Emerging fungal diseases: Understanding their emergence to combat the threat

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Nicola Mira Fischer

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Dekan:	Prof. Dr. Gerald Kerth
1. Gutachter:	Prof. Dr. Gerald Kerth
2. Gutachter:	Prof. Dr. Stukenbrock
3. Gutachter:	Dr. Ropars
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“The fact remains that man has unprecedented control over the world and everything in it. And so, whether he likes it or not, what happens next is very largely up to him”

David Attenborough (Life on Earth, 1979)

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Abstract (English)

Emerging infectious diseases are among the greatest threats to human, animal and plant health as well as to global biodiversity. They often arise following the human-mediated transport of a pathogen beyond its natural geographic range, where host species are typically not well adapted due to a lack of co-evolutionary host-pathogen dynamics. One such pathogen is the fungus *Pseudogymnoascus destructans* (*Pd*), which causes White-Nose disease in hibernating bats. While *Pd* was first observed in North America where it has led to mass-mortalities in some bat species, the pathogen originates from Eurasia where infection is not associated with mortality. Most of the *Pd* research has focused on the invasive North American range, which likely underestimated the genetic structure of the pathogen and the role it might play in the disease dynamics.

In my work, I therefore evaluated the genetic structure of *Pd* in its native range with the aim of uncovering cryptic diversity and further use population genetic data to address some key ecological aspects of the disease dynamics. With an extensive reference collection of more than 5,000 isolates from 27 countries I first demonstrated strong differentiation between two monophyletic clades across several genetic measures (multi-locus genotypes, full genome long-read sequencing and Illumina NovaSeq on isolate pools). These findings are consistent with the presence of two cryptic species which are both causative agents of bat White-Nose disease ('*Pd*-1', which corresponds to *P. destructans sensu stricto*, and '*Pd*-2'). Both species exist in the same geographic range and co-occur in the same hibernacula (i.e., in sympatry), though with specialised host preferences. I further described the fine-scale population structure in Eurasia which revealed that most genotypes are unique to single hibernacula (more than 95% of genotypes). The associated differences in microsatellite allele frequencies among hibernacula allowed the use of assignment methods to assign the North American isolates (exclusively *Pd*-1) to regions in Eurasia. Hence, a region in Ukraine (Podilia) is the most likely origin of the North American introduction.

To gain further insights into the spatial and temporal dynamics of White-Nose disease on a localised scale, several hibernacula were sampled with high intensity (artificial hibernaculum in Germany and natural karst caves in Bulgaria). Low rates of *Pd* gene flow were observed even among closely situated hibernacula. This indicates that *Pd* does not remain viable on bats over summer or it would be frequently exchanged among bats (and hence hibernacula) resulting in a homogenous distribution of genotypes. Instead, bats need to become re-infected each

hibernation season to explain the yearly re-occurrence of White-Nose disease. Given the distribution and richness of *Pd* genotypes on hibernacula walls and infected bats of the same hibernacula, bats become infected from the hibernacula walls when they return after summer. This means that environmental reservoirs exist within hibernacula (i.e., the walls) on which *Pd* spores persist during bat absence and which drive the yearly re-occurrence of White-Nose disease. In an experimental setup, I confirmed the long-term viability of *Pd* spores on abiotic substrate for at least two years and furthermore discovered temporal variations in *Pd* spores' ability to germinate. In fact, these variations followed a seasonal pattern consistent with the timing of bats absence (reduced germination) and presence (increased germination) and could indicate adaptations of *Pd* to the bats' life-cycle. The infection of bats from environmental reservoirs hence seems to be a central aspect of White-Nose disease dynamics and *Pd* biology.

*Pd*s ability to remain viable for extended periods outside the host increases its risk of being anthropogenically transported and might have played a role in the emergence of White-Nose disease in North America. The existence of a second species (*Pd*-2) poses a great additional danger to North American bats considering that its introduction there could lead to deaths and associated population declines in so-far unaffected species given what is known about differing host species preferences in Eurasian bats. Even within the native range of *Pd*, the movement of *Pd* between differentiated fungal populations could facilitate genetic exchanges (e.g., through sexual reproduction) between genetically distant genotypes. Such genetic exchanges could lead to phenotypic jumps in pathogenicity or host-species preferences and should hence be prevented.

The native range of a pathogen holds great potential to better understand the genetic and ecological basis of a (wildlife) disease. My work informs about the dangers associated with the accidental transport of *Pd* (and other pathogens) and highlights the need for 'prezootic' biosecurity-oriented strategies to prevent disease outbreaks globally. Once a pathogen has arrived in a new geographic range, and particularly if it has environmentally durable spores (as demonstrated for *Pd*), it will be difficult/impossible to eradicate. Furthermore, a pathogen's ability to remain viable outside the host and infect them from environmental reservoirs has been associated with an increased risk of species extinctions and needs to be considered when designing management strategies to mitigate disease impact.

Abstract (German)

Neu auftretende Infektionskrankheiten gehören zu den größten Bedrohungen für die Gesundheit von Menschen, Tieren und Pflanzen sowie für die weltweite Artenvielfalt. Oft liegt ihnen der anthropogene Transport eines Krankheitserregers in neue geografische Verbreitungsgebiete zugrunde, wo aufgrund fehlender historischer Wirt-Pathogen Koevolution eine schlechte Anpassung des Wirtes vorliegt. Einer dieser Erreger ist der Pilz *Pseudogymnoascus destructans* (*Pd*), welcher bei überwinternden Fledermäusen die Weißnasenkrankheit (engl. White-Nose disease) verursacht. *Pd* wurde erstmals in Nordamerika entdeckt, wo er durch das mit ihm assoziierte Massensterben einiger nordamerikanischer Fledermausarten auffällig wurde. Erst später stellte man fest, dass *Pd* aus Eurasien stammt, wo der Pilz zwar weit verbreitet ist aber nicht mit einem vergleichbaren Fledermaussterben in Verbindung steht. Der Großteil der bisherigen Forschung zur Weißnasenkrankheit konzentrierte sich auf das invasive nordamerikanische Verbreitungsgebiet, wo die genetische Diversität des Erregers, und deren Rolle in der Krankheitsdynamik, vermutlich unterschätzt wurde.

In meiner Arbeit untersuchte ich daher zunächst die genetische Struktur von *Pd* in seinem natürlichen Verbreitungsgebiet. Mithilfe einer umfangreichen Referenzsammlung von mehr als 5.000 *Pd*-Isolaten aus 27 Ländern konnte ich anhand verschiedener genetischer Maße (Multilokus-Genotypen, Long-Read-Sequenzierung des gesamten Genoms und Illumina NovaSeq an Isolatpools) eine starke Differenzierung zwischen zwei monophyletischen Kladen nachweisen. Diese Ergebnisse weisen auf das Vorhandensein zweier kryptischer Arten hin, beide von ihnen Erreger der Weißnasenkrankheit („*Pd*-1“, welcher *P. destructans sensu stricto* entspricht, und „*Pd*-2“). Beide Arten kommen im gleichen geografischen Gebiet und sogar in denselben Winterquartieren (d. h. in Sympatrie) vor – allerdings mit Art-spezifischen Wirtspräferenzen. Eine feinmaschigere Untersuchung der eurasischen Populationsstruktur zeigte eine hohe Diversität an Genotypen (innerhalb jeder Art) und größtenteils genotypische Individualität jedes Winterquartiers, wobei der Großteil der Genotypen nur jeweils in einem Quartier zu finden war (mehr als 95 % der Genotypen). Die damit verbundenen lokalen Variationen in den Allelfrequenzen der Mikrosatellitenmarkern konnten angewandt werden, um die nordamerikanischen Isolate (die ausschließlich zu *Pd*-1 gehören) einer Region in der Ukraine (Podilia) zuzuordnen, welche somit der wahrscheinlichste Ursprung der nordamerikanischen Einschleppung ist.

Um nähere Einblicke in die räumliche und zeitliche Dynamik der Weißnasenkrankheit auf lokaler Ebene zu gewinnen, wurden mehrere Winterquartiere mit hoher Intensität beprobt (künstliches Quartier in Deutschland und natürliche Karsthöhlen in Bulgarien). Selbst zwischen nahe-gelegenen Quartieren wurde nur sehr geringer Genfluss (Austausch von Genotypen) beobachtet. Dies deutet darauf hin, dass Fledermäuse außerhalb der Überwinterungszeit kein lebensfähiges *Pd* tragen, ansonsten würde es zwischen Fledermäusen und Winterquartieren ausgetauscht werden und in einer homogenen Verteilung von Genotypen resultieren. Um das jährliche Auftreten der Weißnasenkrankheit zu erklären, müssen sich Fledermäuse also nach dem Sommer neu infizieren. Die Verteilung und Anzahl von *Pd* Genotypen auf Fledermäusen und den Wänden von Winterquartieren deuten darauf hin, dass sich Fledermäuse aus der Umwelt, und zwar von den Wänden, anstecken. Das bedeutet, dass *Pd* den Sommer in einem Umweltreservoir in den Winterquartieren überlebt, welches die jährlich wiederkehrenden Neuinfektionen von überwinternden Fledermäusen verursacht. Die Langlebigkeit der *Pd*-Sporen wurde zudem mithilfe eines experimentellen Versuchsaufbaus untersucht, welcher zeigte, dass die Sporen von *Pd* mindestens 2 Jahre lang auf abiotischem Substrat überleben. Darüber hinaus zeigten sich im Laufe des Experiments Schwankungen in der Keimungsfähigkeit von Sporen, welche mit der zeitlichen Dauer von Fledermaus-Abwesenheit (verringertes Wachstum) und -Anwesenheit (erhöhtes Wachstum) verknüpft zu sein schienen. Dies könnte eine Anpassung von *Pd* an den Lebenszyklus von Fledermäusen darstellen und deutet darauf hin, dass die Infektion von Fledermäusen aus dem Reservoir (von den Wänden) einen zentralen Aspekt der Krankheitsdynamik und der Biologie von *Pd* darstellt.

Die Fähigkeit von *Pd*, über längere Zeiträume außerhalb seines Wirts lebensfähig zu bleiben, erhöht das Risiko einer anthropogenen Verschleppung und könnte beim Auftreten der Weißnasenkrankheit in Nordamerika eine Rolle gespielt haben. Das Vorhandensein einer zweiten Art (*Pd*-2) stellt eine große zusätzliche Gefahr für nordamerikanische Fledermäuse dar, da ihre Einschleppung zu Todesfällen und damit verbundenen Populationsrückgängen bei bisher nicht betroffenen Arten führen könnte (Im Hinblick auf die Wirtspräferenzen von *Pd*-1 und *Pd*-2 in Eurasien). Selbst anthropogene Bewegungen von *Pd* (intraspezifisch in *Pd*-1 oder *Pd*-2) zwischen differenzierten Populationen innerhalb des natürlichen Verbreitungsgebietes sind zu vermeiden, da sie zu genetischem Austausch (z. B. durch sexuelle Reproduktion) mit resultierenden

phänotypischen Veränderungen im Hinblick auf Pathogenität oder infizierten Wirtsarten führen könnten.

Das natürliche Verbreitungsgebiet eines Krankheitserregers birgt großes Potenzial, um ein besseres Verständnis der genetischen und ökologischen Basis einer (Wildtier-) Krankheit zu erhalten. Meine Arbeit informiert über die Gefahren, die mit dem unbeabsichtigten Transport von *Pd* (und anderen Pathogenen) einhergehen und verdeutlicht, dass "präzootische", auf Biosicherheit ausgerichtete Strategien erforderlich sind, um Krankheitsausbrüche weltweit zu verhindern. Wenn ein Erreger erst einmal in einem neuen geografischen Gebiet angekommen ist, und vor allem, wenn er über umweltbeständige Sporen verfügt (wie für *Pd* gezeigt), wird es schwierig bis unmöglich sein, ihn auszurotten. Die Fähigkeit eines Erregers, Wirte aus Umweltreservoiren zu infizieren wird zudem mit einem erhöhten Risiko des Artensterbens in Verbindung gebracht und muss bei der Entwicklung von Managementstrategien von Krankheiten berücksichtigt werden.

1. Introduction

1.1 Wildlife pathogens in the Anthropocene

As a species, humans have altered natural ecosystems and climatic conditions to such an extent that a new term has been proposed to describe the current epoch – the Anthropocene (Steffen et al., 2011; Waters et al., 2016). Humans and their livestock now account for 96% of global mammal biomass and we are currently experiencing Earth's 6th mass extinction (Barnosky et al., 2011; Pörtner et al., 2021). These severe population declines, extirpations and extinctions imperil human civilisation through the break-down of ecosystem services – a threat which has been termed "biological annihilation" (Ceballos et al., 2017). Maintaining human health and securing food production therefore depend on preserving the functionality of Earth's fragile ecosystems and preserving biodiversity ("One Health", see e.g., Cunningham et al., 2017; Jenkins et al., 2015). In this context, the increasing incidence of newly emerging infectious diseases (EIDs) affecting wildlife is a worrying development that puts pressure on populations that are already stressed through habitat destruction and climate change (Daszak et al., 2000; Jones et al., 2008; see also Box 1). In most cases, the occurrence of an EID is linked to a pathogen's appearance in a novel geographic range (see also Box 2), though increases in pathogen incidence or virulence within the existing geographic range may also occur (Daszak et al., 2000; Morse & Schluederberg, 1990).

Fungal pathogens (including fungal-like organisms such as oomycetes), in particular, have been responsible for some of the most severe die-offs – both in wild populations and in agricultural settings (Fisher et al., 2012). Examples include *Phytophthora infestans* , the pathogen responsible for the Irish potato famine, which is estimated to have led to the death of approximately one million people (12% of the Irish population between 1845 and 1852; Mokyr & Ó Gráda, 1999; Ó Gráda, 2007), as well as *Batrachochytrium dendrobatidis* and the closely related *B. salamandrivorans* which are responsible for the declines of at least 500 amphibian species in recent decades (Berger et al., 1998; Martel et al., 2013; Scheele et al., 2019; Stuart et al., 2004). Fungi form a highly diverse group of eukaryotic organisms and play key roles in all ecosystems, from single-celled yeasts, to colony-forming moulds and fruiting mushrooms (Stajich et al., 2009). Only a fraction of the estimated 11.7 – 13.2 million fungal species have been described (Wu et al., 2019), and the ability to cause disease is often dependent on host condition (i.e., opportunistic)

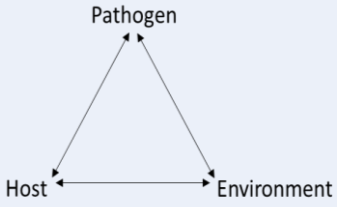
and can be found among a variety of genera (Rokas, 2022; Shang et al., 2016). This makes it very difficult to predict which fungal species might become a risk to humans or wildlife.

Box 1: What is wildlife disease?

In most cases, the cause of disease is multifactorial, for example a combination of climate and pathogen (Omazic et al., 2019), or low host genetic diversity that increases susceptibility to a pathogen (Lively, 2010). Parasitism (both micro- and macro-) plays an integral role in ecosystems, and therefore cannot be the only consideration when determining whether an individual or population is diseased.

Wobeser (1997) describes disease as "*any impairment that interferes with or modifies the performance of normal functions, including responses to environmental factors such as nutrition, toxicants, and climate; infectious agents; inherent or congenital defects; or combinations of these factors*." This definition is widely accepted (see e.g., Rogers, 2010) and appropriately captures the term 'disease' in the context of impaired function – which may result from a multitude of causes and interactions – rather than simply in terms of deaths. It can also be transferred from individuals to populations and even ecosystems.

In the context of pathogens as disease agents, it therefore seems clear that disease and its associated symptoms are the outcome of complex interactions between the pathogen (agent), the host, and the environment (e.g., Wobeser, 2007). This network of interactions is also known as the epidemiological triad or the disease triangle (see adjacent diagram).



This lack of knowledge on the diversity and identity of potential pathogens leads to their (mostly) unencumbered global transport, which increases their likelihood of emerging as problematic in a novel environment. Indeed, in most cases the causative agent of disease is described for the first time only after an outbreak has occurred. Greater efforts are currently being made to create a vigilant global community which could sound the alarm upon the first signs of disease emergence (see e.g., Gao, 2019 which lists and discusses several programmes for coordinated disease monitoring). While these steps are critical in ensuring rapid awareness of pathogen emergences there is also a need for more information on the diversity of host-pathogen interactions and specific dangers associated with known pathogens. This information can help to be better prepared for future emerging diseases and can be used to develop rapid and effective management strategies to successfully contain disease outbreaks (in terms of geographical spread and/or pathogen abundance; see also Wobeser, 2002).

Box 2: Host-pathogen coevolution

Coevolution refers to reciprocal evolutionary changes in response to selective pressures which two species (or populations) exert upon one another (Janzen, 1980). Host-pathogen interactions can result in arms-race dynamics, whereby hosts experience continued selective pressures to evolve resistance to pathogens, while pathogens strive to evade host defences and accomplish successful infection, also referred to as "Red Queen Hypothesis" (Brockhurst et al., 2014; Haldane, 1932; Salathé et al., 2009; Van Valen, 1973). In these host-pathogen interactions, changes in gene frequencies are common, yet the relative fitness of host and pathogen mostly remain constant.

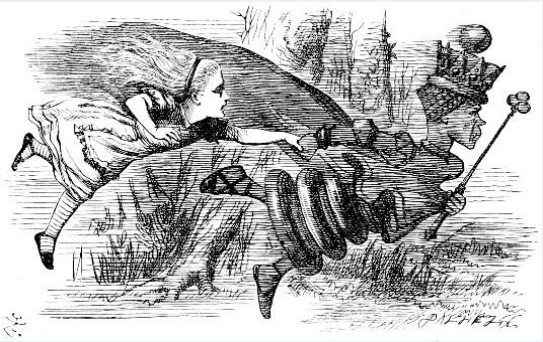


Illustration by Sir John Tenniel from the original edition

The term "Red Queen Hypothesis" was inspired by Lewis Carroll's novel "Through the Looking Glass" (1872), where the Red Queen tells Alice: *"Now here, you see, it takes all the running you can do, to keep in the same place."*

Host-pathogen coevolution has been demonstrated in wild populations (e.g., Australian rabbit populations and their interaction with myxoma virus; Kerr et al., 2017) and in laboratory settings (e.g., foot-and-mouth disease virus coevolving with cell cultures; de la Torre et al., 1988). In biological invasions of generalist pathogens (e.g., through anthropogenic introduction to novel geographic ranges), lack of mutualistic coevolution is a main factor often leading to rapid circulation and severe population declines in naïve host populations. Evidence of this is found in the great number of pathogens described only after their introduction to a novel geographic range and which are later found to be present but less pathogenic in the original host species (e.g., Dunn & Hatcher, 2015). For example, the fungal pathogen *Hymenoscyphus fraxineus,* which is non-pathogenic in its native range (East Asia) but has led to widespread ash dieback (*Fraxinus excelsior & Fraxinus angustifolia*) in Europe (Zhao et al., 2012). In another example, the co-introduction of parapoxvirus to the UK with the invasive grey squirrel (*Sciurus carolinensis*) – for which it is non-pathogenic – causes high mortality in native red squirrels *(S. vulgaris)* and is accelerating their displacement (Tompkins et al., 2003).

1.2 White-Nose disease – a threat to North American bats

Wildlife biologists first observed diseased and dead bats in a cave near Albany, New York, in 2007 and, in response to the white fuzzy growth found mainly on the muzzles, ears and wings of

hibernating bats, called the disease White-Nose syndrome (Blehert et al., 2009; Veilleux, 2008). The term White-Nose syndrome, as opposed to White-Nose disease, is often used synonymously but should be reserved for severe infections where hosts exhibit a range of symptoms consistent with a syndrome (Frick et al., 2016). For simplification, I will use the term "White-Nose disease" in the following, as it refers to all cases in which bats are infected independent of the severity of each individual's symptoms.

Following this first observation, the disease was seen in hibernating bats in an ever-greater geographic range each winter, now covering large parts of the United States and southern Canada (U.S. Fish and Wildlife Service, 2022, Figure 1). The causative agent of the disease was identified as the previously unknown fungal pathogen *Pseudogymnoascus destructans* (*Pd*, Minnis & Lindner, 2013; formerly classified within the genus *Geomyces*, Gargas et al., 2009). Infection with *Pd* leads to the formation of characteristic cup-like epidermal erosions and ulcers, particularly in wing membranes, and growth of fungal hyphae into the hair follicles, as well as into the sebaceous and apocrine glands of hibernating bats (Cryan et al., 2010; Meteyer et al., 2009). While the exact mechanism of death is yet unclear, it seems to be linked to physiological and behavioural changes (e.g., more frequent arousals from hibernation) which increase the energy expenditure of bats and lead to premature depletion of fat reserves towards the end of hibernation (Verant et al., 2014). The death toll reaches up to 90% in some North American populations, and was estimated to amount to at least five million bats within the first five years of disease occurrence (i.e., until 2012; the death toll has risen continuously since, though there are no accurate mortality estimates; U.S. Fish and Wildlife Service, 2022), marking the greatest die-off in bats and possibly all mammals ever documented (Cryan et al., 2010). The pathogen has so far been observed growing on 12 North American bat species, with some species being more susceptible to disease and mortality than others (e.g., Frick et al., 2017). The populations of three once-common species have declined by more than 90% and these species are being listed as threatened under the U.S. Endangered Species Act resulting from White-Nose disease (*Myotis lucifugus*, *M. septentrionalis & Perimyotis sub-flavus; Cheng et al., 2021*). This is not only tragic in the context of local biodiversity, but also directly impacts ecosystem services provided by insectivorous bats. In fact, the extinction, or even drastic population declines, of affected North American insectivorous bats could lead to agricultural deficits of more than 3.7 billion US dollars annually due to the lack of their natural pest control (Boyles et al., 2011).

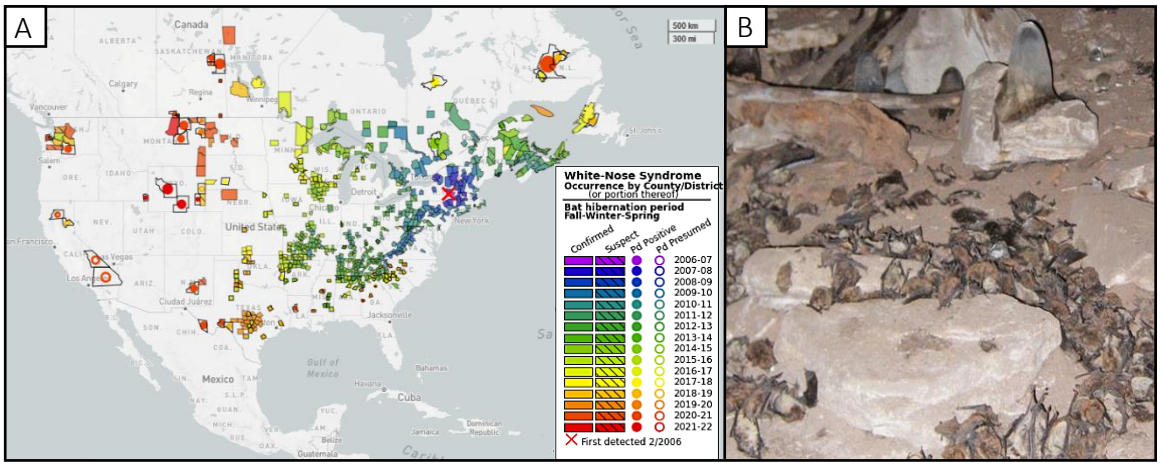


Figure 1: A. Map showing the spatial expansion of White-Nose disease over time in its invasive range in North America (colours indicate when Pd was detected in the respective county or district for the first time). The red cross shows the location of the first observation in winter 2006/07 in upstate New York. This figure was downloaded from https://www.whitenosesyndrome.org/resources/map on 02/06/2022. **B.** Dead *Myotis lucifugus* bats on the floor of Aelus Cave (Vermont). The population experienced a major crash in winter 2008/2009 due to White-Nose disease. Image courtesy of University of Tennessee.

After discovery of the pathogen in North America, it was also isolated from European and, shortly after, Asian bats (Hoyt et al., 2016b; Puechmaille et al., 2010; Puechmaille et al., 2011; Zukal et al., 2016). While infection is associated with similar pathological lesions to those found in infected North American bats, no associated mortality or unusual behaviour accompanies the disease in Eurasia (Fritze & Puechmaille, 2018; Puechmaille et al., 2011; Wibbelt et al., 2013; Zukal et al., 2016). When *Pd* isolates collected in Europe were used to inoculate North American bats (*Myotis lucifugus*) in a laboratory setting they showed the same exacerbated symptoms, including mortality (Warnecke et al., 2012). These findings, corroborated by genetic analyses placing the genetic origin of the North American haplotype of *Pd* within Europe, clearly show that *Pd* is an invasive pathogen introduced to North America from Europe (Leopardi et al., 2015). Bats do not migrate between North America and Eurasia, so the emergence of the pathogen in North America is most likely due to anthropogenic activities. Since its emergence in North America we have learned that *Pd* is broadly distributed in Europe, being particularly common in central and eastern Europe, and likely rarer in Scandinavia and southern Europe (though this remains to be confirmed and may be due to sampling bias or more conspicuous growth of *Pd* in central Europe) and that it is most commonly found on *Myotis myotis* bats (Puechmaille et al., 2011). However, little has been known about the connectivity of *Pd* in its native range, nor how much genetic diversity exists, though it is clearly greater there than in the introduced range of the pathogen (see Drees et al.,

2017a). It had been long believed that microorganisms (including pathogens) have broad geographic ranges without any strong population structure or barriers to dispersal because they are easily transported by other mobile species as well as via wind or water (e.g., Finlay, 2002). However, it is becoming increasingly clear that this assumption was likely driven by the inability to differentiate many species of microorganisms based on their very similar (or even identical) morphological features (i.e., crypticity, Martiny et al., 2006). So the presence of multiple disease agents (as opposed to a single one) is sometimes only discovered years after initial descriptions of a disease (which may be particularly true for fungal pathogens; Taylor et al., 2006). Most cryptic species are discovered using phylogenetic tools instead of (or in addition to) morphological ones. The many examples include the model organism *Neurospora* – which had been well studied for decades before phylogenetic relationships were resolved (Dettman et al., 2003); as well as fungal pathogens of humans – for example *Histoplasma (H. capsulatum* and at least 3 more species*)* which causes Histoplasmosis, the most common respiratory infection of humans worldwide (Sepúlveda et al., 2017); fungal pathogens of plants – for example, *Botrytis* cinereal, whose cryptic relative *B. pseudocinerea* was discovered after causing gray mold in French vineyards; and fungal pathogens of animals, for instance *Ophiocordyceps unilateralis* – the 'zombie ant fungus' which parasitizes ants and changes their behaviour, and which has several closely related host-specific species (Kobmoo et al., 2019).

Up to now it has been assumed that White-Nose disease is caused by a single fungal pathogen (*Pd*) with a broad geographic range across Eurasia, though there has so far been no investigation into the genetic variation of *Pd* at a larger European scale. Speciation largely depends on adaptations to different niches that enable a species to thrive and be maintained within a new niche long term (e.g. Barraclough et al., 2003,). So, when cryptic species of pathogens are discovered, it can be expected that they differ in their adaptive niches (e.g., Stergiopoulos & Gordon, 2014). The discovery of all causative agents of a disease is therefore critical in determining disease progression (including expected outcomes for susceptible hosts) and employing effective management strategies.

1.3 General characteristics of *Pseudogymnoascus destructans*

Pd is haploid and has a heterothallic (outcrossing) mating system with two mating types (MAT1-1, MAT1-2), which are encoded in a single genetic locus (Palmer et al., 2014). While only one mating type is present in North America, both mating types are present in the Eurasian range and can even be found within the same sites, highlighting the potential for sexual reproduction (Palmer et al., 2014). So far, only clonal reproduction – via the production of specialised spores (conidia) – has been observed, and these spores also act as infection propagules (Gargas et al., 2009, Figure 2). However, given the biology of *Pd* and what we know about the commonness of sexual reproduction in pathogenic fungi (Box 3), it seems prudent to expect the occurrence of sexual reproduction in *Pd* (at least until evidence of its true asexuality can be provided).

Pd is slow-growing and psychrophilic ('cold-loving'), which means it exhibits optimal growth in high humidity and at temperatures between 12.5 and 15.8°C, while growth ceases above 21°C (Marroquin et al., 2017; Verant et al., 2012). Like other members of the genus *Pseudogymnoascus*, *Pd* can grow saprophytically (i.e., is able to obtain its resources from dead or decaying organic material) and has been demonstrated to grow on wood, bat guano and in sediments, though with reduced saprophytic ability compared to con-generic species (Reynolds & Barton, 2014; Reynolds et al., 2015; Urbina et al., 2021). Furthermore, *Pd* possesses an enzymatic catalogue which is assumed to aid in the infection of, and growth on, mammal hosts (e.g., the digestion of collagen; O'Donoghue et al., 2015). It has been suggested that *Pd* evolved from cave-dwelling saprophytic fungi and, through coevolution, has since specialized in the infection of hibernating bats (Veselska et al., 2020).

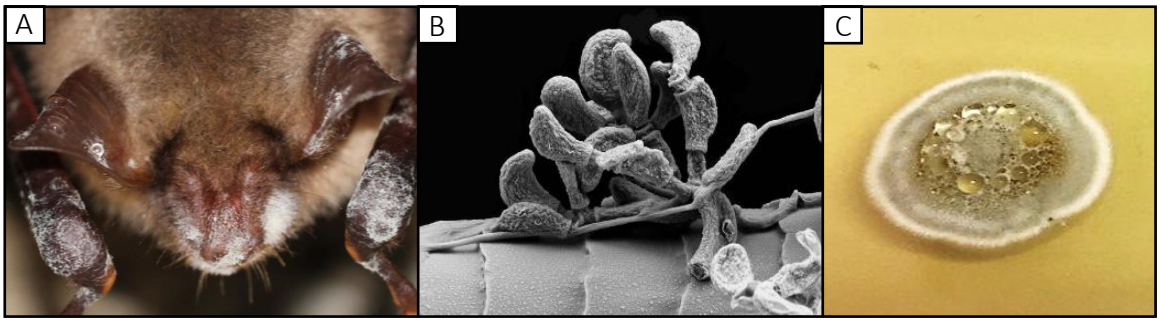


Figure 2: **A**. Image of *Myotis myotis* with White-Nose disease on nose, ears and forearms (picture by S.J. Puechmaille). **B**. Scanning electron microscopy image of *Pseudogymnoascus destructans* hyphae and curved asexual spores on the hair of a bat (from Wibbelt, 2018). **C**. Radial growth of *Pseudogymnoascus destructans* culture on artificial growth medium (diameter roughly 1 cm).

Box 3: Reproductive systems in fungi

Fungi exhibit a range of reproductive strategies, of which asexual (the formation of mitotic spore stages) and sexual reproduction (the formation of meiotic spore stages following recombination) are most common (though some rarer forms exist, see e.g., Nieuwenhuis & James, 2016). In filamentous ascomycetes (to which *Pd* belongs), in particular, a mixture of asexual (clonal) reproduction interspersed with events of sexual reproduction can regularly be observed, also known as facultative sexual reproduction. Prominent examples are found in the human fungal pathogens *Aspergillus fumigatus* and *Cryptococcus neoformans* (Dyer & Paoletti, 2005; Lin et al., 2005). Sexual stages often occur under specific environmental conditions or stressors, and have been linked to fitness, whereby individuals experiencing lower fitness in their current environment allocate disproportionately more resources towards sexual reproduction (Schoustra et al., 2010). Clonal reproduction involves low costs in terms of resources, is rapid, and preserves current well-adapted genotypes, while recombination, on the other hand, allows pathogens to purge deleterious alleles and build up the standing genetic variation needed to evolve and respond to unfavourable conditions (such as the host immune response in evolutionary arms-race dynamics; e.g., Paterson et al., 2010,). Hence, fungal pathogens with mixed reproductive systems involving both asexual reproduction and recombination pose a greater threat to hosts and have higher epidemic potential (McDonald & Linde, 2002). However, it should be mentioned here, that fungi with all types of reproductive morphologies are capable of evolutionary change and speciation (Taylor et al., 1999).

Until very recently, fungal species for which no sexual cycle had been observed were assumed to be asexual. But with the advancement of genetic tools and targeted research into potential recombination, many species previously believed to be strictly clonal were found to have sexual cycles (e.g., coffee rust fungus *Hemileia vastatrix*, Carvalho et al., 2011; wheat stripe rust fungus *Puccinia striiformis*, Jin et al., 2010). In fact, several authors have suggested that in pathogenic fungi, reproductive strategies involving recombination might be the rule – with examples of strict clonal reproduction representing an exception or being absent altogether (Billiard et al., 2012; Drenth et al., 2019; Heitman et al., 2013). In this case, instead of aiming to prove recombination in newly emerged fungal pathogens, recombination should be assumed until evidence of strict clonality can be provided.

1.4 The seasonality of White-Nose disease and its implications

Insectivorous bats found in temperate regions benefit from a reduction of energetic and survival costs during times of low insect availability (mostly during winter; Turbill et al., 2011). This is achieved through hibernation, during which the metabolic rate and body temperature are lowered for extended periods of time interspersed by short euthermic arousals (short bouts of

high body temperatures and metabolic rates; Geiser, 2013). The body temperature of bats during hibernation is usually within 1 to 2°C of the ambient air temperature (McNab, 1974) which is typically between 2 and 15°C and also depends on the bat species (Webb et al., 1996). The selected hibernacula (sing. hibernaculum; roosts used during the winter hibernation season) are characterised by relatively constant cool temperatures and high humidity, and provide protection from external weather conditions and predators – usually underground sites such as caves, cellars and bunkers (Kunz & Fenton, 2005). Bats also use hibernacula during the swarming (mating) period prior to hibernation (van Schaik et al., 2015). The lifecycle of temperate bats can therefore be separated into two main phases, which I will refer to as 'winter' (roughly October through April in the northern hemisphere) in which hibernation takes place and 'summer' during which bats are active, with transition periods (e.g., swarming) in between.

