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Genetic diversity and population structure of the fungus *Pseudogymnoascus destructans*, the causative agent of white-nose disease: From large scale differentiation to locally homogeneous populations and genetically diverse infections in bats

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Abstract

White-nose disease (WND), caused by the psychrophilic fungus *Pseudogymnoascus destructans*, represents one of the greatest threats for North-American hibernating bats. Research on molecular data has significantly advanced our knowledge on various aspects of the disease, yet more studies are needed regarding patterns of *P. destructans* genetic diversity distribution. In the present study, we investigate three sites within the native range of the fungus in detail: two natural hibernacula (karst caves) in Bulgaria, South-Eastern Europe, and one artificial hibernaculum (disused cellar) in Germany, Northern Europe, that we intensively surveyed between 2014 and 2019. Using 18 microsatellite and two mating type markers, we describe how *P. destructans* genetic diversity is distributed between and within sites, the latter including differentiation across years and seasons of sampling; across sampling locations within the site; and between bats and hibernaculum walls. We found significant genetic differentiation between hibernacula, but we could not detect any significant differentiation within hibernacula based on the variables examined, which indicates that most of the pathogen's movement happens within sites. Genotypic richness of *P. destructans* varied between sites within the same order of magnitude, being approximately two times higher in the natural caves (Bulgaria) in comparison to the disused cellar (Germany). Within all sites, the pathogen's genotypic richness was higher in samples collected from hibernaculum walls than in samples collected from bats, which corresponds with the hypothesis that hibernacula walls represent the environmental reservoir of the fungus. Multiple pathogen genotypes were commonly isolated from a single

bat (i.e., from the same swab sample) in all study sites, which might be an important feature to take into consideration when studying disease progression.

Keywords

Chiroptera, emerging infectious disease, fungal pathogen, wildlife disease, white-nose syndrome, Leotiomycetes, Thebolales, Pseudeurotiaceae

Introduction

White-nose disease (WND) represents one of the greatest challenges for North-American bat conservation. It has been estimated that the disease has caused between 5.7 and 6.7 million casualties for the first 5 years after its emergence (White Nose Response Team 2011), the colonies of the most affected species suffering up to more than 90% population declines (Cheng et al. 2021). Such unexpected and rapid onsets pose serious challenges to conservation and management as the causative agent is often unknown or difficult to diagnose, its dispersion mechanisms are not well understood, and its transmission routes and origin can only be speculated upon (Puechmaille et al. 2011b). Given the difficulties associated with pathogens' morphological identification (e.g., small size, cryptic diversity), molecular tools are becoming the gold diagnostic standard, including in the research of WND and its associated causative agent, the fungus *Pseudogymnoascus destructans* (Blehert et al. 2009). The pathogen was first characterised as a novel species by combining morphological and genetic data (Gargas et al. 2009); soon after, genetic data was used to report its presence in Europe (Puechmaille et al. 2010). Based on patterns of genetic diversity and similarity (Rachowicz et al. 2005), Europe was quickly identified as the source population where *P. destructans* in North America was introduced from (Leopardi et al. 2015, Drees et al. 2017b), thereby explaining the pervasive mortality due to WND in the naïve North American species and the lack of mass morbidity or mortality in European species that co-evolved with the fungus (Fritze and Puechmaille 2018, Puechmaille et al. 2010, Puechmaille et al. 2011a, Puechmaille et al. 2011b, Zukal et al. 2016). Subsequently, large sections of WND research, especially on fungal load and identification of *P. destructans* (e.g., Chaturvedi et al. 2011, Muller et al. 2012, Niessen et al. 2022), have relied on molecular tools. Yet, more studies are needed on patterns of *P. destructans* genetic variation at large scale (continental-wide) and on its dispersal/gene flow between established populations (though see Fischer et al. 2022, Forsythe et al. 2021).

At small geographical scales (within individual hibernacula), the use of genetic data and characterisation of levels of genetic variation have been central in elucidating *P. destructans* life cycle and transmission routes. Indeed, Fischer et al. (2022) demonstrated that bats become infected with *P. destructans* anew each autumn when they return to their hibernacula, acting as a long-term reservoir of the fungus (see also Puechmaille et al. 2011a, Hoyt et al. 2021). During the winter, the pathogen undergoes clonal reproduction on its hosts and large amounts of spores are shed back into the reservoir in late hibernation. During the summer, bats clear *P. destructans* and the cycle is repeated the next year (see

also Hoyt et al. 2021). However, many questions remain regarding *P. destructans* levels of genetic diversity and its distribution within a site, such as: Is the population homogeneous within a site, suggesting ample pathogen movement? Or are there any factors acting as barriers for *P. destructans* spread at small spatial scales? Are patterns of genetic diversity distribution similar across sites? Do bats become infected by multiple fungal individuals?

