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ORIGINAL ARTICLE

Population genetics as a tool to elucidate pathogen reservoirs: Lessons from *Pseudogymnoascus destructans*, the causative agent of White-Nose disease in bats

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Abstract

Emerging infectious diseases pose a major threat to human, animal, and plant health. The risk of species-extinctions increases when pathogens can survive in the absence of the host. Environmental reservoirs can facilitate this. However, identifying such reservoirs and modes of infection is often highly challenging. In this study, we investigated the presence and nature of an environmental reservoir for the ascomycete fungus *Pseudogymnoascus destructans*, the causative agent of White-Nose disease. Using 18 microsatellite markers, we determined the genotypic differentiation between 1497 *P. destructans* isolates collected from nine closely situated underground sites where bats hibernate (i.e., hibernacula) in Northeastern Germany. This approach was unique in that it ensured that every isolate and resulting multilocus genotype was not only present, but also viable and therefore theoretically capable of infecting a bat. The distinct distribution of multilocus genotypes across hibernacula demonstrates that each hibernaculum has an essentially unique fungal population. This would be expected if bats become infected in their hibernaculum (i.e., the site they spend winter in to hibernate) rather than in other sites visited before they start hibernating. In one hibernaculum, both the walls and the hibernating bats were sampled at regular intervals over five consecutive winter seasons (1062 isolates), revealing higher genotypic richness on walls compared to bats and a stable frequency of multilocus genotypes over multiple winters. This clearly implicates hibernacula walls as the main environmental reservoir of the pathogen, from which bats become reinfected annually during the autumn.

KEYWORDS
Chiroptera, disease reservoir, emerging infectious disease, fungal pathogen, White-Nose syndrome, wildlife pathogen

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1 | INTRODUCTION

Emerging infectious diseases pose a major threat to the health of humans, animals and plants, and consequently, to global biodiversity (Daszak et al., 2000; Schmeller et al., 2020). Host-pathogen relationships are ubiquitous in nature. One of the main reasons why such relationships only rarely lead to species extinctions is due to density-dependent transmission in many disease systems, in which transmission decreases when population sizes are low, allowing populations to recover (McCallum, 2012). However, if pathogens can survive in the absence of a host in environmental reservoirs, the risk they pose to biodiversity is markedly increased. This ability is often found in fungal pathogens, including *Batrachochytrium dendrobatidis* (causing amphibian chytridiomycosis; e.g., Johnson & Speare, 2003), the *Fusarium solani* species complex (causing disease in a range of hosts, e.g., plants, humans, sea turtles; e.g., Zhang et al., 2006) and *Puccinia graminis* (causing wheat stem rust e.g., Rowell & Romig, 1966).

The ability of a pathogen to spread among host populations and survive on alternative hosts or in environmental reservoirs is a key determinant in disease management (De Castro & Bolker, 2004). Unfortunately, it is precisely this information that is often lacking when dealing with newly emerging infectious diseases. This scenario was encountered during the sudden appearance of the ascomycete fungus *Pseudogymnoascus destructans* in North America in 2006, causing the generally lethal White-Nose disease (the disease associated with White-Nose syndrome) in hibernating bats (Blehert et al., 2009). Although the fungus was first described in North America, it was subsequently found to be present across Europe and parts of Asia (Frick et al., 2016; Hoyt et al., 2016; Kovacova et al., 2018; Puechmaille et al., 2011). Population genetic analyses and a lack of mass mortality in European bats indicate that the fungus was introduced from Europe to North America (Drees et al., 2017; Fritze & Puechmaille, 2018; Leopardi et al., 2015; Puechmaille et al., 2011). In its introduced range, *P. destructans* became an invasive pathogen killing more than 5 million bats within the first 4 years of discovery (Frick et al., 2015).

There are still large gaps in our understanding of *P. destructans* biology. So far in nature, active growth of the fungus (after germination of spores) and spore production have only been observed on bats, which are the only known hosts of *P. destructans* (e.g., Fischer et al., 2020; Langwig, Frick, et al., 2015; Palmer et al., 2018; Puechmaille et al., 2011; Reynolds & Barton, 2014). Only asexual reproduction has been observed in this haploid species (e.g., Gargas et al., 2009), and the resulting spores are likely to remain viable in the environment (dormancy) for several years at least (Fischer et al., 2020). Therefore, it becomes important to integrate information about host behaviour and life cycle to better understand and characterise the location and mode of transmission of *P. destructans* (e.g., Fuller et al., 2020; Hoyt et al., 2020; Langwig, Frick, et al., 2015; Puechmaille et al., 2011; Figure 1).

Given the strong seasonality in temperate regions, the yearly life cycle of bats living there can be divided into two main seasons (summer and winter), separated by transition periods (spring and autumn). Many temperate insectivorous bats spend the coldest months hibernating in underground sites (i.e., hibernacula; Ransome, 1990). During this period, bats exhibit a reduced body temperature making them susceptible to infection with the cold-loving fungus *P. destructans* (Verant et al., 2012; Whiting-Fawcett et al., 2021). In spring, bats become active and leave hibernacula to form summer colonies. Given that *P. destructans* is unable to grow above 20°C and that spores do not survive long periods at elevated temperatures (viability up to 15 days at 37°C), bats clear the infection over the summer period (Campbell et al., 2020; Kunz & Fenton, 2005; Langwig, Frick, et al., 2015; Verant et al., 2012).

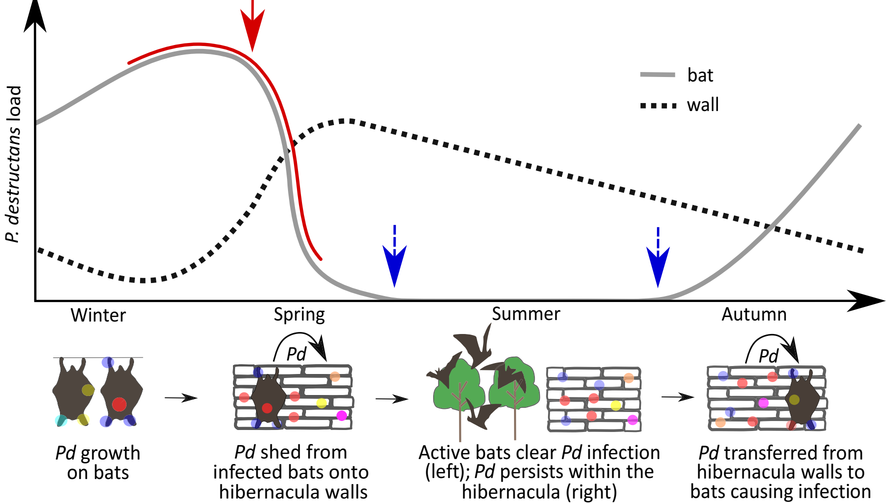

FIGURE 1 Schematic representation of the life cycle of *Pseudogymnoascus destructans* ("*Pd*") on bats (solid grey line) and in an environmental reservoir (hibernaculum; dotted line) throughout a year as predicted from existing literature (e.g., Fuller et al., 2020; Hoyt et al., 2020; Langwig, Frick, et al., 2015; Puechmaille et al., 2011). Timing of sampling is indicated by a red line and arrow (bat sampling) and blue dashed arrows (wall sampling). Seasonality of the bat and fungal life cycles are illustrated; coloured dots represent different fungal multilocus genotypes