While hibernation protects temperate bats from starvation during low insect availability in winter, it also makes them susceptible to the infection by *Pd*, which can only grow on bats when their immune functions and body temperature are reduced (Bouma et al., 2010; Verant et al., 2012. The normothermic body temperature and high temperatures found in maternity roosts throughout summer (temperatures greater than 40°C have been documented; e.g., Webber & Willis, 2018) suggest that bats purge viable *Pd* during this period. Indeed, while spores of *Pd* are more durable than hyphal growth, experimental data have shown that even spores can only survive for up to 15 days at 37°C (roughly the body temperature of active bats) and up to 60 days at 30°C (the temperature expected further from the body, e.g., on fur) making it unlikely that spores remain viable throughout summer (Campbell et al., 2020). Though *Pd* might still be detectable via qPCR-based methods (i.e., quantification of *Pd* DNA without information about viability; see e.g., Huebschman et al., 2019). Interestingly, once diseased bats have been observed in a particular hibernaculum, the bats returning to this site each winter will become infected, even though they are not expected to carry the pathogen themselves after summer. Several authors have therefore suggested that direct transmission (via bat-to-bat contacts shown to occur by Lorch et al., 2011) may not be the only pathway, and that environmental reservoirs may exist within hibernacula from which bats become infected – explaining the recurring infection of bats after summer (e.g., Hoyt et al., 2015; Puechmaille et al., 2011). In fact, viable spores of *Pd* have been found both on the walls and the sediments of bat hibernacula (e.g., Lorch et al., 2013; Puechmaille

et al., 2011). Contact between bats and sediments is likely to be rare owing to bat behaviour, making the sediments an unlikely effective reservoir. So if an environmental reservoir exists, the most likely source would appear to be the walls of the hibernacula, with which bats have a lot of contact during swarming and hibernation.

2. Main objectives

Objective 1 – Cryptic genetic diversity

When dealing with a newly emerging infectious disease, one of the first steps is determining the causative agent of the disease. The biology of the pathogen may be critical for inferring how it multiplies, is spread, and how dangerous it might be to hosts. What is often overlooked, however, is that finding a causative agent of disease may reflect a simplified picture, and that disease could be caused by several pathogenic species. So the first aim of my work was to investigate the presence of cryptic species in *Pseudogymnoascus destructans* (i.e., the presence of two or more species which had previously been assumed to be a single species). Differentiation between species should be seen as an ongoing process following a gradient of genetic differentiation – with randomly mating (panmictic) populations at one end and fully separated species, which never exchange genetic material, at the other (e.g., Harrison, 1991; Wu, 2001). In reality, most closely related species will not be at the extreme end of the gradient (full differentiation) making it difficult to delineate species boundaries (e.g., Ting et al., 2000). This is further complicated in fungi by the fact that mating experiments are often unsuccessful (even within the same species) and morphological features do not reflect speciation events – meaning that molecular speciation cannot be confirmed by other means (e.g., Koufopanou et al., 2001; Taylor et al., 2006). Ensuring that signatures of cryptic species are neither missed nor over-interpreted therefore requires multiple approaches which, taken together, provide unambiguous evidence (Table 1 and see Matute & Sepulveda, 2019).

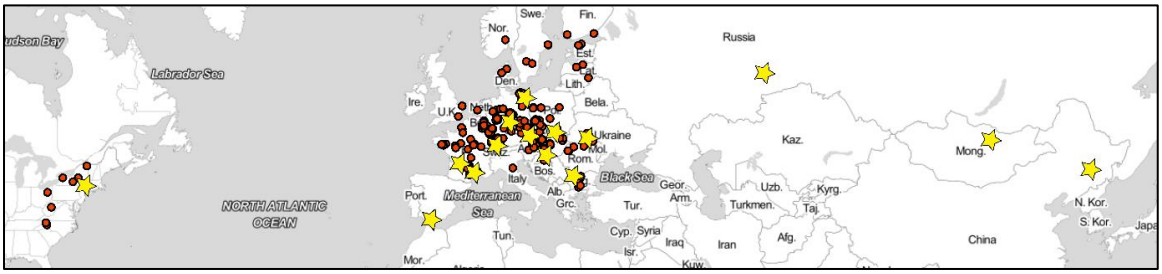


Figure 3. Sampling locations of *P. destructans*. In total, 5,054 isolates were collected and genotyped from 242 hibernacula, which are indicated by red points. Yellow stars indicate hibernacula from which full genomes of isolates were obtained (18 isolates from 15 sites). Except for the hibernacula in Mongolia and China, hibernacula from which full genomes were obtained also yielded multi-locus genotypes. Isolates for pooled sequencing were picked to maximise geographical and genetic distances based on genotype data. The map was generated using stamen maps in R software (R Development Core Team, 2022).

To achieve this for *Pd*, I used an extensive reference collection of over 5,000 *Pd* isolates collected from bats and hibernacula in 26 countries, mostly from the native range of the pathogen (Figure 3, obtained through the help of an invaluable network of collaborators and scientists contributing to sample collection and laboratory work; see list of authors and acknowledged contributors in Manuscript 1). Isolates were genotyped using 18 microsatellite markers (Dool et al., 2020; Drees et al., 2017b) and population genetic differentiation was evaluated with regard to geographical distances among sampling locations, bat species identity (from which the sample was collected), and infection intensity (using a visual score that characterises fungal growth intensity on bats, Fritze et al., 2021). Following results from this dataset, I used 700 conserved genes (BUSCO, Manni et al., 2021) from 18 sequenced isolates sampled in Europe, Asia and North America to see whether the differentiation was present across a larger and more conserved proportion of the genome. Results were additionally confirmed by comparing pooled sequences of a total of 132 isolates (Illumina sequencing; pooling done separately for each of the putative species) and determining the differentiation (via F_{ST} values) across variable regions (SNPs) of the genomes. Taken together, this combination of approaches covering short as well as long evolutionary timeframes and including an extraordinary number of samples allowed me to reliably address the presence of cryptic species in *Pd* and put the findings into an ecological context (e.g., host preference).

This work is presented in **Manuscript 1** (titled "Uncovering cryptic fungal diversity reveals a second causative agent of bat White-Nose disease with distinct host specialisation", which – at the time of writing – was being considered for publication in '*Science*', pages 25-62).

Table 1. Overview of genetic techniques used to address research aims including the number of isolates and markers used as well as each method's main characteristics.

Method	Isolates	Markers	Main characteristics of each method
Genotyping	5,054	18 microsatellites*	• Many isolates (cheap and easy) • Highly polymorphic (allow identification of unique multi-locus genotypes and clones) • Indicates genetic differentiation (though with less reliability and temporal depth than other methods)
Full genomes	18	700 BUSCO genes**	• Fewer isolates (high-quality sequencing is significantly more expensive and time-intensive) • Highly universal genes (present in > 90% of species) and present in single copies • Determination of phylogenetic relationships on a species-level
Pooled sequencing	68 + 72 per pool	41,951 SNPs***	• Pooled sequencing allows for a good number of isolates to be compared • Good representation of the genome and highly variable • Confirmation of differentiation between pools (though prior information required to design pools)

* Microsatellites (also known as simple sequence repeats [SSRs]) are genetic regions in which short motifs of bases (typically between 2 and 10 bases) are repeated and which mutate rapidly. When a sufficient number of markers is used (which was the case here), each individual has its own distinct combination of microsatellite alleles (which are determined via the number of times each motif is repeated).

** BUSCO (Benchmarking Universal Single-Copy Orthologs) genes are highly conserved genes and can be extracted from sequences using a bioinformatic pipeline. Robust phylogenies can be obtained by building a tree for each gene and calculating concordance (i.e., how many of the trees support a given branch).

*** SNPs (single-nucleotide polymorphisms) are single base positions in the genome that are variable among isolates following point mutations. They are distributed across the entire genome and sequences must be aligned (using a reference genome) to detect them and analyse differences among (pools of) sequences.

Objective 2 – Population structure in the native range

Few studies exist in which the intra-specific diversity and distribution of introduced pathogens is investigated thoroughly in their native range – the geographical region of disease emergence usually being central to determining pathogen presence, ecology, and genotypic diversity (see also Sreedharan & Vasudevan, 2021). This a prudent choice early on, given the need for fast responses to emerging infectious diseases and limitation of their further distribution. However, this selective geographic investigation often leads to an under-representation of the genetic diversity present at the species level since it is usually much lower in the introduced range due to invasion bottlenecks (see e.g., Miura et al., 2006 for comparison of genetic diversity in the native and invasive range of a parasite). Hence, studies relying on the identification of unique genotypes have reduced power in the invasive range of a pathogen and might limit the questions that can be addressed using molecular tools (e.g., in phylogeography; Avise, 2009; Rasmussen & Grünwald, 2021). Furthermore, the amount of standing genetic variation at the species level also indicates the ability of a species to evolve and adapt within the framework of host-pathogen-environment interactions (e.g., Fisher & Garner, 2020; Stukenbrock, 2016).

I used the Eurasian genotype data (see also Objective 1 and Figure 3; 5,033 isolates) to evaluate the diversity and distribution of unique multi-locus genotypes (in the following simply referred to as 'genotypes') and allele frequencies among regions and hibernacula. These analyses were done after elucidating the presence of cryptic species in *Pseudogymnoascus* (c.f., Objective 1; in the case of several species being found they would be treated separately for this examination). If significant population genetic differentiation is present, I would expect to find genotypes with limited geographic ranges which would be evidenced through few genotypes being shared among hibernacula, particularly across larger distances. The associated lack of considerable movement of isolates would lead to hibernacula primarily having their own genotypic communities with differing allele frequencies. The potential presence of population genetic structure in *Pd* could therefore also be applied as a tool to determine the most likely source population of the North American introduction. Variable allele frequencies among regions in the native range of *Pd* could be used to assign the alleles observed in the North American isolates to one of these regions. This has been a topic of great interest to researchers working on White-Nose disease but could not be addressed until now.

This work is presented in **Manuscript 1** (titled "Uncovering cryptic fungal diversity reveals a second causative agent of bat White-Nose disease with distinct host specialisation", at the time of writing the manuscript was being considered for publication in '*Science*', pages 25-62).

Objective 3 – Environmental reservoirs as drivers of White-Nose disease seasonality

It has been suggested that environmental reservoirs of *Pd* might exist, leading to the infection of healthy bats after summer. Their presence could influence the expected long-term outcome for affected North American bat species because environmental reservoirs have been associated with an increased risk of species extinctions (De Castro & Bolker, 2004). So far, most studies investigating *Pd* presence in hibernacula environments and/or seasonal dynamics of the disease have used qPCR methods (e.g., Hoyt et al., 2020; Langwig et al., 2015a). While valuable in other ways, these data do not provide information on pathogen viability, a factor clearly required for *Pd* to infect and grow on bats. The third objective addressed in my work is therefore to investigate the presence of environmental reservoirs and associated transmission pathways based on viability (not merely presence). A population genetic approach, combined with a laboratory experiment, was chosen to address this question.

Firstly, to explain the annual re-occurrence of the disease I intended to confirm that bats do not carry viable *Pd* after summer and therefore become re-infected after returning to the hibernacula. To show this, genotypic data was obtained from samples collected in 9 hibernacula (artificial hibernacula close to each other in north-eastern Germany, 523 samples, 1,497 isolates). The significant genotypic diversity present in the native range of the pathogen (see also Objective 2) was key to this analysis, since it allowed me to reliably identify multi-locus genotypes (based on 18 microsatellite loci; Dool et al., 2020) and follow their distribution within and among hibernacula, as well as through time (sampling several times a year for five years). If bats become infected within the hibernacula that they are hibernating in – rather than still being infected after summer or becoming infected at a different site prior to hibernation (e.g., used for swarming only) – movement of viable *Pd* between hibernacula should be limited. In that case, I would expect to find an essentially unique fungal population in each hibernaculum and few shared genotypes among them.

Secondly, I investigated whether spores of *Pd* could remain viable for at least 6 months – the duration of bat absence over summer. I inoculated small cement blocks with *Pd* spores and collected them over the course of two years to investigate changes in germination success (as a proxy of viability and to examine seasonal changes). To support these findings, samples were collected from hibernacula walls before the bats' arrival after summer. If there were viable spores on the walls of hibernacula from which bats could become infected upon contact, these samples should yield many isolates of *Pd*.

Thirdly, I specifically investigated hibernacula walls as the source of infection using data from one intensively sampled hibernaculum in north-eastern Germany where *Pd* was sampled from bats (788 isolates) and walls (274 isolates) over a period of 5 years. If the walls of hibernacula were the main source of bat infection after summer, genotypic richness (the number of unique genotypes relative to the number of samples) should be lower on bats than on walls. This finding would be consistent with a transmission bottleneck, which describes the reduction of genotypic richness from a source population (walls) to its sink (infected bats). There should also be many genotypes found both on bats and on the walls of the same hibernaculum with little or no difference in MLG composition or allele frequencies (i.e., genotypes that are particularly abundant on the walls should infect bats more frequently). The existence of environmental infection of bats would be critical to disease dynamics and was therefore additionally addressed in a collaboration using large natural karst caves in Bulgaria (2 hibernacula, 863 isolates). Taken together, this work could provide clear support of environmental reservoir dynamics and the extended survival of spores in hibernacula environments, as well as provide information on the characteristics of the reservoir itself.

The work on *Pd* spore viability using an experimental approach is presented in **Manuscript 2** (titled "Seasonal patterns of *Pseudogymnoascus destructans* germination indicate host-pathogen coevolution", accepted by '*Biology Letters'* and published in 06/2020, pages 63-77). The study of environmental reservoir dynamics using a population genetic approach is found in **Manuscript 3** (titled "Population genetics as a tool to elucidate pathogen reservoirs: Lessons from *Pseudogymnoascus destructans*, the causative agent of White-Nose disease in bats", accepted by '*Molecular Ecology*' and published online in 10/2021, pages 78-107) and its confirmation based on natural karst caves in Bulgaria is presented in **Manuscript 4** (titled "Genetic diversity and

population structure of *P. destructans*, the causative agent of White-Nose disease in bats. From large scale differentiation to locally homogeneous populations" at the time of writing, the manuscript was under revision for publication by '*The Journal of Wildlife Diseases*', pages 108-138).

3. Publications

3.1. Contributions to this thesis and the publications herein

Abstract/Zusammenfassung – Solely and independently written by Nicola M. Fischer

Introduction – Solely and independently written by Nicola M. Fischer

Main objectives – Solely and independently written by Nicola M. Fischer

Publications

Manuscript 1. Nicola M. Fischer, Imogen Dumville, Violeta Zhelyazkova, Ruth-Marie Stecker, Anna Blomberg, Serena E. Dool, Marcus Fritze, Marie-Ka Tilak, Andriy-Taras Bashta, Clothilde Chenal, Anna-Sophie Fiston-Lavier, Sébastien J. Puechmaille (**under review**): *"*Uncovering cryptic fungal diversity reveals a second causative agent of bat White-Nose disease with distinct host specialisation".

> Sébastien J. Puechmaille conceived the study. Sébastien J. Puechmaille, Violeta Zhelyazkova, Anna Blomberg, Marcus Fritze, Serena Dool, Andriy-Taras Bashta and Nicola M. Fischer collected samples. Nicola M. Fischer, Anna Blomberg, Serena Dool, Marcus Fritze, Violeta Zhelyazkova, Ruth-Marie Stecker, Marie-Ka Tilak, and Sébastien J. Puechmaille carried out the laboratory analyses. Nicola M. Fischer, Imogen Dumville, Anna-Sophie Fiston-Lavier, Clothilde Chenal and Sébastien J. Puechmaille analysed the data. Nicola M. Fischer and Sébastien J. Puechmaille wrote the original draft of the manuscript and both wrote the supplementary material together with Imogen Dumville and Anna-Sophie Fiston-Lavier. All Authors, including Nicola M. Fischer, were involved in data interpretation and critically discussed and edited the manuscript. All authors gave their approval for publication.

Manuscript 2**.** Nicola M. Fischer, Serena Dool, Sébastien J. Puechmaille (**published**): "Seasonal patterns of *Pseudogymnoascus destructans* germination indicate host–pathogen coevolution".

> Sébastien J. Puechmaille conceived the study. Nicola M. Fischer, Serena Dool and Sébastien J. Puechmaille carried out the laboratory analyses. Nicola M. Fischer performed data analyses. Nicola M. Fischer and Sébastien J. Puechmaille interpreted the results and wrote the original manuscript. All Authors, including Nicola M. Fischer, critically discussed and edited the manuscript and gave their approval for publication.

Manuscript 3. Nicola M. Fischer, Andrea Altewischer, Surendra Ranpal, Serena Dool, Gerald Kerth, Sebastien J. Puechmaille (**published**): "Population genetics as a tool to elucidate pathogen reservoirs: Lessons from *Pseudogymnoascus destructans*, the causative agent of White-Nose disease in bats".

> Sébastien J. Puechmaille conceived the study. Sébastien J. Puechmaille, Serena Dool and Nicola M. Fischer collected samples. Nicola M. Fischer, Andrea Altewischer, Surendra Ranpal, Serena Dool and Sébastien J. Puechmaille carried out the laboratory analyses. Nicola M. Fischer performed data analyses. Nicola M. Fischer and Sébastien J. Puechmaille interpreted the results. Nicola M. Fischer and Sébastien J. Puechmaille wrote the original manuscript (including supplementary materials). All Authors, including Nicola M. Fischer, critically discussed and edited the manuscript and gave their approval for publication.

Manuscript 4. Violeta Zhelyazkova, Nicola M. Fischer, Sébastien Puechmaille (**under revision**): ***"***Genetic diversity and population structure of *P. destructans*, the causative agent of White-Nose disease in bats. From large-scale differentiation to locally homogeneous populations".

> Violeta Zhelyazkova & Sébastien J. Puechmaille conceived the study. Violeta Zhelyazkova collected samples. Violeta Zhelyazkova, Nicola M. Fischer and Sébastien J. Puechmaille carried out the laboratory analyses. Violeta Zhelyazkova performed data analyses. Violeta Zhelyazkova, Nicola M. Fischer and Sébastien J. Puechmaille interpreted the results and Violeta Zhelyazkova and Sébastien J. Puechmaille wrote the original manuscript (including supplementary material). All Authors, including Nicola M. Fischer, critically discussed and edited the manuscript and gave their approval for publication.

Synthesis – Solely and independently written by Nicola M. Fischer

Uncovering cryptic fungal diversity reveals a second causative agent of bat White-Nose disease with distinct host specialisation

Nicola M. Fischer[1,2], Imogen Dumville[2], Violeta Zhelyazkova[3], Ruth-Marie Stecker[1], Anna Blomberg[4], Serena E. Dool[1,5], Marcus Fritze[1], Marie-Ka Tilak[2], Andriy-Taras Bashta[6], Clothilde Chenal[2,7,8], Anna-Sophie Fiston-Lavier[2,9], Sebastien J. Puechmaille[1,2,9]*

[1]Zoological Institute and Museum, University of Greifswald, Greifswald, Germany

[2]Institut des Sciences de l'Évolution Montpellier (ISEM), University of Montpellier, CNRS, EPHE, IRD, Montpellier, France

[3]National Museum of Natural History, Bulgarian Academy of Sciences, 1 Tsar Osvoboditel Str., 1000 Sofia, Bulgaria

[4]Department of Biology, University of Turku, Turku, Finland

[5]CBGP, INRAE, CIRAD, IRD, Institut Agro, University of Montpellier, Montpellier, France

[6]Institute of Ecology of the Carpathians, National Academy of Sciences of Ukraine, Lviv, Ukraine

[7]DIADE, University of Montpellier, CIRAD, IRD, 911 Avenue Agropolis, 34934/34830 Montpellier Cedex 5,France

[8]MIVEGEC, University of Montpellier, CNRS, IRD, 911 Avenue Agropolis, 34934/34830 Montpellier Cedex 5,France

[9]Institut Universitaire de France, 75005 Paris, France

*corresponding author (sebastien.puechmaille@umontpellier.fr)

Abstract

White Nose, caused by a fungal pathogen, is the top infectious disease devastating bat populations. Here, using an extensive reference collection of 5,054 isolates from 27 countries, we demonstrate that its widespread causative agent is actually composed of two sympatric cryptic species with specialised host preference and pathogenicity. Each cryptic species contains geographically differentiated populations, allowing us to pinpoint the source population of the North American introduction to a region in Ukraine. Further anthropogenic movement of the pathogens to new areas or between the differentiated fungal populations, could lead to mass mortality through infection of naïve hosts and/or increased virulence, as commonly encountered in fungi. This can be avoided if appropriate 'prezootic' biosecurity-oriented strategies are put in place without delay.

One sentence summary: Discovery of a second widespread fungal species causing White-Nose disease in bats, with distinct host preferences and pathogenicity.

Main text

Emerging infectious diseases, particularly fungal pathogens, are appearing at an unprecedented rate, posing serious threats to public and wildlife health, food security and ecosystem stability worldwide (*1*). For an infectious disease to develop, three conditions must be satisfied: there must be a pathogen, a host, and suitable environmental conditions (*2*). Whilst many studies have investigated host-fungus interactions and their response to environmental factors, the critical component of pathogen genetic variability has often been neglected. The few studies which have investigated this aspect across a species' range have uncovered unforeseen cryptic diversity and incidentally demonstrated that pathogenic fungal species believed to be widespread were in reality composed of several closely related cryptic species - each with a narrower distribution range, and often specific hosts and pathogenicity (e.g., *3-5*). These discoveries of cryptic diversity are probably only the tip of the iceberg and have major consequences for our understanding and prevention of diseases and their emergence. Indeed, large phenotypic leaps leading to host jumps and increased virulence can be precipitated by anthropogenic mixing of previously allopatric fungal species/populations; i.e., when two species, or different populations within the same species, are brought into contact and start exchanging genes (*6-8*). Nevertheless, limited knowledge on cryptic diversity in pathogens and their population structure precludes the

establishment of 'prezootic' biosecurity-oriented strategies that could prevent further movement of pathogens. Among the notorious fungal pathogens is the ascomycete *Pseudogymnoascus destructans* [*Pd*], a bat-specific pathogen which causes White-Nose disease in hibernating bats. It is currently recognised as a single species, native to Eurasia where it is widely distributed, and recently introduced to North America (*9, 10*). Prior research has revealed important insights into the interaction between the fungus and environmental conditions and between the fungus and its hosts, with different species of bats showing varying levels of susceptibility (*11*). However, importantly, this literature has entirely developed under the postulate that *Pd* is a single fungal species with a broad distribution range.

Based on an extensive reference collection of 5,054 isolates originating from 27 countries (5,022 & 32 isolates from Eurasia and North America, respectively), we used molecular data to test for the presence of cryptic diversity in *Pd*, to further describe fine-scale population structure across Europe, and to identify the source population of the North American introduction. We combined the molecular data with field-based ecological data to investigate host specialisation and pathogenicity.

Isolates obtained from bats and/or hibernacula environments of 242 sites were genotyped at 18 polymorphic microsatellite loci (Fig 1A, Tab S1; see *12*). Principal component analysis (PCA) was used to visualise variation among multi-locus genotypes and revealed a clear separation into two clusters with no intermediate genotypes (Fig 1B; hereafter called *Pd*-1 and *Pd*-2, where *Pd-1* corresponds to *P. destructans sensu stricto*). Inferences on individual ancestry coefficients based on a sparse nonnegative matrix factorization algorithm confirmed this finding (Fig 1C). At the broad scale, these two clusters appear geographically sympatric and were found present together at 24 of the sites we studied, making it unlikely that geographic factors currently play a significant role in their differentiation (Fig 1A). Occasionally, isolates from both clusters were found to be syntopic (i.e., isolated from the same body part of the same bat, n=17). The North American isolates, which are known to have a single clonal origin (*9*), cluster with Eurasian isolates of *Pd*-1 (Fig 1B). Besides geography, host preference is known to act as a driver of population differentiation (*13*), hence we investigated which bat species isolates originated from. Out of the samples collected from a total of 15 bat species, the majority of those containing *Pd*-1 isolates originated from *Myotis myotis* bats (80%, with 0% from *M. daubentonii*), while the majority of

swabs containing *Pd*-2 isolates originated from *M. daubentonii* (42% while only 32% originated from *M. myotis*; see also Fig S1). These contrasting data reveal a significant association between the fungal clades (*Pd*-1&*Pd*-2) and the bat host species identity (Chi-squared test, chi^2=2141, df=19, p<.001). These differences cannot be attributed to species distributions, as at least 54% of the sites containing *Pd*-1 isolates are also used as hibernacula by *M. daubentonii* bats (based on bat monitoring available for 175 sites). The latter are therefore expected to come into contact with *Pd*-1 isolates, yet do not appear to be susceptible to infection by them. Such findings suggest that *M. daubentoni i*s resistant to *Pd*-1. In addition to their frequency of occurrence on host species, we investigated infection levels using the visual *Pd*-score (scale from 0 to 4 [maximum]), an index characterising fungal colonisation and wing damage (*14*). We detected variations in *Pd* infection levels between clades with a significantly higher score for *Pd*-1 (median=2) compared to *Pd*-2 (median of 1; Chi-square test, χ^2=18.23, df=3, p<.001, see also Fig S2), suggesting that besides host species, the two clades are also different in their pathogenicity. However, interestingly, *Pd*-1 and *Pd*-2 share similar environmental preferences, i.e., cold (ca. 7-8°C) with high absolute humidity (ca. 6-8g/m^3; Fig S3).

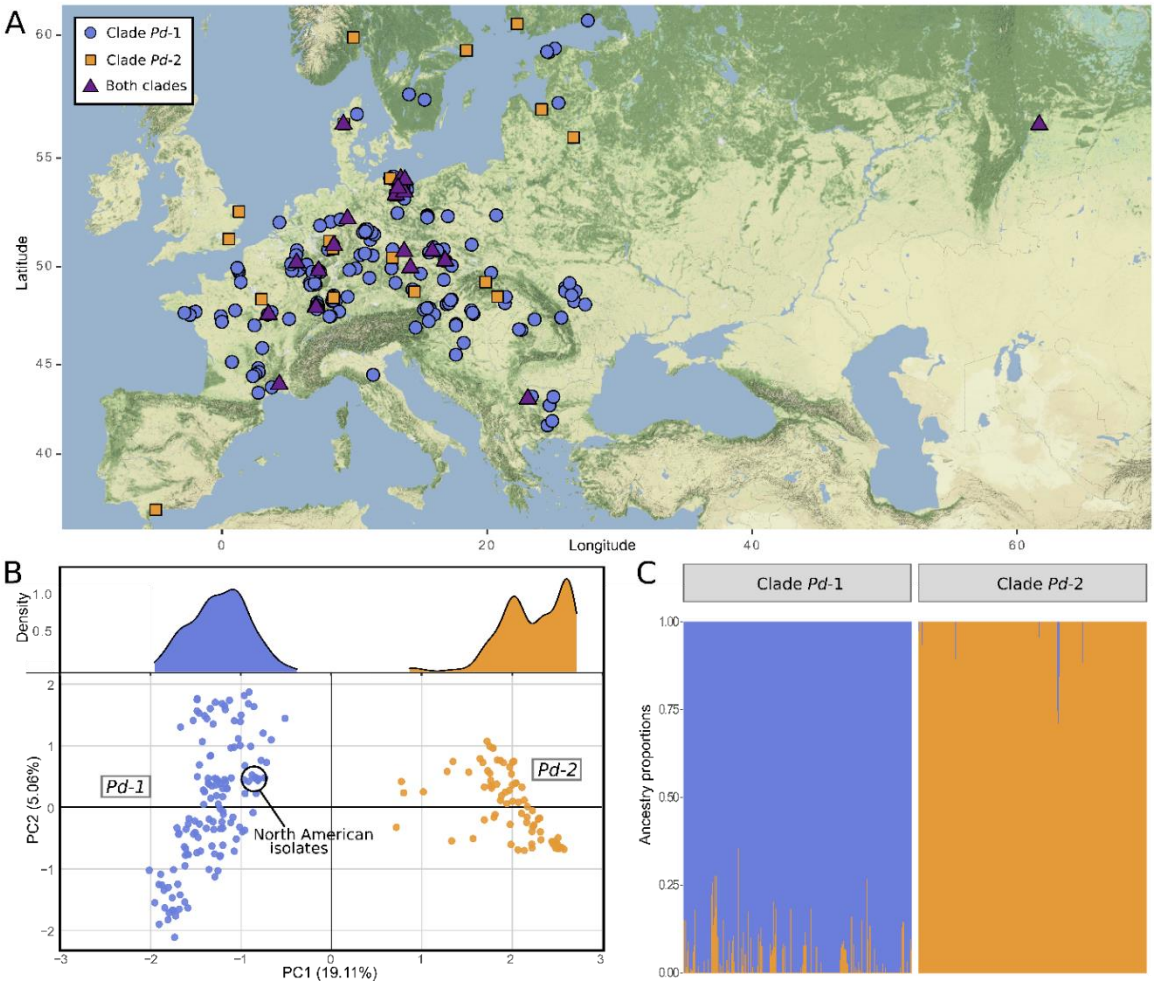


Figure 1. Microsatellites reveal two clades (*Pd*-1, *Pd*-2; 5,054 isolates, 242 sites)**. A.** Sampling locations in Eurasia (North American sites not shown). **B.** Principal Component Analysis (bottom) of isolates (*Pd*-1 was subsampled to ensure even sampling between clades and maximise geographic coverage). Density (top) of PC1 coefficients after random subsampling of *Pd*-1 to get the same number of sites as in *Pd*-2 (48 sites, replicated 100,000 times). **C.** Individual ancestry coefficients calculated for isolates from 48 sites per clade (same individuals as for the PCA; Supplementary information S3.1).

To gain further evidence that the two clades identified via microsatellite analyses are strongly differentiated and to better characterise these differences across the genome, we selected ten Eurasian *Pd* isolates (5 from each clade, *Pd*-1&*Pd*-2, based on microsatellite analyses), one North American *Pd* isolate (*Pd*-1), and one *Pseudogymnoascus sp*. outgroup, for full genome long-read sequencing (MinION nanopore; Tab S2). High quality (>98% complete BUSCO genes) and contiguous genomes (median of 39 contigs; range 18-132) were assembled using Flye v.2.9 (*15*) and polished using 150bp paired-end Illumina reads (Illumina NovaSeq; Table S3). To infer the

relationship between isolates, we extracted the 700 single-copy BUSCO genes (2,176,512 bp alignment) common to 18 isolates: 11 sequenced in this study and 7 previously published (5 from Europe, 1 from Mongolia, 1 from China;*9*). The high bootstrap (100%), site concordance factors (*Pd*-1: 93%; *Pd*-2: 66%), and congruence between gene trees (Fig S5) confirmed the monophyly of the two clades identified with microsatellites (Fig 2A&B). Both clades included samples from Europe and Asia further confirming that the separation is not driven by geography. For example, isolate Gd4986 (clade *Pd*-2) from the Ural Mountains in Russia was more similar to isolates from Spain, Mongolia and China than to another isolate from the same cave but from clade *Pd*-1 (Gd4985). Microsatellite data provide clear evidence that *Pd*-1 is largely dominant in Europe (95% of samples). Despite the limited number of genomes sequenced from East Asia (n=2), the fact that we only identified *Pd*-2 in this region suggests that *Pd*-2 might be dominant in East Asia while the presence of *Pd*-1 remains to be demonstrated in this region. Across 700 BUSCO genes, inter-clade sequence divergence (median of 0.16%) was 3-5 times greater than intra-clade divergence (0.03-0.057%; Fig 2C), while the inter-clade divergence across the full genomes averaged 1.6% (cf. Fig S4). To gain further insights into clades' genomic differentiation across the full genome of a larger number of isolates, we sequenced (Illumina NovaSeq) pools of DNA from 69 and 63 isolates from *Pd*-1 &*Pd*-2 respectively (as determined by microsatellite analyses; cf. Tab S4). Short reads of each pool were mapped to the reference genome of *Pd*-2 (isolate Gd45) allowing us to calculate the F_{ST} between pools. Out of 41,951 SNPs detected, 23,534 (56%) had a different allele fixed in the two clades, 16,361 (39%) had a SNP fixed in one clade but variable in the other, and only 2,056 (5%) were variable in both clades (shared polymorphism). The differentiation between clades was very strong with an average F_{ST} across a window size of 10 SNPs reaching 0.94 (median=0.96; Fig 2D) and a minimum F_{ST} of 0.31. Running the same analysis but using the reference genome of *Pd*-1 instead (isolate Gd_293), provided very similar results (55% had a different allele fixed in the two clades, 40% had a SNP fixed in one clade but variable in the other, and only 5% were variable in both clades - Mean and median F_{ST} were 0.92&0.95 respectively). These data provide strong evidence that, despite sympatry and syntopy, the two clades are not exchanging genetic material, at least in the recent past.