To address these questions, we intensively studied *P. destructans* at three bat hibernacula in Europe: two karst caves in Bulgaria and a disused cellar in Germany (cf. Fischer et al. 2022). We applied the fine-scale approach developed by Fischer et al. (2022) whereby fungal individuals are genetically distinguished as multilocus genotypes (MLGs) and tracked through time and space. This allowed us to test for genetic differentiation between and within sites, the latter being the main goal of the study. Within sites, we tested for differentiation across years and seasons of sampling; across sampling locations within the site (rooms); and between bats and site walls. We also calculated the size of fungal populations for each site, estimated the frequency of multiple infections (involving different MLGs) in bats, and interpreted our results in the light of host-pathogen interactions and disease transmission dynamics. We expected that genetic differentiation would be much stronger between than within sites; that genetic diversity would be variable between sites but within the same order of magnitude; and that multiple infections in bats would be common in all sites.

Material and methods

Environmental reservoir and infections in bats

By comparing genotypic richness of the fungus isolated from bats and from hibernacula walls, Fischer et al. (2022) demonstrated that yearly re-infection of bats originates from hibernaculum walls (environmental reservoir) containing viable spores of *P. destructans*. To corroborate these results, we tested the expectation that a transmission bottleneck would exist between the reservoir and the hosts, whereby genotypic richness of the pathogen would be reduced on the hosts as not all MLGs are successfully transferred from the source to the host population. Therefore, we compared genotypic richness of the fungus isolated from bats and from hibernacula walls by estimating the number of eMLGs at the smallest shared sample size based on rarefaction with 1000 permutations. We additionally ran the analyses when randomly selecting exactly one SSI/swab and when randomly selecting exactly two SSIs/swab (run 1000 times). To compare the genotypic richness per wall or bat swab, we calculated the following ratio for each swab: $(G-1)/(N-1)$, where G is the number of MLGs/swab and N is the number of SSIs/swab (Dorken and Eckert 2001). This ratio varies between 0 when all isolates from a sample harbour the same MLG, to 1 when all isolate from a sample harbour a different MLG. The average of this ratio was calculated per swab type (wall versus bat). To estimate the frequency of multiple infections in bats, we calculated the percentage of cases where more than one MLG or more than one mating type were found on a single bat swab, after taking exactly three SSIs/swab.

Sample collection

For Bulgaria, swab samples were collected from bats and walls in two karst caves, Balabanova dupka (N43.13, E23.04) and Ivanova voda (N41.89, E24.88; Suppl. material 1), representing two of the largest known hibernacula for *Myotis myotis/blythii* in the Balkans. Around 2500 *M. myotis/blythii* hibernate in Balabanova dupka, (personal data), while a maximum of 5,600 and 2,500 *M. myotis/blythii* and *Myotis capaccinii* respectively hibernate in Ivanova voda (maximum counts 5600 and 2500 respectively; EUROBATS 2011). Differentiating *M. myotis* from *M. blythii* during hibernation is challenging (without handling), hence we used the species complex *Myotis myotis/blythii*.

Swab samples were collected from the muzzle, ears or wings of freely hanging and visibly infected bats (classically *M. myotis/blythii*) towards the end of the hibernation season (March – June) between 2015 and 2019, without the need to handle bats (Fritze et al. 2021). Bats with visible fungal growth are only present during this period. Depending on the number of visibly infected body parts, one or more swabs were collected from each bat, i.e., one per infected body part. Infection is herein defined as the acquisition of a microbe by a host (Casadevall and Pirofski 2000). Two times per year, in late winter/spring (March – June) and autumn (October), wall swabs were collected 1) in close proximity (a few centimetres) to the main bat roosting places and 2) further away from them (around 50 meters, see Suppl. material 2). For accuracy, we used plastic markers that stayed in the caves for the full period of the study, and we collected 4 swabs at 1 meter up, 1 meter down, 1 meter left, and 1 meter right of each marker. Each swab touched the cave wall 9 times covering an area of approximately 10 cm². Within each site, sampling locations were distributed in different rooms as presented in Suppl. material 2.

For Germany, we used data collected in the same way over 5 years (2015 – 2019) from the Eldena hibernaculum (N54.09, E13.44) situated in the North-Eastern federal state of Mecklenburg-Vorpommern (Suppl. material 1) described in detail in Fischer et al. (2022). This site is a disused beer cellar with 12 rooms, where a total of 300-400 bats hibernate, mostly *Myotis daubentonii*, *M. nattereri* and *M. myotis*, the latter being the most commonly infected species. A map of the site is presented in Suppl. material 3.