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In autumn, individuals from several species of bats may meet in prehibernation swarming sites (typically also used as hibernacula), where mating occurs (Norquay et al., 2013; van Schaik, Janssen, et al., 2015). During swarming, bats land and crawl on the walls of hibernacula. This behaviour is hypothesised to result in bats becoming infected from the environment (Puechmaille et al., 2011). The fact that *P. destructans* spores persist on hibernacula walls (Vanderwolf et al., 2016), even when bats are absent, and that the seasonal pattern of *P. destructans* germination is synchronised with the presence of bats in hibernacula (Fischer et al., 2020), lends support to the inferred wall-to-bat mode of infection after summer (see also Hoyt et al., 2020). Additionally, sharp increases in *P. destructans* prevalence (estimated via qPCR) on bats in autumn further supports the inferred timing of infection (swarming behaviour at swarming/hibernation sites; see also Langwig, Frick, et al., 2015) although viability of the fungus on bats during this period remains to be confirmed. Indeed, as is the case in most newly emerged wildlife pathogens, a large amount of the available data on the prevalence and load of the pathogen on hosts and their environment comes from the quantification of DNA contained in samples (i.e., qPCR; Bohuski et al., 2015; Boyle et al., 2004; Muller et al., 2013). This information on its own provides no information on pathogen viability (Urbina et al., 2020). Infection cannot occur unless pathogens are viable. Therefore knowing the viability status of pathogens is critical for correctly elucidating transmission routes and disease dynamics (Fritze et al., 2021). In the case of White Nose disease, the seasonal patterns in *P. destructans* prevalence/load have been estimated by qPCR and indicate that the fungal loads on bats and on walls are associated. However, the directionality of the exchange between bats and walls cannot be elucidated from the currently available data which characterises DNA presence/quantity only. Furthermore, no study has yet investigated if bats become infected before or after they have arrived at the site where they hibernate nor the amount of fungal material that is needed to infect bats.

Here, we test the hypothesis that yearly re-infection of bats originates from an environmental reservoir containing viable spores of *P. destructans*, namely the walls of the hibernaculum, and assess the location (i.e., site) of the initial infection of the bat population after summer and quantify the amount of pathogen associated with this infection. The clonal mode of reproduction of *P. destructans* (e.g., Blehert et al., 2009) means that it is possible to follow the pathogen through time (i.e., seasons across several years) and space (environment/bat) using population genetic tools, allowing the elucidation of transmission routes. Specifically, the pathogen population of a host will be very similar to the pathogen population it became infected from, and more dissimilar to other pathogen populations from which it is not connected via transmission routes. These comparisons may be classically inferred from phylodynamics or population differentiation (using allele frequencies). Such approaches may suffer from lack of resolution however. To overcome this issue, we developed a novel fine-scale approach allowing fungal individuals to be distinguished and tracked through time and space. As fungal individuals cannot be distinguished phenotypically, genetic tools were used to determine the genetic fingerprint (i.e., multilocus genotypes) of fungal individuals (classically referred to as genets; Burnett, 2003). Though viability is a key factor for successful infection of a host, it remains rarely considered in the literature. In the current study, we use an innovative population genetic approach on cultures of *P. destructans*, allowing the presence and viability of fungal individuals to be followed in detail.

2 | MATERIALS AND METHODS

2.1 | Sampling

Swab samples were collected from bats in nine bat hibernacula in Northeastern Germany, which is situated within the native range of *P. destructans*. These hibernacula are not used in summer by maternity colonies. Swab samples were collected from freely hanging bats (*Myotis myotis*, *M. nattereri*, *M. dasycneme*) between January and April (without handling bats). On average, 3.65 body parts (i.e., left/right ear, left/right wing, nose, uropatagium) were sampled per bat at each date (1 sampled body part = 1 sample). Visible infection of bats is a good proxy for *P. destructans* load and therefore we sampled the full range of infection statuses, from visibly uninfected to high fungal loads (see Fritze et al., 2021). Bats from eight hibernacula (21–83 km from the main study site Eldena) were surveyed only a few times (between 1 and 3 sampling events per hibernaculum, see Table S2) to obtain a snapshot of *P. destructans* populations to compare with the main study site, Eldena, for which the sampling was more extensive as explained below.

In the main study site, Eldena, both bats and walls were sampled for a period of five calendar years (2015–2019). The walls of the Eldena hibernaculum were sampled by swabbing areas within 10 cm from where bats usually hung during hibernation. The surface of the walls swabbed was comparable to the area swabbed for the bats' body parts. Wall swabbing was conducted over 4 consecutive years, twice a year; once in late April, when most hibernating bats had left the hibernaculum, and once in mid-October to sample just before the arrival of the majority of bats for the next swarming and hibernation season (see Figure S1). In addition to sampling the walls (twice a year) and bats (between January and April), the Eldena hibernaculum was visited biweekly from 2015 to 2018 to document the number and species of bats present (see Supporting Information S1).

2.2 | Culturing

Given that a swab sample, whether collected from a bat or a wall, often contains multiple multilocus genotypes (hereafter simply referred to as "genotypes"), it is important to first isolate single spores before proceeding with culture and DNA extraction (Dool et al., 2020). To do so, each sample was cultured on dextrose-peptone-yeast agar (DPYA) following Vanderwolf et al. (2016) and upon germination of spores (visible under the microscope within 2–4 days after

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plating), individual spores were physically separated and moved into new petri dishes to obtain single-spore isolates (hereafter referred to as "isolates"). All isolates were sealed in petri dishes and stored upside down at 10°C for at least 6 weeks. Depending on availability, in Eldena 1–3 (mean = 2.75, median = 3) and 1–5 (mean = 3.51, median = 4.5) isolates were cultured for bat and wall swabs respectively, while 1–4 (mean = 2.74, median = 3) isolates were cultured from the bat swabs obtained at other hibernacula.

2.3 | Molecular analyses

In preparation for DNA extraction, fungal material was harvested from the isolates and dried (Vacuum centrifuge, V-aq, 30°C, 2.5 h) before homogenization in a tissue lyser with 2 mm glass beads (3 × 15 s at 25 Hz; 2 beads per tube) after 20 min at −80°C. The DNA was then extracted using a KingFisher Flex extraction robot (Thermo Scientific) and the MagMAX Plant DNA Isolation Kit (Thermo Scientific) utilizing magnetic-particle technology with the addition of 40 µM dithiothreitol (DDT) to lysis buffer A and 16.33 µM RNase A to lysis buffer B. To genotype isolates of *P. destructans*, 18 microsatellite markers (Drees et al., 2017) were used in four PCR multiplexes as described in Dool et al. (2020). Genotyping was carried out using an ABI 3130 Genetic Analyser (Applied Biosystems) and GeneMapper Software v.5 (Applied Biosystems) was used for fragment analysis.

2.4 | Data analysis

Most of our analyses use genotypic data based on the identification of genotypes which are defined by a distinct combination of alleles at the 18 microsatellite loci (a schematic representation of the analyses can be found in Figure S2). To ensure that the marker set was powerful enough to distinguish genotypes, we calculated 1/probability of identity (P_{ID}), which gives the theoretical number of different genotypes that can be distinguished (Waits et al., 2001). To determine the genotypic richness (diversity based on quantity of different genotypes), we used the measure of eMLGs which is the number of expected unique genotypes at the smallest shared sample size across several groups/populations based on rarefaction, as classically used to calculate allelic richness (Leberg, 2002). All results for Eldena were obtained by using up to 3 isolates per swab for bats and 5 isolates per swab for walls. This was done to obtain large sample sizes to better estimate genotypic richness overall as well as for each sampling event. To ensure that patterns of genotypic richness were not influenced by differences in number of isolates per swab, we also analysed the data using exactly 3 isolates per swab independent of substrate, which resulted in the same patterns of genotypic richness (see Figures S9, S10 and S11).