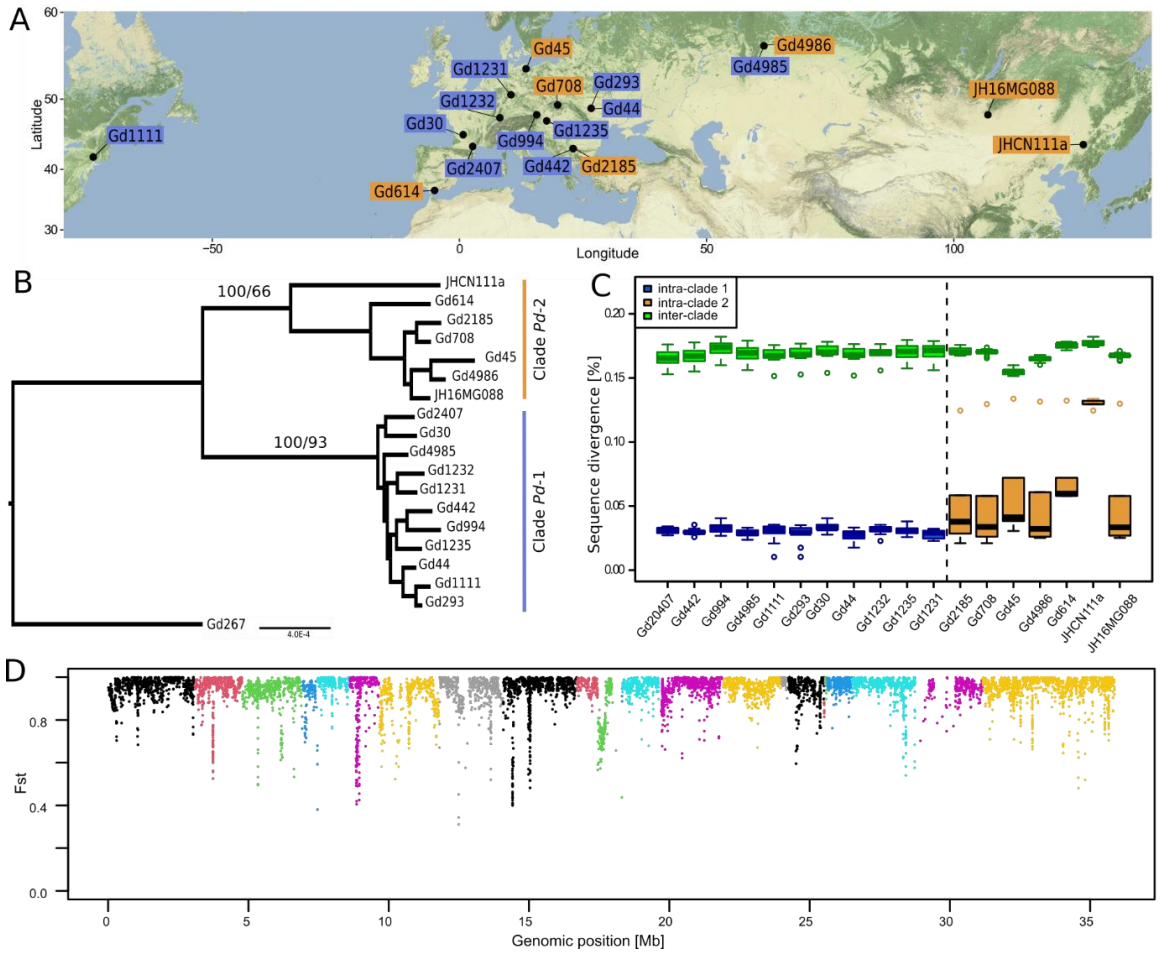


Figure 2. Genomic differentiation between clades. A. Sampling locations of isolates used for phylogeny and sequence divergence analyses **B.** Phylogenetic tree of 700 BUSCO genes with 1000 bootstraps and site concordance factor for nodes of interest (as percentages). The branch to the outgroup has been shortened for visualisation purposes. **C.** Boxplot of the pairwise distance between isolates for the 700 BUSCO genes, partitioned between intra- and inter-clade distance. **D.** Genomic differentiation between pools of individuals from clades *Pd*-1 &*Pd*-2 estimated via F_{ST} across a window size of 10 SNPs, using Gd45 (*Pd*-2 clade) as the reference genome with its 29 contigs coloured successively.

Determining species boundaries is especially challenging in fungi, as distinct morphological characteristics or observations of successful mating among individuals can sometimes be impossible to obtain/measure (e.g.,*16*). Therefore, in recent years, the delimitation of species - especially for microorganisms but not limited to them - has been based mostly on phylogenetic species concepts using molecular data to identify highly supported monophyletic groups that are

reproductively isolated from other such groups (e.g.,*17*). Recently, Matute and Sepulveda (*18*) proposed a standard set of four criteria that are sufficient and necessary to identify species boundaries from genomic data in fungi. We were able to provide evidence for all four criteria; namely (I) both clades are reciprocally monophyletic (Fig 2B), (II) there is a high concordance of this differentiation among genomic partitions (Fig 2B& S5), (III) inter-clade differentiation is higher than intra-clade differentiation (Fig 2C), and (IV) there is a low level of shared polymorphism between clades (main text). Combining the molecular, ecological (host preference), and pathogenicity data, we conclude that there are two pathogenic cryptic species in the *Pseudogymnoascus* genus, native to Eurasia, which are both able to infect hibernating bats and cause White-Nose disease. Whether speciation occurred in allopatry or in sympatry, possibly via host specialisation as the barrier to gene flow (*13*), cannot be elucidated with the present dataset. Nevertheless, our data clearly identify *Pseudogymnoascus destructans* and its cryptic relative as a novel system in which to investigate speciation via host specialisation in a mammalian fungal pathogen.

Host-switching or increased virulence has often been reported when the exchange of genetic material occurs among differentiated populations (*19, 20*). Hence, a first step towards preventing such contact between differentiated populations is to characterise population structure and map regions of high differentiation, a task that can be achieved with the microsatellite dataset given its extensive geographic coverage and large sample sizes. This information is central to inform the establishment of 'prezootic' biosecurity-oriented strategies to prevent the movement of pathogens. In the dataset from Eurasia (total of 5022 isolates), we detected 1,638 distinct multi-locus genotypes (called 'genotype' hereafter) belonging to *Pd*-1 (4765 isolates, 209 sites) and 87 genotypes in *Pd*-2 (out of 257 isolates, 48 sites). More than 95% of genotypes were unique to a single site (see also *21*) and for those present at two or more sites, the average distance between sites is only 58 and 56 km for *Pd*-1 and *Pd*-2 respectively, demonstrating that genotypes generally have a very limited distribution range. To test if an isolate can be genetically assigned to its site of origin, we performed discriminant analysis of principal components (DAPC) based on observed allele frequencies. As many as 66% of isolates from *Pd*-1, and 71 % from *Pd*-2 from Eurasia were successfully re-assigned to their exact site of origin. In terms of distances, isolates were re-assigned at an average distance of only 58.0 km (*Pd*-1) and 45.8 km (*Pd*-2) from their site of origin, which is very near given that samples originated from up to several thousands of kilometres apart.

These very high re-assignment rates cannot be attributed to chance as they sharply contrast with a 'null-DAPC' where information on sites was randomised prior to running the DAPC and where only 0.55% and 1.57% of isolates were correctly assigned respectively (Fig 3B and S6B). To obtain a spatially-informed overview of the genetic discontinuities of each clade, we conducted an estimation of effective migration surfaces (*22*). The analysis revealed three genetic discontinuities for *Pd*-1; one between the Balkans and the rest of Europe, one dividing Europe in two (from Poland through to Slovenia) and the last one dividing France and Iberia from the rest of Europe (Fig 3A and see Fig S6A for clade *Pd*-2). Interestingly, none of these discontinuities are associated with discontinuity in the main host species, *Myotis myotis* (e.g.,*23, 24*) suggesting that the main host population structure is probably not a key driver of the pathogens population structure (see also*21*). Although we currently do not have a specific explanation for the genetic discontinuities for *Pd*-1, these regions are clearly the regions between which anthropogenic movement of isolates should be avoided.

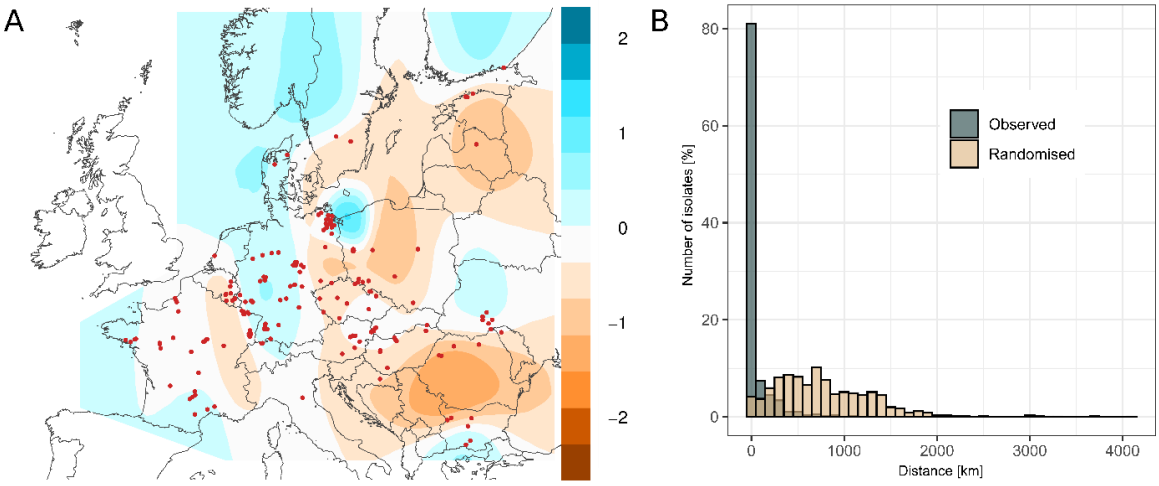


Figure 3. Strong population differentiation in *Pd*-1. A. Estimation of effective migration surfaces based on 1743 isolates from *Pd*-1 in Europe (all sites excluding Russia and the USA after clone correction). For visualisation, results from eight independent runs (each with 8 million iterations and between 100 and 450 demes), were combined. Different shades of colour represent variable levels of high (blue) or low (orange) effective migration rates, while white (0) represents the average. Sampling locations are represented by red dots. **B.** Distribution of the distance between the true and assigned site of each Eurasian isolate of *Pd*-1 (4765 isolates) for the observed and randomised datasets of DAPCs (binwidth = 100 km).

The presence of a strong population structure in the native range of *Pd* also allows us to elucidate the source population of the North American introduction. The genotypes obtained from North America (and hence the descendants of the introduced isolate) unambiguously belong to *Pd*-1 (Fig 1B, 2B & 2C) and can further be assigned with high confidence to a site in western Ukraine (DAPC assignment). This geographic region (Podillia) hence likely represents the region of origin of the introduction.

The elucidation of the region of origin and the discovery of a second causative agent were only possible due to an unprecedented rapid and extensive sampling across a large part of the species native range. Such intensive and synchronous sampling (~February-March each year) at a continental scale was only possible through the combined effort of hundreds of generous volunteers (see supplementary text), demonstrating the great potential of tapping into the synergism of citizen engagement for future surveillance of emerging pathogens. The presence of another pathogenic fungus able to cause White-Nose disease poses a great threat to bat conservation. This danger is clearly present for North American bats (which have never experienced *Pd*-2) but also for Eurasian bats, which might in the future be exposed to more virulent strains originating from inter- and intra-clade genetic exchanges. Preventing the anthropogenic movement of pathogens must therefore become accepted as a critical necessity and a significant part of future conservation planning and disease prevention to ensure the health of wildlife, plants and humans.

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Supplementary Materials for

Uncovering cryptic fungal diversity reveals a second causative agent of bat White-Nose disease with distinct host specialisation

Nicola M. Fischer, Imogen Dumville,Violeta Zhelyazkova, Ruth-Marie Stecker, Anna Blomberg, Serena E. Dool, Marcus Fritze, Marie-KaTilak, Andriy-Taras Bashta, Clothilde Chenal, Anna-Sophie Fiston-Lavier, Sébastien J. Puechmaille

Correspondence to: sebastien.puechmaille@umontpellier.fr

This PDF file includes:

Other Supplementary Materials for this manuscript include the following:

Microsatellite dataset (.csv)

Materials and Methods

1. Sample and field data collection

Swab samples of *Pseudogymnoascus destructans* (*Pd*) were collected from within bat hibernacula. Sampling from hibernating bats was done without handling the animals, but by simply slightly touching infected areas lightly with a sterile dry swab (Polyester swab 164KS01, COPAN). This method can be considered as minimally invasive or even non-invasive (*1, 2*). The timing of sample collection was usually between January and April when the highest numbers of bats with visible infection have been reported (*3*). When no bats were present, or sampling them was not possible, wall swabs were collected, by touching the swab to hibernacula walls (ideally close to where bats usually hang to hibernate; see Fischer et al. 2022 for more detail). Additionally, four isolates were obtained from sediment samples collected from inside hibernacula and 22 were collected from caving gear (i.e., caving suits and harnesses), which most likely originated from contact with hibernacula environments (*5*).

When a sample was taken from a bat or in close proximity (within ca. 10 cm), the bat species was also recorded. For a subset of the sampled bats, the visual *Pd*-score (scale from 0 to 4 [maximum]) quantifying the intensity of infection by the fungus, was estimated in the field. This score has been shown to simultaneously represent fungal colonisation and wing damage (*1*). Temperature and relative humidity were measured in the hibernacula. Absolute humidity was then calculated from measures of relative humidity and temperature by applying the formula presented by Wagner and Pruß (2002).

This work was conducted under permission from the following authorities:

Regional Speleological Federation of Emilia-Romagna (F.S.R.E.R.), and the Management Bodies of the Parks of Emilia-Romagna; Genarny Dyrektor Ochrony Środowiska (General Director for Environmental Protection); Kantonaler Fledermausschutz Aargau; Umweltamt, Veterinäramt; Untere Landschaftsbehörde Siegen-Wittgenstein; Untere Naturschutzbehörde Umweltamt Landkreis Harz & Referat Verbraucherschutz, Veterinärangelegenheiten Landesverwaltungsamt Sachsen-Anhalt; Untere Naturschutzbehörde des Landkreises Vorpommern-Greifswald; Regierung von Unterfranken, Regierung von Mittelfranken; Pest Megyei Kormányhivatal, Országos Környezetvédelmi, Természetvédelmi és Hulladékgazdálkiodási Főosztály (Pest County Government Office, National Department of Environment Protection, Nature Conservation and Waste Management); Bulgarian Ministry of the Environment and Water; DDTM-Morbihan; DREAL; Nature Conservation Agency; The Nature Agency and Daugbjerg Kalkgruber; Speleological Heritage Commission; Regional Directorate for Environmental Protection in Gorzów Wielkopolski (Regionalna Dyrekcja Ochrony Środowiska w Gorzowie Wielkopolskim); Estonian Environmental Board; Natural England; Scottish Natural Heritage; Natural Resources Wales; Southwest Finland Centre for Economic Development, Transport and the Environment; Uppsala djurförsöksetiska nämnd; Swedish board of Agriculture; Swedish Environmental Protection Agency; Miljødirektoratet; Ministère du Développement durable et des Infrastructures du Luxembourg; Croatian Ministry of Environment and Nature.

2. Laboratory materials and methods

2.1 Cultures and genotyping

The DNA extraction and genotyping protocols followed Dool et al. (*7*) and Fischer et al. (*4*), but are briefly outlined here. *Pd* was collected from hibernating bats and the walls of sites where bats hibernate using sterile swabs. The collected fungal material was cultured on dextrose peptone yeast agar (DPYA, *8*) following classical mycological procedures and sterilization of tools between each use. After growth was observed, single spores (colonies which grow outwards from a single germinating spore) were physically separated and stored between 10 and 15°C until enough material had grown to extract DNA (usually within several weeks to months). DNA extraction was done using a KingFisher Flex extraction robot (Thermo Scientific). After DNA extraction, isolates were genotyped using 18 microsatellite markers in four multiplexes. Genotyping was carried out on an ABI 3130 Genetic Analyser (Applied Biosystems) and GeneMapper® Software v.5 (Applied Biosystems) was used for fragment analysis.

2.2 DNA extraction for MinION and Illumina reads

Material was harvested from *Pd* cultures using sterilized tweezers. We used a sorbitol wash buffer (100 mM Tris-HCl pH 8.0, 0.35 M Sorbitol, 5 mM EDTA pH 8.0, 1% (w/v) Polyvinylpyrrolidone [PVP-40]) to clean the fungal material and remove most of the culture medium from the hyphae (the wash was repeated twice with 5 minutes incubation at room temperature each time). After removing the sorbitol wash buffer the second time (through centrifugation), 500 µL CTAB lysis buffer (preheated to 65°C; 0.01M Tris HCl pH 7.5, 25 mM EDTA pH 8.0, 1.5 M NaCl, 2% CTAB powder [w/v]), 30 µL Proteinase K and 5 µL 1M DTT were added for digestion and incubated overnight at 56°C, mixing material after the first hour. After letting samples cool for 5 minutes at room temperature 4µL RNase A were added and left to incubate at room temperature for 10 minutes. 1 volume chloroform - isoamyl alcohol (24:1 v/v) was added after which tubes were inverted 30 times and centrifuged for 5 minutes at maximum speed, keeping the supernatant. We then added a second step of proteinase K (30 µL) and RNase A (4 µL) treatment with an incubation for 30 minutes at 56°C, as it was found to reduce the presence of RNA and result in better quality DNA. To remove these enzymes, we performed a second chloroform - isoamyl alcohol (24:1 v/v) extraction step by adding 1 volume, inverting 30 times and centrifuging at maximum speed for 5 minutes after which the supernatant was kept. Precipitation of DNA was achieved with the use of 1/10 volume sodium acetate, 2 volumes ethanol (> 99% purity), and centrifugation for 20 minutes at maximum speed. After gentle removal of the sodium acetate–ethanol mixture the resulting pellet (containing the DNA) was washed twice with 70% ethanol. DNA was then eluted in ddH_2O and stored in the fridge. The DNA content was determined using Qubit (ThermoFisher).

2.3 Sample preparation for MinION nanopore sequencing

We performed long-read ONT sequencing of 12 isolates (five Eurasian per clade, one North American and one outgroup) using MinION flowcells (FLO-MIN-106) using libraries prepared with the ONT Ligation Sequencing kit SQK-LSK109, following the manufacturer's instructions.

2.4 Sample preparation for Illumina sequencing (PoolSeq)

We combined DNA from 69 *Pd*-1 isolates and 63 *Pd*-2 isolates in sample pools (one pool per clade). Isolates were chosen to maximize both the geographic distance among sites and the genotypic richness within each clade. After DNA extraction (and quantification) of each isolate, DNA was combined resulting in equal concentrations of isolates with a total of 500 ng DNA in a volume of 60 µL per pool. Illumina indexed libraries were prepared according to the protocol detailed in Meyer and Kircher (*9*) with modifications as proposed by Tilak et al. (*10*). Libraries were then sequenced by Novogene on an Illumina NovaSeq.

3. Analyses

3.1 Analyses of multi-locus genotypes

The analyses of multi-locus genotypes (MLGs) were run in R (except for EEMS*;* R version 4.1.1, *11*) using packages for specific analyses. Particularly, the package *poppr* (V2.9.3, *12*) was extensively used as it provides the tools needed for population genetic analyses of haploid species with clonal reproduction (such as *Pd*).

MLGs were defined by their unique combination of alleles across the 18 polymorphic microsatellite loci. This set of markers is sufficient to reliably identify MLGs' identity both among and even within sites, where MLGs are usually less differentiated (see *4*). Across all isolates, the allelic richness was high, ranging from 10 - 88 alleles per locus (mean = 36), however, we found that some alleles were fixed within the *Pd*-2 clade (Table S1).

Principal-component analysis (PCA) was used to visualize the differentiation among isolates (package *adegenet*, V2.1.5, *13*). Since the outcome of PCAs is dependent on sampling intensity (*14*), it was important to select roughly equal sample sizes among clades to capture their differentiation in Eurasia. To achieve this, we chose 48 sites (the same number of sites with*Pd*-2) from *Pd*-1 in a way that maximized geographic distance among them (i.e., thinning sites) and used up to 20 isolates per site (again, to ensure that sampling among sites was not significantly uneven). This resulted in a dataset containing 165and 94isolates for *Pd*-1 and *Pd*-2 respectively over 48 sites each (using unique MLGs only). The PCA was then calculated using this subset for *Pd*-1 combined with the unchanged data for *Pd*-2 revealing two clusters which were completely differentiated. The position of the North-American isolates on the PCA plot was simply determined *a posteriori* by projecting/predicting their coordinates using the function suprow. Considering that maximizing distance among sites may also have an influence, we confirmed these findings on the Eurasian dataset by randomly subsampling 48 sites of Pd-1 repeatedly (100,000 times, number of isolates per site still capped at 20) without considering the geographic distance between chosen sites. After running these 100,000 subsampled PCAs (with *Pd*-2 unchanged), the density of values observed for principal component axis 1 were consistent with the values obtained for the thinned dataset, hence indicating that the signal of differentiation between *Pd*-1 and *Pd*-2 is strong and independent from geography or identity of chosen sites (Fig. 1). These findings were further supported by the calculation of individual ancestry coefficients based on sparse nonnegative matrix factorization with an alpha of 150 (function *snmf* from package *LEA*, V3.6.0, *15*). Since the calculation of ancestry coefficients is particularly susceptible to variations in sample sizes among

groups (*16*), we used the same geographically thinned dataset of 48 sites for *Pd*-1 combined with the unchanged *Pd*-2 as described for the PCA above.

We also used the microsatellite dataset to investigate the presence of population differentiation within each of the discovered clades. For this purpose, we investigated only Eurasian isolates (i.e., excluding the 32 isolates from the US) and treated *Pd*-1 and *Pd*-2 separately.

Firstly, we used Discriminant Analysis of Principal Components (DAPC, *17*; using package *adegenet*). Here, each isolate was probabilistically assigned to sites based on the observed allele frequencies (no assumptions are made, e.g., about independence of loci). Each isolate was run in an independent DAPC resulting in a set of 1954 and 255 DAPCs for *Pd*-1 and *Pd*-2 respectively (excluding all isolates from North America and with a limited number of 20 isolates per site; 120 PCA axis and 100 DA axis retained in all runs). Since some isolates will always be correctly assigned by chance it was important to quantify the percentage of correct assignments by chance compared to correct assignments based on observed allele frequencies. Hence, we ran the same sets of DAPCs after randomizing the site names (independently for each run), to ascertain the frequency of correct assignments occurring by chance and the expected distances between the isolates site of origin and their assigned sites if assignment was no better than random ("randomized DAPC"). The distances between assigned sites and sites of origin were presented in the main manuscript for *Pd*-1 and can be found in figure S4B for *Pd*-2.

The visualization of estimated effective migration surfaces (EEMS) was used to evaluate geographic barriers linked to patterns of gene flow (*18*). This method differs from principal-components analysis (PCA) and the calculation of ancestry coefficients in that genetic differentiation is visualized as a function of migration rates rather than via genetic/genotypic distance. This method uses a population genetic model to compare expected pairwise genetic dissimilarities in relation to their geographic distances (i.e., under a model of isolation-by-distance) with observed patterns across the sampled area. More specifically, a triangular grid with specific density (number of demes) is built over the area containing geo-referenced samples. For the edge of each grid the migration parameter is estimated by Bayesian inference and Markov chain Monte Carlo sampling (MCMC), meaning that migration is estimated in an approximated stepping-stone model between neighboring grid cells. Since sampling locations will fall into the same or neighboring cells depending on grid cell size, the number of demes (defining the overall density of grid cells and hence their size) influences the outcome of estimated migration rates (particularly at small geographic scales). For this reason, we calculated the EEMS for a range of deme sizes (N = 8, 100 - 450 demes) in independent runs of 8 Million iterations (after a burn-in of 1 Million iterations), as recommended by Petkova et al. (*18*). Runs were combined in a single figure for visualization of robust migration rates. It should be mentioned that estimated migration rates are most accurate closer to sampling locations and less accurate in sparsely sampled geographic areas. For this reason, the Russian isolates were excluded before calculating EEMS for both clades.

3.2 Analyses of genome sequences

3.2.1 Basecalling

Basecalling was performed on a GPU computer (hosted by the Montpellier Bioinformatics Biodiversity platform) by running the software Guppy v5.0.7, a state-of-the-art neural network basecaller (*19*).

3.2.2 Genome Assembly

In order to produce high quality genomes (*P. destructans* is haploid), we carried out adaptor trimming with Porechop on all basecalled reads (https://github.com/rrwick/Porechop, *20*). These were then parsed into Flye v2.9 (*21*) with the --nano-hq flag designed specifically for Guppy 5+. Other arguments were left as default (including automatic minimum overlaps). Genomes were polished once using pre-trimmed Illumina reads with HyPo after initial mapping using paired-end mapper bwa mem v.0.7.17-r1188 (*22, 23*). HyPo arguments included approximate genome length of 35 megabases (-s 35m; based on *24*) and the average coverage of each genome (-c) (calculated with SamTools v1.1 depth, *25*) .

To remove possible contamination, contigs with exceptionally low GC content (identified with infoseq EMBOSS v6.6.0.0, *26*) were individually compared to the nucleotide BLAST database (*27, 28*). We removed contamination observed in four isolates (which contained clear contamination by *Cellulosimicrobium cellulans*, *Penicillium solitum*, *Pyrenophora teres f. teres* and *Shiraia bambusicola*).

Mitochondrial contigs, with a characteristic lower GC content and known length (ca. 32 kb), were also identified through BLAST and removed from all further analyses. To remove noise in our genomes from spurious assembly, all contigs below 10,000 basepairs were removed using SeqKit v0.16.1 (-m 10000; *29*).

3.2.3 Repeat Annotation

We annotated repeat content of each genome with RepeatModeler and RepeatMaskertools. BuildDatabase was followed by RepeatModeler v2.0.1, which we individually ran on all 11 *Pd* isolates' genomes to identify repeat regions with default parameters. All novel consensus repeats sequences were combined using CD-HIT est v4.8.1 to remove redundancy in the clustered library (-aS 1 -c 1 -r 1 -g 1 -p 0; *30*). All final repeat sequences of unknown identity were then removed from the repeat library. Further, BLASTN v.2.9.0+ searches of the repeat library were carried out against the reference *P. destructans* RNA file downloaded from NCBI (GCF_001641265.1_ASM164126v1). Any repeats with a percentage of identical matches over 80% of over 80% of query length (-outfmt 6, -perc_identity 80, manual removal of qcovs> 80) were also removed (*31*). Finally, we removed duplicated fasta entries with samtools (faidx). Softmasked assemblies as used downstream were produced using RepeatMasker v.4.1.2. (*32*) (-xsmall), with the curated repeat library (-pa 5 -a -s -gff -no _is). RepeatMasker produced tabulated files of individual repeat sites per isolate. The same pipeline was then used for the outgroup (264) though in separate runs to *Pd* isolates.

3.2.4 BUSCO genes

Each isolate assembly was benchmarked with BUSCO v5.2.2. (hmmsearch v3.1 and metaeuk v5.34c21f2) comparisons using the option -m genome flag amongst Fungal, Ascomycota and Onygenales odb10 databases from orthoDB v10, which correspond to *P. destructans'* kingdom, division and sister order and contain 758, 1706 and 4862 orthologous gene sequences each respectively (*33, 34*). Only Fungal BUSCO orthologs were used downstream. Basic statistics of reads were obtained with NanoStat and NanoPlot (*35*).

3.2.5 Tree

To produce the phylogenetic tree of sequenced and public isolates' BUSCO genes, we used all single copy, complete orthologs as identified from the fungi odb10 database that were common to all 18 isolates (Tab. S2) and the *Pseudogymnoascus sp.* outgroup (isolate 267). We extracted the genes from each assembly with BEDTools v2.30.0-8 (*36*) using the command etfasta before individually aligning with MAFFT v7.453 (--auto, --adjustdirection) (*37*). In IQ-TREE2 v2.0.4 (*38*), a concatenation-based species tree with edge-linked proportional partition model with 1000 ultrafast bootstrap (-B 1000 -T AUTO) was produced (*39*). In order to produce the species concordance factor, the ortholog and species trees were used (--scf 100--prefix concord -T 10) by IQ-TREE2. The final tree was manually rooted at the outgroup, 267, in FigTree v1.4.4. (http://tree.bio.ed.ac.uk/software/figtree/).To represent conflicting phylogenetic signal between gene trees (e.g., due to recombination and/or incomplete-lineage sorting) we used the function consensusNet from the phangorn R package (*40*) and computed the consensus network from the splits occurring in the different gene trees. Only splits occurring in at least 10% of trees were represented in the network.

3.2.6 Sequence divergence

Sequence divergence for BUSCO genes was calculated from the MAFFT alignment (described above), in R, via the function 'dist.dna' from the ape package (*41*).

For the full genomes, for each of the assemblies, sub-contigs were obtained by deleting the repeated and low-complexity sequences detected using RepeatModeler and RepeatMasker pipelines (as described above). A local alignment of the sub-contigs was carried out with NUCmer4 (*42*) for all the individuals against each other and interpreted using the show-coords program by applying a minimum percentage of sequence identity of 80%.The aligned regions were used to calculate the weighted average identity.

3.3 Analyses of pooled sequences (Illumina reads)

To calculate Fst between pools, we mapped *Pd*-1 and *Pd*-2 separately onto the *Pd*-2 (isolate Gd_45) reference genome with minimap2 v2.1 (-axsr -t4; *43*). SamTools (v1.14; htslib, bcftools) was used to convert mapped poolseq into vcf files with the use of mpileup (--redo-BAQ, --max-depth 8000, --min-BQ 30, --per-sample-mF, --annotate FORMAT/AD, FORMAT/ADF, FORMAT/ADR, FORMAT/DP, FORMAT/SP, INFO/AD, INFO/ADF, INFO/ADR), followed by bcftools call (--multiallelic-caller --variants-only --ploidy 1 -Ov). Indels were then removed from the vcf file via a custom script in R. The vcf file was then imported into R via the poolfstat v2.0.0. package (*44*)

in R with vcf2pooldata (designating the pool size as 69 and 63 for clade 1 and 2 respectively, other arguments including max.cov.per.pool=100, min.cov.per.pool=10, min.maf=0.01, min.rc=2). The multilocus F_{ST} was then calculated across 10 SNPs with compute.F_{ST} function (with 'sliding.window.size=10').

3.4 Maps and Plotting

Unless otherwise mentioned, figures were produced in R software using functions from base R (*11*) and ggplot2 (*45*). Maps (Figures 1A and 2A) were downloaded as tiles from stamen designs (see maps.stamen.com) with data by OpenStreetMap (under ODbL, under CC BY 3.0) and plotted using the ggmaps package (*46*). They represent maps of type "terrain" with colors representing natural vegetation colours and elevation (via shading). Inkscape v1.1.1 was used to optimize visualizations.

Supplementary Text

List of contributors who helped with sample collection in the field:

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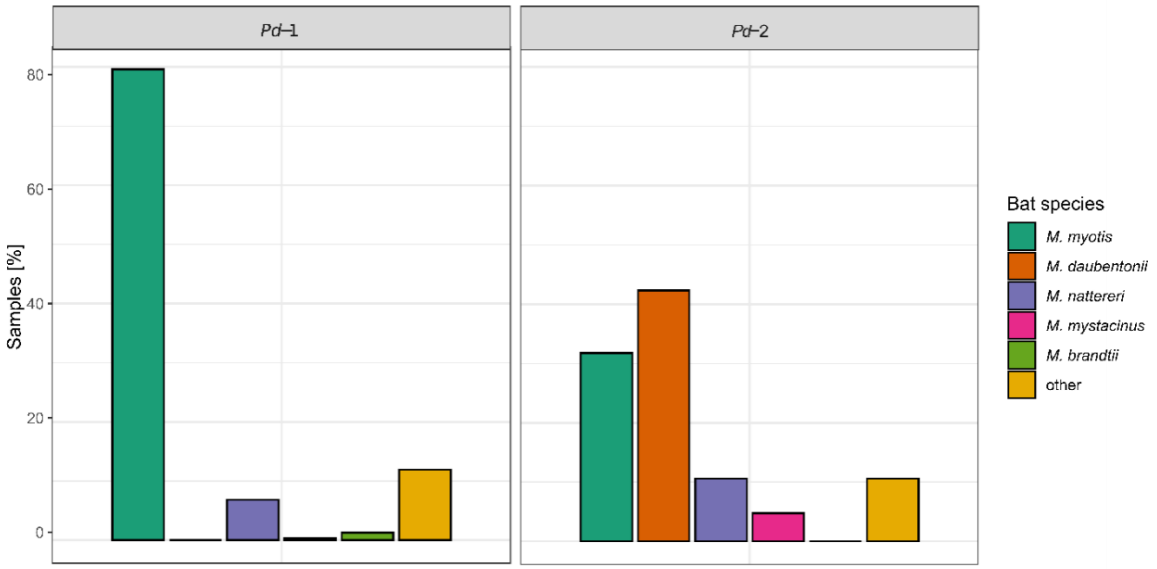
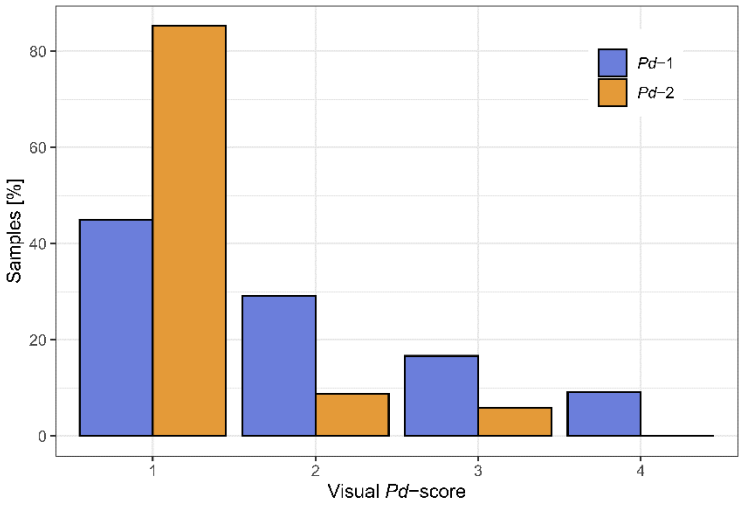
Fig. S1.



