of non-native micro-organisms, e.g. forest pathogens (Liebhold *et al.* 2012). Microbial invasions are thus clear evidence that microbial cosmopolitanism (the absence of dispersal limitations) postulated in the "everything is everywhere" hypothesis is not the general rule (Green and Bohannan 2006, Martiny *et al.* 2006).

One acute problem in studying the spatial distribution of microbes, including fungi, which may explain apparent species cosmopolitanism, is the relatively low taxonomic resolution provided by morphological characters (Green and Bohannan 2006). For example, molecular analyses have shown that many 'morpho-species' of fungi hide a complex of genetically divergent species, called cryptic species, with follow-up studies providing evidence for some differentiation in their ecology and biogeography (Taylor *et al.* 2000). In particular, many plant diseases that were formerly believed to be caused by a single pathogen species were later shown to be due to a complex of multiple cryptic species (Fitt *et al.* 2006). For example, Eucalyptus leaf spot is associated with more than 60 species of *Mycosphaerella* (Crous and Groenewald 2005), and grapevine downy mildew is caused by five *Plasmopara* species in North America (Rouxel *et al.* 2013).

The existence of cryptic species may have important implications in the context of invasions, notably by causing so-called cryptic invasions (Geller *et al.* 2010). Invasions may be unrecognized due to the morphological similarity between native and introduced species (Geller 1999). In the recent European ash dieback-, it took years to identify the exotic origin of the fungal pathogen (*Hymenoscyphus fraxineus*), closely related to a native non-pathogenic species, impeding the implementation of quarantine measures (Gross *et al.* 2014). Moreover, some invasions regarded as caused by a single species may hide several invasion events of different species (Mackie *et al.* 2012). For example, fungal chytridiomycosis has been identified as one of the major drivers of the decline of amphibians worldwide and was initially thought to be caused by the single species *Batrachochytrium dendrobatidis* (Fisher *et al.* 2009). However, a closely related species that preferentially infects salamanders has been recently detected and described (Martel *et al.* 2013). Cryptic species, although morphologically similar, often display some level of ecological divergence which could play a role in the invasion process. For example, differences in terms of environmental tolerance

may lead to some geographical segregation in introduced areas (Mackie *et al.* 2012). In the case of pathogens, as shown in the *Batrachochytrium* complex, adaptation to the plant or animal host is a major driver of ecological divergence and speciation (Le Gac *et al.* 2007, Giraud *et al.* 2010). Moreover, the ability to perform host shifts to non co-evolved hosts in the introduced area is a key process of pathogen invasions in wild communities (Woolhouse *et al.* 2005, Slippers *et al.* 2005). Differences in levels of host specialization within a species complex may cause different invasion patterns between cryptic species (Saleh *et al.* 2012).

Here we focus on oak powdery mildew, a foliar disease first recorded in the beginning of the 20th century in Europe, causing seedling mortality and tree decline, especially in the two most common European oaks, *Quercus robur* and *Q. petraea* (Marçais and Desprez-Loustau 2014). Recent molecular studies suggest a multiple invasion with three closely related fungal species in the *Erysiphe* genus putatively originating from Asia (Takamatsu *et al.* 2007, Mougou *et al.* 2008). The most common species, known as *E. alphitoides*, was described as a new species in 1912 (under the name of *Microsphaera alphitoides*) and regarded as the causal agent of the new invasion in Europe (Griffon and Maublanc 1912). The second species, *E. hypophylla*, tentatively identified from slightly different symptoms and morphology, was assumed to cause an independent invasion, starting a few decades later from northern Europe (Roll-Hansen 1961). The third species, *E. quercicola*, was first described in Asia (Limkaisang *et al.* 2006, Takamatsu *et al.* 2007) and recently detected in France (Mougou *et al.* 2008) and later Spain (Desprez-Loustau *et al.* 2017). Its date of introduction in Europe remains unknown.

The overarching aim of this study is to understand the multi-scale spatial distribution of the cryptic species forming the invasive complex with the ultimate goal of understanding their invasive behaviour. Our working hypothesis is that species show some degree of niche

separation, which may translate into differences in their distribution across multiple spatial scales. More specifically, we address the following questions:

- What is the spatial distribution of the three *Erysiphe* species, ranging from a large scale (i.e. the European continent) and local scale (i.e. stand level) to the micro-scale (i.e. leaf level)? At which spatial scales do the different species co-occur?
- Do the fungal species differ in their biogeographical distribution at the continental scale due to differences in their climatic niche and their ability to attack different oak species?
- At the stand scale, is the pathogen complex the same on trees of different developmental stages?
- At the scale of single leaves, where direct and plant-mediated interactions between the pathogen species are expected to occur, do species segregate between and within leaf surfaces?