Cultures

Each swab was cultured on a petri dish containing DPYA growing medium (Vanderwolf et al. 2016). After a few days, individual spores (single spore individuals or SSIs) were physically separated and transferred into new petri dishes (as described in Fischer et al. 2022). These were sealed with parafilm and stored upside down in a fridge for at least 3 months until material was harvested for DNA extraction. We limited the number of SSIs obtained from a single swab sample to six, rather than isolating a large number of SSIs from a few samples

Molecular analyses

DNA extraction was performed following the protocol in Fischer et al. (2022). We then genotyped *P. destructans* isolates using 18 microsatellite markers (Drees et al. 2017a) and 2 mating type markers in 4 PCR multiplexes as described in Dool et al. (2018), see also Fischer et al. (2022). Genotyping was carried out using an ABI 3130 Genetic Analyser (Applied Biosystems) and the GeneMapper Software v.5 (Applied Biosystems).

Data analysis

The genotypic analysis was based on the identification of multilocus genotypes (MLGs) which are defined by the distinct combination of alleles at the 18 microsatellite loci. As *P. destructans* is a haploid and clonally reproducing organism (no sexual reproduction has yet been demonstrated), these MLGs can be used to track fungal individuals in space and time (Fischer et al. 2022). Thus, detection of the same MLG in different hibernacula or in different substrates/rooms means that *P. destructans* has moved between those. Missing data was not used as information to define MLG identity, and MLGs containing more than 20% of missing data (7 MLGs in total) were excluded from the analysis. Calculations of allelic diversity and differentiation were performed on clone-corrected data, meaning that only a single SSI of each MLG was retained per site or per analysed within-site groups. All analyses were performed in R software (version 4.0.1, R Core Team 2019).

Population differentiation

To estimate population structure between and within hibernacula, we traced shared MLGs in space and time, and between bats and walls (genotypic differentiation), and we applied AMOVA (analysis of molecular variance, Excoffier et al. 1992), based on allele frequencies, with 1000 permutations to test for significance (allelic differentiation). The factors analysed by the AMOVA were: site, hibernaculum room, year and season of sampling (winter-spring/autumn), and substrate (bat/wall). All factors were analysed both separately and in combination and all tests were carried out twice, with (using a 5% threshold) and without removing loci containing missing data. When testing allelic differentiation between rooms, rooms with a number of swab samples below 10 (see Suppl. material 5) were excluded. Genotypic differentiation was evaluated as the number of shared MLGs between factors considered divided by the total number of MLGs, after removing MLGs appearing only once throughout the dataset as by definition, these cannot be shared. Given that both genotypic differentiation (based on MLGs) and allelic differentiation (based of allele frequencies) are proxies for genetic differentiation, we hereafter use the term 'genetic differentiation' to refer to both measures.

Genotypic richness and population size

The number of expected MLGs (eMLGs) at the smallest shared sample size (based on rarefaction with 1000 permutations) was used to compare genotypic richness (number of

MLGs) between hibernacula. For estimating population size (= expected genotypic richness or expected number of MLGs), we used the CMRPopHet functions implementing a capture-mark-recapture (CMR) model based on a single sampling event (Petit and Valiere 2006). One assumption of the CMR model is that each MLG has the same probability of being sampled, which in its simplest form, means that all MLGs are assumed to have the same relative frequency of occurrence in the population. In order to test this assumption in the studied hibernacula, we used the heterogeneity test developed by Puechmaille and Petit (2007). Considering that the exact number of SSIs isolated per swab was not identical, we validated that the sampling design/intensity did not influence our estimates by running the analyses after randomly selecting exactly one SSI/swab (run 1000 times to factor in stochastic effects).

Environmental reservoir and infections in bats

By comparing genotypic richness of the fungus isolated from bats and from hibernacula walls, Fischer et al. (2022) demonstrated that yearly re-infection of bats originates from hibernaculum walls (environmental reservoir) containing viable spores of *P. destructans*. To corroborate these results, we tested the expectation that a transmission bottleneck would exist between the reservoir and the hosts, whereby genotypic richness of the pathogen would be reduced on the hosts as not all MLGs are successfully transferred from the source to the host population. Therefore, we compared genotypic richness of the fungus isolated from bats and from hibernacula walls by estimating the number of eMLGs at the smallest shared sample size based on rarefaction with 1000 permutations. We additionally ran the analyses when randomly selecting exactly one SSI/swab and when randomly selecting exactly two SSIs/swab (run 1000 times). To compare the genotypic richness per wall or bat swab, we calculated the following ratio for each swab: $(G-1)/(N-1)$, where G is the number of MLGs/swab and N is the number of SSIs/swab (Dorken and Eckert 2001). This ratio varies between 0 when all isolates from a sample harbour the same MLG, to 1 when all isolate from a sample harbour a different MLG. The average of this ratio was calculated per swab type (wall versus bat). To estimate the frequency of multiple infections in bats, we calculated the percentage of cases where more than one MLG or more than one mating type were found on a single bat swab, after taking exactly three SSIs/swab.