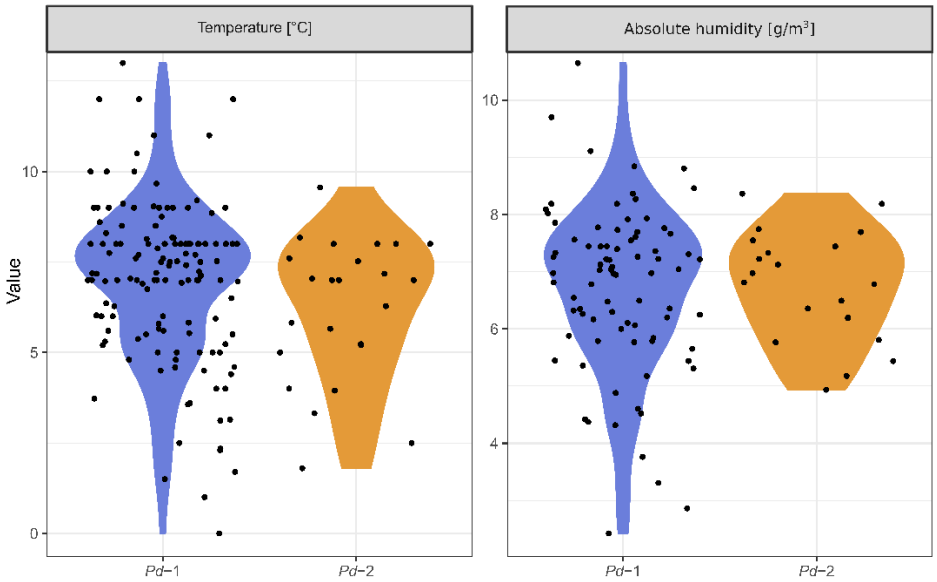
Percentages of swab samples collected (Eurasian sites only) from the 5 most frequently sampled bat species (*Myotis myotis*, *M. daubentonii*, *M. nattereri*, *M. mystacinus*, *M. brandtii*) and all other species combined ("other") per clade. Samples from the walls or with missing bat species information were removed for this figure, resulting in data from 1214 and 85 swabs for *Pd*-1 and *Pd*-2, respectively.

Fig. S2.



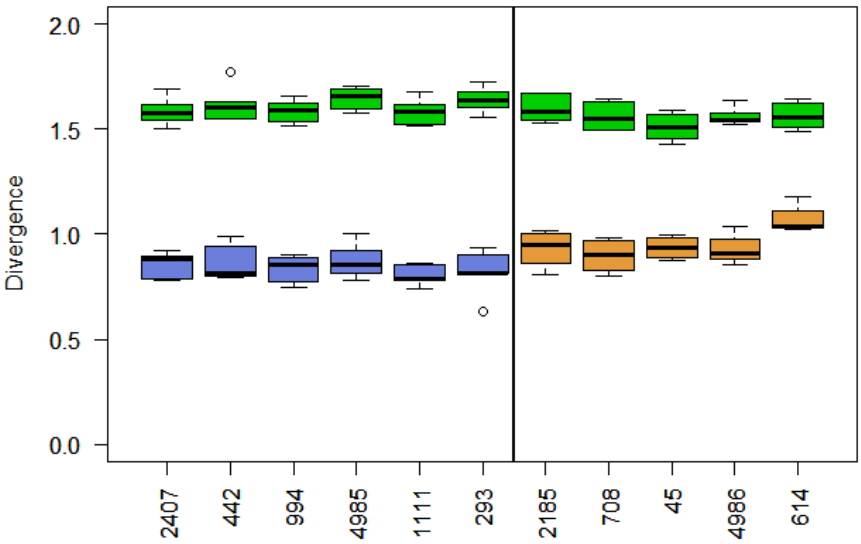
Percentage of samples obtained from bats exhibiting visual *Pd*-scores ranging from 1 to 4 (highest) for *Pd-1* and *Pd-2*. The visual *Pd*-score of infected bats represents a reliable index of disease severity of *Pd* (*1*). To ensure that the sampling procedure itself was not biased, we only used samples from a region in north-eastern Germany, where most samples were collected by the same person (454 samples from *Pd*-1, 26 samples from *Pd*-2).

Fig. S3.



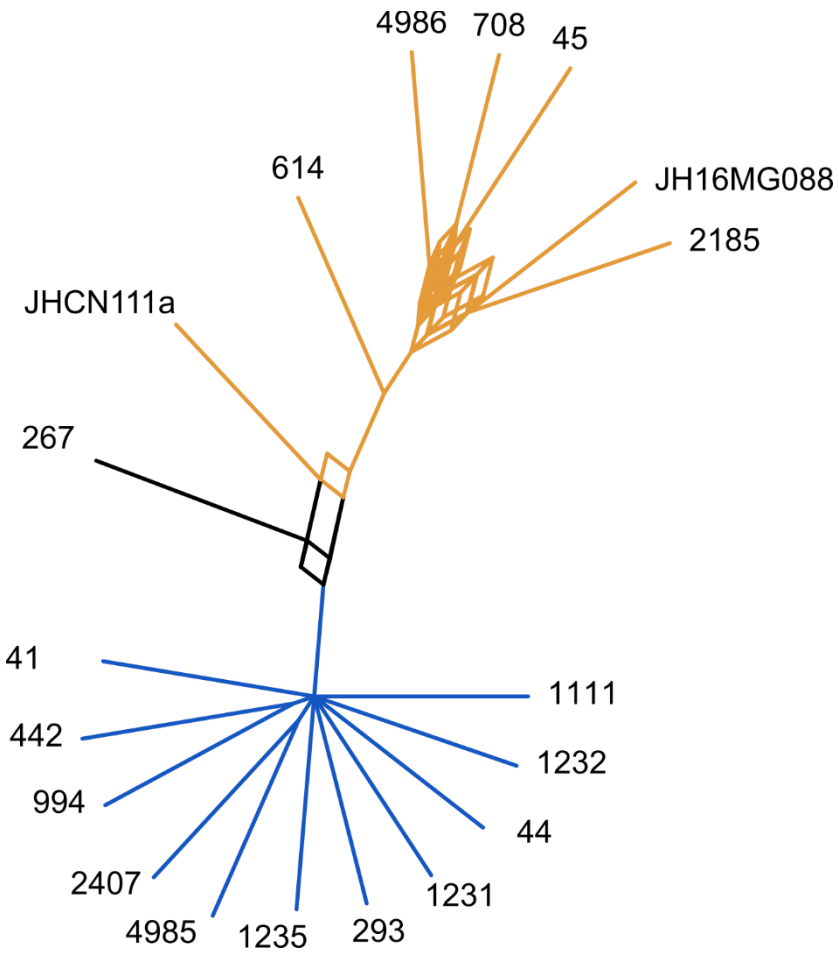
Temperature and absolute humidity recorded in Eurasian hibernacula. Results are shown as raw data (dots) and as violin plots (coloured shading) showing the probability density estimate of the variables per clade. Temperature data was obtained from 133 and 24 sites in which clades *Pd*-1 and *Pd*-2 were sampled while absolute humidity was recorded in 82 and 21 sites per clade. There was no significant difference between the clades, neither for temperature (t-test, t = 1.71, df = 34.5, p = 0.10) nor humidity (t-test, t = -0.11, df = 46.4, p = 0.91).

Fig. S4.



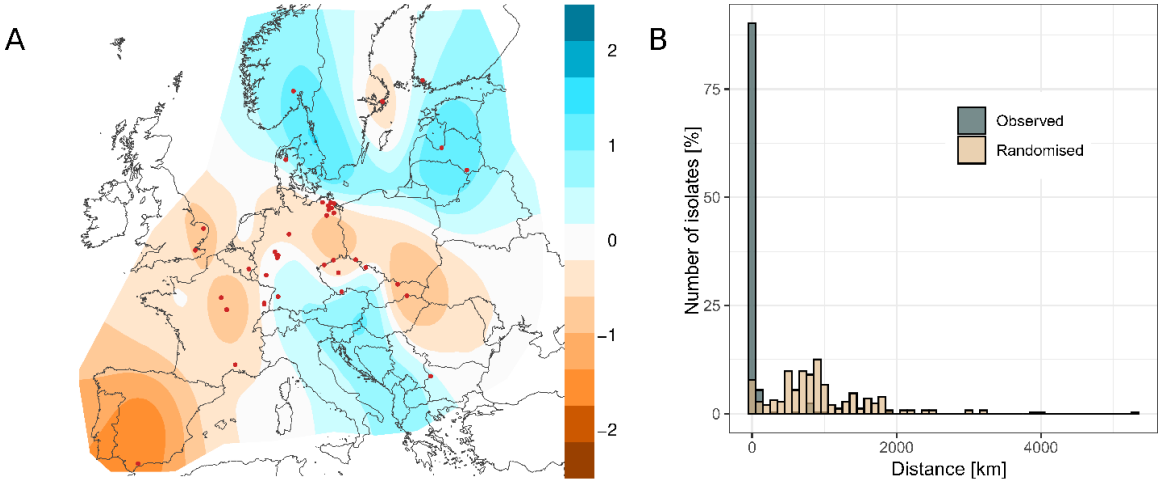
Boxplot of the pairwise distance between isolates, for the full genomes, partitioned between intra- and inter-clade distance. Intra-clade *Pd*-1 &*Pd*-2 divergence are colored in blue and orange respectively while inter-clade divergence is colored in green (as per Fig. 2C).

Fig. S5.



Consensus network depicting high congruence between the 700 BUSCO gene trees (same dataset as in Fig. 2B). The network was produced with proportion 0.10, where a split is present in 10% of trees in order to be represented in the network. *Pd*-1 is represented in blue, *Pd*-2 in orange, and the outgroup in black.

Fig. S6.



Strong population differentiation in *Pd*-2. **A.** Estimation of effective migration surfaces based on 93 isolates from *Pd*-2 in Europe (all sites excluding Russia after clone correction). For visualization, results from eight independent runs (each with 8 million iterations and between 100 and 450 demes), were combined. Different shades of a colour represent variable levels of high (blue) or low (orange) effective migration rates, while white (0) represents the average. Sampling locations are represented by red dots. **B.** Distribution of distance between true and assigned site of each isolate for the observed and randomized datasets (binwidth = 100 km). The mean distance of assignment was 45.82 km with a median of 0 km (in the Null-DAPC with randomization of sites before assignment mean and median were 938.95 km and 812.57 km, respectively).

Table S1.

Allelic richness (number of different alleles) for each of the microsatellite markers used in the study overall and for each of the clades separately.

Locus	Allelic richness overall	Allelic richness *Pd*-1	Allelic richness *Pd*-2
Pd1	60	48	22
Pd2	86	86	7
Pd3	35	34	4
Pd4	50	42	10
Pd5	93	71	40
Pd6	10	10	2
Pd7	55	55	7
Pd9	11	11	6
Pd10	15	15	1
Pd11	38	38	5
Pd12	27	11	19
Pd13	24	24	7
Pd14	27	27	1
Pd17	14	14	2
Pd19	29	21	20
Pd21	13	13	4
Pd22	47	42	5
Pd23	28	28	5
Mean	36.8	32.8	9.3

Table S2.

Information on sequences and their origin including the isolates' full name, country of origin, substrate they were recovered from (wall and bat with information on bat species) and Sequence Read Archive Number (SRA). Contaminants were found via irregularities in GC content and identified through BLAST database, leading to the exclusion of seven contigs. CCF numbers refer to the accession number of culture obtained from the Culture Collection of Fungi, Prague, Czech Republic.

* SRA available in the BioProject no. PRJNA862744.

Short Name	Clade	Full name	Country	From	SRA
Gd293	*Pd*-1	Gd_00293-aad	Ukraine	Bat (*Myotis myotis*)	*
Gd442	*Pd*-1	Gd_00442-ba	Bulgaria	Bat (*M. myotis/M. blythii*)	*
Gd994	*Pd*-1	Gd_00994-aaa	Austria	Bat (*M. myotis*)	*
Gd1111	*Pd*-1	Gd_01111-aaa=20631-21 (type isolate)	USA	Bat (*Myotis lucifugus*)	*
Gd2407	*Pd*-1	Gd_02407-aa	France	Bat (*M. myotis*)	*
Gd4985	*Pd*-1	Gd_04985-ea=CCF-4985	Russia	Bat (*Myotis dasycneme*)	*
Gd1231	*Pd*-1	GU999986=Tesa-OT-8-09	Germany	Bat (*M. myotis*)	SRR6011467
Gd1232	*Pd*-1	GU350433=Tesa-OT-10-09	Switzerland	Bat (*M. myotis*)	SRR6011468
Gd1235	*Pd*-1	GU350434=Tesa-OT-14-09	Hungary	Bat (*M. myotis*)	SRR6011465
Gd30	*Pd*-1	Gd30	France	Bat (*M. myotis*)	SRR6011497
Gd44	*Pd*-1	Gd44	Ukraine	Bat (*M. myotis*)	SRR6011496
Gd45	*Pd*-2	Gd_00045-a2ab	Germany	Bat (*M. myotis*)	*
Gd614	*Pd*-2	Gd_00614-baa	Spain	Wall	*
Gd708	*Pd*-2	Gd_00708-ba	Poland	Wall	*
Gd2185	*Pd*-2	Gd_02185-ab	Bulgaria	Wall	*
Gd4986	*Pd*-2	Gd_04986-cc=CCF-4986	Russia	Bat (*M. dasycneme*)	*
JH15CN0111a	*Pd*-2	JH15CN0111a	China	Bat (*Myotis petax*)	SRR6011485
JH16MG088	*Pd*-2	JH16MG088	Mongolia	Bat (*Plecotus ognevi*)	SRR6011486
Gd267	Out-group	Gd_00267-ac	Germany	Bat (*Myotis daubentonii*)	*

Table S3.

All statistics, including the number of contigs and length of genome is noted after polishing and removal of contigs below 10,000 base pairs in length. Complete BUSCO is presented. The isolate in bold denotes the outgroup used in the phylogeny.

Isolate	BUSCO gene number	N50	Number of contigs	Length	Number of repeats in library	Length of repeats in library	% of repeats
1111	745	2,177,740	29	34,598,246	698	698,241	37.07
994	746	1,810,452	38	34,860,382	701	698,898	36.89
442	746	1,442,842	53	37,097,739	784	695,809	35.27
4986	747	616,158	132	40,418,191	870	786,120	36.14
4985	745	1,943,489	61	38,708,551	782	721,395	36.64
614	745	1,855,880	42	37,190,968	707	709,228	40.67
708	745	630,186	99	35,092,322	717	658,606	36.15
2407	746	2,184,225	35	35,426,190	683	697,985	37.35
2185	745	2,649,086	27	37,296,702	722	691,397	39.10
293	744	2,399,949	18	38,552,003	797	794,881	37.19
45	747	2,271,831	29	36,800,457	710	679,033	38.96
267	**749**	**2,204,431**	**18**	**31,995,477**	**109**	**123,601**	**3.70**

Table S4.

Information on samples used in the PoolSeq, including the isolates full name, country of origin, substrate they were recovered from (wall and bat with information on bat species). CCF numbers refer to the accession number of isolates obtained from the Culture Collection of Fungi, Prague, Czech Republic.

No.	Clade	Full name	Country	From
1	*Pd*-1	Gd_00015-l2aea	Luxembourg	Bat (*Myotis myotis*)
2	*Pd*-1	Gd_00026-2aeaaa	France	Bat (*M. myotis*)
3	*Pd*-1	Gd_00030-2aaaa	France	Bat (*M. myotis*)
4	*Pd*-1	Gd_00048-caaa	France	Bat (*Myotis mystacinus*)
5	*Pd*-1	Gd_00085-baa	Germany	Bat (*M. myotis*)
6	*Pd*-1	Gd_00161-dgbaa	Bulgaria	Wall
7	*Pd*-1	Gd_00177-accaaa	Austria	Bat (*M. myotis*)
8	*Pd*-1	Gd_00185-abeaa	Germany	Bat (*M. myotis*)
9	*Pd*-1	Gd_00194-a2abaa	Netherlands	Bat (*Myotis dasycneme*)
10	*Pd*-1	Gd_00198-bdac	Germany	Bat (*M. myotis*)
11	*Pd*-1	Gd_00221-bbaa	Croatia	Wall
12	*Pd*-1	Gd_00281-aadaa	Ukraine	Bat (*M. myotis*)
13	*Pd*-1	Gd_00537-adaa	Germany	Bat (*M. myotis*)
14	*Pd*-1	Gd_00559-abaa	Germany	Bat (*M. myotis*)
15	*Pd*-1	Gd_00562-bbaa	Germany	Bat (*M. myotis*)
16	*Pd*-1	Gd_00593-ccaa	Germany	Bat (*M. myotis*)
17	*Pd*-1	Gd_00619-baaa	Poland	Wall
18	*Pd*-1	Gd_00630-blaa	Sweden	Bat (*Myotis brandtii*)
19	*Pd*-1	Gd_00642-aeaa	Hungary	Wall
20	*Pd*-1	Gd_00661-bdaa	Germany	Wall
21	*Pd*-1	Gd_00709-aaaaa	Poland	Wall
22	*Pd*-1	Gd_00759-aaac	Poland	Bat (*M. myotis*)
23	*Pd*-1	Gd_00819-ceaa	Germany	Wall
24	*Pd*-1	Gd_00886-aaaa	Italy	Wall
25	*Pd*-1	Gd_01038-bdaa	Slovakia	Bat (*M. myotis*)
26	*Pd*-1	Gd_01064-aaaa	Hungary	Bat (*M. myotis*)
27	*Pd*-1	Gd_01077-bbaa	France	Bat (*M. myotis*)
28	*Pd*-1	Gd_01094-bbaa	France	Bat (*M. myotis*)
29	*Pd*-1	Gd_01095-acaa	France	Bat (*M. myotis*)
30	*Pd*-1	Gd_01144-baaa	Switzerland	Bat (*M. myotis*)

No.	Clade	Full name	Country	From
31	*Pd*-1	Gd_01148-bcaaa	Hungary	Bat (*Myotis blythii*)
32	*Pd*-1	Gd_01244-acaab	Belgium	Bat (*M. mystacinus*)
33	*Pd*-1	Gd_01248-acaaa	Germany	Bat (unknown)
34	*Pd*-1	Gd_01253-acbaa	Ukraine	Bat (*M. myotis*)
35	*Pd*-1	Gd_01393-abaa	Germany	Bat (*M. myotis*)
36	*Pd*-1	Gd_01719-acaa	Poland	Bat (*M. myotis*)
37	*Pd*-1	Gd_01770-aaaa	Germany	Bat (*M. myotis*)
38	*Pd*-1	Gd_01794-abaa	Germany	Bat (*M. myotis*)
39	*Pd*-1	Gd_01880-aaaa	Germany	Bat (*M. myotis*)
40	*Pd*-1	Gd_01882-adaa	Germany	Bat (*M. myotis*)
41	*Pd*-1	Gd_01933-aaacaaa	Finland	Bat (*M. brandtii*)
42	*Pd*-1	Gd_01952-afaa	France	Bat (*M. myotis*)
43	*Pd*-1	Gd_01990-adaa	Romania	Bat (*M. myotis/M. blythii*)
44	*Pd*-1	Gd_01992-adaa	Romania	Bat (*M. myotis/M. blythii*)
45	*Pd*-1	Gd_02008-aaaaac	Republic of Moldova	Bat (*M. blythii*)
46	*Pd*-1	Gd_02032-aaaa	Romania	Bat (*M. myotis/M. blythii*)
47	*Pd*-1	Gd_02056-aaaaa	Slovakia	Bat (unknown)
48	*Pd*-1	Gd_02330-abaa	France	Bat (*M. myotis*)
49	*Pd*-1	Gd_02392-aaaa	France	Bat (*M. myotis*)
50	*Pd*-1	Gd_02441-aaaaa	France	Bat (*M. myotis*)
51	*Pd*-1	Gd_02455-aaaac	France	Bat (*M. myotis/M. blythii*)
52	*Pd*-1	Gd_02465-acaa	France	Bat (*M. myotis*)
53	*Pd*-1	Gd_02472-abaa	Poland	Bat (*M. myotis*)
54	*Pd*-1	Gd_02473-aaaa	France	Bat (*M. myotis*)
55	*Pd*-1	Gd_02486-aaaa	France	Bat (*M. myotis*)
56	*Pd*-1	Gd_02496-aaaa	Denmark	Bat (*M. dasycneme*)
57	*Pd*-1	Gd_02501-aaab	Denmark	Bat (*M. dasycneme*)
58	*Pd*-1	Gd_02601-aaaa	Austria	Bat (*M. myotis*)
59	*Pd*-1	Gd_02627-aca	Estonia	Bat (*M. brandtii*)
60	*Pd*-1	Gd_02638-aaa	Sweden	Bat (*Myotis nattereri*)
61	*Pd*-1	Gd_02648-abb	Latvia	Bat (*Plecotus auritus*)
62	*Pd*-1	Gd_02702-aeaa	Bulgaria	Bat (*M. blythii*)
63	*Pd*-1	Gd_02746-afaa	Bulgaria	Bat (*M. blythii*)
64	*Pd*-1	Gd_02915-aaa	Germany	Bat (*M. myotis*)
65	*Pd*-1	Gd_04129-caaa=CCF-4129	Czech Republic	Bat (*M. myotis*)
66	*Pd*-1	Gd_04994-cbab=CCF-4994	Czech Republic	Bat (*M. myotis*)
67	*Pd*-1	Gd_05003-daaa=CCF-5003	Czech Republic	Bat (*M. myotis*)

No.	Clade	Full name	Country	From
68	*Pd*-1	Gd_05149-cbaa=CCF-5149	Czech Republic	Wall
69	*Pd*-1	Gd_05163-caaa=CCF-5163	Czech Republic	Bat (*M. myotis*)
1	*Pd*-2	Gd_00045-a2abea	Germany	Bat (*Myotis daubentonii*)
2	*Pd*-2	Gd_00164-bacaaaa	England	Bat (*M. daubentonii*)
3	*Pd*-2	Gd_00259-acbaa	Germany	Bat (*M. mystacinus*)
4	*Pd*-2	Gd_00436-baaa	Bulgaria	Bat (*M. myotis/M. blythii*)
5	*Pd*-2	Gd_00518-cbaaa	Germany	Bat (*Myotis nattereri*)
6	*Pd*-2	Gd_00528-beaaa	Hungary	Bat (*M. myotis*)
7	*Pd*-2	Gd_00539-abaa	Germany	Bat (*M. myotis*)
8	*Pd*-2	Gd_00539-acaa	Germany	Bat (*M. myotis*)
9	*Pd*-2	Gd_00573-aaaa	Poland	Bat (*M. myotis*)
10	*Pd*-2	Gd_00614-baacaaa	Spain	Wall
11	*Pd*-2	Gd_00708-baaaa	Poland	Wall
12	*Pd*-2	Gd_00708-caaab	Poland	Wall
13	*Pd*-2	Gd_00763-aaabb	Poland	Bat (*Myotis nattereri*)
14	*Pd*-2	Gd_00785-abaaa	Germany	Bat (*M. daubentonii*)
15	*Pd*-2	Gd_00953-aaaa	Germany	Bat (*M. myotis*)
16	*Pd*-2	Gd_00956-bcaa	Germany	Bat (*M. myotis*)
17	*Pd*-2	Gd_01031-aaaa	Germany	Bat (*M. myotis*)
18	*Pd*-2	Gd_01056-adaaaaa	Germany	Bat (*M. myotis*)
19	*Pd*-2	Gd_01089-abab	France	Bat (*M. mystacinus*)
20	*Pd*-2	Gd_01238-acaaaaa	Germany	Bat (*M. myotis*)
21	*Pd*-2	Gd_01249-aabaa	Germany	Bat (unknown)
22	*Pd*-2	Gd_01250-aaaaa	Germany	Bat (unknown)
23	*Pd*-2	Gd_01370-acaa	Germany	Bat (*Myotis nattereri*)
24	*Pd*-2	Gd_01395-aaaa	Germany	Bat (*M. daubentonii*)
25	*Pd*-2	Gd_01416-aaaaa	Germany	Bat (*M. daubentonii*)
26	*Pd*-2	Gd_01424-aaaa	Germany	Bat (*Myotis nattereri*)
27	*Pd*-2	Gd_01514-adab	Germany	Bat (*M. myotis*)
28	*Pd*-2	Gd_01561-aaaa	Germany	Bat (*M. daubentonii*)
29	*Pd*-2	Gd_01600-aaab	Denmark	Bat (*M. daubentonii*)
30	*Pd*-2	Gd_01600-abaa	Denmark	Bat (*M. daubentonii*)
31	*Pd*-2	Gd_01604-aaaaa	France	Bat (*M. myotis*)
32	*Pd*-2	Gd_01604-acaaa	France	Bat (*M. myotis*)
33	*Pd*-2	Gd_01636-adaa	Germany	Bat (*M. daubentonii*)
34	*Pd*-2	Gd_01657-adaa	Belgium	Bat (*M. myotis*)
35	*Pd*-2	Gd_01813-abaa	France	Bat (*M. myotis*)

No.	Clade	Full name	Country	From
36	*Pd*-2	Gd_01815-abaa	France	Bat (*M. myotis*)
37	*Pd*-2	Gd_01844-daaa	Spain	Wall
38	*Pd*-2	Gd_01844-dbaa	Spain	Wall
39	*Pd*-2	Gd_01854-acaa	Latvia	Bat (*M. daubentonii*)
40	*Pd*-2	Gd_01855-aaaa	Latvia	Bat (*M. daubentonii*)
41	*Pd*-2	Gd_01937-abbaa	Finland	Bat (*Myotis nattereri*)
42	*Pd*-2	Gd_01938-acaaa	Finland	Bat (*M. daubentonii*)
43	*Pd*-2	Gd_02185-acaa	Bulgaria	Bat *(Myotis blythii*)
44	*Pd*-2	Gd_02351-aaaa	France	Bat *(Myotis alcathoe)*
45	*Pd*-2	Gd_02450-abaaab	France	Bat (*Myotis capaccinii*)
46	*Pd*-2	Gd_02475-abab	France	Bat (*M. myotis*)
47	*Pd*-2	Gd_02475-acaaaa	France	Bat (*M. myotis*)
48	*Pd*-2	Gd_02506-abaa	Denmark	Bat (*M. daubentonii*)
49	*Pd*-2	Gd_02630-acaab	Sweden	Bat (unknown)
50	*Pd*-2	Gd_02631-abaaa	Sweden	Bat (*Myotis nattereri*)
51	*Pd*-2	Gd_02632-aaaab	Sweden	Bat (*M. mystacinus/M. brandtii*)
52	*Pd*-2	Gd_02633-acaaa	Sweden	Bat (*Myotis nattereri*)
53	*Pd*-2	Gd_02642-aaa	Latvia	Bat (*M. daubentonii*)
54	*Pd*-2	Gd_02642-aba	Latvia	Bat (*M. daubentonii*)
55	*Pd*-2	Gd_02643-aaa	Latvia	Bat (*M. daubentonii*)
56	*Pd*-2	Gd_02644-aaa	Latvia	Bat (*M. daubentonii*)
57	*Pd*-2	Gd_02646-aca	Latvia	Bat (*M. daubentonii*)
58	*Pd*-2	Gd_02647-aaa	Latvia	Bat (*M. daubentonii*)
59	*Pd*-2	Gd_02650-aaa	Norway	Bat (*M. mystacinus/M. brandtii*)
60	*Pd*-2	Gd_02652-aaa	Norway	Bat (*Eptesicus nilssonii*)
61	*Pd*-2	Gd_04993-daaa=CCF-4993	Czech Republic	Bat (*M. myotis*)
62	*Pd*-2	Gd_05012-eaaaa=CCF-5012	Czech Republic	Bat (*M. myotis*)
63	*Pd*-2	Gd_05021-cbaaa=CCF-5021	Czech Republic	Bat (*M. myotis*)

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Author for correspondence:
Sebastien J. Puechmaille
e-mail: sebastien.puechmaille@umontpellier.fr

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Pathogen biology

Seasonal patterns of *Pseudogymnoascus destructans* germination indicate host–pathogen coevolution

Nicola M. Fischer[1,2], Serena E. Dool[1] and Sebastien J. Puechmaille[1,2]

[1]Zoological Institute and Museum, University of Greifswald, 17489 Greifswald, Germany
[2]Institut des Sciences de l'Évolution Montpellier (ISEM), University of Montpellier, CNRS, EPHE, IRD, 34095 Montpellier, France

NMF, 0000-0003-4056-1032; SED, 0000-0003-4728-4154; SJP, 0000-0001-9517-5775

Emerging infectious diseases rank among the most important threats to human and wildlife health. A comprehensive understanding of the mode of infection and presence of potential reservoirs is critical for the development of effective counter strategies. Fungal pathogens can remain viable in environmental reservoirs for extended periods of time before infecting susceptible individuals. This may be the case for *Pseudogymnoascus destructans* (*Pd*), the causative agent of bat white-nose disease. Owing to its cold-loving nature, this fungal pathogen only grows on bats during hibernation, when their body temperature is reduced. Bats only spend part of their life cycle in hibernation and do not typically show signs of infection in summer, raising the question of whether *Pd* remains viable in hibernacula during this period (roughly six months). If so, this could facilitate the re-infection of bats when they return to the sites the following winter. In a laboratory experiment, we determined the germination rate of *Pd* spores kept under constant conditions on a wall-like substrate, over the course of two years. Results showed that the seasonal pattern in *Pd* germination mirrored the life cycle of the bats, with an increased germination rate at times when hibernating bats would naturally be present and lower germination rates during their absence. We suggest that *Pd* is dependent on the presence of hibernating bats and has therefore coupled its germination rate to host availability. Furthermore, we demonstrate that *Pd* spores survive extended periods of host absence and can remain viable for at least two years. There is, however, a strong decrease in spore viability between the first and second years (98%). *Pd* viability for at least two years on a solid mineral-based substrate establishes the potential for environmental reservoirs in hibernacula walls and has strong implications for the efficacy of certain management strategies (e.g. bat culling).

1. Background

Emerging infectious diseases are a severe conservation threat for a variety of plants and animals. Among them, an unprecedented number of fungal diseases have recently caused some of the most severe die-offs and extinctions ever witnessed in wild species [1]. They pose difficult challenges to conservation owing to their typically unanticipated and rapid onset, combined with high mortality rates [2]. The pathogens responsible are generally only identified after disease outbreaks are reported. This puts scientists at a considerable disadvantage, as crucial information on the pathogens' life cycle and mode of infection is entirely lacking when measures to treat and prevent the outbreak are badly needed [3]. One important factor influencing disease outcome is the ability of the pathogen to produce long-lived environmental stages such as spores [4]. This

characteristic has been associated with an increased risk of host extinction and is commonly found in fungal pathogens such as *Fusarium solani* (infecting the eggs of sea turtles [5]) and *Batrachochytrium dendrobatidis* (causing chytridiomycosis in amphibians [6]).

The presence of an environmental reservoir consisting of long-lived spores has also been suggested for the ascomycete fungus *Pseudogymnoascus destructans* (*Pd*), the causative agent of bat white-nose disease [7]. Since 2006, the disease has caused massive die-offs in North-American bats, where the pathogen is an invasive species of European origin [8]. *Pd* is a cold-loving fungus, only able to survive and grow on bats while their body temperature is reduced, mainly during hibernation. It is then purged from active bats, typically during summer [9,10]. Puechmaille *et al*. [7] hypothesized that bats entering swarming and/or hibernation sites in autumn could become contaminated with *Pd* spores left on the walls from bats which were infected during the previous winter. This hypothesis of autumnal contamination is supported by qPCR data demonstrating increases in prevalence of *Pd* on bats in the autumn [9]. However, the source of the contamination, and whether hibernacula walls play a role as environmental reservoirs, remain to be elucidated. If infection from the environment is occurring, it is likely to play a major role in the disease dynamics. One of the main prerequisites for the presence of an environmental reservoir in this host–pathogen system is the ability of *Pd* spores to survive when bats are absent during the summer, and to retain the ability to germinate following subsequent contact with bats.

Here, we tested the ability of *Pd* spores to remain viable in environments resembling the walls of bat hibernacula. We hypothesize that spores remain viable for longer than six months, the period when bats are active and unsuitable hosts for *Pd*. Furthermore, variations in germination rates through time are expected if host-presence shapes the life cycle of the pathogen, as germination at times when bats are active would be maladaptive for the fungus. If this is the case, germination should be highest when bats are in hibernation and lowest during bats' active season.