MATERIAL AND METHODS

Hierarchical sampling design

To assess the multi-scale distribution of the powdery mildew species complex, we sampled more than seven hundred powdery mildew-infected oak trees from across Europe using a series of sampling strategies (see below). The focal oak species was *Quercus robur*, which has the widest geographic range and spans all over Europe (http://www.euforgen.org/species/) but other oak species were also sampled (see below). The infected leaves were pressed dry immediately after field collection, sent to the laboratory and maintained at room temperature until the molecular analyses were performed. Unless otherwise stated, 6 mm diameter leaf discs were taken with a cork borer in sporulating lesions visible on the upper (adaxial) surface. Tools were sterilized between taking each leaf disc.

European scale sampling

The main sampling to assess the distribution of the cryptic powdery mildew species across Europe was completed during the growing seasons of 2014 - 2016 by collecting leaves from 20-80 oak trees, from one or several locations in each of 15 European countries and Turkey. Additional data come from a previous sampling in France in 2007 (Mougou-Hamdane *et al.* 2010) and from previous collections in various countries (see *Leaf scale sampling*). Most samples were collected on *Q. robur*, with some of them identified as "*Q. robur* or *Q. petraea"*, since these two species are closely related and not easily distinguished. Other *Quercus* species with a more restricted range, especially *Q. pyrenaica*, *Q. cerris*, *Q. vulcanica*, *Q. pubescens*, and *Q. frainetto*, were sampled in a limited number of countries (Desprez-Loustau *et al.* 2018). Individual samples for molecular analysis consisted of one leaf disc cut from one leaf per tree.

Stand scale sampling

In order to investigate whether the powdery mildew community is similar in mature trees and in seedlings growing under their canopy, we first sampled four mature trees growing approximately 50 m apart in Le bois des Sources in Cestas, France (lat. 44.76°, long. -0.71°), a location where *E. alphitoides* and *E. quercicola* were previously known to occur (Hamelin *et al.* 2016). For each mature tree, eight leaves from each of four branches were collected at *circa* 4 m high in the canopy using a pole pruner, in June 2014. The presence of *Erysiphe* species in seedlings was studied for two out of the four mature trees by collecting one infected leaf from each of 15 seedlings growing under the canopy of the mature tree at the same date. In June 2015, we sampled again in the same location (Cestas) as well as in Laveyron (lat. 43.76°, long. -0.22°), another naturally regenerating stand in southwestern France where *E. alphitoides* and *E. quercicola* were known to occur. Five trees were sampled in Cestas

(including the four sampled in 2014) and eleven in Laveyron, with five leaves sampled in the canopy of each mature tree and one leaf on each of five seedlings growing underneath each tree. Samples for molecular analyses consisted of one disc per leaf.

Leaf scale sampling

Three different samplings were performed at the leaf scale.

First, because the three species may differ in the location and intensity of sporulation, we tested whether visual sampling may introduce a bias in the detection of species. For this, we compared targeted vs. non targeted sampling in infected leaves, i.e. one leaf disc cut in a visibly sporulating lesion on the upper leaf surface and a second, "non-targeted", leaf disc taken on the same leaf, at the symmetric position from the main leaf vein (i.e., irrespective of any visual selection of lesions). Leaves were taken from the four trees used for canopy sampling in 2014 (total number of samples: 128 leaves and 256 leaf discs).

Second, differences in the occurrence of *Erysiphe* species between the upper and lower leaf sides were investigated on 74 samples collected from Armenia, Czech Republic, Germany, Iran, Israel, Lithuania, Slovakia, Switzerland, Hungary and the United Kingdom, as collected from 1969 to 2006. Mycelium and spores were scraped separately from lesions visible on lower (abaxial) or upper (adaxial) sides of leaves. If present, chasmothecia were also isolated separately for each leaf side.

Third, the fine scale distribution of *E. alphitoides* and *E. quercicola* within leaves, and their possible co-occurrence, was studied on leaves taken from different seedlings in Cestas and Laveyron forests in June 2015. The seven analyzed leaves (four from Cestas and three from Laveyron) showed extensive mycelial development on the upper surface (and not on the lower surface) and were known to harbor both *E. alphitoides* and *E. quercicola* based on previous analyses. Intensive sampling of the whole infected leaf surface was performed by taking many

small leaf discs (4 mm in diameter). Taking leaf discs with a cork borer was preferred over cutting the leaf into quadrats in order to avoid cross contamination while cutting.

Molecular identification

Identification of *Erysiphe* species was based on polymorphisms in the ITS (Internal Transcribed Spacer) sequences of ribosomal DNA, the universal barcode for fungi (Schoch *et al.* 2012).