All analyses were performed in R (version 4.0.2; R Team, 2019) using the packages poppr (version 2.8.6, Kamvar et al.,2015, Kamvar, Tabima, et al., 2014), vegan (version 2.5.6, Oksanen et al., 2019) and adegenet (for DAPCs; version 2.1.4, Jombart, 2008; Jombart & Ahmed, 2011). The tidyverse collection of packages (Wickham et al., 2019) was used to improve ease and efficiency in analyses and the corrplot package (version 0.90, Wei & Simko, 2017) was used for visualization of correlations of relative frequencies of genotypes occurrence. The R-script used for analyses as well as the raw data are available from Dryad (https://doi.org/10.5061/dryad.x0k6djhhx; Fischer et al., 2021).

2.5 | Where do bats become infected with *P. destructans*?

If bats become infected from an environmental reservoir within hibernacula this could either happen where they hibernate or at a different site prior to hibernation (e.g., where they swarm). If successful infection of bats takes place at one or more sites other than their hibernaculum, bats would move among sites while already carrying spores leading to dispersal of *P. destructans* genotypes. As a result, sites should share genotypes that had been transferred, and the spatial pattern of genotypes should be homogeneous. Furthermore, probabilistically, common genotypes would be more likely to be picked up by a bat and transferred, resulting in a correlation in the frequencies of occurrence of genotypes across sites (i.e., genotypes common in site A would be expected to be common in site B if there was frequent exchange of material between sites). We therefore determined genotypic differentiation among the nine hibernacula by correlating the relative frequency of occurrence of each genotype (i.e., how often each genotype was sampled divided by the total number of isolates genotyped) at a given site with every other site (pairwise Pearson product-moment correlation, *p*-values corrected using sequential Holm-Bonferroni method).

Second, we used a discriminant analysis of principal components (DAPC; Jombart et al., 2010) to probabilistically assign isolates into predefined groups (in this case hibernacula) without relying on model assumptions (e.g., independent loci). If no genotypic differentiation is present, the assignment into groups should be no better than random while strong genotypic differentiation should be associated with high assignment of isolates to the hibernacula from which they were sampled. To avoid circularity, the isolate to be assigned to a group (i.e., hibernaculum) in the DAPC was removed from the dataset before assignment (i.e., a leave-one-out procedure). Hence a DAPC was specifically built for each isolate. To investigate if the assignment of isolates to their hibernaculum of origin (i.e., where they were sampled from) was more successful than expected by chance, we performed a second set of DAPCs for which we randomly reshuffled the information on hibernaculum before performing DAPCs (DAPC as described above). This second set of DAPCs corresponds to a data set where assignment is simply due to chance (named null DAPC). To avoid potential issues that could arise from differences in sample sizes, we performed subsampling of isolates from the most intensively sampled hibernaculum, Eldena, keeping only 85 isolates from this

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hibernaculum (Puechmaille, 2016), to match the sample size at other hibernacula. The subsampling of isolates was independently performed for each DAPC (1497 DAPCs per set). For each of the two sets of DAPCs (i.e., one containing the true information on hibernaculum of origin and one with hibernacula randomised – the null DAPC), we then summarised the results by simply calculating the percentage of isolates that were correctly assigned to the hibernaculum that they were originally sampled from.

2.6 | Do individual genotypes persist long-term?

If bats become infected from the walls of their hibernaculum and there is little exchange of genotypes among hibernacula, hibernacula can be considered as closed systems for *P. destructans*. In this case, given the clonal mode of reproduction of the species, we would expect high stability of the relative frequency of occurrence for genotypes through time, with common genotypes in one year remaining common in the next. Therefore, the temporal stability of genotype composition in Eldena was studied across different times of the year (October and January–April) and in different substrates (bat & wall) between 2015 and 2019. The stability of each genotypes' frequency of occurrence in the data set (i.e., how often each genotype was sampled divided by the total number of isolates genotyped for each winter season) was evaluated by pairwise correlations between winter seasons (wall swabs collected in October pooled with bat and wall swabs collected between January and April the next calendar year). For this we calculated a correlation matrix (pairwise correlations based on Pearson product-moment correlation, *p*-values corrected using sequential Holm-Bonferroni method) from the frequency of occurrence of each genotype in Eldena per winter season (2014/15–2018/19, 10 correlations). This was done to determine whether common genotypes remained common or if there was substantial turnover in the pathogen population across five winter seasons.

2.7 | Are genotypic patterns of *P. destructans* consistent with environmental infection?

We investigated environmental infection (i.e., infection from the walls of the hibernaculum) by studying the isolates/genotypes observed in samples obtained from bats ("bat isolates"/ "bat genotypes") as well as from walls ("wall isolates"/ "wall genotypes") in Eldena. If the walls of hibernacula are the main source of bat infection after summer, we expect to find genotypic signatures consistent with a transmission bottleneck. Here, the genotypic richness would be reduced on the hosts as not all genotypes are transferred from the source (here the environmental reservoir) to the host population. Therefore, to examine the existence of such a transmission bottleneck, we examined the difference in eMLG values (expected number of genotypes at equal sample size) calculated for bats and walls. To test for differences in genotypic richness, we then used two complementary methods, a one-sided permutation test (see Supporting Information material, Figure S7), and a one-sided paired *t* test (to test for differences while considering bat and wall isolates from the same winter as paired measures). Furthermore, if the genotypes found on the bats are transferred from the walls, we would also expect to see a lack (or low amount) of genotypic differentiation between bat and wall isolates. If so, the isolates found on the bats would have a similar pool of genotypes with similar relative frequencies of occurrence as those found on the walls. To test if our data are consistent with this expectation, we correlated the relative frequency of occurrence of each genotype found on bats and walls in the dataset using Pearson product-moment correlation.

2.8 | How many spores of *P. destructans* are transferred from the walls to each bat?

The difference in genotypic richness on the donor (walls–environmental reservoir) and receiver (bats–hosts) populations can be used to estimate the strength of the transmission bottleneck (i.e., number of spores passed) at the start of the hibernation season. To examine this, we simulated bottlenecks of different strengths (i.e., sampled isolates; in steps of 1 from 2 to 3100) by subsampling the pool of genotyped isolates from the walls in Eldena, mimicking the subset of spores passed from the walls to the bats (each winter season investigated separately). As previous studies have shown that only a fraction of spores germinates, we subsequently randomly selected 17.5% of the previously subsampled isolates for further analyses (based on the mean germination rate for spores of *P. destructans* from the data in Fischer et al., 2020; mean = 17.5%). For each value of bottleneck strength (i.e., sample size), we then calculated the genotypic richness (i.e., number of genotypes) observed in the subsampled data set. We then compared the results from all simulations to infer the bottleneck size with the average (over 1000 runs) that best matched the observed genotypic richness in bats in Eldena (Figure S5). To obtain an average number of spores passed from the wall reservoir to each individual bat, we simply divided the determined matching bottleneck size (i.e., number of spores transferred to the entire sampled population of bats) by the number of body parts that were sampled (i.e., the number of collected samples) and multiplied by six, the number of body parts that are commonly infected by *P. destructans* (i.e., left/right ear, left/right wing, nose and uropatagium).

Although we isolated and genotyped several isolates per swab sample (see above), it is likely that we did not capture all the genotypes that were present on the samples we analysed. Hence, to be more precise, we used the upper and lower bounds of the 95% confidence interval of the predicted number of genotypes present on bats (per winter season) to calculate the number of transferred spores. These predicted numbers of genotypes were obtained via a Bayesian estimator classically used to estimate population size based on a single sampling session (Petit & Valiere, 2006; Puechmaille & Petit, 2007).