2. Methods

(a) Experimental setup

The experimental setup in the laboratory was designed with respect to the annual life cycle of bats, in order to track the germination pattern of *Pd* in relation to bat presence/absence in the hibernacula. The overall setup consisted of cement pieces (a solid mineral-based substrate mimicking hibernacula walls) inoculated with spores from six *Pd* isolates stored in sealed 50 ml falcon tubes (40 pieces per tube, 5 tubes per isolate; see electronic supplementary material). These were incubated at 10°C in saturated humidity, as typically encountered in bat hibernacula, and 'sampled' at predetermined intervals to assess the germination rate of spores over time. More specifically, the *Pd* isolates that were used in the experiment had been incubated on culture medium for three to five months (depending on the isolate), which corresponds to the expected duration when *Pd* can grow on hibernating bats. After harvest from the culture medium, the spores were transferred onto cement pieces. This process was intended to mimic the transfer of spores from a highly infected bat onto the hibernacula wall at the end of hibernation in late winter. For this reason, the first sampling date (1 day after inoculation of cement pieces) was considered as late winter in terms of the bat life cycle and seasonality followed accordingly (see electronic supplementary material for further information). Thereafter, sampling of cement pieces and estimations of *Pd* germination rate were conducted at three-month intervals to ensure a good temporal coverage of periods of bat absence and presence at hibernacula, which span roughly six months each. The experiment was continued over the course of 24 months with nine sampling dates in total. During each sampling event, 20 cement pieces (four from each of the five falcon tubes) were collected from each of the six isolates. The spores retrieved from cement pieces were diluted in water, plated on dextrose–peptone–yeast agar and stored at 10°C for 14 days, after which the number of resulting colonies was counted (figure 1). This resulted in 120 counts per sampling date and a total of 1080 counts over the course of 2 years (see supplementary material for further details).

(b) Data analyses

A generalized additive mixed model (GAMM) with Poisson distribution was used as counts of *Pd* colonies were nonlinear over time (supplementary material). The 1056 counts (24 counts were removed owing to technical reasons; see electronic supplementary material) were used as a response variable, sampling dates as a smoothed term explanatory variable ($k = 9$) and the origin of spores (i.e. isolate used) and falcon tubes were added as random effects. The smoothing was penalized using cubic regression splines to obtain an optimum amount of smoothness and to avoid over-fitting of data [11]. Additionally, an observation level random effect was added to account for over-dispersion in the count data [12]. All analyses were performed in R version 3.6.0 [13]. The GAMM was fitted using the mgcv package [14] and visualized with the mgcViz [15] and ggplot2 packages [16].

3. Results

Pd germination rates exhibited a nonlinear, bimodal temporal pattern over the course of 24 months (GAMM, intercept = 4.06, s.e. = 0.58, d.f. = 1025, p-value < 0.001; see electronic supplementary material). Although slight variations exist between the six isolates (see electronic supplementary material, table S3), the general pattern consistently shows an increase in germination of spores during the first six months. This is followed by a plateau between 6 and 12 months, after which germination drops sharply (figure 2; electronic supplementary material, figure S4). Germination rates rise again at 18 months and then decline towards 24 months. When *Pd* germination rates are considered in the context of the bat life cycle, there is a clear seasonal pattern of higher germination rates when bats would naturally be present in hibernacula, with lowered rates when they would be absent (figure 2).

Our results also demonstrate that some *Pd* spores remain viable on hibernacula substrates for at least 2 years. However, germination rates decreased by 98% between the first and second years, indicating an important decrease in viability after an annual cycle (electronic supplementary material).

4. Discussion

We were able to demonstrate the viability of *Pd* for at least 2 years on a substrate identical to or closely resembling bat hibernacula surfaces. Although temporal differences in viability of *Pd* spores could be expected within and between hibernacula owing to variations in abiotic and biotic factors, our results clearly indicate that the pathogen can remain

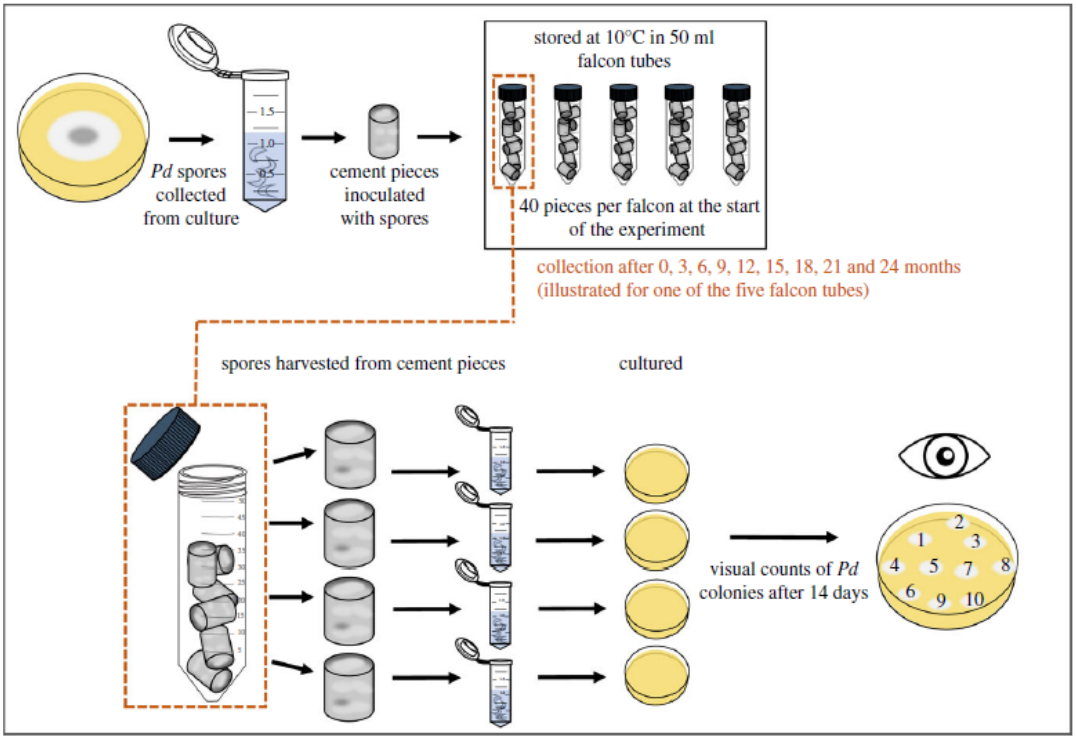

Figure 1. Schematic of experimental setup and procedure used to obtain germination rates of *Pd* over time. Shown for one of the six isolates.

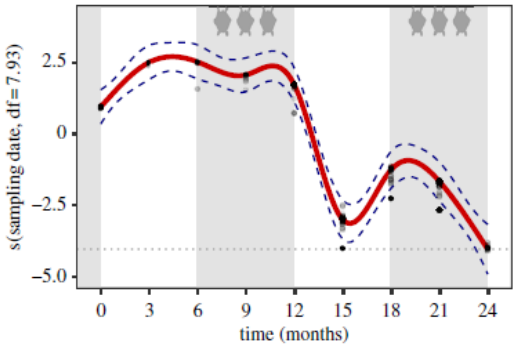

Figure 2. Generalized additive mixed model (solid red line) fitted to germination counts over time. Smoothed term of the counts (*Y*-axis) over time (*X*-axis); 95% confidence intervals (blue dashed lines) and distribution of residuals (semi-transparent points) depicted. Periods in which bats would naturally be hibernating are represented by grey shaded background, while white background represents absence of hibernating bats. The dotted grey line represents observed count of zero. The model is based on counts of intrinsic germination rates with stable temperature and humidity.

viable, as a dormant free-living infectious stage (spore), for longer than the annual life cycle of the host.

Our experimental setup represents a simplified version of hibernacula walls and does not consider differences in temperature and humidity, either temporally or spatially, within and between hibernacula; nor does it include interactions between *Pd* spores and other organisms. However, given that abiotic factors (temperature and humidity) were kept constant in the course of the laboratory study, the obtained germination rates are regarded as intrinsic and would be expected to be similarly expressed under natural conditions in bat hibernacula. We therefore hypothesize that the high germination rate of *Pd*, synchronized with bat hibernation, is the result of a long-term adaptation of the fungus to the presence of suitable hosts that are only present during half of the year. This conclusion is corroborated by field data that clearly demonstrate that *Pd* grows on bats during the hibernation season, while reports of *Pd* growth on bats during the active season are extremely scarce [7]. The observed synchronization between pathogen germination rate and host availability is consistent with long-term coevolution between *Pd* and bats.

While our results indicate a dependence of *Pd* on hibernating bats, it is still unclear if additional biotic hosts play a role in the disease dynamics. However, the presence of an abiotic pathogen reservoir is now undisputable. We have demonstrated the viability of *Pd* spores on hibernacula-like substrates for a period greater than bats absence, showing that hibernacula walls play a role as environmental reservoirs from which bats may become infected. In other studies, PCR assays revealed the presence of *Pd* DNA in hibernacula sediments up to two years after the extirpation of bats [17] and *Pd* has also been found to grow in sediments under laboratory conditions [18], showing that sediments may represent an additional *Pd* reservoir. However, biotic interactions between fungal spores and other microorganisms are likely to be pronounced in sediments relative to hibernacula walls [19] and the ratio of *Pd* to other fungal spores is magnitudes higher on walls compared to in sediments.

Attempts to isolate *Pd* from sediments in a hibernaculum had a less than 1% success rate, whereas roughly 50% of samples collected from the walls of the same site contained viable *Pd* (NM Fischer, SJ Puechmaille 2017, unpublished data), suggesting that bats are far more likely to get infected with *Pd* from hibernacula walls rather than sediment. Based on what is currently known about bat ecology and behaviour, physical contact between bats and sediments is a rare event in most species, suggesting that sediments would be unlikely to play an important role in *Pd* infection or disease dynamics. Collectively, the available evidence indicates that hibernacula walls are the main reservoir responsible for infecting bats with *Pd* at the start of each hibernation period.

Classical theories of host–pathogen dynamics predict that diseases do not usually result in host extinction, as virulence is expected to be density dependent and to decrease once a sufficiently low population threshold is reached [4,20,21]. For this reason, a common management strategy to deal with infectious wildlife diseases is to cull infected or susceptible individuals to attain this sufficiently low population threshold and reduce the number of infectious individuals [22]. However, it has been shown that density dependence is not applicable when transmission to the host is indirect via pathogen reservoirs, which may be biotic (i.e. alternative host/reservoir species) or abiotic (i.e. environmental reservoirs) [4]. When pathogen reservoirs exist, the culling of individuals would not lead to a reduction of disease occurrence but would rather decrease the host population size even further and hence exacerbate the progression and impact of the disease [23]. It is, however, important to mention that some form of density dependence could still be acting even if transmission is indirect via a pathogen reservoir (e.g. walls). For example, smaller bat populations could be associated with a lowered rate of spore transfer from the bats to the hibernacula walls in late winter. This would decrease the amount of *Pd* spores in the environmental reservoir and hence the probability for bats to get infected from the walls the following autumn. Nevertheless, a study based on empirical field data from Europe did not detect a correlation between the number of hibernating bats and the proportion of individuals visibly infected by *Pd* [24]. Future studies should therefore clarify the importance of density dependence in the disease dynamics.

Informed decision-making and management strategies require a comprehensive understanding of all existing disease reservoirs and their respective contribution to disease transmission. Therefore, the results of our experimental work have major implications for conservation and emphasize the need to better characterize the contribution of walls as environmental reservoirs and/or passive vectors of infection. The available data suggest that infection is happening late in the season (autumn, early winter) [9]. However, the exact timing, location (swarming versus hibernation), mode (indirect versus a combination of direct and indirect) and dynamics (single versus multiple infections) of infection are not clearly known. Such knowledge would greatly strengthen our understanding of the dynamics involved in white-nose disease and inform evidence-based conservation management strategies.

Data accessibility. The dataset supporting this article has been uploaded as part of the electronic supplementary material.

Authors' contributions. S.J.P conceived the study. N.M.F. and S.J.P. designed the study; S.J.P. acquired funding and supervised the project; N.M.F. (80%), S.E.D (15%) and S.J.P (5%) carried out the laboratory analyses; N.M.F. performed data analyses. N.M.F. and S.J.P. interpreted the results and wrote the original manuscript. All authors critically discussed and edited the manuscript, approved its final version, and agree to be held accountable for the content therein.

Competing interests. We declare we have no competing interests.

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Supplementary information accompanying the manuscript

Seasonal patterns of *Pseudogymnoascus destructans* germination indicate host-pathogen coevolution

Nicola M. Fischer[1,2], Serena Dool[1], Sébastien J. Puechmaille[1,2]*

[1]Zoological Institute and Museum, University of Greifswald, Greifswald, Germany
[2]Institut des Sciences de l'Évolution Montpellier (ISEM), University of Montpellier, CNRS, EPHE, IRD, Montpellier, France

*corresponding author

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This appendix is structured into two parts: Supplementary information accompanying materials and methods used in the experimental setup (Section A1: Methods) and additional results obtained from the data (Section A2: Results). In addition to this text, the R script used for analyses as well as a csv file containing the raw data (i.e. counts) are provided.

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APPENDIX A1: Methods

In this study, spores of *Pseudogymnoascus destructans (Pd)* were transferred onto cement pieces to be harvested, cultured and quantified at 3-month intervals to elucidate intrinsic germination rates over time. The first section of the supplementary methods (A1.1) describes the processes involved in the setup of the experiment including further information on the isolates used and the steps needed to inoculate cement pieces with their spores. These steps were carried out only once before the start of the experiment. The second section (A1.2) explains how the steps conducted during the setup of the experiment correspond to the natural life cycle of the fungus in the wild, ensuring that the experimental findings are biologically relevant. Section A1.3 describes the protocol used during each of the nine sampling dates and explains in detail how the counts for viability over time were obtained.

A1.1: Setup of the experiment prior to sampling

A1.1.1: Isolates used

We used six isolates which had one of four multilocus genotypes (based on 18 microsatellite markers and 3 mating type markers [1]); see Table S1. We used several different isolates to ensure that the true trend in germination rates was captured for this fungal species and not the germination rates specific to particular isolates or geographic regions. The isolates came from two sites separated by more than 1400 km (Table S1). These sites harbour some of the most divergent European populations of this fungus. Thus, by using these isolates, an important part of the diversity of the fungus in its European range is captured (authors' unpublished data). Additionally, both mating types (*MAT1-1* and *MAT1-2*) are represented by these isolates. All isolates were obtained in 2015 from sampling the walls of hibernacula and had been cultured on DYPA medium for 3-5 months before spores were harvested. The isolates used in the experiment can be obtained from the corresponding author (S. J. Puechmaille) upon request.

Table S1. Information on the 6 isolate cultures from which spores were harvested for the experiment.

Solution	Isolate	Genotype	Mating type	Origin	Stored in Falcons
1	Gd_00603-cda	1	MAT1-2	Germany (lat. 54.08, lon. 13.45)	1-5
2	Gd_00533-aaa	2	MAT1-2	Germany (lat. 54.08, lon. 13.45)	6-10
3	Gd_00455-baa	3	MAT1-1	Bulgaria (lat. 43.13, lon. 23.04)	11-15
4	Gd_00454-aba	4	MAT1-1	Bulgaria (lat. 43.13, lon. 23.04)	16-20
5	Gd_00473-aca	1	MAT1-2	Germany (lat. 54.08, lon. 13.45)	21-25
6	Gd_00476-aca	2	MAT1-2	Germany (lat. 54.08, lon. 13.45)	26-30

A1.1.2: Making uniform spore solutions and the inoculation of cement pieces

In order to inoculate cement pieces with equal numbers of spores from the isolates (see A1.1.1), it was necessary to prepare solutions which contained the same concentration of spores, i.e. uniform spore solutions. These spore solutions were made following standard microbiological sterile techniques (i.e. flaming off or autoclaving of used materials and use of sterile filter tips) in a laminar flow hood (Heraeus Instruments). Spores were collected from cultures grown on DYPA medium following Verant *et al.*[2] by flooding the culture with 2 mL phosphate buffered saline containing 0.5% Tween® 20 (PBST). The Tween® 20 prevents the formation of large spore clumps. The surface of the colony was gently rubbed with a sterile inoculation loop until spores became suspended in the liquid. Spores were homogenized using a 2.5 mL syringe without a needle.

Cement pieces needed to be inoculated with 14000 spores to ensure that there would be sufficient spores for the duration of the experiment. To achieve this, each of the homogenized spore solutions was counted 20 times in a counting chamber (Thoma, depth 0.02 mm, smallest square 1/400 mm2). Then the six solutions were diluted so that 10 µL of each solution contained 14000 spores, which was the volume used for the inoculation of each of the 1,080 cement pieces. The use of custom-made cement pieces facilitated the easy storage and regular sampling of the isolates from independent falcons.

The custom-made cement pieces were made as follows: cylindrical cement pieces (width = 0.75 cm, height = 0.91 cm) were made from 44.78% model plaster ("Modellbauergips" Toom, Germany), 29.85% dried builders sand and 25.37% distilled water. Before inoculation, the cement pieces were autoclaved and subsequently dried at 60°C. After inoculation with 10 µl of the diluted spore solutions (1400 spores/µl), the pieces were transferred into 50 mL falcon tubes (numbered from 1-30, Table S1) which were sealed with ParafilmM® (Pechiney Plastic Packaging) and stored in the dark at a constant temperature of 10°C. Thermochron iButtons (DS 1922L-F5#) were placed in a control falcon containing cement pieces inoculated only with distilled water to record relative humidity throughout the experiment. However, saturation of humidity caused the iButton sensors to fail. Nevertheless, drops of water were seen on the walls and/or lids of the falcon tubes during each sampling date, indicating that relative humidity continued to be saturated.

We used a high number of replicates, per isolate and per sampling date (30 falcons containing a total of 1080 cement pieces) to ensure that trends in the data would be revealed despite the possibility of stochastic variations introduced during inoculation and culturing (see section A1.3). Additionally, long-term experiments often experience unforeseen events. Using many different falcons ensured that bacterial contamination or other problems were contained and that the experiment could be continued following such an event.

A1.1.3: Negative Control

In addition to the large number of falcons containing inoculated cement pieces, two falcons with cement pieces were designated as controls for *Pd* cross-contamination and contamination with other fungi or bacteria when plating the spores. Thus, control cement pieces were treated with 10 µL sterile Milli-Q-H_2O at the beginning of the experiment (performed at the same time as the inoculation of cement pieces with *Pd* spores).

A1.2: Seasonality

To define the timing of the experiment, we followed the key events in the phenology of the fungus in the wild. The same key events were imitated in the lab but adjusted to lab conditions:

Table S2. Key events in the phenology of *P. destructans* in the wild and how the setup of the experiment corresponds to them with regard to timing.

In the wild	In the experiment
1 - The large majority of bats get infected by the fungus in autumn/early winter.	1 - Spores were plated on culture medium.
2 - During hibernation (3-6 months), the fungus develops on bats and produces large amounts of asexual spores (see [3] and [4]).	2 - Cultures grew for 3-5 months at 10°C and saturated humidity (conditions commonly encountered in bat hibernacula) on sealed petri-dishes containing DYPA medium. They produced large amounts of asexual spores.
3 - When spring returns, bats emerge from hibernation and a fraction of the spores they carry are transferred (by contact/gravity) to the walls (and ground) of hibernacula.	3 - Spores were transferred from the culture onto the cement pieces.

As a result, the designation of late winter/spring at the start of the experiment (point '0' in Fig. 2 and Fig. S3) is based on the transfer of spores onto the cement pieces (step 3 in the table above), which represents a discrete event when bats arouse from hibernation and emerge from the hibernacula. Hence, the first sampling date can be seen as late winter/spring and seasonality follows from there with sampling events every 3-months.

A1.3: Experimental procedure

A1.3.1: Protocol performed on each sampling date

After the setup of the experiment (A1.1), spores were harvested from cement pieces every three months for two years (nine sampling events in total) using the following protocol:

1. **Sampling of cement pieces.** Four cement pieces were retrieved from each falcon resulting in 20 pieces per isolate and 120 pieces per sampling date overall. (Each of the six isolates had five falcon tubes).
2. **Spore retrieval.** Cement pieces were individually incubated at room temperature in 500 µL sterile Milli-Q-H_2O in a 5 mL tube for 30 minutes. After vortexing for 30 sec. at medium speed (Vortex Genie 2, Scientific Industries), the soaked cement piece was moved to a sterile 1.5 mL conical tube, while the liquid containing spores was retained in the 5 mL. In 1.5 mL conical tubes, the cement piece is too bulky to descend into the base of the tube and remains physically lodged above the 500 µL graduation. The tube can then be centrifuged at 5000 rpm for 1 min. to retrieve any spores/solution remaining on the cement piece, which can collect in the tube below the cement piece. After this step the cement piece was removed and discarded. The two aliquots (from the 5 mL and the 1.5 mL) were then combined.
3. **Spore culture.** The spore solutions from step 2. were cultured on 6 cm DPYA petri dishes in two dilutions (detailed in A1.3.2). Petri-dishes were sealed using ParafilmM® (Pechiney Plastic Packaging) and stored upside down in the dark at 10°C.
4. **Spore germination**. Spores were allowed to grow for fourteen days after plating, after which the number of colonies on each petri dish was counted. These counts were used in the analyses and are available as electronic supplementary material ("Viability.csv").
5. **Controls.** Control treatments (see also A1.1.3) consisted of cement pieces 'inoculated' with water (i.e. the negative control) kept in falcon tubes and treated identically to experimental falcons, except that three cement pieces were retrieved on each sampling date instead of four. That is, steps 2-4 were followed using the same equipment (e.g. tweezers, spreaders) used for the experimental falcons.

A1.3.2: Culturing details – volume adjustments and replicates to ensure accuracy and precision

Germination rates were expected to vary over the course of the experiment. However, the timing and magnitude of such changes was unknown *a priori*. Using the same volumes for plating (e.g. A1.3.1 step 3) throughout the experiment would allow the estimation of the concentration of germinating spores to be accurate but not precise. With changing rates of germination, this strategy would run the risk of plating too few spores when germination rates are low or too many spores when germination rates are high, and both would result in imprecise growth estimates. Therefore, to obtain precise estimates of germination rates, plating volumes were adjusted throughout the experiment. Combined with the high number of replicates (20 cement pieces per isolate/date), this strategy ensured a high precision and accuracy in the estimation of the concentrations of germinating spores.

The ideal plating volume can be expected to differ depending on isolate and date. As these ideal values cannot be known *a priori*, we used two different dilution volumes based on the number of germinating spores counted during the previous sampling date; one anticipating increased growth and one decreased. In this way, both an increase and decrease in viability from the previous sampling date would be quantifiable. These dilutions were adjusted at each sampling date for each isolate.

On the first sampling date, the two dilution volumes used were chosen based on previous experiments and author's unpublished data. These were: no dilution (100 µL solution plated), and 1/5 dilution (20 µL solution + 80 µL sterile Milli-Q-H_2O).

This strategy resulted in two counts for each cement piece at each sampling date. For the analyses, we used the count which was closer to 100 colonies per culture as it provided the optimal conditions for reliable counts. This is because too many colonies on a plate result in colonies growing in close proximity or over one another which leads to less accurate data; too few colonies (close/equal to zero) result in imprecise estimates (unless all spores are dead in both dilutions).

Counts were then adjusted to take into consideration the volume plated (i.e. the dilution factor). For example, the count of a plate from a 1/5 dilution (20 µL solution) was multiplied by 5 to obtain the number of germinating spores per 100 µL, while the count of a plate with no dilution was left unchanged. In this way, all counts were directly comparable. Therefore, the counts used in the analyses reflect the concentration of viable spores (based on the counts from cultures) in 100 µL of spore harvest for each of the cement pieces. In the csv file containing the raw data we supply the count obtained using a dilution specific to the isolate and the date as well and the factor by which we multiplied this count to obtain the data used in the model (also supplied).

A1.3.3: Counting details

Colony counts during the first and second time points (0 and 3 months) were solely carried out microscopically (10 - 40x). Subsequently, plates were photographed and colonies in the photos were counted visually. Photographs were taken with a digital camera using a customized photo-box (Canon EOS 600 D with 60 mm Canon Macro lens EF-S, shutter speed = 1/6000, aperture = 3.2, ISO = 400, evaluative metering). This approach was validated the first time it was used on the third sampling date at 6 months. Both microscopic and photographic methods were used and while small differences between counts were observed, these never exceeded 7 % of the count (see Fig. S1). In more than half of the cases, the counts were exactly equal.

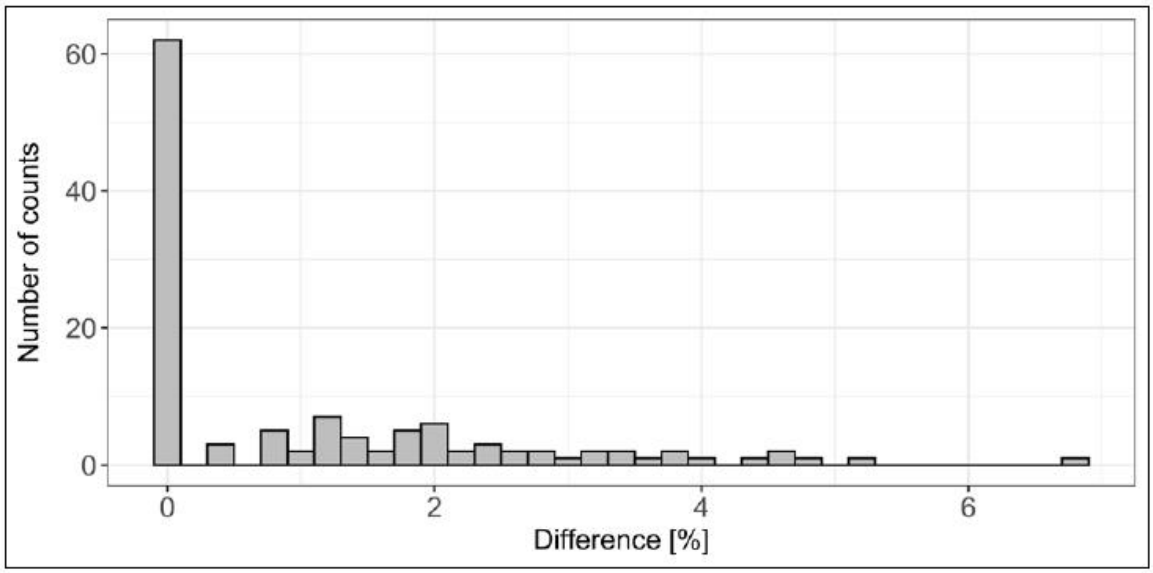

Figure S1. Histogram showing differences (%) between counts of *P. destructans* colonies based on visual counts using a microscope versus photographs (n=120).

All counts were conducted by the same person to avoid observer biases. Colonies were allowed to grow for 14 days as most (if not all) spores had germinated by this time, yet fungal colonies remained small enough not to overgrow each other, facilitating the easy discrimination of distinct colonies (Fig. S2).

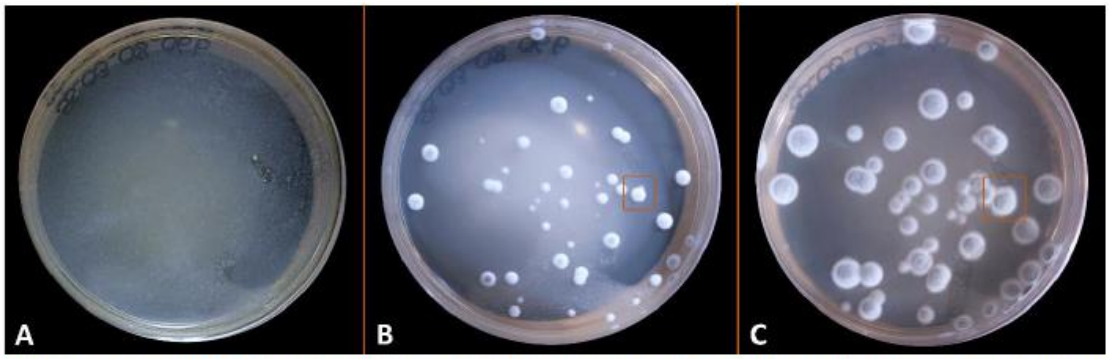

Figure S2. Photographs of the same culture after 7 days (A), 14 days (B) and 21 days (C) of plating. Area highlighted by an orange box in B and C shows 2 colonies of *Pd* which could be discriminated after 14 days, but not after 21 days due to overgrowth.

APPENDIX A2: Additional results

A2.1: Detailed results of viability for each of the isolates

There was no evidence for contamination with either *Pd* or other fungi or bacteria in the control cultures, indicating that the experiment was successfully conducted under sterile conditions. There was however some bacterial contamination on cultures from experimental falcons later in the experiment, which was localized to three falcons. These falcons were completely removed from the experiment on the date the bacterial contamination was observed and were not used subsequently. To avoid biases, the removal affected all cultures (contaminated or not) from the falcon. These removals concerned: falcon 4 (solution 1, removal at 18 months); falcon 16 (solution 4, removal at 21 months); and falcon 18 (solution 4, removal at 24 months). These removals only slightly reduced the number of available counts from 1080 to 1056.

Table S3. Mean number of viable spores per sampling date calculated for each of the isolates, adjusted by dilution factor (i.e. all numbers correspond to a volume of 100 µL/cement piece).

Sampling date [months]	Isolate 1	Isolate 2	Isolate 3	Isolate 4	Isolate 5	Isolate 6
0	46.4	23.8	159.2	214.4	623.0	485.5
3	1131.9	188.5	1072.3	3237.8	3099.5	726.5
6	2219.5	120.3	1769.5	4931.5	1900.5	401.3
9	1542.8	97.7	626.3	6580.5	1577.0	278.2
12	1928.5	60.5	433	5279.0	1049.3	195.1
15	27.5	24.1	12.3	38.1	3.9	0.6
18	229.1	0.1	53.5	1333.2	31.0	3.5
21	518.6	0.0	38.6	769.7	24.3	3.1
24	67.1	0.0	2.95	175.1	0	0.1

Counts of colonies were transformed into 'increase factors' (count at time X/mean count at time 0) to represent the relative change in number of colonies compared to the initial culturing (time point 0). The increase factors revealed non-linearity in counts over time as well as differences between the isolates (Fig. S3). For this reason, the generalized additive mixed model (GAMM) was fitted to the data to allow the determination of the general trend in germination over time.

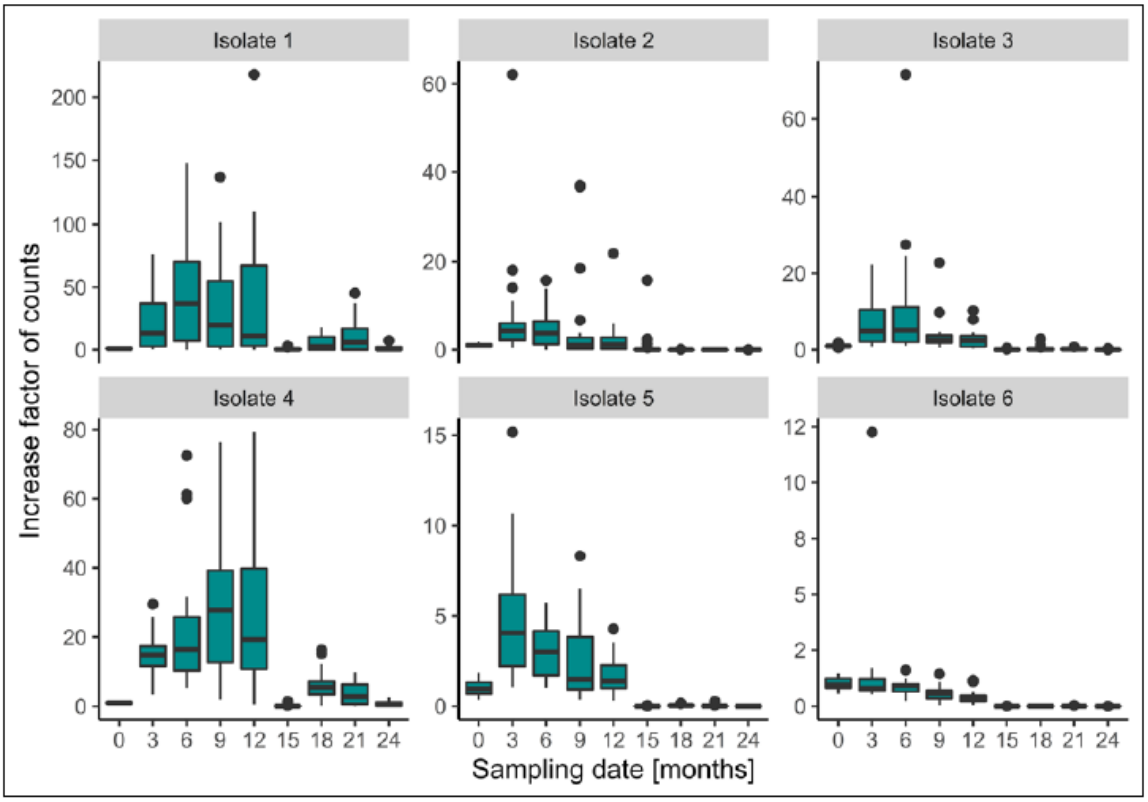

Figure S3. Increase factors (relative increase/decrease of counts compared to initial culturing at the start of the experiment) over time for each of the 6 isolates. Visualization follows classical Tukey representation: bars show interquartile range between 25th and 75th percentile; line in box shows the median of the distribution; upper and lower whiskers represent the largest and smallest value within 1.5 times the interquartile range; and dots show outlier values between 1,5 and 3 times the interquartile range.