Results

We used here a data set containing a total of 1925 *P. destructans* single spore isolates (SSIs), of which 608 came from Balabanova dupka, 255 SSIs from Ivanova voda, and 1062 from Eldena. The average number of SSIs per bat swab was 4.84 (range 2-6, median 5) for Balabanova dupka, 4.5 (range 1-6, median 5) for Ivanova voda, and 2.76 (range 1-4, median 3) for Eldena. For wall swabs, we obtained an average of 2.78 SSIs/swab (range 1-6, median 2) for Balabanova dupka, 2.21 (range 1-6, median 1) for Ivanova voda, and 3.51 (range 1-5, median 4.5) for Eldena. We successfully amplified all microsatellite and mating type markers, with an overall amount of missing data of 1.2%.

Population differentiation

The AMOVA detected no significant allelic differentiation within sites with regard to any of the factors considered: hibernaculum room, time of sampling (year and season), and substrate (bat or wall). Additionally, within a site, MLGs were often shared between times of sampling and substrates, especially in Balabanova dupka and Eldena. Across sampling years and seasons, 61.7%, 22.7%, and 80.2% of MLGs found at least twice were shared in Balabanova dupka, Ivanova voda, and Eldena, respectively (Fig. 1). Shared occurrences were encountered both in the presence (in winter-spring) and absence (in autumn) of hibernating bats with visible fungal infection. Across hibernaculum rooms, 54.2%, 6.8%, and 80.2% of MLGs were shared in Balabanova dupka, Ivanova voda, and Eldena respectively, including rooms where bats were not encountered during our hibernation surveys. Between bats and walls, 45%, 11.4%, and 65.4% of MLGs were shared in Balabanova dupka, Ivanova voda, and Eldena respectively (Fig. 1).

As shown by AMOVA (see Suppl. materials 4, 5 for sample sizes), there was significant allelic differentiation among sites explaining 26.88% of total variance ($p < 0.001$). Results were the same with or without removing missing data. The strong allelic differentiation was corroborated by the genotypic differentiation showing that none of the 615 *P. destructans* MLGs detected were shared between any of the three study sites.

Genetic diversity and mating types

All studied loci were polymorphic, their mean richness being highest in Ivanova voda and lowest in Eldena (Table 1). Within sites, allelic richness widely varied per locus (range: 2-64 alleles; Suppl. material 6). A total of 301 unique MLGs were obtained from Balabanova dupka, 165 from Ivanova voda, and 149 from Eldena. At equal sample size, genotypic richness was more than twice higher in Balabanova dupka and Ivanova voda in comparison to Eldena (Table 1). Differences in allelic and genotypic richness between the study sites were consistent when we repeated the analysis with a single SSI/swab (Suppl. material 8).

Within sites, genotypic richness of *P. destructans*, estimated as the number of eMLGs, was consistently higher in swab samples taken from walls than in swab samples taken from bats (Table 2). Results were consistent (bar one exception) when we repeated the analysis with one SSI/swab or two SSIs/swab (Suppl. material 9). The mean genotypic richness per swab, estimated as $(G-1)/(N-1)$, was also higher in wall swabs (Table 2).

Multiple MLGs were very commonly found on the same bat swab. Indeed, after taking exactly three SSIs/swab, multiple MLGs were found in 83.6%, 80.9%, and 83.3% of bat swabs in Balabanova dupka, Ivanova voda, and Eldena respectively. Both mating types of the pathogen were found in 44%, 39.3% and 46.4% of bat swabs in Balabanova dupka, Ivanova voda, and Eldena respectively.

Both mating types were present in all hibernacula although with different proportions whereby the Bulgarian sites were more similar to each other than to the German site (Table 1).

Population size estimates

According to the CMR model, *P. destructans* population size (or the total number of MLGs predicted to the present) was 377 MLGs (Highest Probability Density [HPD] 95% 352-401) for Balabanova dupka, 274 MLGs (HPD95% 233-317) for Ivanova voda, and 150 MLGs (HPD95% 150-151) for Eldena (Table 1). Although moderate, some heterogeneity was observed in the probability of MLG sampling for Balabanova dupka and Ivanova voda while a seemingly stronger heterogeneity was present in Eldena (Suppl. material 7).