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3 | RESULTS

3.1 | Geographic genotypic differentiation

For Eldena, (the focal hibernaculum), 286 bat swabs and 78 wall swabs were processed over the course of five winters (2014/15 until 2018/19), resulting in a total of 1062 *P. destructans* isolates. The number of swabs collected from bats from the eight other hibernacula was at least 10 and resulted in 27 to 85 isolates per hibernaculum, for a total of 435 isolates (Table 1). All isolates were genotyped at 18 microsatellite loci with an overall level of missing data <1% (across all hibernacula and loci). Each locus had between three and 17 different alleles (mean = 8.67, median = 7.5; see Table S1). The P_{ID} (probability of identity) across hibernacula was 6.63 $*10^{-7}$, indicating that more than 1.5 million different genotypes could theoretically be distinguished by our marker set, clearly exceeding the obtained number of genotypes (see Supporting Information S4 for P_{ID} at each hibernaculum).

To evaluate the genotypic differentiation among sites, we calculated summary statistics such as the percentage of shared genotypes

TABLE 1 Information on *Pseudogymnoascus destructans* sample sizes, genotypic richness, and number of shared multilocus genotypes across hibernacula

Hibernaculum	Distance (km)	Swabs	Isolates	Genotypes	Shared genotypes
Eldena	–	364	1062	149	13
Wolgast	21	22	63	18	3 (2)
Anklam	30	12	33	16	3 (0)
Richtenberg	39	12	35	7	5 (4)
Altentreptow	46	27	76	31	8 (5)
Bad Sülze	52	10	31	11	2 (1)
Strasburg	67	34	85	28	8 (6)
Burg Stargard	67	10	27	18	3 (1)
Neustrelitz	83	32	85	32	9 (1)

Distance in kilometres refers to the distance from each hibernaculum to Eldena. “Swabs” refers to the number of swab samples collected and cultured with “isolates” giving the number of obtained single spore isolates and “genotypes” giving the total number of unique multilocus genotypes per hibernaculum. Shared genotypes are given as all genotypes shared with any other hibernaculum; the number of genotypes shared with Eldena is given in brackets.

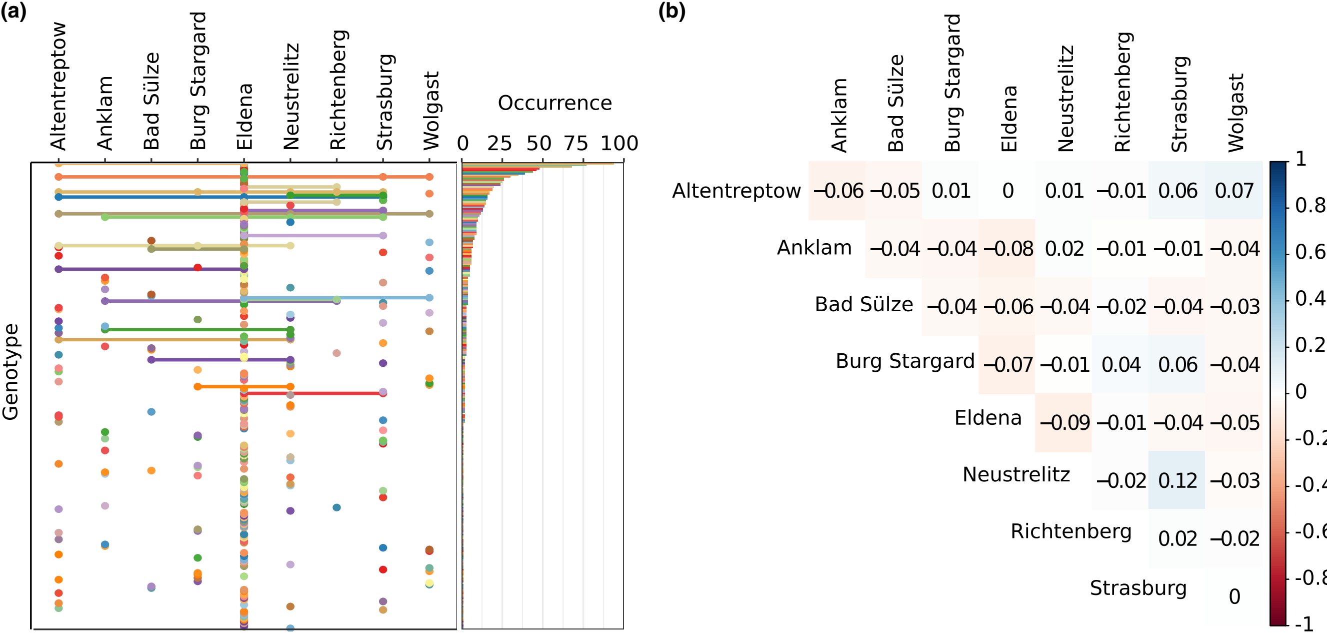

FIGURE 2 *Pseudogymnoascus destructans* genotypic differentiation across hibernacula. (a) Observation of genotypes (y-axis) at different hibernacula (x-axis; dots represent observed genotypes) on the left, and each genotypes frequency of occurrence (“occurrence”) across all hibernacula on the right. The figure was created based on an R script provided by Kamvar, Larsen, et al. (2014). (b) Correlation of genotype relative frequency of occurrence among pairs of hibernacula. Values represent Pearson's product-moment correlation coefficients. None of the correlations were significant after sequential Holm-Bonferroni correction (see text for further details)

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between sites and tested for correlations between the relative frequency of occurrence of each genotype for pairs of hibernacula.

Irrespective of how the data were partitioned (see below), the results consistently show a small percentage of genotypes shared between hibernacula. Each hibernaculum had a nearly unique collection of genotypes with 92% of genotypes not shared between hibernacula (278 genotypes in total, of which only 22 were shared among two or more hibernacula; see Figure 2a). Of the 142 genotypes observed at the eight other hibernacula (bar Eldena, 435 isolates), 8.5% were shared among these eight hibernacula (12 genotypes), and 9.2% were shared with Eldena (13 genotypes, 1062 isolates).

The correlation between the relative frequency of occurrence of each genotype for pairs of hibernacula was low (36 pairwise correlations ranging from −0.09 to +0.12) and nonsignificant (all $p > .05$; see Figure 2b). When looking at the correlation of relative frequency of genotype occurrence between Eldena and Strasburg as well as between Eldena and Neustrelitz (which both had the greatest numbers of isolates apart from Eldena; see Table 1) values were −0.04 and −0.09, respectively. Even the relative frequencies of occurrence of genotypes pooled for all eight hibernacula excluding Eldena (435 isolates) were not significantly correlated with the observed relative frequency of genotype occurrence in Eldena (all years pooled, 1062 isolates; $r = -0.05$, $p = .80$). The null DAPC (assignment of isolates expected by chance) correctly reassigned only 14% of isolates to their hibernaculum of origin, while the DAPC on observed data correctly reassigned 74% of isolates (Figure 3 and Figure S3), demonstrating the genotypic differentiation between hibernacula (see also Figure S4 for Hedrick's G_{ST} among pairs of hibernacula).