In the case of leaf disc samples, total DNA was extracted from leaf samples using the Invisorb Spin Plant mini kit, according to manufacturer's instructions (Stratec Molecular GmbH, Berlin). Amplification of the ITS1 region was performed using the ITS1-Fungi (Gardes and Bruns 1993) and o-micro-rev primers (Heuser and Zimmer 2002), as previously described (Desprez-Loustau *et al.* 2017). Overall, 94% of powdery mildew lesions produced the expected amplicon. Sequencing of the amplicon was done by Sanger technology (Genewiz, England). Sequences were aligned with BioEdit and *Erysiphe* species identified thanks to six fixed SNPs (Takamatsu *et al.* 2007, Mougou *et al.* 2008). Bad quality sequences or those that could not be assigned to one of the *Erysiphe* species were discarded (less than 3%).

Kommentert [M1]: make a final check with table

In the case of leaf scrapings, DNA was extracted using the chelex method (Hirata and Takamatsu 1996). The rDNA ITS region including 5.8S rDNA was amplified by two sequential PCR reactions using partially nested primer sets according to the procedures of Takamatsu *et al.* (2008). The amplified ITS regions were digested with *Pvu*II or *Alw*26 I (TaKaRa, Tokyo, Japan) according to the manufacturer's recommendation. Digestion mixtures were prepared as 10 units of enzyme, 1.5 µl of enzyme buffer (supplied by the manufacturer), 7.5 µl of sterile distilled water and 5 µl of the PCR product per tube. Digestions were run for two hours at 37˚C. Restricted DNA was then analyzed on 2% agarose

gels in TBE buffer. *Erysiphe* species were identified according to band patterns. The ITS of *E. quercicola* is cleaved by neither of the two enzymes. The ITS of *E. alphitoides* is not cleaved by *Alw*26 I, but is split into two bands (ca 450 and 100 bp length) by *Pvu*II treatment. The ITS of *E. hypophylla* is split into two bands (ca 450 and 100 bp length) by *Pvu*II treatment and also generates *c.* 300 and 250 bp bands by *Alw*26 I.

Climatic data

Climatic data were retrieved for each sampling location from WorldClim (Fick and Hijmans 2017) for the period 1970-2000, with a 2.5 minute resolution. Mean monthly temperature and rainfall were extracted and seasonal bioclimatic variables were computed by averaging temperature and summing rainfall for the spring (April to June), summer (July to September), autumn (October to December) and winter (January to March) over the 30-year period.

Statistical analyses

Statistical analyses were performed with the SAS 9.4 TS1M1 software (SAS Institute Inc., Cary, NC, USA). The probability of occurrence of the three *Erysiphe* species at the different spatial scales was studied by generalized linear models with binomial distribution and logit link. The effect of a set of predefined predictor variables on this probability was assessed by Wald X^2 probabilities and odds ratio (OR) estimates. The OR quantifies the strength of the association between the dependent and independent variables, where OR>1 indicates a positive association and OR<1 indicates a negative association (Rita and Komonen 2008). Analyses were either performed at the level of the individual sample (i.e. the leaf discs) or by grouping samples as explained below. We used a scale parameter (estimated by the ratio of

Pearson Chi-2 to degrees of freedom) to model the overdispersion in the data along with an AIC corrected for overdispersed data, i.e. QAIC (Burnam and Anderson 2003).

The analysis of the distribution of the three mildew species at the European scale (N = 689) was performed by pooling all samples within a country. To identify large scale geographic factors independently of host effects, only samples collected from *Q. robur*, *Q. petraea* and unidentified species within this complex were retained in the first statistical analysis (N = 498). For each *Erysiphe* species, a first model was run with proportion of positive samples per country explained by latitude and longitude (averaged among samples within a country). In order to investigate potential climatic effects underlying the geographic effects, the model was then run with the seasonal climatic variables (averaged within a country over 1970-2000) as predictor variables. We thereby assumed that the probability of detecting a given species at the national scale was mainly related to the establishment of a population of this species, itself depending on the long-term average climate. Due to the strong correlations existing between climatic variables, we first considered models with only one climatic variable. To determine the most influential climatic variables, we ranked models according to QAIC differences between the simple regressions (with a single climatic predictor) and the corresponding intercept models (with the same value of scale parameter for each pair of models, thus enabling QAIC comparisons). In a second stage, we ran models with all combinations of one temperature variable and one precipitation variable. The impact of host species on the occurrence of each of the fungal species was investigated separately for the four countries where sufficient samples had been collected from multiple oak species (at least 13 samples per oak species), i.e. Austria and Germany for *Q. robur* and *Q. petraea*, and Spain and Portugal for *Q. robur* and *Q. pyrenaica*. For each fungal species, we modelled the presence-absence on a single leaf disc cut from one leaf per tree as a function of the oak species.

At the stand scale, the proportion of positive samples for each *Erysiphe* species was computed for the 18 trees sampled over the two years and locations, separately for the canopy and the seedlings underneath the mature trees. We then modelled the proportion of leaves with each *Erysiphe* species as a function of tree developmental stage (mature *vs.* seedling), year (2014 *vs.* 2015) and location (Cestas *vs.* Laveyron).