The dataset and the R script used for the analysis are presented in Suppl. materials 10, 11, respectively.

Discussion

We discovered no significant genetic differentiation in *P. destructans* population within sites contrasting with the strong genetic differentiation observed between sites. This indicates that the rate of pathogen movement is magnitudes higher within compared to between hibernacula and underlines the importance of studies investigating spatial and temporal changes at individual sites to better understand the intricacies of host-pathogen interactions. Additionally, we highlight the considerable genotypic richness of the pathogen within any of our study sites, which in turn leads to a high frequency of multiple infections in bats with potentially important biological consequences. Altogether, our results not only advance fundamental knowledge on *P. destructans* and WND but also provide critical information to design studies and suggests novel directions for future research.

Lack of significant *P. destructans* genetic differentiation within sites

The present study is the first one to characterize *P. destructans* genetic diversity and its distribution at multiple spatial scales in natural hibernacula within the native range of the fungus. As expected, none of the factors considered within a site, including substrate (bats or walls), time (across sampling years and seasons), or space (hibernaculum rooms), were associated with significant allelic or genotypic differentiation of *P. destructans*. The lack of significant differentiation between bats and hibernaculum walls is consistent with the known life cycle of *P. destructans* whereby yearly re-infection of bats originates from the environmental reservoir, which is in turn replenished by bats shedding fungal spores towards the end of the hibernation season (Hoyt et al. 2021, Fischer et al. 2022). The lack of significant differentiation across sampling seasons is consistent with the long-term survival of the pathogen in hibernacula, demonstrated by Fischer et al. (2020), Fischer et al. (2022) and previously suggested in other studies (e. g., Puechmaille et al. 2011a, Lorch et al. 2012, Raudabaugh and Miller 2013, Reynolds et al. 2015, Vanderwolf et al. 2016,

Lindner et al. 2017), as well as by its clonal mode of reproduction. If sex was the dominant mode of reproduction, a higher turnover of MLGs would be expected throughout the years due to the generation of new MLGs following recombination, which is not what we observe in Eldena and Balabanova dupka. The apparent higher turnover observed in Ivanova voda is most likely explained by the lower number of SSIs obtained from this cave and spring flooding potentially washing away or killing fungal spores (Suppl. materials 2, 4). Nevertheless, considering the genetic machinery needed for sexual recombination is present in *P. destructans* (Palmer et al. 2014) and that the two mating types co-occur within the same site, sexual reproduction is still possible, and more studies are needed on the subject.

On the other hand, population structure of *P. destructans* based on different rooms of the same hibernaculum had never been studied before and the absence of significant genetic differentiation is consistent with the ecology of hibernating bat species. In autumn, when bats arrive at their hibernaculum, they often engage in mating or other social interaction, flying around, landing and crawling on different places/rooms of the roost walls, the so-called swarming behaviour. Elevated activity of bats within the site is also observed in spring, when the animals prepare for moving out and can hang in different places across the hibernaculum. Even in winter, bats regularly interrupt their torpor bouts (Blažek et al. 2019) and may engage in grooming or contact other individuals or areas of the roost environment (Bartonička et al. 2017, Hoyt et al. 2021). Together, such behaviours are expected to lead to ample movement of the fungus across hibernacula used by bats. Yet, we cannot exclude the role of other factors such as air currents or movement of arthropods (Bastian et al. 2009, Kokurewicz et al. 2016) although no research has demonstrated their implication so far.

Altogether, our results show that *P. destructans* populations are not genetically differentiated within a site, suggesting ample pathogen movement. This does not rule out the possibility that certain factors (different microclimates or the segregation of bat species within a site) could act as barriers for *P. destructans* or lead to the selection of certain pathogen genotypes. However, even if such barriers/selection pressures exist, it seems that bat movement within a site is sufficient to mask their effects.

Significant *P. destructans* genetic differentiation at large scale

In agreement with our expectation, we detected significant allelic differentiation in *P. destructans* populations and we did not find a single pathogen MLG shared between sites for the full duration of our study. Although our number of sites is low (n=3), it is worth noting that our results are in complete agreement with the results of Fischer et al. (2022) who included a higher number of sites (n=9), closely situated to each other and without apparent barriers to movement for the bat hosts. These results could appear to contradict research from North America where population structure is limited and MLGs can be shared over large distances (Forsythe et al. 2021). However, the limited population structure observed in North America is most likely the result of the recent expansion (colonisation) rather than a signal of ongoing extensive gene flow between established *P.*