A2.2: Verification of strong decline in viability at 15 months

The sampling at 15 months revealed a strong decrease in viability of *Pd* spores which was far more pronounced than the decline at previous sampling dates. Therefore, we wanted to ensure that this was not due to errors in diluting before culturing or any other step of spore harvest for the 15 months' time point. We therefore repeated the culturing for isolates 1 - 4 for this time point, exactly following the protocol always used for these steps (see section A1.3.1). We observed the same pattern (i.e. a sharp reduction in germination). The difference between the two counts was negligible. The most precise estimate (with lowest dilution) is presented in the manuscript. The results from the less precise count (i.e. with high dilution) from this sampling date (Fig. S4) show the same trend as is illustrated in Fig. 2. Hence, a sampling or lab artefact can be ruled out and the results can confidently be inferred to have biological relevance.

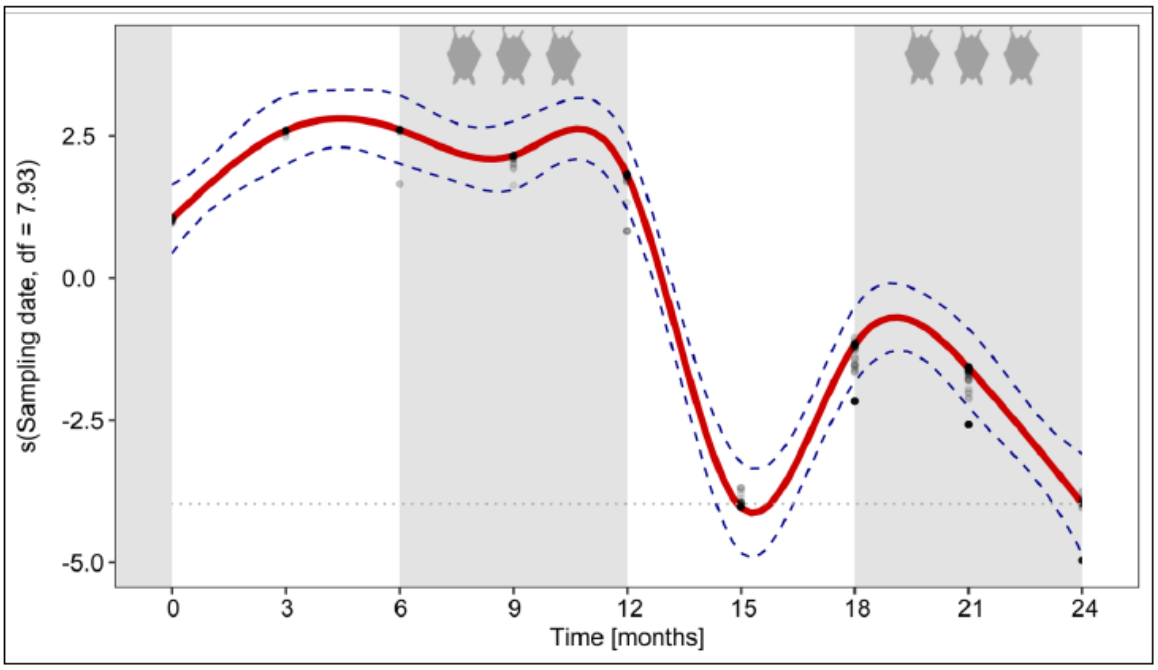

Figure S4. Generalized Additive Mixed Model (solid red line) fitted to germination counts over time. Smoothed term of the counts (Y-axis) over time (X-axis); 95% confidence intervals (blue dashed lines) and distribution of residuals (semi-transparent points) depicted. Periods in which bats would naturally be hibernating are represented by grey shaded background while white background represents absence of hibernating bats. The dotted grey line represents observed count of zero. The model is based on counts of intrinsic germination rates with stable temperature and humidity. The data for the 15 months sampling was obtained from a second round of culturing and quantifying viability to investigate the possibility of sampling artefacts. The data for the other sampling dates is identical to that presented in the manuscript.

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ORIGINAL ARTICLE

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

Nicola M. Fischer[1,2] | Andrea Altewischer[1] | Surendra Ranpal[1] | Serena Dool[1,3] | Gerald Kerth[1] | Sebastien J. Puechmaille[1,2,4]

[1]Zoological Institute and Museum, University of Greifswald, Greifswald, Germany

[2]Institut des Sciences de l'Évolution Montpellier (ISEM), University of Montpellier, CNRS, EPHE, IRD, Montpellier, France

[3]CBGP, INRAE, CIRAD, IRD, Institut Agro, University of Montpellier, Montpellier, France

[4]Institut Universitaire de France, Paris, France

Correspondence
Sebastien Puechmaille, Institut des Sciences de l'Évolution Montpellier (ISEM), University of Montpellier, CNRS, EPHE, IRD, Montpellier, France.
Email: sebastien.puechmaille@umontpellier.fr

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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

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).

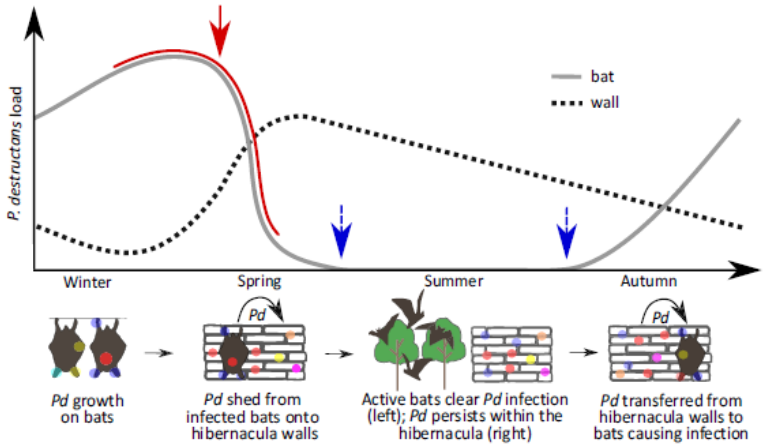

FIGURE 1 Schematic representation of the life cycle of *Pseudogymnoascus destructans* ("*Pd*") on bats (solid grey line) and in an environmental reservoir (hibernaculum; dotted line) throughout a year as predicted from existing literature (e.g., Fuller et al., 2020; Hoyt et al., 2020; Langwig, Frick, 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

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

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

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).

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.

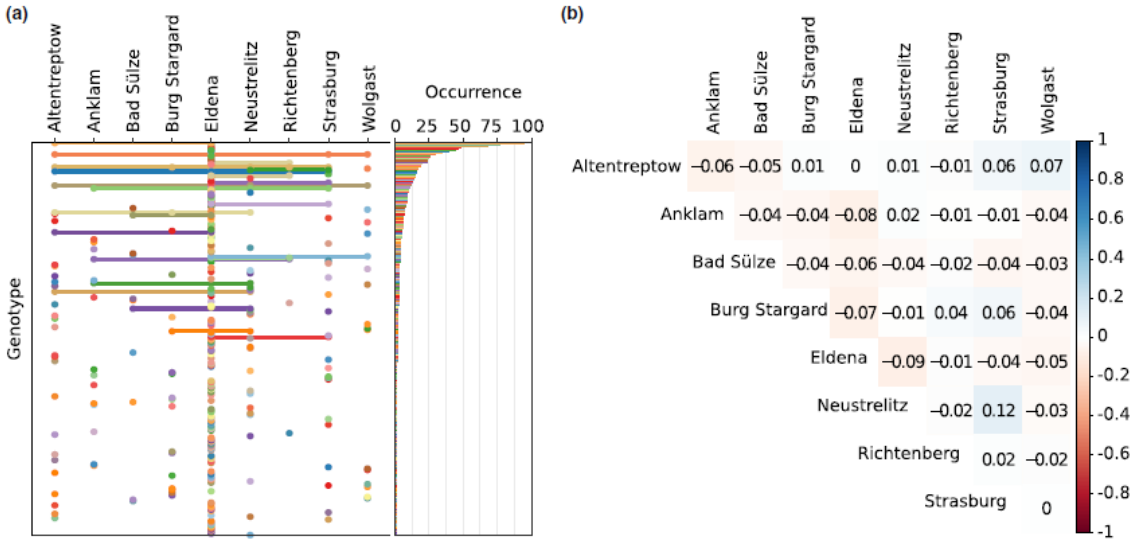

FIGURE 2 *Pseudogymnoascus destructans* genotypic differentiation across hibernacula. (a) Observation of genotypes (y-axis) at different hibernacula (x-axis; dots represent observed genotypes) on the left, and each genotypes frequency of occurrence ("occurrence") across all hibernacula on the right. 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)

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 ($r = 0.88$, $p < .001$).

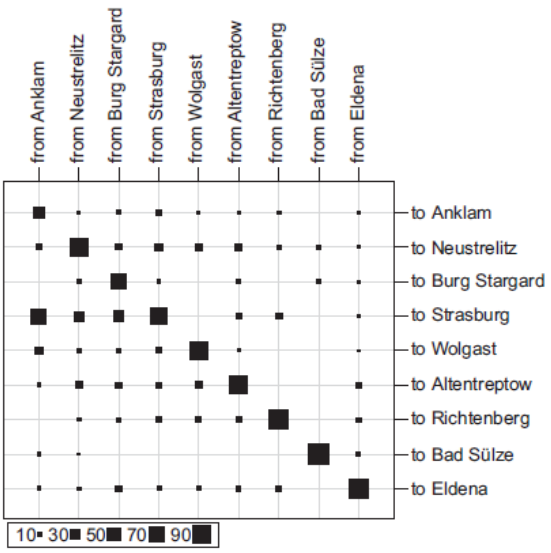

FIGURE 3 Assignment of *Pseudogymnoascus destructans* isolates to hibernacula using a discriminant analysis of principle components (DAPC) approach. Columns correspond to sampled hibernacula while rows correspond to inferred hibernacula (assignment result). Size of filled squares indicates the percentage 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)

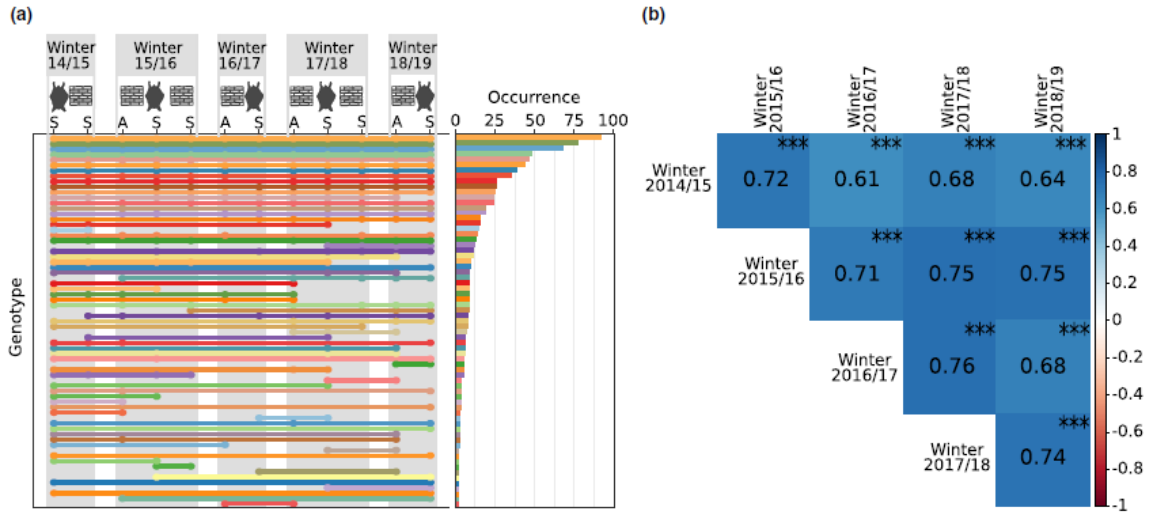

FIGURE 4 *Pseudogymnoascus destructans* genotypic differentiation across time in Eldena hibernaculum. (a) Observation of genotypes (y-axis) at different sampling events (x-axis; dots represent observed genotypes, sampled either in spring [S] or autumn [A] on the substrate [bat or wall] represented above 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)

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.

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 ($t = -2.83$, $df = 4$, $p = .038$)

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

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).

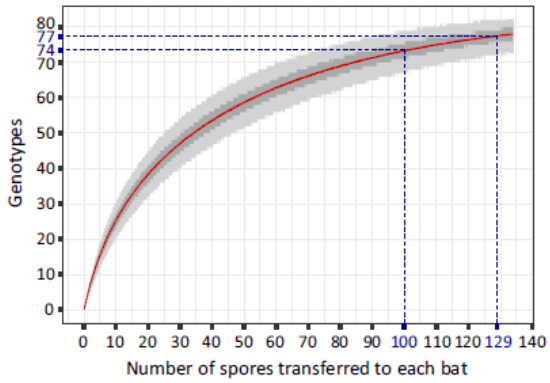

FIGURE 5 Estimated number of *Pseudogymnoascus destructans* spores transferred from walls to each bat in Eldena in the winter 2014/2015 (dashed blue lines). The red line represents the estimated mean number of spores transferred from the walls to each bat (x-axis) in relation to the total number of genotypes 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

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

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.

OPEN RESEARCH BADGES

This article has earned an Open Data badge for making publicly available the digitally-shareable data necessary to reproduce the reported results. The data is available at: https://doi.org/10.5061/dryad.x0k6djhhx.

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).

ORCID

Nicola M. Fischer https://orcid.org/0000-0003-4056-1032
Serena Dool https://orcid.org/0000-0003-4728-4154
Sebastien J. Puechmaille https://orcid.org/0000-0001-9517-5775

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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Supplemental Information for:

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

Nicola M. Fischer[1,2], Andrea Altewischer[1], Surendra Ranpal[1], Serena Dool[1,3], Gerald Kerth[1], Sebastien J. Puechmaille[1,2,4]*

[1]Zoological Institute and Museum, University of Greifswald, Greifswald, Germany
[2]Institut des Sciences de l'Évolution Montpellier (ISEM), University of Montpellier, CNRS, EPHE, IRD, Montpellier, France
[3]CBGP, INRAE, CIRAD, IRD, Institut Agro, University of Montpellier, Montpellier, France
[4]Institut Universitaire de France, 75005 Paris, France

*corresponding author (sebastien.puechmaille@umontpellier.fr)

Content

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1. Counts of bats in Eldena

Eldena was visited bi-weekly from July 2015 to July 2018 to establish the typical seasonality of bat presence and absence (Figure S1). Previous visits (less regular, hence not reported) were used to elucidate the best timing of sampling from bats and walls in Eldena. Most bats were sampled during March, when visible growth of *Pseudogymnoascus destructans* on bats is greatest (e.g., Puechmaille et al., 2011). Samples from the walls were collected in April and October just after and before hibernation when most bats were absent from the hibernaculum.

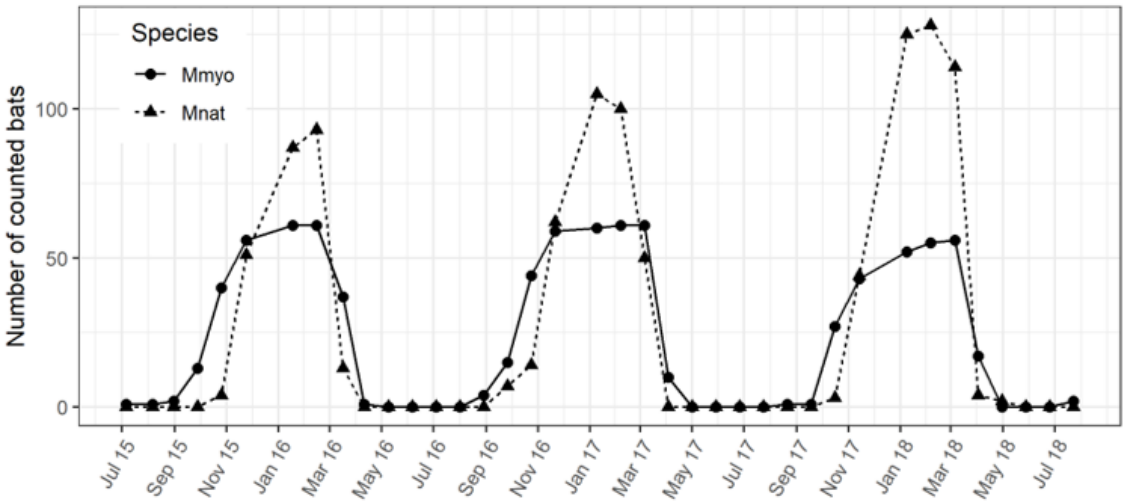

Figure S1. Number of *Myotis myotis* (dots) and *Myotis nattereri* (triangles) bats counted in Eldena every 14 days for 3 years. Counts data are shown for both species with symbols; the lines are only used to help visualise the patterns of changes.

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2. Overview of data analyses

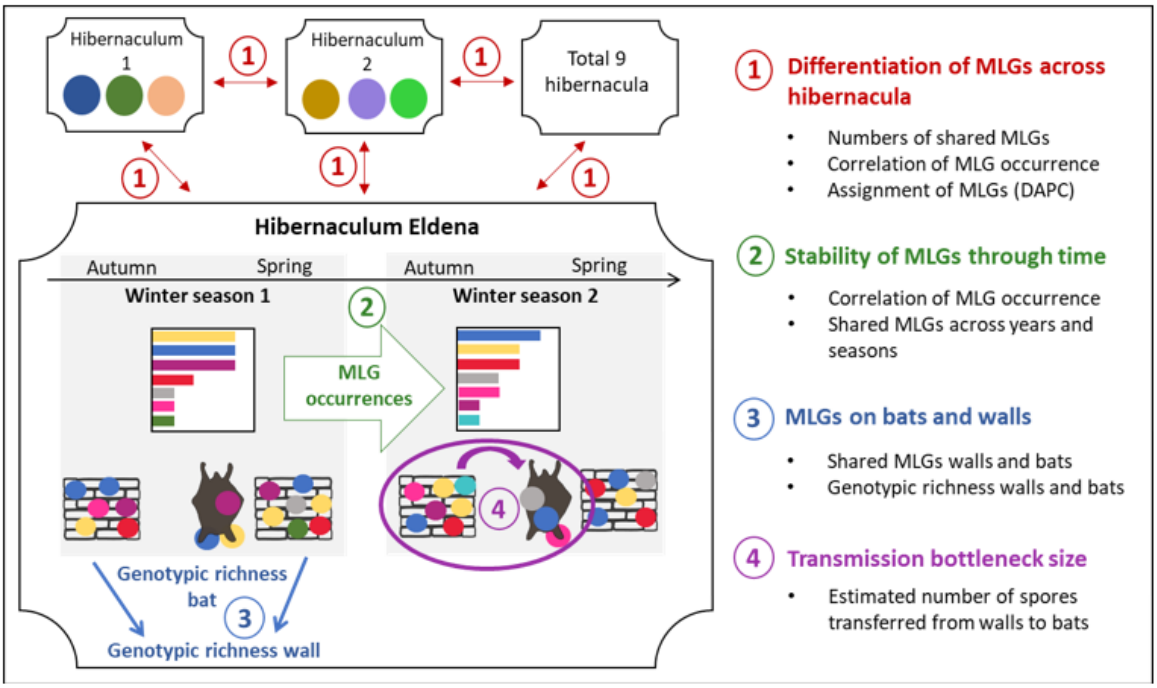

Figure S2. Summary of analyses used. Coloured dots and bars represent the identity of multi-locus genotypes ('MLG', quantity of genotypes underrepresented for visual purposes). A reduced number of hibernacula and winter seasons are shown to illustrate the overall framework in a concise manner.

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3. Allelic richness of microsatellite markers

The allelic richness (number of different alleles in the dataset) ranged from 3 – 17 for each of the microsatellite markers (mean = 8.67, median = 7.5, Table S1).

Table S1. Allelic richness (number of different alleles) for each of the microsatellite markers used in the study.

Locus	Allelic richness
PD1	17
PD2	8
PD3	3
PD4	14
PD5	17
PD6	4
PD7	7
PD9	5
PD10	10
PD11	10
PD12	3
PD13	15
PD14	11
PD17	6
PD19	6
PD21	7
PD22	8
PD23	5

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4. Probability of Identity (P_{ID}) across hibernacula

The P_{ID} is used to estimate the number of multi-locus genotypes which can theoretically be distinguished using a specific marker set. It uses the sum of squares of the probabilities of all alleles, or in other words, it calculates the likelihood of two individuals sharing the same genotype (i.e., having the same alleles in all examined loci) even though they are actually different.

This information is helpful in determining the reliability of genotype-identification across and between the studied hibernacula. The overall P_{ID} between hibernacula was 6.63 $*10^{-7}$ which results in a 1/PID (number of genotypes which are expected to be distinguished) greater 1.5 million. Considering, that allelic diversity per locus was different across hibernacula it was also important to estimate the P_{ID} for each of the hibernacula to ensure that it was possible to determine reliable genotype identities for each one (used to find matches across the dataset to determine, e.g., the number of shared genotypes among hibernacula). The number of $1/P_{ID}$ by far extends the number of observed genotypes in this study (Table S2).

Table S2. Probability of identity (P_{ID}) and $1/P_{ID}$ as well as the observed number of unique genotypes of *Pseudogymnoascus destructans* for the studied hibernacula. Furthermore, the number of sampling events and the temporal range in which this sampling happened are given for each hibernaculum.

Hibernaculum	P_{ID}	$1/P_{ID}$	Genotypes	Sampling events	Sampling period
Eldena	$1.51*10^{-6}$	661,245	149	12	2014/15 – 2018/19
Wolgast	$1.82*10^{-6}$	550,847	18	2	2016/17 – 2017/18
Anklam	$2.73*10^{-6}$	366,594	16	3	2013/14 – 2018/19
Richtenberg	$7.95*10^{-4}$	1,257	7	1	2017/18
Altentreptow	$3.20*10^{-7}$	3,129,561	31	1	2017/18
Bad Sülze	$7.11*10^{-5}$	14,073	11	1	2017/18
Strasburg	$7.45*10^{-7}$	1,341,568	28	3	2016/17 – 2018/19
Burg Stargard	$2.39*10^{-6}$	417,365	18	1	2016/17
Neustrelitz	$2.26*10^{-6}$	468,499	32	2	2016/17 – 2018/19

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5. Additional analyses on *Pseudogymnoascus destructans* genotype distribution among hibernacula

5.1 Discriminant Analysis of Principle Components (DAPC) assigning isolates to hibernacula

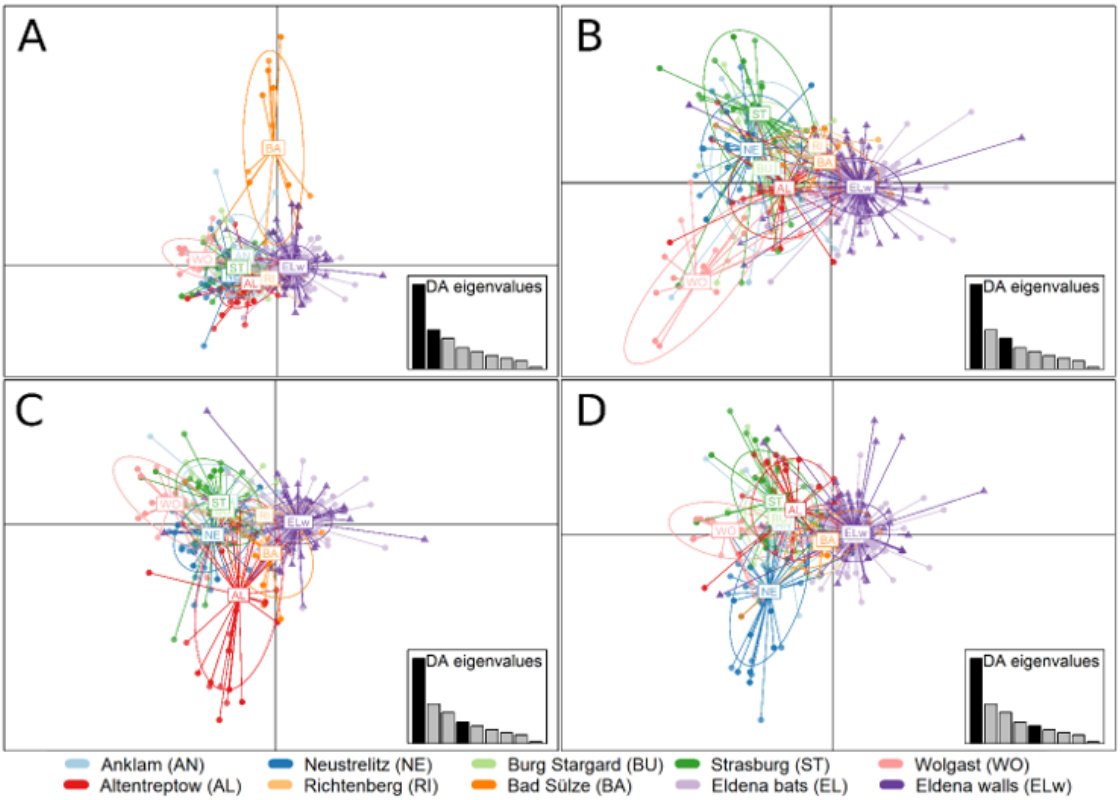

Figure S3. Discriminant Analysis of Principle Components (DAPC) of *Pseudogymnoascus destructans* isolates from nine hibernacula in Northeastern Germany. Isolates are coloured by the hibernaculum they were sampled from. While only bat swabs were collected from eight hibernacula, both bats and walls were sampled from Eldena (light purple with round symbol and dark purple with triangular symbol, respectively; the label for Eldena bats is behind the label for Eldena walls as bats and walls from this site cluster together [irrespective of the axes combination looked at], hence only one label can be seen). The results of the Discriminant Analysis (DA) are presented for a subset of the axes explaining together 52.7% of the global variance, **A.** axis 1 (19.1% of variance explained) and 2 (10.4 % of variance explained). **B.** axis 1 and 3 (9.5% of variance explained). **C.** axis 1 and 4 (7.5% of variance explained). **D.** axis 1 and 5 (6.2% of variance explained). Although sites are overlapping along two axes, their samples can largely be assigned to the correct site (see main text & Fig. 2).

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5.2 Differentiation of isolates from different hibernacula using Hedrick's G_{ST}

We used a measure of population differentiation summary statistics (Hedrick's standardised G_{ST}) to assess the differentiation among pairs of hibernacula (Figure S4). Values of Hedrick's G_{ST} range from 0 to 1 whereby 0 indicates no differentiation and 1 indicates that populations are fully segregated (Hedrick, 2005).

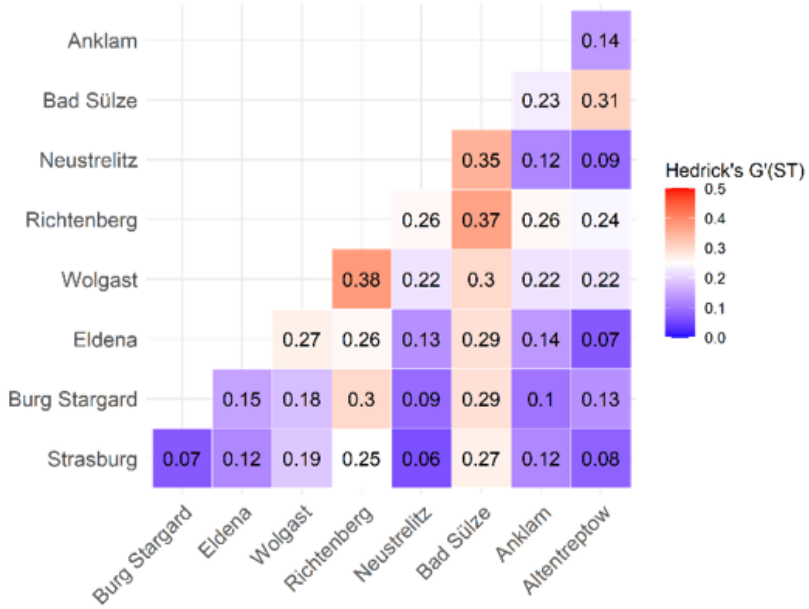

Figure S4. Genetic differentiation of *Pseudogymnoascus destructans* between pairs of hibernacula. Differentiation is calculated using Hedrick's G_{ST} which is represented as number and colour between each pair of hibernacula (i.e., each hibernacula's differentiation from every other hibernaculum is shown).

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6. Additional analyses on *Pseudogymnoascus destructans* genotype distribution between bats and walls in Eldena

6.1 Number of spores transferred from walls to each bat (bottleneck size)

The determination of bottleneck size (i.e., how many spores were successfully transferred from walls to bats) in different winters was obtained by simulating the transmission of spores from walls to bats and comparing it to the observed data using as proxy the number of unique genotypes.

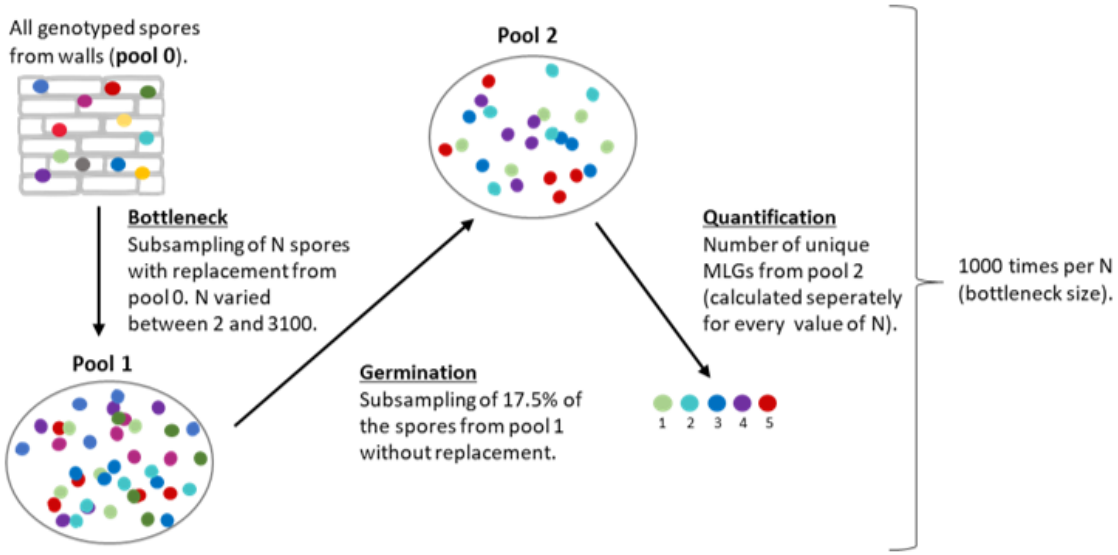

Figure S5. Schematic visualization of the steps used to estimate the number of spores transferred from walls to bats. Coloured circles represent different genotypes (underrepresented for visual purposes).

Specifically, the 'Pool 0' was composed of all isolates from wall swabs collected in Eldena hibernaculum (across all years, with genotype occurrences exactly as observed). We sampled (with replacement) different quantities of spores (*N* in the range 2-3100) from the 'pool 0' and obtained a new 'Pool 1' (Figure S5, 'Bottleneck'). We then sampled 17.5% of spores from 'Pool 1' to take into consideration the germination rate calculated for European isolates (4 isolates; Mean= 0.175; Based on data in Fischer et al., 2020; Figure S5, 'Germination'). For each bottleneck size, we then quantified the number of genotypes after the two successive samplings (i.e., for 'Pool 2'; Figure S5, 'Quantification'). This procedure was repeated 1000 times for each bottleneck size, resulting in 1000 values of genotypic richness (number of unique genotypes) for each value of *N* (see Figure S6).

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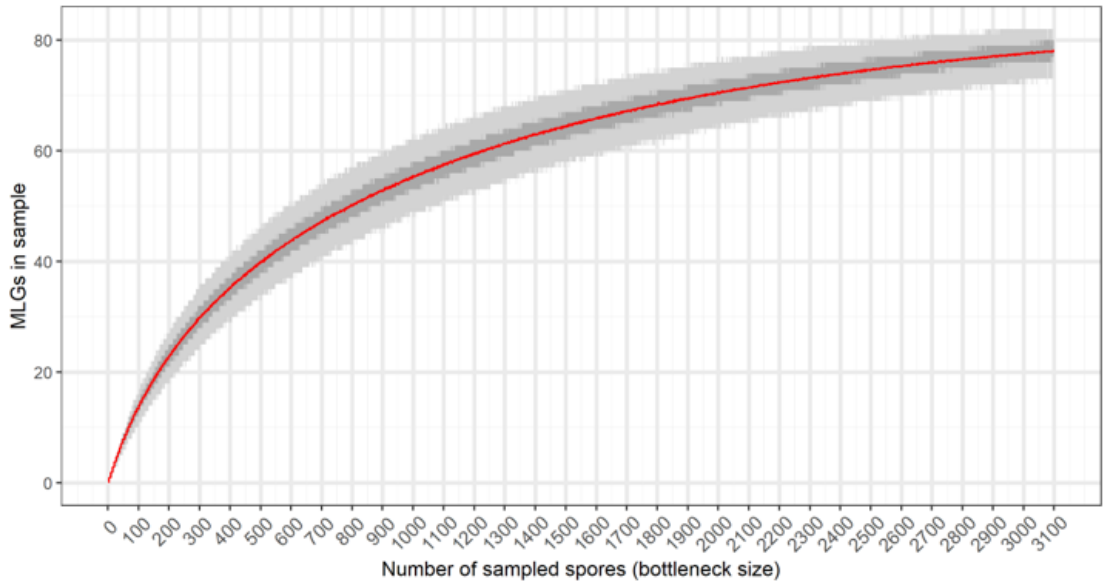

Figure S6. Number of unique multi-locus genotypes ('MLGs', y-axis) for a given number of sampled isolates (x-axis; size of bottleneck, steps of 1 between 2 and 3100) from all isolates collected from Eldena walls. The red line shows the mean number of genotypes for each bottleneck size across 1000 runs, while the area shaded in dark grey and light grey show the 50% and 95% confidence interval, respectively.