Within leaves, the effect of targeted vs. non targeted sampling on detection of a given *Erysiphe* species was tested by modeling presence-absence as a function of sampling method for the 128 sampled leaves. To test for differences of occurrence of the fungal species on the two sides of a leaf, presence-absence of each species was modelled as a function of leaf side (upper vs. lower) for a total of 74 leaves.

To investigate the potential (positive or negative) interactions between species (*E. alphitoides* and *E. quercicola*) within a single leaf, we used the checkerboard score (C_{score}) as an indicator of species segregation, as proposed by Stone and Roberts (1990). A low C_{score} indicates a high randomness, i.e. a greater likelihood that the distribution of one species has not been directly affected by the presence of the other species.

First we calculated the C_{score} from observed data:

$$C_{score} = (N_{EA} - N_{EA\text{–}EQ})(N_{EQ} - N_{EA\text{–}EQ})$$

where N_{EA} is the total number of leaf discs yielding *E. alphitoides*, N_{EQ} is the total number of leaf discs yielding *E. quercicola*, $N_{EA\text{-}EQ}$ is the total number of leaf discs yielding both *E. alphitoides* and *E. quercicola*. The C_{score} was calculated for each sampled site independently and by pooling all samples.

Second we simulated the distribution of C_{score} under the null hypothesis of randomness. Each C_{score} was obtained by randomizing occurrences of *E. alphitoides* and *E. quercicola* among all

discs. To take into account potential differences in relative frequencies of *Erysiphe* species among sites (Cestas and Laveyron), the total number of each species occurrence was kept at the observed value in each site. This was repeated 1000 times.

Third we performed a two-tailed test by comparing the observed C_{score} to the lower (α_{low}=0.025) and higher (α_{high}=0.975) critical values of the null distribution of C_{score}. The null hypothesis was rejected if the observed C_{score} was lower or higher than the corresponding critical values, indicating more or less frequent co-occurrence than predicted by chance, respectively. These analyses were implemented in R software v. 3.3.3 (R Core Team 2017).

RESULTS

European scale

Clear geographical patterns emerged at the continental scale (Fig. 1). *E. alphitoides* was detected and was dominant in all European countries (Fig. 1). In contrast, the ranges of *E. quercicola* and *E. hypophylla* were more limited, with almost non-overlapping distribution areas. *E. quercicola* was mostly restricted to the southern part of Europe and particularly common in the Iberian peninsula. It was also detected at very high frequency in Turkey and southwestern Asia, and was the sole species in the few samples from Israel and Iran. *E. hypophylla* was most abundant in northern and central Europe, and almost absent from southwestern Europe, with the exception of a few leaves in Portugal and southern France. A significant proportion (22%) of samples (i.e. leaf discs) yielded mixtures of *E. alphitoides* with one of the two other species, whereas *E. quercicola* and *E. hypophylla* were only detected once on the same leaf (also with *E. alphitoides*).

Samples from *Q. robur* and/or *Q. petraea* covered a latitudinal gradient ranging from 40.29° to 66.02° and a longitudinal gradient ranging from -8.37° to 25.61°. While we did not detect any significant effect of latitude or longitude on the distribution of the three *Erysiphe* species, climatic variables were shown to have a significant effect on the presence of *E. hypophylla* and *E. quercicola* (Table S1). Temperature, especially in fall and winter, had the most significant and important effects but in opposite directions for the two species. *Erysiphe hypophylla* was more common in a cold climate (OR around 0.75), while the reverse was true for *E. quercicola*, with OR around 1.5, meaning that the odds of finding *E. quercicola* are increased by *c.* 50% for each additional degree in mean winter temperature (Fig. 2; Table S1). Among the precipitation variables, spring rainfall showed a significant effect, slightly negative for *E. hypophylla* and positive for *E. quercicola*. No better model with two variables was obtained for either species.

The probability of finding *E. alphitoides*, *E. hypophylla* or *E. quercicola* differed among oak species in areas where several species were present (Fig. 3). In Germany and Austria, *E. hypophylla* was detected more frequently on *Q. petraea* than on *Q. robur* (ORs of 6.7 and 5.0 in 66 and 46 samples, respectively; P = 0.005 and 0.16, respectively). In Spain and Portugal, *E. quercicola* was detected much more frequently on *Q. pyrenaica* than on *Q. robur* (ORs of 6.3 and 9.6, in 51 and 85 samples, respectively; P = 0.03 and <0.0001, respectively). For *E. alphitoides*, a host effect could only be tested for Portugal, with lower odds for *Q. pyrenaica* than for *Q. robur* (OR = 0.05, P = 0.0004).