3.2 | Stability of genotypes through time (Eldena)

We used two complementary methods to determine if genotypes remain temporally stable within a site. Firstly, we found a great number of genotypes in Eldena shared across winters (Figure 4a). Secondly, to investigate the turnover of genotypes in Eldena in more detail, we calculated a correlation matrix based on the relative frequency of occurrence of each genotype in the data set for pairwise winter seasons (five winters: 10 pairwise correlations). We found high and significant correlations of the relative frequencies of occurrence of genotypes between each pair of winters, ranging from 0.61 to 0.76 (Pearson product-moment correlation, $p < .001$ for all pairs; Figure 4b). The correlation between the first and last sampled winters (2014/15 and 2018/19) was 0.64.

3.3 | Presence of a transmission bottleneck from walls (the reservoir) to bats (the hosts)

The sampling of Eldena spanned a period of 5 years with sampling in regular intervals yielding 788 and 274 bat and wall isolates, respectively. To determine whether there is a difference in genotypic richness between the two sampled substrates (bats and walls) we calculated eMLG values for each, giving the number of expected unique genotypes at equal sample size, hence allowing a simple comparison of richness even with different yields of isolates. We found that genotypic richness of *P. destructans* was significantly higher on samples collected from walls than from bats ($eMLG_{WALLS} = 85$, $eMLG_{BATS} = 76.5$; permutation test, $p = .021$, see Figure S8). In addition to determining genotypic richness for all samples of a substrate (bat/wall) pooled we also investigated genotypic richness of the substrates in each winter season. For each winter season respectively, eMLG values of wall samples (pooled per winter, i.e., October and April) were higher than those of bat samples with the exception of winter 2014/15 when wall sampling occurred in April only (one-sided paired t test, $t = -2.38$, df = 4, $p = .038$; Table 2).

We detected considerable numbers of *P. destructans* genotypes shared between bats and walls in Eldena and a high quantity of shared genotypes across the five different winter seasons (Figure 4a; 78% of genotypes observed at a minimum of two sampling events were shared between bats and walls). When data from the five winter seasons were pooled together for each substrate (i.e., all isolates from walls across years and bats across years), the Pearson correlation of genotypes relative frequency of occurrence on bats and walls was high ($r = 0.88$, $p < .001$).

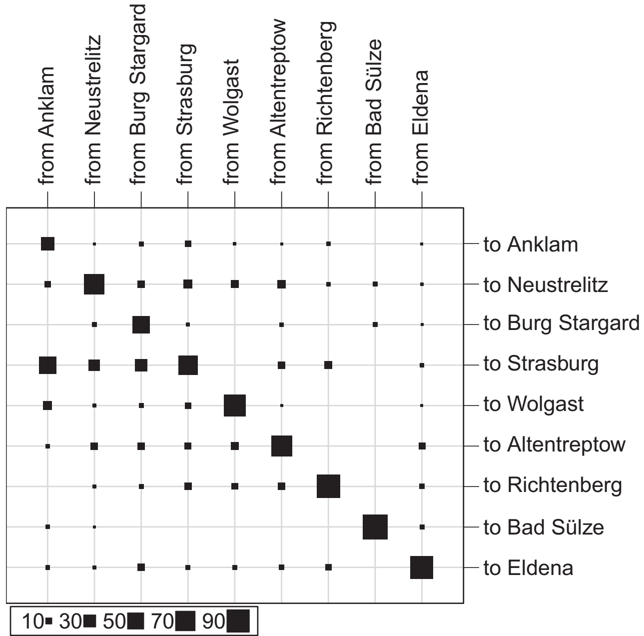

FIGURE 3 Assignment of *Pseudogymnoascus destructans* isolates to hibernacula using a discriminant analysis of principle components (DAPC) approach. Columns correspond to sampled hibernacula while rows correspond to inferred hibernacula (assignment result). Size of filled squares indicates the percentage of isolates from one site (x-axis, "from") which were assigned to another site (y-axis, "to"). Squares on the diagonal (top left to bottom right) show the rates of successful assignment as a percentage. To avoid potential issues that could arise from differences in sample sizes, isolates from Eldena were subsampled prior to the DAPCs, keeping only 85 isolates at a time (see Materials and Methods for more details). Successful assignment from the empirical and null-DAPCs were 74% and 14%, respectively (overall)

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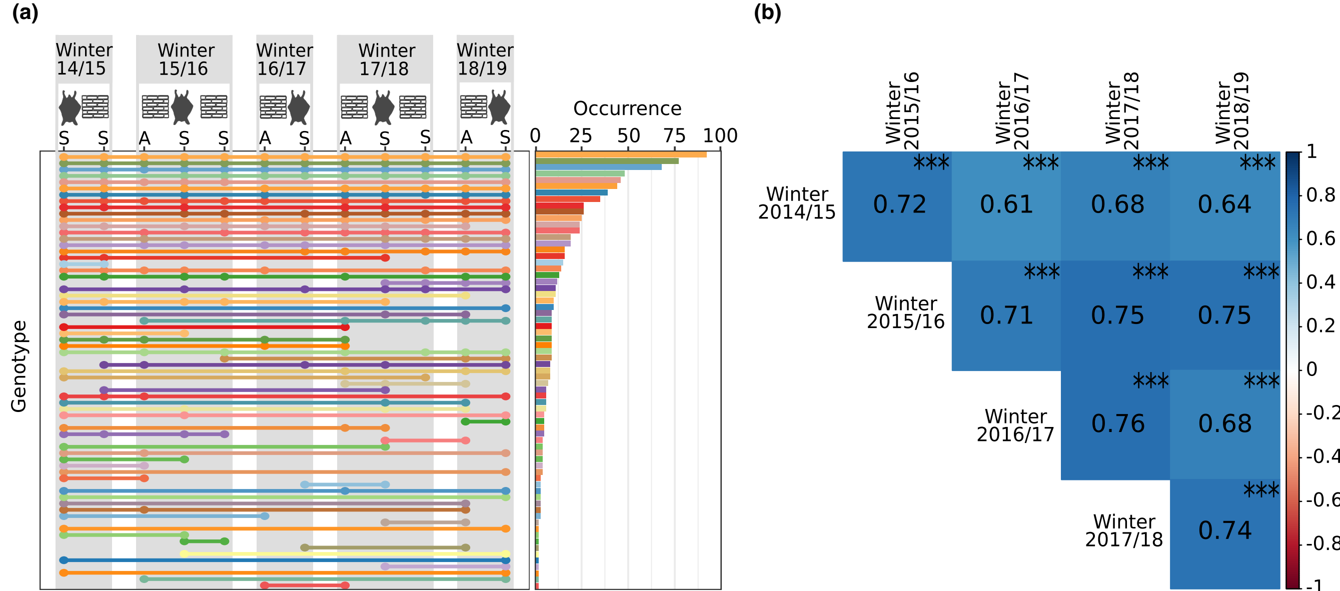

FIGURE 4 *Pseudogymnoascus destructans* genotypic differentiation across time in Eldena hibernaculum. (a) Observation of genotypes (y-axis) at different sampling events (x-axis; dots represent observed genotypes, sampled either in spring [S] or autumn [A] on the substrate [bat or wall] represented above the A or S letter) on the left, and each genotypes frequency of occurrence ('occurrence') across all sampling events on the right. The figure was created based on an R script provided by Kamvar, Larsen, et al. (2014). Only genotypes which occurred at more than one sampling event are shown. (b) Correlation of genotypes relative frequency of occurrence between winter seasons. Values represent Pearson's product-moment correlation coefficients while stars indicate level of significance after sequential Holm-Bonferroni correction (***$p < .001$; see text for further details)

TABLE 2 Information on *Pseudogymnoascus destructans* sampling success ("swabs", number of cultured swab samples; "isolates", number of single spore isolates) and genotypic richness ("genotypes", number of unique multilocus genotypes; eMLG, Number of expected multilocus genotypes at equal sample size) in winters in 5 years and from different substrates (bats/walls) for Eldena. To test for the difference in genotypic richness (eMLG) between bats and walls of each year a one-sided, paired t test was calculated ($t = -2.83$, df = 4, $p = .038$)

Winter and substrate	Swabs	Isolates	Genotypes	eMLG
2014/15 bats	139	391	74	16.9
2014/15 walls[a]	8	34	21	16.3
2015/16 bats	31	80	34	15.3
2015/16 walls	25	77	44	17.7
2016/17 bats	10	26	16	14.4
2016/17 walls[b]	6	22	17	17.0
2017/18 bats	53	149	37	14.5
2017/18 walls	24	89	37	15.7
2018/19 bats	53	142	47	15.7
2018/19 walls[b]	15	52	32	16.9

[a]Contains isolates from wall sampling in April only.
[b]Contains isolates from wall sampling in October only.