We then compared the quantity of genotypes from all simulated bottleneck sizes with the genotypic richness (number of genotypes) isolated from bats in Eldena for different winters. To get more precise results we used a predicted number of genotypes for bats (obtained via a Bayesian estimator classically used to estimate population sizes based on a single sampling session; Petit & Valiere, 2006; Puechmaille & Petit, 2007). This predicted number of genotypes accounts for genotypes that were present in the samples but had not been observed (e.g., not cultured and/or not genotyped). We used the 95% confidence interval (more specifically, the "quantile 95%", containing all remaining values after removing values below the 2.5% and above the 97.5% quantiles) of genotype richness estimates for each of the winters, resulting in a range of estimated numbers of genotypes which was slightly different for each winter (ranging from 19-77 genotypes). The bottleneck size (*N*) with the closest mean (calculated across the 1000 values for each N) to the observed/predicted numbers of genotypes on bats were considered as the estimated numbers of spores transferred from walls to bats (to the entire sampled population of bats). To obtain an average number of spores passed from the wall reservoir to each single bat, we then simply accounted for sampling intensity in each winter by dividing the determined matching bottleneck sizes (i.e., numbers of spores transferred to the entire sampled population of bats) by the number of body parts that were sampled (i.e., the number of 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). Hence, while working with the same initial distribution of genotypes and corresponding values of N (Figure S6) the number of estimated genotypes as well as the sampling intensity of bats per winter resulted in different values for the estimated numbers of spores transferred from the walls to each bat in each winter (see Table 3). Given that the most intensively sampled winter was that of 2014/15 this is likely the most accurate estimate of transferred spores (Figure 5).

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6.2 Testing for differences in genotypic richness using a permutation test

In addition to giving the eMLG values (genotypic richness at smallest shared sample size) for samples collected from walls and bats, we also wanted to test the difference in genotypic richness between the substrates using a permutation test (Figure S7). For this, the sample size obtained for bats (788 isolates) was reduced through subsampling to 274 isolates (the same sample size as obtained from wall swabs). In this way, the resulting subsampled dataset from bat samples has exactly the same sample size as obtained from walls and can be used to compare genotypic richness. The proxy used for genotypic richness here was simply the absolute number of unique genotypes.

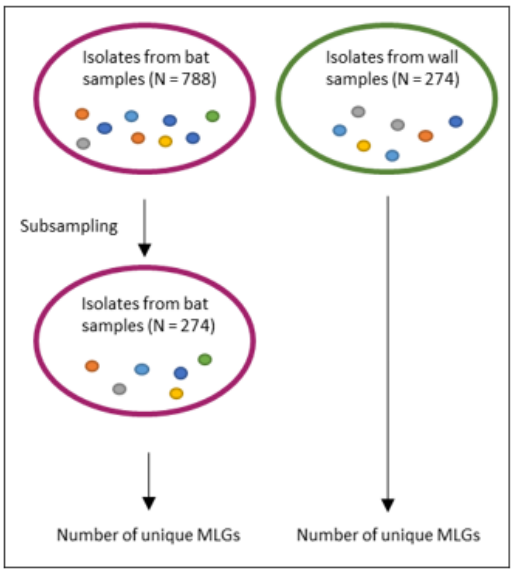

Figure S7. Schematic principle of permutation (i.e., subsampling) to test for differences in genotypic richness between samples collected from bats and walls. Shown exemplary for a single run.

Considering that the subsampling itself can have an impact on the resulting number of unique genotypes, this step was repeated 1000 times, resulting in 1000 values for unique genotypes from bat swabs (isolates from wall swabs remain unchanged, hence their genotypic richness remains fixed).

The numbers of unique genotypes obtained after subsampling of isolates from bat swabs to 274 isolates ranged from 63 to 90 (across 1000 runs, Figure S8) with a mean of 76.5 and a median of 77. The observed number of unique genotypes in isolates obtained from the walls was fixed at 85. Therefore, the genotypic richness of isolates obtained from bats, after subsampling to the same sample size of 274 isolates, was lower than the genotypic richness observed in isolates sampled from the walls of the hibernaculum (One-sided permutation test, $df = 999$, $p = 0.021$).

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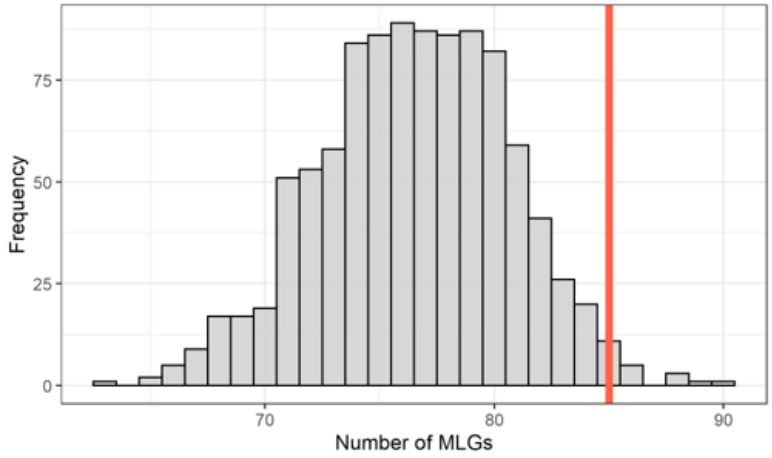

Figure S8. Histogram of number of unique *Pseudogymnoascus destructans* genotypes in subsamples from bat isolates (grey bars); Number of isolates = 274; 1000 subsamples. Red line at 85 indicates the number of observed unique MLGs in wall isolates (also with a sample size of 274).

7. Genotypic richness of *P. destructans* on bats and walls with 3 isolates per swab (Eldena)

We decided to show the results obtained from using 3 isolates per bat swab and 5 isolates per wall swab for all analyses on patterns of *P. destructans* between bat and wall in Eldena. This was done to obtain large sample sizes to best estimate genotypic richness overall as well as at each sampling event.
In the following, we present the results for analyses on genotypic richness between bats and walls under the same sample size (considering exactly 3 isolates per swab independent of the sampled substrate).

After excluding all samples for which we had obtained less than 3 isolates, all swabs collected from bats yielded exactly 3 isolates per swab. However, usually 5 isolates were obtained from wall swabs resulting in the need to subsample the isolate from these swabs down to also obtain 3 isolates. Because the selection of isolates per swab could influence the observed genotypic richness, the subsampling of isolates from wall swabs was done 1000 times to obtain estimates independent of which isolate combination was picked. The resulting dataset contained 150 isolates from wall samples and 699 isolates from bat samples.

7.1 eMLG of isolates from walls and bats (overall and temporal)

The eMLG for *P. destructans* isolates from bat swabs with exactly 3 isolates per swab was 56.47 while the mean eMLG from wall swabs (subsampled to 3 isolates per swab, 1000 runs) was 60.48 (at smallest shared sample size of 150; See Figure S9). Therefore, at the same number of isolates per swab and independent of sample size per substrate, *P. destructans* isolates from walls have greater genotypic richness than those from bats.

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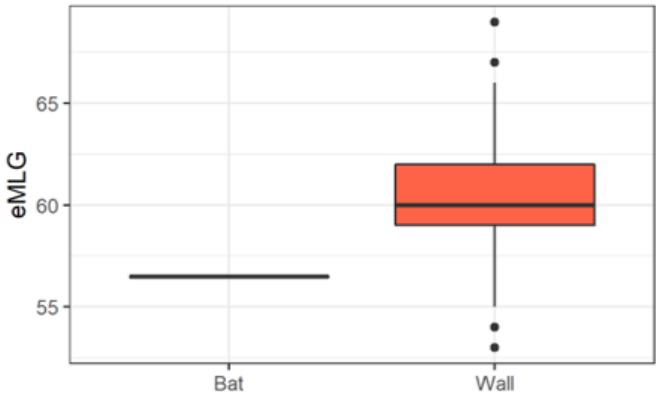

Figure S9. Genotypic richness of *Pseudogymnoascus destructans* from bat and wall samples at exactly 3 SSIs per swab. The smallest shared sample size was 150 (SSIs from wall swabs). Bars show interquartile range between the 25th and 75th percentile; Line shows the median; Whiskers represent the largest and smallest value within 1.5 times the interquartile range; Points show outliers between 1.5 and 3 times the interquartile range.

In most cases, the mean eMLG of *P. destructans* from wall swabs was higher than the eMLG for isolates from bat swabs collected the same winter season (Figure S10). The exception to this was the winter of 2014/15, when genotypic richness on bats was slightly higher than that on walls.

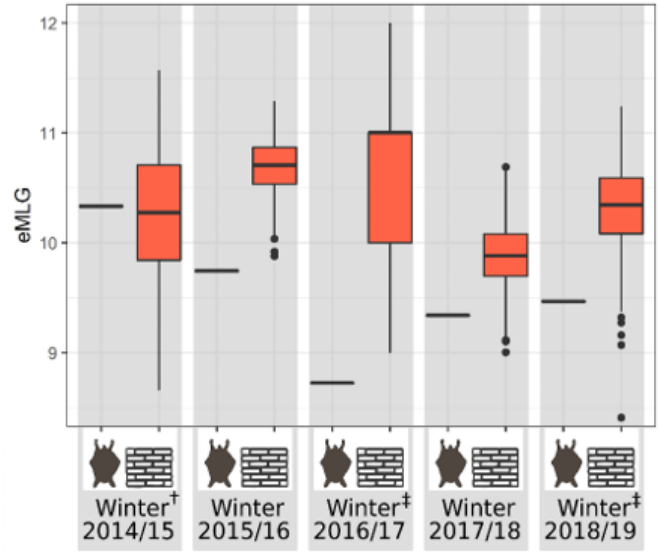

Figure S10. Genotypic richness (eMLG) of *Pseudogymnoascus destructans* for each substrate (indicated by bat and wall symbols) by winter season at a smallest shared sample size of 12 (wall winter 16/17). Bars show interquartile range between the 25th and 75th percentile; Line shows the median; Whiskers represent the largest and smallest value within 1.5 times the interquartile range; Points show outliers between 1.5 and 3 times the interquartile range.

† contains isolates from wall sampling in April only.

‡ contains isolates from wall sampling in October only.

7.2 Testing for differences in genotypic richness using permutation test

This analysis follows the same principle as shown in section S6.2 but on the dataset of wall and bat isolates subsampled to exactly 3 isolates per swab. To quantify the genotypic richness on bats and walls in a permutation test (in addition to the eMLGs obtained in S7.1), we subsampled the isolates obtained from bat samples (N = 699, at exactly 3 isolates per swab) down to the sample size obtained from wall samples (N = 150, at exactly 3 isolates per swab). We then compared the obtained number of unique genotypes in the subsample to the observed number of unique genotypes from wall swabs (i.e., at the same sample size and the same number of isolates per

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swab). This resulted in 1000 values for bat swabs (which did not have to be previously subset to obtain 3 isolates per swab) and 1.000.000 values for wall swabs (which were first subset to 3 isolates per swab in 1000 runs before running this permutation test).

The mean number of unique genotypes from bats was 56.47 while the mean number of unique genotypes from wall swabs was 60.50 (Figure S11). At the same sample size, samples from the walls of hibernacula show a greater number of unique genotypes than samples collected from bats (Two-sample Kolmogorov-Smirnov test, $D = 0.48$, $p < 0.001$).

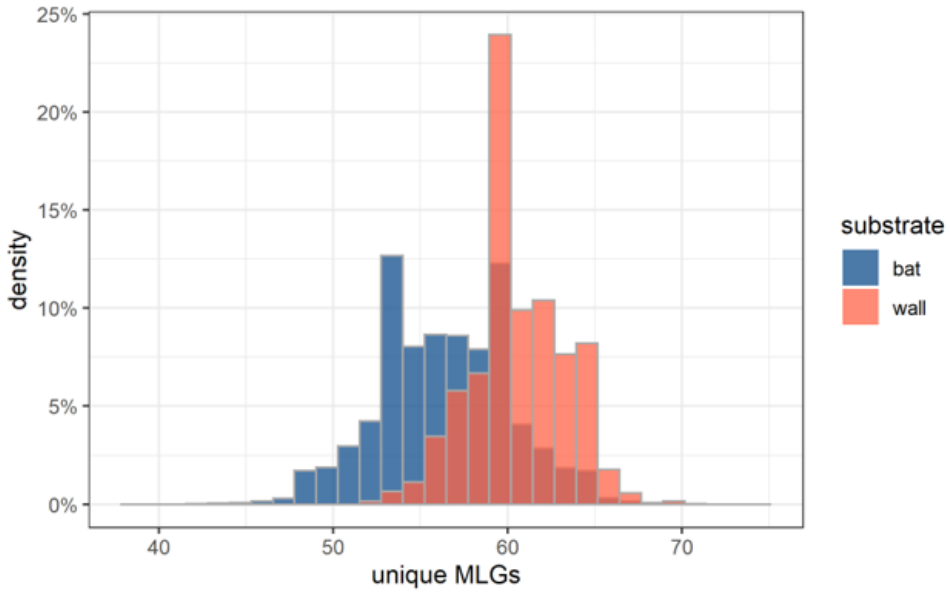

Figure S11. Density distribution of the number of unique genotypes of *Pseudogymnoascus destructans* following subsampling of bat isolates (N=699) to the number of isolates from walls (N = 150) with exactly 3 isolates per swab sample.

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Genetic diversity and population structure of *P. destructans*, the causative agent of White-Nose disease in bats. From large scale differentiation to locally homogeneous populations

Violeta Zhelyazkova[1], Nicola M. Fischer[2,3], Sébastien Puechmaille[2,3,4,*]

[1]National Museum of Natural History, Bulgarian Academy of Sciences, 1 Tsar Osvoboditel Str., 1000 Sofia, Bulgaria

[2]Zoological Institute and Museum, University of Greifswald, Soldmannstr. 14, 17489 Greifswald, Germany

[3]ISEM, University of Montpellier, CNRS, EPHE, IRD, Place Eugène Bataillon, 34095 Montpellier Cedex 05, France

[4]Institut Universitaire de France, Paris, France

*Corresponding author: sebastien.puechmaille@umontpellier.fr

Abstract

White-nose disease represents one of the greatest threats for North-American bat conservation. Its causative agent is the psychrophilic fungus *Pseudogymnoascus destructans* that affects bats during hibernation in winter but also survives for multiple years in the substrate of bat hibernacula. The pathogen was recently introduced from Europe, where it does not cause mortality, to North America, where it is lethal to bats leading to more than 90% declines in some populations. Considering these differential outcomes, most of White-nose disease research comes from North America where mitigation strategies are most needed, however, genetic richness of *P. destructans* there is magnitudes lower in comparison to Eurasia. This reduces the power of genetic markers, rendering several related questions challenging to address within the invasive range of the fungus. In the present paper, we describe genetic diversity, population size, and fine scale population structure of *P. destructans* at three sites in its native range: two natural hibernacula (karst caves) in Bulgaria, South-Eastern Europe, and one artificial hibernaculum (disused cellar) in Germany, Northern Europe, that we surveyed between 2014 and 2019. We then use these data to describe how *P. destructans* genetic diversity is distributed at various scales showing significant genetic differentiation between hibernacula and no differentiation within hibernacula. Such patterns could be explained by the specificity of *P. destructans* biology and the bat hosts' behaviour. Genetic richness of *P. destructans* was approximately two times higher in the caves in comparison to the disused cellar, and within all sites, genetic richness of the pathogen was higher in samples collected from hibernaculum walls than in samples collected from bats. Infections with multiple pathogen genotypes were very common in bats from all study sites, which might be important for White-nose disease progression and is worth future research.

Keywords: Chiroptera, emerging infectious disease, fungal pathogen, wildlife disease, White-Nose Syndrome

Introduction

Genetic diversity not only provides the raw material for the evolution of species but significantly influences the size, dynamics and fitness of a population, species interactions, and ecosystem functions (Hughes et al. 2008). It also plays a crucial role in pathogen-host interactions, virulence, and transmission, and determines the potential for the emergence of more (or less)

adaptative and dangerous pathogen variants. Thus, information on a pathogen's genetic diversity and its distribution are important for understanding infectious diseases, including wildlife diseases such as White-nose disease in hibernating bats.

Currently, White-nose disease represents one of the greatest threats for North-American bat conservation and is seriously impacting both natural and agricultural ecosystems. Its causative agent, the psychrophilic fungus *Pseudogymnoascus destructans* (Lorch et al. 2011; Warnecke et al. 2012), affects bats during hibernation in winter, when their temperature is low enough to support the growth of a psychrophilic organism for extended periods of time and when the function of their immune system is suppressed (Whiting-Fawcett et al. 2021). Additionally, the substrate of bat hibernacula (cave, mines, or other underground roosts where temperate bats spend the winter) serves as a long-term reservoir for *P. destructans* (Puechmaille et al. 2011; Hoyt et al. 2020; Fischer et al. 2022). This allows the pathogen to survive even in the absence of its hosts, further complicating its removal from the environment and disease eradication. During the first 5 years after its emergence, White-nose disease was estimated to have caused between 5.7 and 6.7 million casualties (White-nose Syndrome Response Team 2011), and the colonies of the most affected species *Myotis septentrionalis*, *Myotis lucifugus*, and *Perimyotis subflavus* suffered more than 90% population decline within the newly colonised geographic range of the pathogen (Cheng et al. 2020). Such pervasive mortality is unarguably related to the fact that *P. destructans* was only recently introduced to North America (Leopardi et al. 2015; Drees et al. 2017b) where it encountered large numbers of naive hosts lacking appropriate defences against it (Whiting-Fawcett et al. 2021). In Europe and Asia where *P. destructans* is native, no mass morbidity or mortality has been observed in bats (Puechmaille et al. 2010; Puechmaille et al. 2011a,b; Zukal et al. 2016a; Fritze et al. 2018), indicating long-term coevolution between the hosts and the pathogen (Fischer et al. 2020; Fritze et al. 2021).

Considering these differential outcomes, it is not surprising that most of White-nose disease research comes from North America where disease mitigation strategies are most needed. However, 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). As a consequence, the power of genetic marker sets is drastically reduced in North America,

therefore, several questions related to the disease that require genetic data (see for example Fischer et. al. 2022) are challenging to address within the invasive range of the fungus.

In the present paper, we describe genetic diversity, population size, and fine scale population structure of *P. destructans* at three sites in its native range: two natural hibernacula (karst caves) in Bulgaria, South-Eastern Europe, and one artificial hibernaculum (disused cellar) in Germany, Northern Europe. We then use this data to describe how *P. destructans* genetic diversity is distributed at various scales going from differences across hibernacula to within hibernacula spatial and temporal differences and interpret these data in light of the interactions between the pathogen, its bat hosts, and the underground environment.

Materials and Methods

Sample collection

For Bulgaria, swab samples were collected from bats and walls in two karst caves only reachable with caving equipment, Balabanova dupka (N43.134, E23.040) and Ivanova voda (N41.894, E24.880). Both caves are cold (air temperature usually below 5℃) and are used as winter roosts by bats. In Balabanova dupka, around 2500 *Myotis myotis/blythii* hibernate (personal data), and in Ivanova voda the hibernation colony consists of *Myotis myotis/blythii* and *Myotis capaccinii* (maximum counts 5600 and 2500 respectively; EUROBATS 2011). Swab samples were collected from the muzzle, ears or wings of freely hanging and visibly infected bats (*Myotis myotis, M. 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 were 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. Wall swabs were collected from different cave rooms: both in close proximity (a few centimetres) to the main bat roosting places and further away from them (around 50 meters, see Supplement 1) two times per year in spring (March to May) and autumn (October). 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 см^2. The sampling locations within each site are presented in Supplement 1.

For Germany, we used the data collected in similar ways (sampling bats and walls) over 5 years (2015-2019) from the Eldena hibernaculum (N54.090, E13.445) situated in the North-Eastern federal state of Mecklenburg-Vorpommern described in details in Fischer et al. (2022). Briefly, 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*.

Cultures

Each swab was plated on a petri dish containing DPYA growing medium (Vanderwolf et al. 2016) and individual spores (single spore individuals or SSIs) were physically separated and cultured in 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 bat or wall swab sample to six.

Molecular analyses

Fungal material digestion and DNA extraction were performed following Fischer et al. (2022). We then genotyped isolates of *P. destructans* using 18 microsatellite markers (Drees et al. 2017a) and 2 mating type markers in 4 PCR multiplexes as described in Dool et al. (2020). 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 mostly clonally reproducing organism, these MLGs can be used to track the fungus in space and time (Fischer et al. 2022). 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 (genic 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 combined models. One locus containing more than 5% missing data was excluded from the analysis. When testing differentiation between rooms, rooms with a very low number of swabs or unidentified rooms (see Supplement 2) were also excluded.

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 between hibernacula. For estimating population size or, in other words, the total number of unique *P. destructans* MLGs present within a hibernaculum, we used the CMRPopHet functions implementing a capture-mark-recapture (CMR) model based on a single sampling event (Petit & Valière, 2006). One assumption of the CMR model is that each MLG has the same probability of being sampled (i.e., has the same frequency and distribution in the population). In order to test if this was the case in the studied hibernacula, we used the heterogeneity test developed by Puechmaille & Petit (2007). Considering that each swab sample, whether from a bat or wall, yielded variable numbers of SSIs, it was important to validate that this variation did not influence measures of genotypic richness and population size. For this purpose, we additionally ran the analyses when randomly selecting exactly one SSI per swab (run 1000 times to avoid strong stochastic effects).

Population dynamics

In order to further test the hypothesis of the environment being the source of *P. destructans* infection (see Fischer et al. 2022), 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 per swab and when randomly selecting exactly two SSIs per swab (run 1000 times). To compare genotypic diversity per wall or bat swab, we calculated the following ratio for each swab: (G-1)/(N-1), where G is the number of MLGs per swab and N is the number of SSIs per swab. To estimate the frequency of genetically diverse infections in bats, we calculated the percentage of cases where more than one MLG or more than one mating type was found in a single bat swab, after taking exactly three SSIs per 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 SSI 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%.

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 percentage 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%

Population differentiation

As shown by AMOVA (see Supplement 2 for sample sizes), there was significant genic differentiation between sites explaining 26.88% of total variance ($p<0.001$) when we considered all three sites, and explaining 9.31% of total variance ($p<0.001$) when Eldena was excluded. No significant differentiation was detected within a site with regard to any of the three factor levels considered: hibernaculum room, time of sampling (year and season), and substrate (bat or wall). None of the 615 *P. destructans* MLGs detected was shared between any of the study sites. Within a site, however, MLGs were often shared. Across sampling years and seasons, 61.7%, 22.7%, and 80.2% of MLGs found more than once were shared in Balabanova dupka, Ivanova voda, and Eldena respectively (Figure 1), both in the presence and absence of bats. Across hibernacula rooms, 54.2%, 6.8%, and 80.2% of MLGs found more than once 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 found more than once were shared in Balabanova dupka, Ivanova voda, and Eldena respectively (Figure 1).

Table 2: Comparison of *P. destructans* genotypic diversity found on bats and walls in the study sites. Abbreviations are as defined for Table 1. (G-1)/(N-1) is the mean genotypic diversity 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

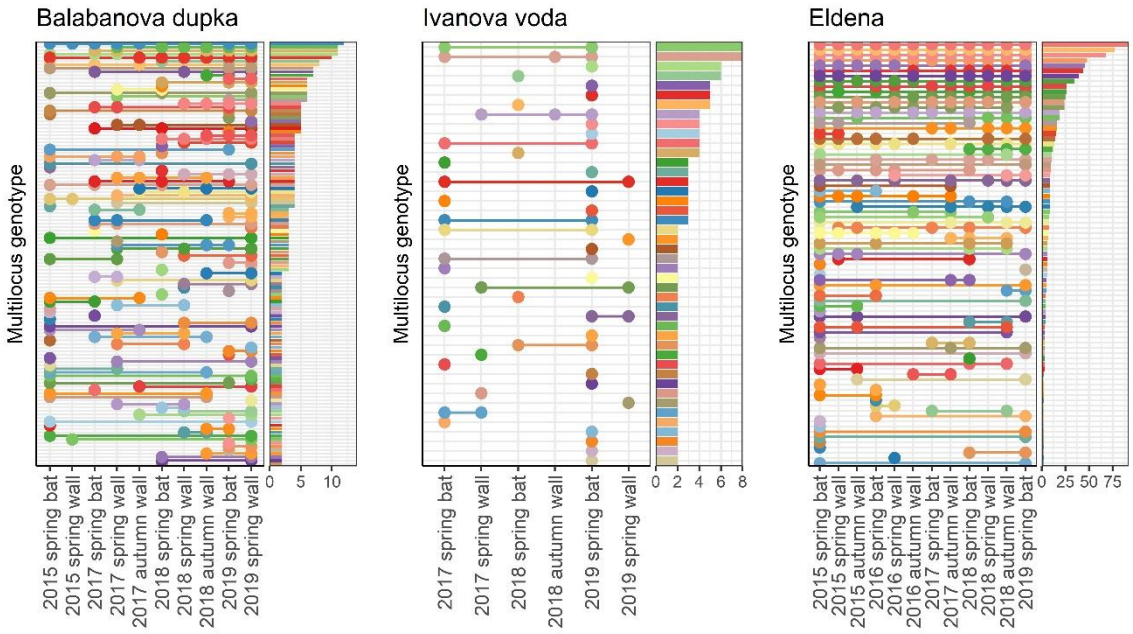
Genetic diversity and population size

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; Supplement 3). We obtained a total of 301 unique multilocus genotypes (MLGs) from Balabanova

dupka, 165 from Ivanova voda, and 149 from Eldena. At equal sample size, genotypic richness was more than two times 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 1 SSI/swab (Supplement 5). 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). According to the CMR model, *P. destructans* population size 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 strong heterogeneity was present in Eldena (Supplement 4), meaning that capture probability for MLGs was variable in all sites.

Within sites, genotypic richness of *P. destructans* 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 (Supplement 6). Mean genotypic diversity per swab was also higher in wall swabs (Table 2). Infections with multiple MLGs were dominant in bats. After taking exactly three SSIs per swab, more than one MLG was 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.

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.



Discussion

Significant P. destructans genetic differentiation at large scale

Our research revealed significant allelic differentiation in *P. destructans* populations between sites in Eurasia. Additionally, for the whole duration of the study, and despite intensive sampling, not a single pathogen MLG was shared between any of the three studied sites, including between Balabanova dupka and Ivanova voda, which are situated approximately 200 km apart. This confirms the result from our previous work on the pathogen's genetic differentiation between several hibernacula with established *P. destructans* populations (Fischer et al. 2022) and further supports the hypothesis that *P. destructans* is rarely successfully moved by bats between hibernacula, including after the end of the hibernation season when bats start moving between winter and summer roosts. Direct comparison with results obtained for the North American *P.*

destructans population is challenging due two important differences: 1) the current population structure in North America may not have reached equilibrium following its recent colonisation, and 2) given the reduced diversity of the North American population, as a consequence of the introduction bottleneck, the power to detect population structure is accordingly reduced (see for example Drees et al. 2017b; Forsythe et al. 2021). To overcome these issues, a recent study used a partitivirus, specifically infecting *P. destructans*, as a proxy to infer *P. destructans* connectivity between hibernacula in North America. The mutation rate of the partitivirus is orders of magnitude higher than typical fungal mutation rates (Zhu et al. 2014; Thapa et al. 2021), hence the partitivirus can provide a much finer resolution, both in time and space. Using this highly variable partitivirus, Thapa et al. (2021) found very strong partitivirus population differentiation across the studied region in North America, a result that is completely consistent with the strong population structure recovered in the established European populations of *P. destructans* (Fischer et al. 2022; this study). These studies combined clearly demonstrate that once populations have established, in Europe or North America, the successful movement of the pathogen is only occasional (Thapa et al. 2021; Fischer et al. 2022; this study). Although beyond the scope of the present paper, it would be enlightening to study the factors that are associated with the successful/unsuccessful movement of the pathogen at different spatial scales, from local (between nearby hibernacula) to global scales (across continents) (Petit & Puechmaille, 2015).

Lack of P. destructans genetic differentiation within sites

Within sites, we found neither allelic, nor genotypic differentiation in *P. destructans* populations for any of the factors considered: time, hibernaculum room, or substrate (bats or walls). Despite the fact that we could not obtain an equal number of samples for each factor combination in the AMOVA, the absence of differentiation is most probably a biological reality rather than a statistical artefact due to a lack of power. Indeed, the presence of the same MLGs and the lack of population structure across sampling seasons (both in the presence and absence of bats) is consistent with the clonal mode of reproduction of the pathogen and its long-term survival in the hibernacula, demonstrated in Fischer et al. (2020; 2022) and previously suggested in several other studies (e. g. Lindner et al. 2011; Puechmaille et al. 2011a; Lorch et al. 2013; Raudabaugh & Miller 2013; Reynolds et al. 2015a; Vanderwolf et al., 2016). Furthermore, given that bats become infected each year (in autumn) from the hibernacula walls and shed spores back

onto the walls (most likely in Spring before emergence from hibernacula; Fischer et al. 2022), the lack of differentiation between bats and walls, and through time, were both expected. The lower number of shared MLGs through time/substrate in Ivanova voda in comparison to Balabanova dupka and Eldena, is possibly due to the overall lower number of SSIs obtained from this cave combined with the more unequal sampling, and/or the fact that spring flooding is occurring at this site, substantially washing away/killing *P. destructans*, leading to a higher MLG turnover (see Supplement 2). The population differentiation of *P. destructans* between different rooms of the same hibernaculum has never been studied before but the absence of differentiation observed here is completely 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 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 often be seen hanging in different places from where they typically hibernate during the previous months. Additionally, even in winter, bats regularly interrupt their torpor bouts (Blažek et al. 2019) when they may engage in grooming behaviour or leave their groups and contact other individuals or areas of the roost environment (Hoyt et al. 2018). Torpid bats have also been shown to change position without elevating their body temperature (Bartonička et al. 2017). Accordingly, there are no obvious reasons to expect *P. destructans* population differentiation within a hibernaculum, at least, for hibernacula of comparable size (a few hundred meters between sampling points), and our data supports this expectation.

Altogether, considering our results on *P. destructans* genetic differentiation between and within sites and previous findings in Fischer et al. (2022), it seems clear that the full cycle of the host-pathogen interaction happens within the hibernacula, which can be viewed as predominantly closed systems for *P. destructans*. Hence, better understanding the disease dynamics, the host-pathogen interactions, and the factors affecting their outcome will greatly benefit from studies investigating in further details the dynamics in such a system. Indeed, fine scale longitudinal studies are greatly needed to better understand when the fungus starts growing on bats, how much fungal material is effectively exchanged between individual bats during hibernation, how important multiple infections are, etc. The level of genetic diversity within a hibernaculum and the distribution of this diversity (Fischer et al. 2022; this study) provide critical information to design

studies (i.e., using genetic tools as in Fisher et al. 2022) to address the abovementioned questions in a natural setting.

P. destructans genetic diversity between and within sites

When we compared different sites, both allelic and genotypic richness of *P. destructans* populations were significantly higher in the Bulgarian caves Balabanova dupka and Ivanova voda in comparison to the German cellar Eldena. In fact, we described the most genetically diverse populations of *P. destructans* known so far, pointing to the presence of up to at least 400 different MLGs in a single hibernaculum. Considering the presence of population heterogeneity, which could bias CMR results, most often towards underestimation (Puechmaille & Petit, 2007), the real pathogen population size is most likely even higher. These results are in agreement with the hypothesis that *P. destructans* diversity varies between sites, although we cannot point out the most significant factor (e.g., natural vs artificial roosts, larger bat colonies, more diverse environment, longer host-pathogen evolutionary history, geographical location, hibernation duration, etc.) accounting for this difference. To address this, further studies with data on many more sites distributed across the continent as well as their associated variables would be needed. Nevertheless, the information on *P. destructans* population size in two different types of hibernacula presented here will be valuable for planning future studies and defining the optimal samples sizes needed to answer various research questions.

Within sites, we found consistently higher genotypic richness of *P. destructans* in swab samples taken from hibernaculum walls in comparison to swab samples taken from bats. This is in agreement with the hypothesis that hibernacula walls represent the multi-year reservoir of the pathogen while bats become infected from this reservoir anew each year (Fischer et al. 2020; 2022). This means that the *P. destructans* population on the walls is a composite of fungal MLGs shed by bats during the last hibernation season and MLGs surviving on the same walls from previous seasons. The latter would have been shed by bats during the hibernation seasons before the last (Fischer et al. 2020), or might eventually occur through environmental growth (potentially followed by reproduction) although such growth has not yet been documented in nature. Further studies are needed to better understand *P. destructans* life history strategy, especially in relation to its dormancy and survival on hibernacula substrates (Fischer et al. 2020).

Genetically diverse infections in bats

Despite the lower genotypic richness found in bat swab samples in comparison to the swab samples taken from hibernacula walls, infections with multiple MLGs were dominant in bats, with more than 80% of the animals harbouring multiple *P. destructans'* MLGs. However, we only considered three fungal isolates per swab for this analysis and the estimated 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. This 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 (Smith et al. 1999; Mercereau-Puijalon 1996) 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 & Taylor 2001) and possibly represent a powerful determinant for pathogen evolution and disease epidemiology (Susi et al. 2014). 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. 2008). 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 (Lopez-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) will be of paramount importance to address these questions.

Acknowledgements

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Supplementary Information for

Genetic diversity and population structure of *P. destructans*, the causative agent of White-Nose disease in bats. From large scale differentiation to locally homogeneous populations.