Stand scale – Erysiphe *species in the canopy of mature trees compared to seedlings growing under their canopies*

In both sampling locations in southwestern France, almost all samples belonged to *E. alphitoides* or *E. quercicola.* However, the relative proportion of species varied widely between tree canopies and seedlings, with *E. quercicola* much more frequently detected (alone or in mixture with *E. alphitoides*) in seedlings than in the canopy of overhanging mature trees (Fig. 4, OR = 6.2, Wald's X^2 = 23.6, P < 0.001). There was also significant variation in the frequency of *E. quercicola* among the two years (OR = 3.59 for 2015 *vs.* 2014), but no variation among the two locations.

Leaf scale – visual sampling effect and differences among the upper and lower leaf sides

There was a marked difference between discs taken from sporulating powdery mildew lesions on the upper leaf surface and discs taken randomly from the same leaves (Fig. 5). Although not as high as in the targeted sampling (91.3%), the detection rate of *Erysiphe* in leaf discs taken without targeting lesions reached 78.7%. The detection of *E. hypophylla* was higher in these non-targeted samples (i.e. irrespective of any visual selection) than in targeted samples (i.e. with visibly sporulating lesion), with 22% compared to 2%, respectively, and a very high odds ratio value of 15.8.

Infections and successful detection of *Erysiphe* spp. were more frequent for the upper leaf side than for the lower leaf side (71 vs. 39 out of a total of 74 leaves). Whereas *E. alphitoides* and *E. quercicola* were detected on both sides of the leaves, *E. hypophylla* was only detected from lesions sampled on the lower leaf surfaces, either as mycelium and conidia or chasmothecia (Fig. 6). *Erysiphe alphitoides* was present on the upper surface of most leaves where *E. hypophylla* was detected on the lower surface.

Within leaf co-occurrence patterns

Among the 177 leaf discs analyzed on the seven leaves, almost half (88, i.e. 48%) corresponded to a mixed infection by both *E. alphitoides* and *E. quercicola* (i.e. with both ITS detected, Fig. 7). In five out of the seven leaves, either *E. alphitoides* (for leaves C3, L7) or *E. quercicola* (for leaves C1, C2 and L5) occupied the whole infected surface (i.e. was detected in all leaf discs, or almost for L5), with some of the discs also infected by the other species, i.e. showing mixed infection (Fig. 7). The leaves C4 and L6 were almost equally colonized by the two species, with most discs showing mixed infections and a few discs with only *E. alphitoides* and others with only *E. quercicola* (Fig. 7).

The observed C_{scores} were 240, 816 and 1972, for Cestas, Laveyron and the whole set of leaves, respectively. These values were higher than the upper critical values for the C_{scores} generated under the null hypothesis of randomness ($P = 0.012$, <0.001 and <0.001, respectively), suggesting that *Erysiphe* species were more segregated than expected by chance (Fig. S1).

DISCUSSION

Multi-scale distribution patterns are less well documented for invasive species than for native species, although they can offer crucial insights into their invasive potential and ecology (Allen and Shea 2006, Brown *et al.* 2008). For micro-organisms, such information is even scarcer because difficulties in species identification have long hampered biogeographical studies (Taylor *et al.* 2000, Geller *et al.* 2010; but see Linde *et al.* 2002). Our investigation provides the distribution and co-occurrence data of three closely-related fungal invasive species, across spatial scales ranging from an oak leaf to the European continent. We show

that both environmental and host factors explain the different invasion patterns and the coexistence of the three species through niche segregation at the various spatial scales.

The continental scale: one broadly distributed species and two species with a restricted geographical range- a predominant effect of climate?

Only one of the three *Erysiphe* species, namely *E. alphitoides*, was found to be spread throughout Europe, thus showing high invasive success. Numerous studies have seeked to identify inherent traits or characteristics explaining species invasiveness (Sakai *et al.* 2001, Philibert *et al.* 2011), among which climate match has been shown to be a consistent predictor across biological groups (Hayes and Barry 2008). While the ecophysiological requirements for *E. alphitoides* are not precisely documented, its spatial distribution encompasses a wide range of geographic and climatic gradients, in agreement with previous studies along altitudinal gradients (Desprez-Loustau *et al.* 2010, Dantec *et al.* 2015), which suggests tolerance to a wide range of environmental conditions. In contrast, *E. quercicola* and *E. hypophylla* showed more restricted and largely non-overlapping geographical distributions, and we found significant climatic variables explaining their distribution, which may reflect sensitivity to temperature, albeit in opposite direction for the two species. The most important variables for both species were temperature during the off-season (fall and winter), while there was no effect of the summer temperature, a pattern previously reported at a regional scale in France (Marçais *et al.* 2017). Hence, while many epidemiological studies focus on pathogen performance during the growing season, it may be the off-season that limits the distribution of plant pathogen species (e.g. Redondo *et al.* 2015), especially for obligate pathogens like powdery mildews, for which overwintering represents a critical stage (Tack and Laine 2014). One explanation for the success of *E. hypophylla* in colder climates may be the prolific production of efficient overwintering structures consituted by chasmothecia (the sexual fruiting bodies) (Roll-Hansen 1961, Sucharzewska 2009), in strong contrast to the