destructans populations (see also Fischer et al. 2021). Besides, due to the founder effect whereby genetic richness in the invasive population of a species is reduced due to the introduction of a single or just a few individuals, genetic diversity of *P. destructans* in North America is magnitudes lower in comparison to Eurasia (Drees et al. 2017b), which reduces the power of genetic markers to detect population differentiation at such short timescales. To overcome this issue, Thapa et al. (2021) used the fast-evolving mycovirus PdPV-pa as a proxy to infer the *P. destructans* population structure and found very strong differentiation across the studied region in North America. Although there are different factors that can decouple the mycovirus (PdPV-pa) population structure from its host (*P. destructans*) population structure (Mazé-Guilmo et al. 2016), the specificities of the current system point towards a situation where both structures should be correlated. Indeed, PdPV-pa seems to specifically infect *P. destructans* (Ren et al. 2020), all North-American *P. destructans* isolates seem to be infected with PdPV-pa (Ren et al. 2020, Thapa et al. 2021, Thapa et al. 2016), and mycoviruses lack an extracellular life stage and are transmitted only through cell division, sporulation, or cell fusion (Pearson et al. 2009, Son et al. 2015, Kotta-Loizou 2021). Taken together, these studies (Thapa et al. 2021, Fischer et al. 2022; the present study) strongly suggest that once *P. destructans* populations have established, the successful establishment of newly arrived pathogen MLGs is rare in comparison to *in situ* recruitment (see also Fischer et al. 2022). Thus, a detailed characterisation of the continental population structure of *P. destructans* across Europe may predict its population structure in North America after reaching equilibrium.

***Pseudogymnoascus destructans* genetic diversity between and within sites**

Our results confirmed the expectation that *P. destructans* genotypic richness at different sites in Europe are comprised within the same order of magnitude, pointing to the presence of up to 400 different MLGs in a single hibernaculum, possibly even more. No hibernacula have been intensively surveyed in North America to allow the calculation of genotypic richness within an individual site but patterns observed across many sites with a limited number of isolates characterised and a reduced number of loci (9 versus 18 in the present study) suggest a much-reduced genotypic richness with MLGs shared across much larger distances than in Europe (e.g., Forsythe et al. 2021 vs. Fischer et al. 2022). Consistently, the number of alleles per locus is much reduced in North America compared to Europe (Table S2 in Forsythe et al. 2021 vs Table S1 in Fischer et al. 2022 and Suppl. material 6 herein; Table 2 in Drees et al. 2017a). This is consistent with the population bottleneck, resulting from the pathogen's recent introduction to North America and subsequent geographical expansion (Leopardi et al. 2015, Drees et al. 2017a, Drees et al. 2017b).

Within the same site, we found shared MLGs between walls and bats and consistently higher genotypic richness of *P. destructans* in walls in comparison to bats. Thus, our results corroborate the hypothesis tested in Fischer et al. 2022 stating that hibernaculum walls represent the environmental reservoir of the pathogen and the initial source of bat infection.

Genetically diverse infections in bats

Infections with multiple MLGs were prevalent in bats, with more than 80% of the bat swabs harbouring multiple *P. destructans* MLGs. However, we only considered three fungal isolates per swab and the number of *P. destructans* spores that infect a bat at the start of hibernation is estimated to be roughly between 50 and 500 (Fischer et al. 2022). Thus, it is expected that practically every bat infected with *P. destructans*, at least within its native range, will be infected with numerous pathogen MLGs. To our knowledge, our study is the first to investigate multiple infections in *P. destructans*, a finding that is important to consider for two reasons. First, significant variation in pathogenicity can exist even between closely related variants of the same fungal pathogen species (Raabe 1972). Second, due to various interactions between pathogen MLGs, genetically diverse infections (co-infections) often behave differently from clonal ones. For example, competition for the host resources between different MLGs can lead to infection suppression and protection against superinfection (Mercereau-Puijalon 1996, Tanner et al. 1999) or, on the contrary, increase pathogen burden by stimulating pathogens to occupy broader niche space or raising the cost of the immune response (Taylor et al. 1998). Additionally, interactions between pathogen MLGs can alter the density of more or less virulent MLGs (Read and Taylor 2001) and possibly represent a powerful determinant for pathogen evolution and disease epidemiology (Susi et al. 2015). In some cases, hosts co-infected with multiple pathogen MLGs exhibit more severe disease symptoms and more intense transmission of the pathogen as shown in the host plant *Plantago lanceolata* and its fungal parasite *Podosphaera plantaginis* (Susi et al. 2015). In other cases, genetically diverse infections seem to reduce pathogen load as in the snail *Gasterosteus aculeatus* and its trematode parasite *Diplostomum pseudospathaceum* (Rauch et al. 2007). Furthermore, the characteristics of multiple infections might be different depending on the degree of relatedness between pathogen MLGs with competitive exclusion of distant MLGs and tolerance towards closely related MLGs (López-Villavicencio et al. 2007). As a consequence, it would be interesting to study how infections with multiple *P. destructans* MLGs influence the outcome of the disease. Knowledge on the pathogen's genetic richness within individual hibernacula and on individual bats (presented here) combined with information on *P. destructans* inoculum size on bats at the start of hibernation (presented in Fischer et al. 2022) are of paramount importance to address these questions as they provide an estimate of the number of different MLG interacting on individual bats.