3.4 | Strength of wall to bat transmission bottleneck in Eldena (numbers of transferred spores)

Subsampling of different numbers of isolates obtained from wall swabs allowed us to estimate how many isolates from the walls were needed to explain the genotypic richness (number of unique genotypes) observed on bats each winter. Based on the subsampling of isolates (which mimics the bottleneck occurring when bats become infected from the walls), the germination rate and the sampling scheme/intensity, we estimated that between 48 and 530 spores were transferred from walls to each individual bat (Table 3; for further information, see Supporting Information S6.1 & Figure S6). The estimate obtained for the winter season with the most intense sampling (i.e., 2014/15; 139 swab samples, between 74 and 77 predicted genotypes) is between 100 and 129 spores transferred from the walls to each individual bat (Figure 5).

4 | DISCUSSION

4.1 | Population structure as a prerequisite for identifying sites where infection occurs

Using genetic information to identify the site of infection requires some level of population structure within the pathogen. Stronger population structure will enable more accurate estimates of the

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TABLE 3 Number of *Pseudogymnoascus destructans* swab samples collected from bats ("swabs"), predicted number of multilocus genotypes ("genotypes"), as well as number of spores transferred to a single bat per winter season in Eldena ("spores per bat")

		Predicted	
Winter season (bat samples only)	Swabs	Genotypes	Spores per bat
Winter 14/15	139	74–77	100–129
Winter 15/16	31	35–48	74–143
Winter 16/17	10	19–52	88–530
Winter 17/18	53	37–41	48–61
Winter 18/19	53	47–56	77–121

The numbers of transferred spores were determined based on predicted numbers of multilocus genotypes ("genotypes"; lower and upper bounds of the 95% confidence interval) and their match with genotypic richness observed for different bottleneck sizes (see Supporting Information S6.1).

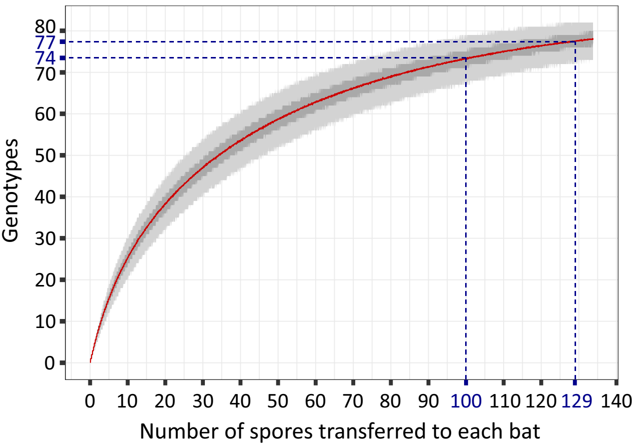

FIGURE 5 Estimated number of *Pseudogymnoascus destructans* spores transferred from walls to each bat in Eldena in the winter 2014/2015 (dashed blue lines). The red line represents the estimated mean number of spores transferred from the walls to each bat (x-axis) in relation to the total number of genotypes on bats (y-axis). The areas shaded in dark grey and light grey depict the 50% and 95% confidence intervals of the estimates, respectively. The x-axis (spores transferred to each bat) has been adjusted to account for sampling effort in the winter 2014/2015. Results for other winter seasons are shown in Table 3

site of infection. In general, it is expected that the population structure of a pathogen will be strongly linked to the population structure of the host with dispersal routes of the pathogen following host movements. However, while pathogen and host differentiation are often linked (Mazé-Guilmo et al., 2016), there are many exceptions to this (see e.g., McCoy et al., 2005; van Schaik et al., 2015). This can be explained by the vast diversity of host and pathogen life cycles found in nature as well as how they interact with each other (Barrett et al., 2008). The deviation of pathogens from their host population structure is mainly determined by the reproductive mode of the pathogen (sexual/asexual) and the presence of free-living stages (e.g., spores) relative to total developmental stages (Mazé-Guilmo et al., 2016).

In our study system, *P. destructans* predominantly reproduces clonally and has a free-living stage (spores). These factors are expected to have a greater impact on pathogen population structure compared to the absence of structure in the host. Owing to the strong differentiation of the pathogen between hibernacula, it is possible to identify where (at which site) bats become infected. The bats most often infected by *P. destructans* (*Myotis myotis*) show seasonal movements between summer roosts, swarming sites and hibernacula, where they regularly interact with conspecifics and sometimes with bats of other species (Kunz & Fenton, 2005; Steffens et al., 2007). While these bat-to-bat contacts offer opportunities for the exchange of pathogens, including *P. destructans* (Lorch et al., 2011; Webber & Willis, 2016), we found that genotypes of *P. destructans* were mostly site-specific and did not reflect the genotypic patterns expected if *P. destructans* was readily exchanged between bats at different periods of the year. If bats became infected before they arrive in the hibernaculum where they spend winter (i.e., their hibernaculum), their movement between sites while already infected should cause significant transfer of genotypes leading to a homogenous population of *P. destructans* with low rates of differentiation, low rates of correct assignment, and a greater number of shared genotypes between hibernacula. In contrast to this, we detected a strong pattern of differentiation, and high rates of correct assignment of *P. destructans* genotypes, even between geographically close hibernacula, which bats could feasibly visit during a single swarming season. Thus, our results suggest that the hibernaculum where bats spend the winter is also the source of successful bat infection. The timing of infection cannot be precisely elucidated with our data but is most likely during the autumn swarming season as previously hypothesised (Puechmaille et al., 2011), when contact between bats and walls is frequent. This period only briefly precedes the onset of hibernation, allowing spores to remain viable and later cause infection upon the onset of hibernation when bat body temperature is reduced (Whiting-Fawcett et al., 2021). The exchange of genotypes between hibernacula may be limited, as most bats are faithful to their swarming site and they also hibernate where they swarm. This pattern has already been documented in several temperate *Myotis* species (Glover & Altringham, 2008; Humphrey, 1971; Norquay et al., 2013; Parsons & Jones, 2003; Rivers et al., 2006; van Schaik, Janssen, et al., 2015).

4.2 | Fungal spores' viability as a key factor determining movements of pathogen

Fungal pathogens carry a higher than average danger of causing host extinctions which, among other factors, is due to their ability to produce very durable free-living stages (Fisher et al., 2012). For example, spores of the genus *Aspergillus*, a globally distributed fungal

pathogen with multiple hosts including humans, might be the most resilient studied so far; able to survive for decades when dehydrated (Kwon-Chung & Sugui, 2013), and tolerating temperatures above 85°C (Wyatt et al., 2015). However, although fungi are more durable than viral and (most) bacterial pathogens, their geographic distribution and range of hosts are still largely determined by a combination of biotic and abiotic filters (e.g., Crowther et al., 2014).