absence or limited number of such structures in *E. quercicola* (Feau *et al.* 2012). The importance of sexual overwintering structures in the pattern of invasions was also shown for *Phytophthora infestans*, the agent of potato late blight. This plant pathogen was restricted to European regions with oceanic climates in the first decades after its introduction, when it occurred as a single mating type, but it spread to colder climates when sexual reproduction became possible after the introduction of the second mating type (Yuen and Andersson 2013). Whether the low production of chasmothecia is an inherent characteristic of *E. quercicola*, a consequence of bottleneck effects during the introduction into various regions (as occurred for *P. infestans*), or a secondary loss as observed in other species of invasive fungi (Gladieux *et al.* 2015) remains to be investigated. However, preliminary data suggests that *E. quercicola* is also characterized by a low production of chasmothecia in its putative native area in Asia. Like in Europe, *E. quercicola* is reported to occur on average in warmer regions than *E. alphitoides* in Japan (Takamatsu *et al.* 2007). It has been reported (in the asexual form) in other warm regions in the world, on different mediterranean and sub-tropical species (Takamatsu *et al.* 2007, Desprez-Loustau *et al.* 2017). The absence of *E. hypophylla* in warmer climates may relate to a high sensitivity to elevated temperature and possibly UV radiation (Willocquet *et al.* 1996), which may also explain its absence from the upper leaf surface.

An alternative reason for the widespread distribution of *E. alphitoides*, and, reciprocally, of more restricted distributions for *E. quercicola* and *E. hypophylla*, may be the timing of introduction of the three different species resulting in different residence time, a major factor in invasion success (Wilson *et al.* 2007). *E. alphitoides*, described at the time of the first devastating outbreaks of oak powdery mildew in Europe (Griffon and Maublanc 1912), may have been introduced first. *E. hypophylla* was not reported before the 1960s; our observations show that its distribution has not enlarged much ever since (Roll-Hansen 1961, Cruchet 1962,

Viennot-Bourgin 1968, Braun and Cook 2012). *E. quercicola* has been detected only recently in Europe (Mougou *et al.* 2008, Desprez-Loustau *et al.* 2017), following its description (Takamatsu *et al.* 2007). The findings of *E. quercicola* in this study represent the first records in Armenia, Bulgaria, Hungary, Iran, Israel, Portugal, Slovakia, Sweden and Turkey (Desprez-Loustau *et al.* 2018). However, the species was probably overlooked for a long time when no molecular tools were available for its identification, as suggested by this large distribution and its detection in specimens dating from the late 1960s or early 1970s (Desprez-Loustau *et al.* 2018). The total extent of suitable habitat is another important variable conditioning invasion success (Wilson *et al.* 2007), and this suitable habitat is represeneted by the distribution of susceptible hosts for obligate pathogens such as powdery mildews. Variation in the susceptibility to a given pathogen within a host genus or family is a common observation for both plants and animals (Le Gac *et al.* 2007, Bancroft *et al.* 2011). We here confirmed differential susceptibility of oak species to the three *Erysiphe* species (Takamatsu *et al.* 2007, Marçais *et al.* 2017). The spread of *E. alphitoides* could have been favored by the high susceptibility of *Q. robur*, which is the most widespread oak species in Europe. In contrast, the other two *Erysiphe* species were more frequent on oak species with more restricted ranges, such as *E. quercicola* on *Q. pyrenaica*.

The stand scale: the impact of host developmental stage on the fungal community

Age-related differences in susceptibility or resistance to pathogens have commonly been reported for plants, and are associated with different resistance mechanisms acting in juvenile vs. mature stages (Develey-Rivière and Galiana 2007). Our results may thus suggest that *E. quercicola,* mostly found in seedlings, is an agent of juvenile disease of oaks. This contrasts with *E. alphitoides*, which infects all stages. However, we emphasize that several factors other

than ~~not only~~ age-related resistance sensu stricto may explain the observed patterns, such as ~~but also~~ changes in ~~plant growth patterns and architecture with aging may be involved, e.g. through~~ the amount and distribution of susceptible organs with aging (Calonnec *et al.* 2013), ~~and~~ microclimate, or vertical distribution of inoculum. For tree powdery mildews, overwintering within canopies relies on the presence of chasmothecia, which attach to cracks in the the bark surface (Takamatsu 2004). The absence (or low production) of chasmothecia in *E. quercicola* may thus explain its low frequency in tree canopies. In contrast, polycyclic growth in seedlings (i.e., occurrence of successive shoot flushes separated by the production of new buds within the same season) may favor bud infection and thereby the alternative mode of overwintering (Desprez-Loustau *et al.* 2014).