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Conflicts of interest

The authors have declared that no competing interests exist.

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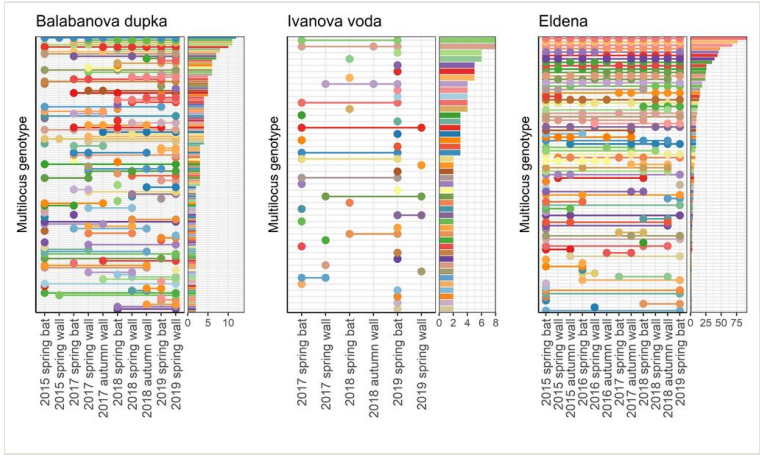


Figure 1. Visual representation of shared multilocus genotypes (MLGs) in the three study sites Balabanova dupka, Ivanova voda, and Eldena across time and between bats and walls. Each row represents a particular MLG and a circle signifies that this MLG was detected during the particular sampling event. The bar graph represents MLG relative frequencies of occurrence. The code used to recreate the graph was obtained from Kamvar et al. (2015). For convenience, only MLGs that were detected more than once are included on the graphs.

Table 1.

Comparison of *P. destructans* genetic diversity in the three study sites. Swab is the total number of swab samples collected from each site, both from bats and hibernacula walls; SSI is the total number of single spore isolates of *P. destructans* (= sample size); Allele is the mean number of alleles per locus; MLG is the total number of multilocus genotypes observed; eMLG is the number of expected multilocus genotype at the smallest shared sample size between the three sites (N=255); Pop size is the estimated population size based on the CMR model; HPD95% is the highest probability density of the population size estimate; M1 & M2 are the percentages of mating type MAT1_1 & MAT1_2 respectively.

	Swab	SSIs	Allele	MLG	eMLG	Pop size	HPD 95%	M1	M2
Balabanova dupka	172	608	12.5	301	170.5	377	352-401	68.5%	31.5%
Ivanova voda	74	255	14.6	165	165	274	233-317	70.2%	29.8%
Eldena	364	1062	6	149	77.3	150	150-151	42.4%	57.6%

Table 2.

Comparison of *P. destructans* genotypic richness found on bats and walls in the study sites. Abbreviations are as defined for Table 1. (G-1)/(N-1) is the mean genotypic richness per swab, where G is the number of MLGs per swab and N is the number of SSIs per swab. As the smallest shared sample size is calculated for bats and walls within each individual site, the reported eMLG values should only be compared within sites.

		Swab	SSI	MLG	eMLG	(G-1)/(N-1)
Balabanova dupka	Bats	63	305	159	158.3	0.58
	Walls	109	303	196	196	0.94
Ivanova voda	Bats	40	180	101	55.1	0.54
	Walls	34	75	69	69	0.97
Eldena	Bats	286	788	118	76.5	0.61
	Walls	78	274	84	84	0.85

Supplementary materials

Suppl. material 1: Study sites

Authors: Nicola Fischer

Data type: picture

Brief description: Geographic location of our study sites. Balabanova dupka and Ivanova voda are natural karst caves only accessible with caving equipment and Eldena is an artificial hibernaculum, a disused cellar.