For *P. destructans*, temperature seems to be a strong abiotic filter as the fungus cannot grow on active bats (Verant et al., 2012) and spores can only survive at elevated temperatures for several weeks (Campbell et al., 2020). These findings, along with temporal patterns of fungal infection on bats (e.g., Langwig, Frick, et al., 2015; Puechmaille et al., 2011), suggest that temperature is a key driver in *P. destructans* seasonality and explains why the pathogen is mostly found on hibernating bats and is not exchanged by bats during the summer months, resulting in the genotypic differentiation observed in the present study. While the majority of observed *P. destructans* genotypes were restricted to single hibernacula, we did observe a small fraction of genotypes present at more than one hibernaculum. We suggest that the late hibernation/spring transition period is the most favourable time for successful transfer of spores between hibernacula, as the fungal load of *P. destructans* on bats is at its highest in spring when the bats are in their final stage of hibernation (Langwig, Frick, et al., 2015; Puechmaille et al., 2011). Indeed, bats very commonly touch the walls of hibernacula during arousal as they stretch their wings and groom themselves (e.g., Brownlee-Bouboulis & Reeder, 2013). At this time, bats typically start moving between sites—from hibernacula to summer roosts—possibly transiting via other hibernacula in between (Zukal et al., 2017). The time *P. destructans* would be exposed to elevated temperatures during such transfers between hibernacula is likely to be short enough for some spores to survive on the active bats.

It remains unclear whether only few spores manage to be transferred and survive between hibernacula or, whether many are transferred, with only a subset leading to successful infection. Furthermore, while transfer of spores is much less likely in autumn (due to lower fungal load on bats) the importance of timing of transfer has yet to be addressed (though see Langwig et al., 2021). It should be mentioned that *P. destructans* gene flow might not be due solely to the movement of bats but could also be facilitated by the mobility of other animals, as well as humans between hibernacula (Zhelyazkova et al., 2020). However, our data suggest that, if successful gene flow between hibernacula with established *P. destructans* populations is happening, it is a relatively rare event (compared to in-situ recruitment), which does not significantly modify the patterns of genotypic richness (at least in the species' native range at the time scale investigated herein).

Although beyond the scope of the current study, factors like local adaptation to environmental conditions as well as intra- and interspecific competition might play important roles that remain to be investigated (Lilley et al., 2018; Susi et al., 2015). Our population genetic data, suggesting low exchange rates of genotypes between hibernacula, might at first seem contradictory with the rapid colonisation of the fungus in North America during the last decade (U.S. Fish and Wildlife Service, 2021). However, the gene flow between hibernacula already occupied by the fungus and the colonisation of novel hibernacula are distinct processes (Kerth & Petit, 2005; Wade & McCauley, 1988). The colonisation of a hibernaculum previously not harbouring *P. destructans* requires that the pathogen has the ability to cope with local abiotic environmental conditions and local biota (possible inter-specific competition). In addition to these factors, genotypes dispersing between hibernacula with already existing *P. destructans* populations also need to contend with possible competition between genotypes (intra-specific competition; e.g., Fraser et al., 2014).

4.3 | Identification and characterisation of the environmental reservoir

Understanding the natural ecological niche of fungi remains a particularly challenging task, including for some of the most well-studied and economically important fungi, for example, *Penicillium roquefortii* (used in the production of blue cheeses worldwide; Dumas et al., 2020) and *Saccharomyces cerevisiae* (baker's yeast; Gallone et al., 2016). For fungal pathogens, key aspects of their ecological niche that must be identified for informing disease management is the ability of the pathogen to survive on alternative hosts or in environmental reservoirs (De Castro & Bolker, 2004). This requires sampling fungal material from ecologically relevant substrates, confirming fungal species identification and spore viability. Here we successfully performed all three tasks and were able to show that genotypic patterns observed in viable *P. destructans* isolates collected from bats and walls are consistent with the presence of an environmental reservoir inside bat hibernacula, which can account for the yearly re-infection of bats after summer. This conclusion is also corroborated by results obtained from disease dynamics modelling showing that environmental transmission is the primary infection mode in *P. destructans* (Meierhofer et al., 2021). Moreover, microbiome studies found similar fungal and bacterial assemblages on bat wings and hibernacula walls, reiterating the strong influence of site on the bat skin microbiome, which results in predominantly site-specific microbiota (Ange-Stark et al., 2019; Avena et al., 2016). These findings suggest frequent exchange of microbial material with a potential source-sink relationship of microorganisms between bats (the potential sink) and their environment (the potential source; directionality of source-sink probably depending on the microbial species, Grisnik et al., 2020). In the case of *P. destructans*, the situation is probably more complex as the environmental reservoir is expected to be replenished from the bats at the end of the hibernation season (Hoyt et al., 2020; Puechmaille et al., 2011).

In addition to walls, sediments as well as bat guano have been suggested to be possible sources of *P. destructans* environmental infection (Lorch et al., 2013; Urbina et al., 2020). However, biotic interactions between fungi and other microorganisms are most probably stronger in sediments/guano compared to hibernacula walls, making sediments/guano a challenging environment for *P. destructans* to

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survive in (Urbina et al., 2021; Wilson et al., 2017; Zhang et al., 2014). Furthermore, physical contact between bats and guano/sediments is a rare event in most bat species, suggesting these potential reservoirs are unlikely to play an important role in *P. destructans* infection. In contrast, contact of bats with the walls of hibernacula occurs often as bats land and crawl on walls during swarming and hibernation and contact is also often observed during grooming in arousal bouts from hibernation (Blažek et al., 2019; Puechmaille et al., 2011). From this, we conclude, that sediments and guano are unlikely to play a significant role as pathogen reservoirs from which bats could become infected.

4.4 | A novel approach for estimating the inoculum load

Knowing the quantity of spores that led to host infection is an important aspect in disease dynamics. For example, considering that disease severity and response might be dependent on inoculum load, using appropriate fungal loads would be essential to obtain accurate results in studies based on artificial infection of hosts (e.g., Carey et al., 2006). However, in general, limited data exist on the number of fungal spores involved in the initial infection of hosts in natural settings (Burnett, 2003). The number of particles (e.g., spores) required to cause successful infection (the "infection dose") will mainly depend on the ability of the host immune system to combat infection (e.g., Ayres & Schneider, 2012). Hence the infection dose is often highly variable among host-pathogen systems. Here, we used a novel approach quantifying the transmission bottleneck (based on genotypic richness) between the environmental substrate and bats, directly providing an empirical estimation of the spore load (the number of viable spores passed from the walls to one bat) of between roughly 50 and 500 spores. While this represents an approximation that could be estimated more accurately in subsequent studies, the order of magnitude of our findings (i.e., approximately several hundred spores per bat) is most likely close to the real figure for several reasons. Indeed, this finding is consistent with the observation that only a limited number of spores is transferred from walls to bats during infection in autumn, based on the difficulty in obtaining viable fungal material from the walls of hibernacula (N.M. Fischer & S.J. Puechmaille, unpublished data). It thus appears unlikely that bats come in contact with large quantities of spores at the end of the summer when they start to visit the hibernacula. Additionally, a study by Johnson et al. (2014) found that the inoculation of *Myotis lucifugus* bats with 500 spores of *P. destructans* reduced the bats survival odds significantly which was not observed for higher inoculum sizes (5000, 50,000 and 500,000 spores tested; potential explanations for this non-intuitive result are discussed by the authors). Our results suggest that future studies aiming to elucidate physiological or behavioural aspects of infection in hosts in an experimental setup might use an adjusted inoculum size (i.e., lower; 500,000 spores per wing is classically used, e.g., Lorch et al., 2011; Warnecke et al., 2012) or ideally several inoculum sizes to more closely match the apparent situation for wild bats naturally infected by *P. destructans*.