The leaf scale: spatial segregation vs. coexistence between and within leaf surfaces

Leaves are increasingly recognised as a complex and heterogenous environment providing habitat for diverse microbial communities (Vorholt 2012). Such within-leaf variation is caused by a diversity of factors, like topography, structural elements such as trichomes, resource aggregation and microclimate, wich operate at different spatial scales on foliar microbes and allow their co-existence (Esser *et al.* 2015). Our study confirmed that leaf side (upper *vs.* lower) is a first important factor structuring the *Erysiphe* complex on oak leaves. In agreement with previous observations (Takamatsu *et al.* 2007) and its Latin binomial (Cruchet 1962), *E. hypophylla* was only found on the lower leaf surfaces. This was in strong contrast to *E. alphitoides* and *E. quercicola*, which colonized both sides but were generally more abundant on the upper leaf surfaces. Comparison of fungal communities among upper and lower leaf surfaces are scarce, but Breeze and Dix (1981) suggested that upper leaf surfaces show higher spore deposition while lower leaf surfaces may provide more favourable

conditions for development due to lower levels of competition and protection from direct sunlight and rain washing. In our study, a lower competitive ability of *E. hypophylla* towards *E. alphitoides* was suggested by the results of the targeted vs. non-targeted sampling experiment, since *E. hypophylla* was much more frequent in randomly sampled discs than in those with profuse sporulation associated with *E. alphitoides*. These findings are in accordance with observations by early mycologists that *E. hypophylla* was often found in trees not infected by *E. alphitoides* and that its sporulation was much less abundant compared to *E. alphitoides* (Roll-Hansen 1961, Cruchet 1962). From a methodological point of view, our study ~~demonstrates~~ demonstrated that sampling the most obvious symptoms, as often done in plant pathology, can induce a bias when studying the frequency of cryptic species associated with the same disease. The relative frequency of *E. hypophylla* may thus have been underestimated in our study at the European scale with targeted sampling, even though this is unlikely to affect the general pattern of geographic distribution.

Spatial segregation within leaves at a smaller scale, on the same leaf side, was suggested to occur between *E. alphitoides* and *E. quercicola*, possibly reflecting competition effects. Although less studied than in plants and animals, competition has been shown to occur within and between foliar pathogen species (Newton *et al.* 1997, Al-Naimi *et al.* 2005, Kozanitas *et al.* 2017). In the particular interaction between *E. quercicola* and *E. alphitoides*, priority effects could be strongly involved, as shown in other systems (Vannette and Fukami 2014), since the two species were shown to exhibit different temporal dynamics during the season, associated with the differences in overwintering modes (buds vs. chasmothecia) (Feau *et al.* 2012, Hamelin *et al.* 2016).

Formatert: Skrift: Kursiv

Further investigations ~~at even finer spatial scales and integrating temporal dynamics, e.g. with sequential and co-inoculation experiments,~~ are now required to test several hypotheses arising from our study, related to competitive ~~elucidate the processes underlying~~ interactions

between *Erysiphe* species ~~on oak leaves. including resource competition (Pozo et al. 2016).~~ (using sequential and co-inoculation experiments, as in Kozanitas *et al.* 2017). Likewise, experimental field and laboratory studies could tease apart the independent and interactive effects of host species, host age, inoculum density and microclimate on the differences in mildew communities observed between trees and seedlings.

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Conclusion

Altogether, our results show that seemingly similar species exhibit different distributions across multiple spatial scales in their invaded area, even though all three *Erysiphe* species successfully completed a host shift to European oaks, especially *Q. robur*. Environmental and host factors (climate and micro-climate, host species, host development, leaf characteristics) acting at each spatial scale ~~appeared~~ were suggested as important drivers in the dynamics of this simple fungal community. Context-dependent mechanisms linked to the history of invasions, such as residence time, genetic drift or loss of sexual reproduction may also be involved. This study highlights the role of environmental and host heterogeneities at scales ranging from a single leaf to a continent as factors shaping invasion patterns in related pathogen species, ~~through~~ with niche differentiation ~~allowing avoidance of~~ limiting competition with other native or invasive species.

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DATA AVAILABILITY

All data are available at ~~Dryad Digital Repository~~ data.inra.fr (DOI :).

Kommentert [M2]: to be done

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Figure captions

Figure 1. Geographic distribution in Europe and neighbouring Asian countries of the three *Erysiphe* species associated with oak powdery mildew. Circle sizes represent the sampling effort per country, ranging from 1-5 samples (smallest circles) to more than 40 samples (largest circles).