[Download file](#) (2.95 MB)

Suppl. material 2: Sampling locations Bulgaria

Authors: Violeta Zhelyazkova

Data type: image (pdf)

Brief description: Sampling locations (rooms) in relevance to the hibernating bat colonies in the two studied Bulgarian caves. In Balabanova dupka, the entire colony of *Myotis myotis/blythii* hibernates in Room 1. Bats are found occasionally in Spring and Autumn in Rooms 2 & 3. The map is modified from Georgiev et al. (2016). In Ivanova voda, the colony of *Myotis myotis/blythii* hibernates mostly in Rooms 2 & 3, and the colony of *Myotis capaccinii* hibernates mostly in Room 4 and 5, above a big lake. The cave can get flooded in Spring, the water reaching up to Room 3. The original map was made by S. Adreeev and H. Delchev (1962).

[Download file](#) (110.41 kb)

Suppl. material 3: Sampling locations Germany

Authors: Nicola Fischer

Data type: image

Brief description: Sampling locations (rooms) in the artificial hibernaculum Eldena in Germany. Here, *Myotis daubentonii*, *M. nattereri* and *M. myotis* hibernate. R stands for Room. Bats hibernate in all rooms although in different numbers. Samples were collected in all rooms except rooms 1 & 12.

[Download file](#) (36.64 kb)

Suppl. material 4: Sample summary per season

Authors: Violeta Zhelyazkova

Data type: table

Brief description: Summary of the numbers of swab samples, isolated single spore cultures (SSIs), and multilocus genotypes (MLGs) of *P. destructans* obtained from bats and from hibernacula walls in the study sites divided by season.

[Download file](#) (17.01 kb)

Suppl. material 5: Sample summary per room

Authors: Violeta Zhelyazkova

Data type: table

Brief description: Summary of the number of swab samples, isolated single spore isolates (SSIs), and multilocus genotypes (MLGs) of *P. destructans* obtained from bats and from hibernacula walls in the study sites divided by rooms.

[Download file](#) (16.84 kb)

Suppl. material 6: Allelic richness

Authors: Violeta Zhelyazkova

Data type: table

Brief description: Allelic richness per locus in the three study sites. Calculations were performed on clone corrected data after SSI with missing data were removed.

[Download file](#) (14.43 kb)

Suppl. material 7: Heterogeneity test

Authors: Violeta Zhelyazkova, Sebastien Puechmaille

Data type: graph

Brief description: Outputs of the heterogeneity test developed by Puechmaille & Petit (2007) for the three study sites. This analysis is used to test the assumption of the capture-mark-recapture (CMR) model that each MLG has the same probability of being sampled. Filled circles show the model distribution of captures or single spore isolates (SSIs) obtained per individual multilocus genotype (MLG) under the assumption that each MLG has the same probability of being sampled (the population is homogenous). Empty circles show the highest probability density of the population size estimate (HPD95%). Triangles show the observed distribution of obtained SSIs per individual MLG.

[Download file](#) (20.69 kb)

Suppl. material 8: *P. destructans* genetic diversity per site

Authors: Violeta Zhelyazkova

Data type: table

Brief description: Comparison of *P. destructans* genetic diversity in the study sites when one SSI only is considered per swab. Swab is the total number of swab samples collected from each site, both from bats and hibernacula walls; SSI is the total number of single spore isolates of *P. destructans* (= sample size); Allele is the mean number of alleles per locus; MLG is the total number of multilocus genotypes observed; eMLG is the number of expected multilocus genotype at the smallest shared sample size between the three sites (N = 255); Pop size is the estimated population size based on the CMR model; HPD95% is the highest probability density of the population size estimate; M1 & M2 are the percentage of mating type MAT1_1 & MAT1_2 respectively.

[Download file](#) (14.08 kb)

Suppl. material 9: *P. destructans* genetic diversity on bats and walls

Authors: Violeta Zhelyazkova

Data type: table

Brief description: Comparison of *P. destructans* genotypic diversity found on bats and walls in the study sites when one (top) or two (bottom) SSIs are considered per swab. Abbreviations are as defined for Supplement 5. For the analysis with two SSIs per swab, all swabs that had given

only one SSI were removed. As the smallest shared samples size is calculated for bats and walls within each individual site, the reported eMLG values should only be compared within sites.

[Download file](#) (14.32 kb)

Suppl. material 10: Dataset

Authors: Violeta Zhelyazkova, Nicola Fischer, Sebastien Puechmaille

Data type: data table

Brief description: This is the dataset combining microsatellite data on *P. destructans* from Bulgaria and Germany analyzed in the present research.

[Download file](#) (447.10 kb)

Suppl. material 11: R script

Authors: Violeta Zhelyazkova, Nicola Fischer, Sebastien Puechmaille

Data type: R script

Brief description: This is the R script used to analyze the data.

[Download file](#) (107.85 kb)