4.5 | Stability of pathogens' genotypes through time

The genetic composition of populations typically changes through time via the action of mutation, selection, gene flow, and drift. In our work, we mostly concentrate on the latter two as these evolutionary forces are often the most relevant at short timescales (some generations). Using an approach comparing the genotypic composition of live *P. destructans* isolates revealed a high similarity in genetic composition observed at Eldena across winters and substrates, suggesting that hibernacula can be seen as predominantly closed systems (i.e., no significant emigration, immigration or extinction) with a very stable environment. In simplified terms, we expect bats to become infected with the fungus from the walls of hibernacula in autumn. Over the winter, the fungus reproduces clonally on bats by producing large amounts of spores. These spores will then be shed into the environment (including the walls of the hibernacula) at the end of the hibernation period when fungal load is highest on bats (Langwig, Frick, et al., 2015; Puechmaille et al., 2011). This predicted shedding of fungal material from bats to the walls at the end of the hibernation period is corroborated by increases in prevalence and load of *P. destructans* DNA on wall surfaces near to where infected bats spent the winter (Hoyt et al., 2020). Fungal shedding on the walls is most likely happening when bats move within the hibernaculum, groom themselves or crawl on walls (Bartonicka et al., 2017; Brownlee-Bouboulis & Reeder, 2013; Puechmaille et al., 2011), leading to a higher prevalence of fungal DNA near bats (Hoyt et al., 2020). Along with this known pattern of *P. destructans* DNA on environmental surfaces, our data further confirm that bats are not simply a dead-end but rather an important host for the fungal pathogen to complete its life cycle (see also Palmer et al., 2018; Reynolds & Barton, 2014). Some spores will survive on the walls of hibernacula through the summer and will be able to infect bats again when they return in autumn. In such a system we expect the frequencies of genotype occurrences to remain fairly constant: all other things being equal, the chances for a genotype to infect a bat in year $n + 1$ will be proportional to its frequency of occurrence on the walls in year n. Examining the correlation of relative frequencies of occurrence of genotypes in the data set across years in Eldena revealed a strong (and highly significant) correlation between pairs of winters, even across multiple years. This finding further indicates that, while relatively rare genotypes might not have been sampled, our sampling probably captures a large proportion of the common and viable genotypes within the hibernaculum.

4.6 | Implications for conservation strategies

Knowing the source of an infectious pathogen is the first step in managing any disease, whether it be human pathogens or those of plants and animals. Only once the source and the mode of transmission are known can one successfully attempt to reduce

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or fully prevent the spread. However, after the emergence of a novel pathogen, this information is often lacking. Therefore, most management strategies to deal with emerging infectious diseases of wildlife have traditionally focussed on targeting the host (see e.g., Langwig, Voyles, et al., 2015). Among these are the controversial culling of hosts either to remove infected hosts or reduce population densities (see e.g., the culling of badgers in England to curb bovine Tuberculosis; Ham et al., 2019) as well as inhibition of pathogen growth on hosts (by applying natural or chemical antifungals to hosts, for example, Boire et al., 2016). Regarding *P. destructans*, our results provide clear evidence that the walls of the bats' hibernacula act as an environmental reservoir containing viable spores from which bats become infected. This suggests that similarly to a case study with *Batrachochytrium dendrobatidis* affecting amphibians (Bosch et al., 2015), it is probably more feasible and effective to target the pathogen (instead of the host) to reduce fungal load in the environment and hence prevent yearly re-infection of the bat hosts.

If we consider White-Nose disease dynamics, reducing the number of spores on the walls of hibernacula could lead to fewer bats becoming infected in the following hibernation seasons. With fewer infected bats in winter, the fungus would reproduce less on bats, and less spores would be shed onto the walls in spring. This could then lead to fewer infections of bats in the following year. Therefore, significantly reducing the number of spores in the environment would be a straightforward way to break the cycle of reinfections, which could include long-term effects which would need to be repeated fewer times compared to methods targeting the host. The concept of reducing *P. destructans* load from hibernacula environments is supported by the work from Hoyt et al. (2020) who predicted that a reduction of fungal load to 20% of its current load over the summer could result in the stabilization of bat populations in North America.

4.7 | Population genetics as a tool for understanding infectious disease dynamics

With an increasing human population as well as travel and the trade of goods across the world, the occurrence of novel pathogens and introductions of pathogens beyond their natural range are expected to increase. Therefore, understanding the processes involved in pathogen emergence and infection of hosts is critical to reduce the threat and manage outbreaks. Population genetics are an important part of the toolbox needed to reach this goal.

So far, population genetics have already been used to answer a broad range of questions related to pathogen biology (including parasitology). Examples of this include the elucidation of clonality in pathogens (e.g., Hill et al., 1995; Morehouse et al., 2003) and understanding the spatial structure and diversity of a pathogen in a geographical range (e.g., Anderson et al., 2000; Mekonnen et al., 2020) which can further be used to identify the source population of a pathogen's introduction (e.g., Jarman et al., 2019). These types of studies investigate patterns over long (evolutionary) time frames, which are an important part of the framework for understanding wildlife diseases (e.g., Vander Wal et al., 2014). Population genetics may also be used to understand disease in the context of short time frames such as the description of pathogen life cycles (including the study of alternative hosts or reservoirs). While such studies on pathogen reservoirs have been frequently conducted with regard to human or livestock pathogens (e.g., Dubey et al., 2020; Venkatesan & Rasgon, 2010) they have scarcely been conducted for wildlife pathogens. However, the knowledge on pathogen life cycles and reservoirs is indispensable if we are to understand wildlife diseases, develop possible management strategies or indeed avoid the further spread of pathogens (e.g., from caving equipment in the case of *P. destructans*; Zhelyazkova et al., 2020, 2019). Our study provides evidence that population genetic approaches provide an invaluable and resource-efficient tool to elucidate cryptic infection pathways, including in the field of emerging infectious diseases of wildlife where resources are often limited.

The current study on the transmission pathways of *P. destructans*, the causative agent of White-Nose Disease, provides clear evidence for environmental infection of bats. These results highlight that population genetic approaches can provide fundamental knowledge on pathogen transmission dynamics; knowledge that is critically needed to adjust management strategies, and that more studies are needed to focus on developing management strategies targeting the environmental reservoir of pathogens.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

AUTHORS CONTRIBUTIONS

Sebastien J. Puechmaille conceived the study. Nicola M. Fischer and Sebastien J. Puechmaille designed the study; Sebastien J. Puechmaille acquired funding and supervised the project; Sebastien J. Puechmaille and G.K. administered the project. Nicola M. Fischer (40%), Andrea Altewischer (25%), Surendra Ranpal (25%), Serena Dool (5%) and Sebastien J. Puechmaille (5%) carried out the laboratory analyses; Nicola M. Fischer performed data analyses. Nicola M. Fischer and Sebastien J. Puechmaille interpreted the results and wrote the original manuscript. All authors critically discussed the results and edited the manuscript, approved its final version, and agree to be held accountable for the content therein.

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DATA AVAILABILITY STATEMENT

The data set supporting this article and the annotated R script to replicate all the analyses are available from Dryad (https://doi.org/10.5061/dryad.x0k6djhhx).

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