Figure 2. Relationship between winter temperature and probability of occurrence of *Erysiphe alphitoides*, *E. hypophylla* and *E. quercicola* in Europe (the shaded area represents the 95% confidence interval)

Figure 3. Distribution of the *Erysiphe* species on different oak species within the same countries. Shown are pooled data for Germany and Austria (top) and Spain and Portugal (bottom). *EA* stands for *E. alphitoides*, *EQ* for *E. quercicola*, *EH* for *E. hypophylla*.

Figure 4. The community composition of *Erysiphe* species in mature tree canopies versus seedlings growing under their canopies. *EA* stands for *E. alphitoides*, *EQ* for *E. quercicola*, *EH* for *E. hypophylla*.

Figure 5. Detection probability of *Erysiphe* species among pairs of discs taken on the same leaves, either cut in a visibly sporulating lesion or in a non-targeted sample (i.e. cut without *a priori* information on the presence of a lesion). *EA* stands for *E. alphitoides*, *EQ* for *E. quercicola*, *EH* for *E. hypophylla*.

Figure 6. The relative frequency of each of the three *Erysiphe* species on the adaxial (upper) and abaxial (lower) surfaces of 74 leaves. *EA* stands for *E. alphitoides*, *EQ* for *E. quercicola*, *EH* for *E. hypophylla*.

Figure 7. Distribution of *Erysiphe alphitoides* and *E. quercicola* at the leaf level, for leaves of *Quercus robur* collected in spring 2015 in Cestas (code 'C') and Laveyron (code 'L') in France. The diameter of leaf discs is 4 mm, with the exception of one or two larger leaf discs per leaf. Note that the scale varies among leaves.

Figure S1. Null distribution of C_{scores} as based on 1000 randomizations, over the whole set of leaves, or independently for each site. The vertical thick line represents the observed C_{score}. Dashed lines represent the upper and lower critical values of the two-tailed test at a level of statistical significance of 5%.

Table S1. Results of generalized linear models with seasonal climatic variables (T = temperature, P = precipitation) tested separately as predictor variables of the presence of *Erysiphe* species. For each species, models are ranked by decreasing values of ΔQAIC = (QAIC without predictor) - (QAIC with predictor). Predictors with a significant effect are indicated in bold.

Erysiphe species	Predictor variable	ΔQAIC *	Wald Chi2	P-value	Odds Ratio (95% CI)
E. alphitoides	Pspring	2 (104-102)	2.7	0.1	1.01 (0.99-1.02)
	Twinter	1 (108-107)	2.7	0.1	1.18 (0.97-1.44)
	Tfall	1 (107-106)	2.9	0.09	1.25 (0.97-1.63)
	Pfall	1 (103-102)	2.2	0.14	1.01 (0.99-1.02)
	Pwinter	0 (97-97)	1.3	0.26	1.00 (0.99-1.01)
	Tspring	0 (95-95)	2	0.16	1.17 (0.94-1.44)
	Tsummer	-2 (84-86)	0.2	0.62	1.17 (0.80-1.71)
	Psummer	-2 (76-78)	0.5	0.46	1.01 (0.99-1.02)
E. quercicola	**Twinter**	**9 (72-63)**	**8.2**	**0.004**	**1.41 (1.12-1.79)**
	Tfall	**8 (64-56)**	**6.8**	**0.009**	**1.68 (1.14-2.49)**
	Tspring	3 (61-58)	2.9	0.09	1.51 (0.94-2.42)
	Pspring	**1 (55-54)**	**4.1**	**0.04**	**1.01 (1.00-1.02)**
	Tsummer	0 (51-51)	1.9	0.17	1.46 (0.85-2.51)
	Psummer	0 (44-44)	1.6	0.21	0.99 (0.97-1.01)
	Pwinter	-1 (45-46)	1.6	0.2	1.00 (0.99-1.01)
	Pfall	-1 (45-46)	0.8	0.36	1.00 (0.99-1.01)
E. hypophylla	**Twinter**	**13 (174-161)**	**11.4**	**0.0007**	**0.78 (0.67-0.90)**

Tfall	**11 (166-55)**	**11.3**	**0.0008**	**0.72 (0.60-0.87)**
Pspring	**11 (169-158)**	**9**	**0.003**	**0.99 (0.98-0.99)**
Tspring	**6 (136-130)**	**7.7**	**0.006**	**0.80 (0.68-0.94)**
Pwinter	**5 (141-136)**	**4.2**	**0.04**	**1.00 (0.99-1.00)**
Tsummer	2 (119-117)	3.7	0.054	0.75 (0.57-1.00)
Pfall	1 (115-114)	1.9	0.17	1.00 (0.99-1.00)
Psummer	-2 (106-108)	0.4	0.53	1.00 (0.99-1.02)

* QAIC values without predictor are different between models for a single species because a (different) scale parameter was included to account for overdispersion for each tested predictor