Violeta Zhelyazkova[1], Nicola M. Fischer[2,3], Sébastien Puechmaille[2,3,4,*]

[1]National Museum of Natural History, Bulgarian Academy of Sciences, 1 Tsar Osvoboditel Str., 1000 Sofia, Bulgaria

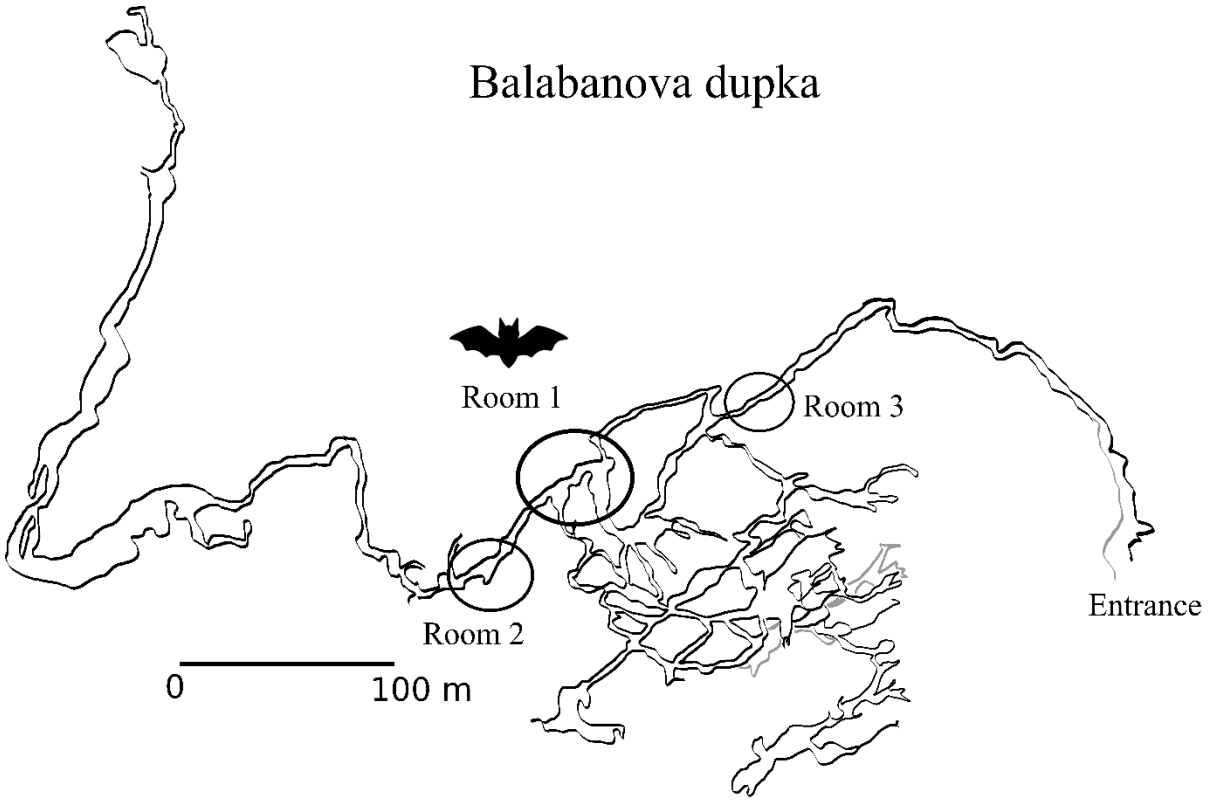
[2]Zoological Institute and Museum, University of Greifswald, Soldmannstr. 14, 17489 Greifswald, Germany

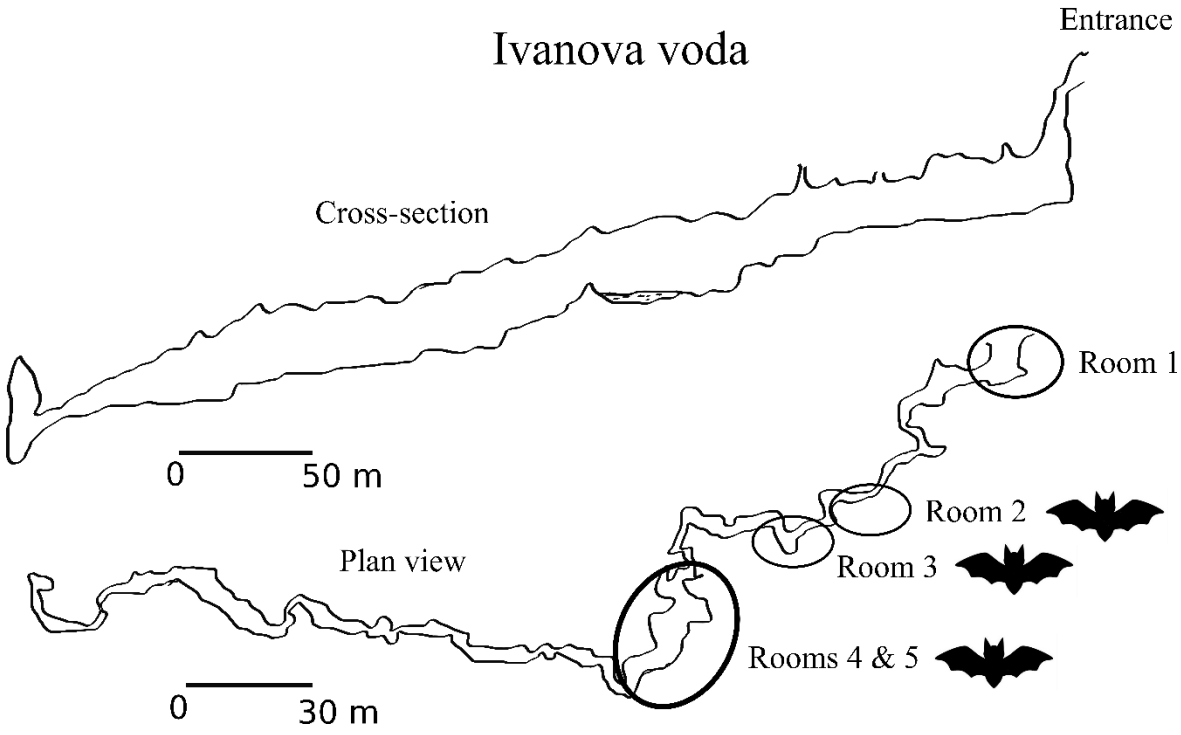
[3]ISEM, University of Montpellier, CNRS, EPHE, IRD, Place Eugène Bataillon, 34095 Montpellier Cedex 05, France

[4]Institut Universitaire de France, Paris, France

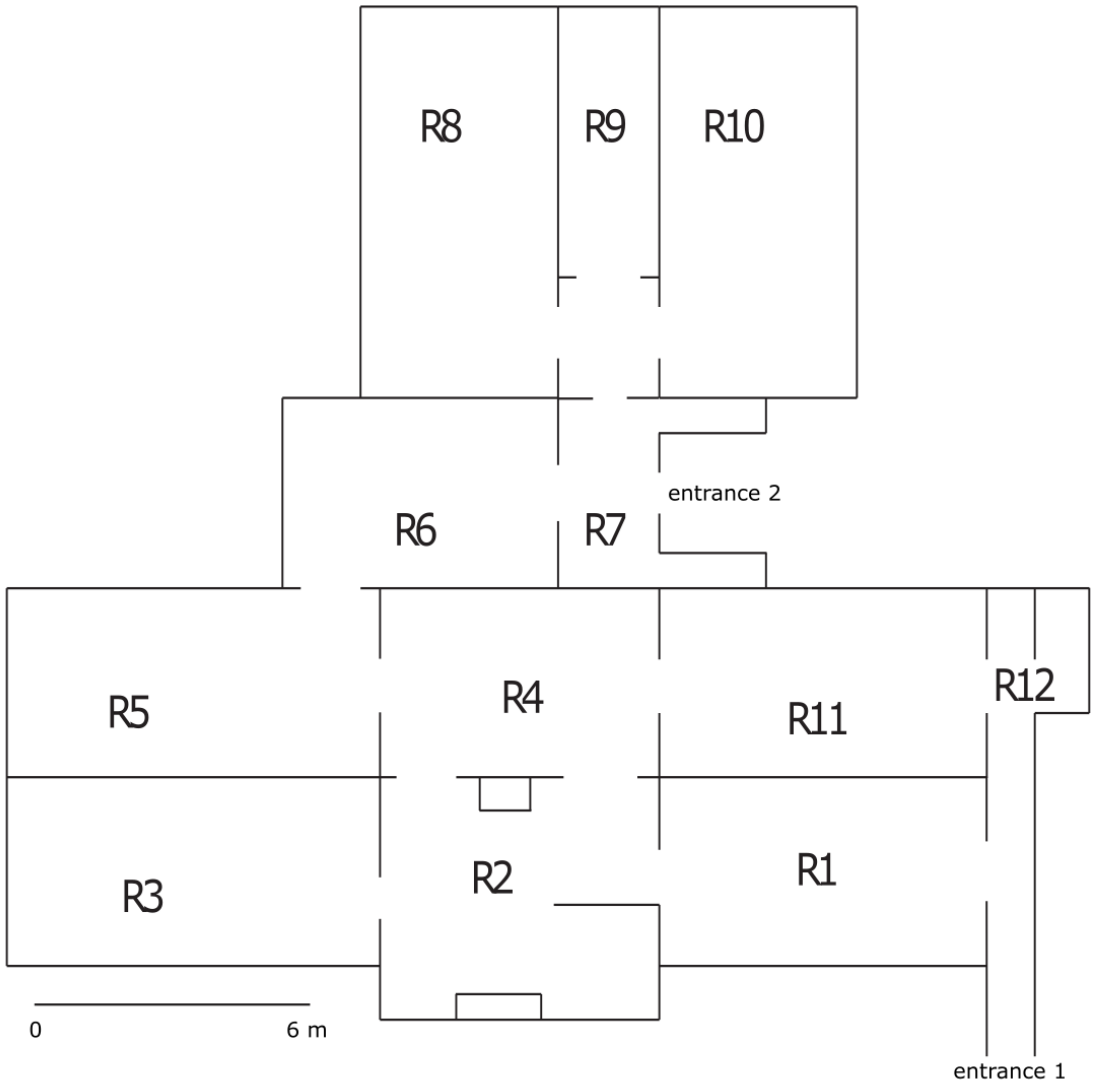
*Corresponding author: sebastien.puechmaille@umontpellier.fr

Supplement 1A: 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/blythi* 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).


Balabanova dupka
Room 1
Room 3
Room 2
Entrance
0
100 m


Ivanova voda
Entrance
Cross-section
0
50 m
Room 1
Room 2
Room 3
Plan view
Rooms 4 & 5
0
30 m

Supplement 1B: Sampling locations (rooms) in the artificial hibernaculum Eldena in Germany, where *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.



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Supplement 2A: Summary of the 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.

Sampling season	Total swab	Bat swab	Wall swab	Total SSI	Bat SSI	Wall SSI	Total MLG	Bat MLG	Wall MLG
Balabanova dupka									
Spring 2015	23	21	2	97	90	7	62	58	6
Spring 2017	34	11	23	99	48	51	72	35	44
Autumn 2017	13	0	13	21	0	21	18	0	18
Spring 2018	27	12	15	95	60	35	62	32	34
Autumn 2018	19	0	19	37	0	37	36	0	36
Spring 2019	56	19	37	259	107	152	151	58	113
Ivanova voda									
Spring 2017	29	15	14	98	62	36	78	45	34
Autumn 2017	1	0	1	3	0	3	3	0	3
Spring 2018	8	5	3	33	25	8	20	12	8
Autumn 2018	8	0	8	8	0	8	8	0	8
Spring 2019	28	20	8	113	93	20	68	51	18
Eldena									
Spring 2015	147	139	8	425	391	34	77	74	21
Autumn 2015	16	0	16	39	0	39	29	0	29
Spring 2016	40	31	9	118	80	38	44	34	22
Autumn 2016	6	0	6	22	0	22	17	0	17
Spring 2017	10	10	0	26	26	0	16	16	0
Autumn 2017	16	0	16	55	0	55	30	0	30
Spring 2018	61	53	8	183	149	34	45	37	19
Autumn 2018	15	0	15	52	0	52	32	0	32
Spring 2019	53	53	0	142	142	0	47	47	0

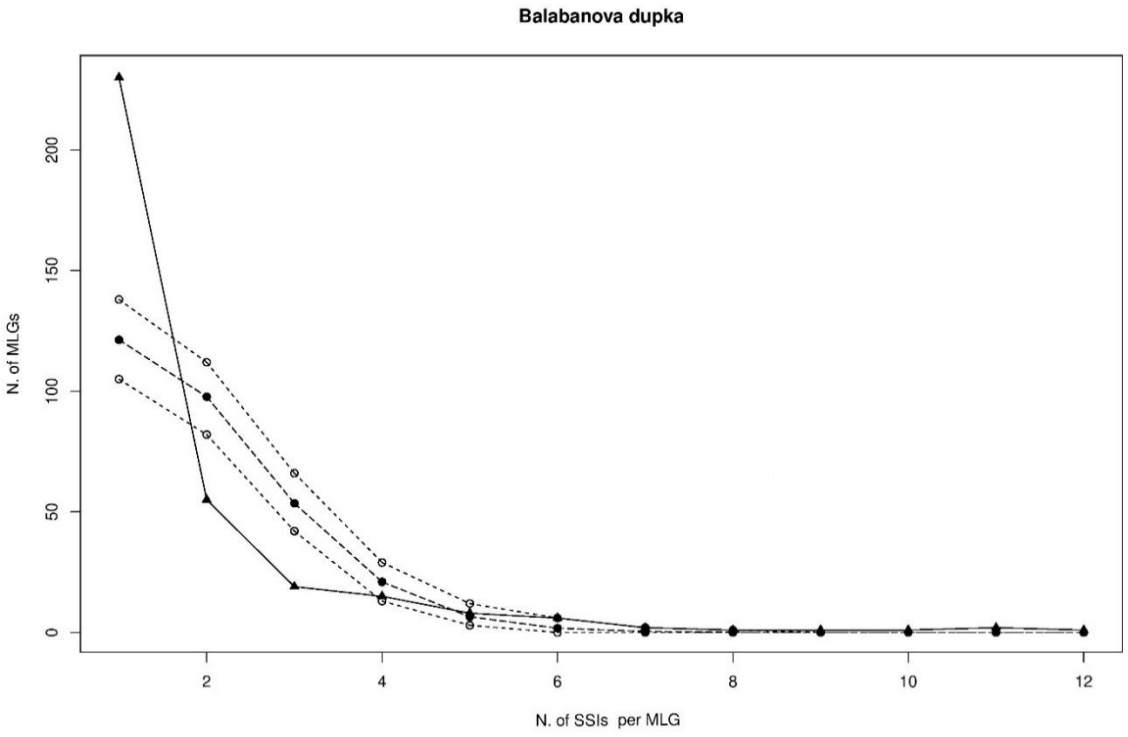
Supplement 2B: Summary of the swab samples, isolated single spore isolates (SSIs), and multilocus genotypes (MLGs) of *P. destructans* obtained from bats and from hibernacula walls in the study sites divided by rooms.

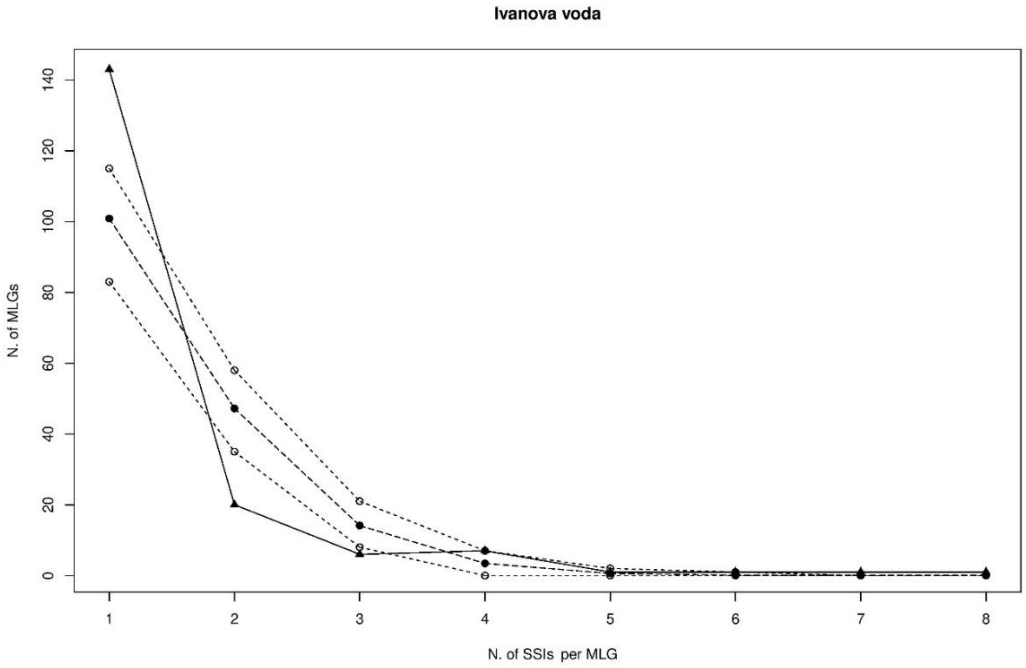
Room	Total swab	Bat swab	Wall swab	Total SSI	Bat SSI	Wall SSI	Total MLG	Bat MLG	Wall MLG
Balabanova dupka									
1	110	56	54	411	273	138	217	144	105
2	55	0	55	165	0	165	129	0	129
3	7	7	0	32	32	0	23	23	0
Ivanova voda									
1	3	0	3	3	0	3	3	0	3
2	60	38	22	230	172	58	147	97	53
3	6	1	5	13	5	8	10	2	8
4	3	0	3	5	0	5	5	0	5
5	1	0	1	1	0	1	1	0	1
NA	1	1	0	3	3	0	3	3	0
Eldena									
2	12	12	0	35	35	0	16	16	0
3	7	7	0	19	19	0	9	9	0
4	4	4	0	12	12	0	3	3	0
5	11	11	0	28	28	0	15	15	0
6	12	11	1	28	27	1	15	15	1
7	1	0	1	4	0	4	3	0	3
8	89	60	29	257	162	95	71	48	47
9	83	66	17	249	183	66	70	59	37
10	107	79	28	318	215	103	76	59	43
11	11	9	2	31	26	5	16	14	3
NA	27	27	0	81	81	0	25	25	0

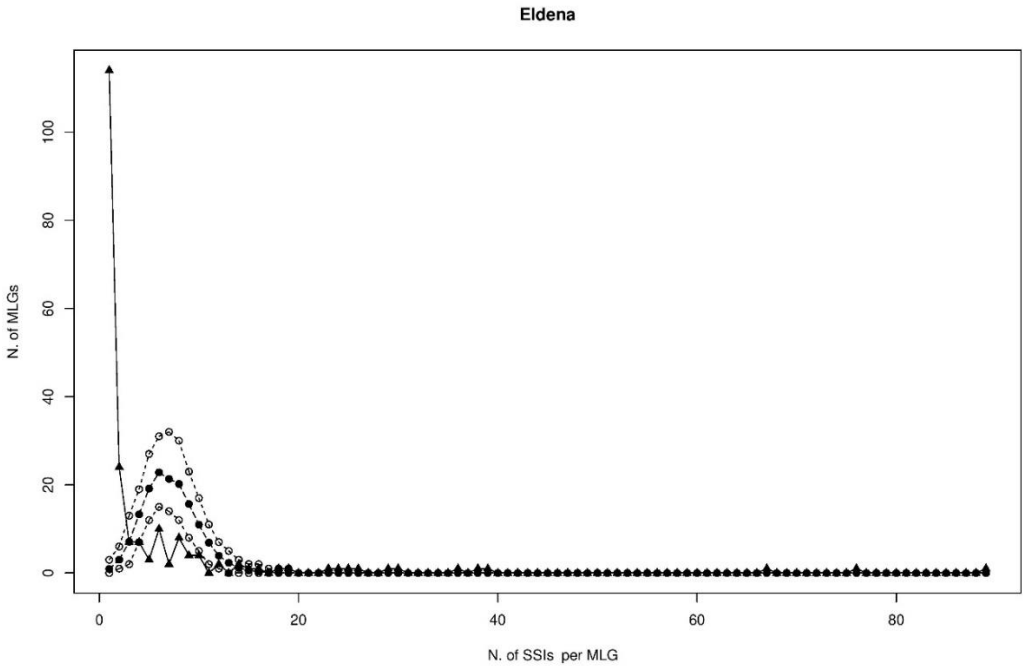
Supplement 3: Allelic richness per locus in the three study sites. Calculations were performed on clone corrected data after SSI with missing data were removed.

	Balabanova dupka	Ivanova voda	Eldena
Locus name	Number of alleles		
Pd1	25	28	14
Pd2	64	49	8
Pd3	13	11	3
Pd4	20	18	13
Pd5	5	31	13
Pd6	6	5	3
Pd7	14	12	5
Pd9	5	8	4
Pd10	3	7	3
Pd11	8	14	4
Pd12	6	5	2
Pd13	10	10	13
Pd14	10	14	5
Pd17	7	8	3
Pd19	11	12	5
Pd21	7	7	3
Pd22	9	14	4
Pd23	2	9	3
Mean	12.5	14.6	6

Supplement 4: 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 singles 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.







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Puechmaille SJ, Petit EJ. 2007. Empirical evaluation of non-invasive capture-mark-recapture estimation of population size based on a single sampling session. *J Appl Ecol* 44:843–852.

Supplement 5: 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.

	Swab	SSI	Allele	MLG	eMLG	Pop size	HPD 95%	M1	M2
Balabanova dupka	172	172	11.2	137.4	66.4	395.6	282-516	68.7%	31.3%
Ivanova voda	74	74	11.6	67.2	67.2	569.2	203-1082	73.8%	26.2%
Eldena	364	364	5.8	93.1	39.3	96.2	94-99	41.9%	58.1%

Supplement 6: 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.

		Swab	SSI	MLG	eMLG
One SSI per swab					
Balabanova dupka	Bats	63	63	57.3	57.3
	Walls	109	109	92.8	57.1
Ivanova voda	Bats	40	40	36.6	31.6
	Walls	34	34	33.2	33.2
Eldena	Bats	286	286	80.6	40.1
	Walls	78	78	42.1	42.1
Two SSIs per swab					
Balabanova dupka	Bats	63	126	93.5	93.5
	Walls	64	128	100.1	98.8
Ivanova voda	Bats	39	78	58.6	28.2
	Walls	16	32	31.3	31.3
Eldena	Bats	268	536	104.4	51.9
	Walls	61	122	52.7	52.7

4. Synthesis

4.1 Main findings

Pseudogymnoascus destructans (*Pd*) is a fungal pathogen causing White-Nose disease in hibernating bats and has been introduced from Eurasia to North America. In Manuscript 1, we detected a second cryptic causative agent of White-Nose disease (*Pd*-2) in the native Eurasian range which was consistently differentiated from *Pd*-1 (*P. destructans sensu stricto*) across different genetic measures (microsatellites, full genomes yielding BUSCO genes, and pooled Illumina sequencing resulting in a SNP dataset). Interestingly, the clade identity of isolates was not determined by geographical distances among sampled hibernacula, but rather both clades exist in sympatry. The genetic data presented fulfil the requirements put forward by Matute & Sepulveda (2019) to delineate fungal species boundaries, and I am therefore able to conclude that two bat-pathogenic *Pseudogymnoascus* species exist in Eurasia. Each of the clades is found in differing frequencies among bat species, with *Pd*-1 being particularly common on *Myotis myotis* bats and *Pd*-2 being most frequently found on *M. daubentonii.* These findings suggest differences in host preferences among clades, with *M. daubentonii* seeming resistant to infection with *Pd*-1. Furthermore, the visual *Pd*-score (an index characterising fungal colonisation and wing damage, Fritze et al., 2021) recorded during sample collection showed significant differences between the clades (higher score observed for *Pd*-1), implying differences in pathogenicity (the term 'pathogenicity' is used here as the relative capacity to cause damage in a host as defined by Casadevall & Pirofski, 1999). *Pd* isolates collected from hibernacula in North America were clearly assigned to clade *Pd*-1 (based on microsatellites, BUSCO genes, and overall sequence divergence) with *Pd*-2 currently not detected outside of Eurasia.

Within each of the clades (*Pd*-1 and *Pd*-2) populations were highly diverse in the native range and every hibernaculum contained numerous multi-locus genotypes (in the following simply referred to as 'genotypes'). Even for each single bat, infections by multiple genotypes were common, which also occasionally included inter-clade infections (by *Pd*-1 and *Pd*-2 together). Furthermore, genotypes were highly differentiated among hibernacula and different regions, with more than 95% of genotypes unique to a single hibernaculum. Even though geneflow was generally low (based on genotypic differentiation), analyses of effective migration revealed areas of higher and lower geneflow across Eurasia (for each of the clades separately), with the latter representing

potential barriers to geneflow. Based on varying allele frequencies, the North American *Pd*-1 isolates could be assigned to hibernacula in Eurasia (based on their microsatellite allele frequencies). This revealed the most likely origin of the introduction to be a region in Ukraine (Podilia), addressing a question of interest since disease emergence in 2006.

The genotypic richness and large number of samples obtained for *Pd*-1 in European hibernacula (95% of isolates were assigned to *Pd*-1) could further be used to quantify the movement of genotypes among several geographically close hibernacula as well as between different substrates within these hibernacula (bats and walls). Given the low rates of shared genotypes among these hibernacula, the infection of healthy bats after summer takes place in the hibernacula that bats hibernate in (as opposed to previously visited sites). These data complement prior knowledge on *Pd'*s low tolerance to elevated temperatures and provide evidence that bats generally purge *Pd* over summer. Walls are the most likely source of infection within hibernacula because there is usually a lot of contact between bats and walls during swarming and hibernation when bats land and crawl on the walls (Blažek et al., 2019; Puechmaille et al., 2011). Indeed, viable *Pd* was easily detected on the walls of hibernacula both in April (just after most bats emerged from hibernation) and in October (just before bats arrived for hibernation) in the hibernacula in Germany and Bulgaria. In a laboratory approach, we further confirmed the longevity of *Pd* spores (at least two years) on abiotic substrate under conditions similar to those found in hibernacula and discovered seasonal variations in *Pd*'s ability to germinate consistent with the timing of bat presences and absences. These findings suggest that *Pd* has adapted to the bat life cycle (i.e., the bat presences and absences in hibernacula), and further confirm the central role of environmental infection in the disease dynamics. Indeed, bat and wall samples shared a large proportion of genotypes, and together formed an undifferentiated population. Moreover, the number of unique *Pd* genotypes relative to the number of sampled isolates revealed a greater genotypic richness on walls compared to bats. This decrease of genotypic richness from walls to bats is consistent with the presence of a transmission bottleneck. Based on the observed patterns of genotypic richness, only an estimated 500 spores are transferred from walls to each bat during hibernation, a value much lower than the 1 million spores frequently used in experimental setups involving artificial inoculation of bats (e.g., Lorch et al., 2011). Consistent findings (regarding the presence of environmental *Pd* and patterns of genotypic richness between bats and walls) were obtained from

the two south-east European hibernacula (Bulgaria), showing that the presence of environmental reservoirs is most likely a general characteristic of the host-pathogen dynamics rather than specific to habitat types, geographic ranges, or bat population sizes. This work provided evidence of an environmental reservoir of *Pd* from which bats become infected, and which is likely to be replenished with *Pd* spores from infected bats towards the end of hibernation when bats exhibit maximum fungal loads (Figure 4; see Puechmaille et al., 2011 for temporal patterns in visual fungal loads). Given the greater number of isolates available for *Pd*-1, current work on environmental reservoirs focuses on genotypes from this clade as I was better able to reliably describe genotypic movements between and within hibernacula. However, given the ecological similarities between *Pd*-1 and *Pd*-2, as well as between their hosts, the main infection pathway causing the infection of healthy bats after summer (i.e., environmental infection) is expected to be the same between both clades.

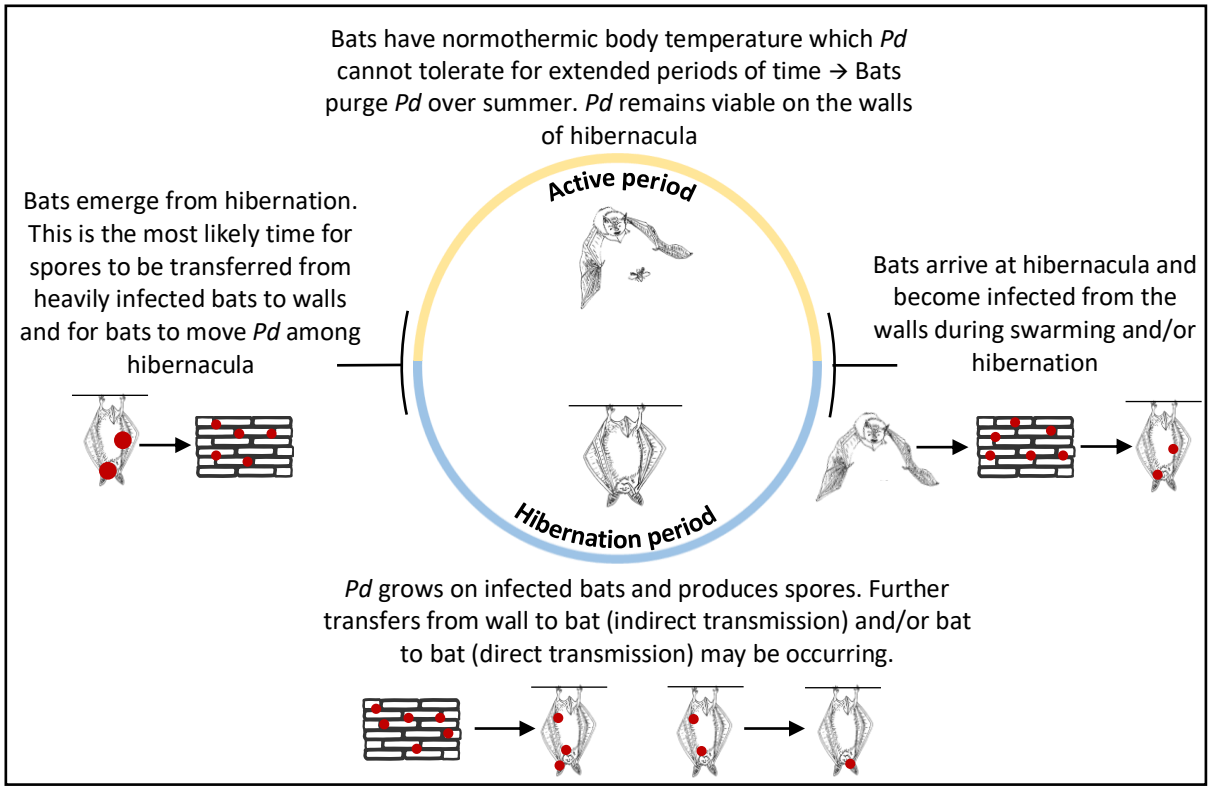

Figure 4: Seasonal patterns of White-Nose disease. The diagram shows the relevant characteristics of the bats' and pathogens' lifecycles, as elucidated by my work and previously hypothesised by other authors (e.g., Fuller et al., 2020; Hoyt et al., 2020; Langwig et al., 2015a; Puechmaille et al., 2011)

4.2 Additional considerations and suggestions for future research

The impact of a second White-Nose disease agent on past and future research

Knowing how different groups of organisms are structured can provide a considerable amount of information on their evolutionary histories and species-specific adaptations – with every species being adapted to their specific ecological niche. For pathogens, this means that species might behave differently in terms of environmental durability, dispersal rates, infection ability, and damage caused to hosts. Therefore, when two cryptic species have unknowingly been studied together, it is important to re-evaluate past findings in the context of possible inter-specific differences. My work investigated the genomic differentiation of *Pd* in its native range (Eurasia) and detected two sympatric monophyletic clades, *Pd*-1 and *Pd*-2, consistent with the presence of two cryptic species. Both are pathogens of bats and cause the characteristic growth associated with White-Nose disease. Most data on *Pd* exist for *Pd*-1 because only this clade is currently present in the invasive North American range, and it has also been more frequently sampled in Europe (Manuscript 1). So, much less is known about infections caused by *Pd*-2, for example whether North American bats are also susceptible to it and whether there are differences in histopathology and immune response elicited by bats experiencing the different clades (see e.g., Davy et al., 2017 for transcriptome responses of Eurasian and North American bats to *Pd*-1 only). Furthermore, to obtain an accurate risk assessment for bats in areas not currently harbouring White-Nose disease (e.g., Australia, Holz et al., 2019; Turbill & Welbergen, 2020), model priors (i.e., the underlying input data of models) will need to be re-investigated and possibly updated to include the potential introduction of not only *Pd*-1 but also *Pd*-2. Indeed, the current information on preferred humidity range only exists for North American *Pd*-1 isolates (Marroquin et al., 2017) while it is currently unknown whether the temperature-dependent growth limits were elucidated using only *Pd*-1 isolates or both clades (Verant et al., 2012). Regarding some work based on the previously valid assumption of a single *Pd* species, the presence of both *Pd* clades in Eurasian hibernacula means that findings need to be re-evaluated to ascertain whether they are valid for *Pd*-1, *Pd*-2, or both. This includes several observational studies comparing White-Nose disease in the invasive and native ranges, whereby both *Pd*-1 and *Pd*-2 are likely included in the native range – possibly skewing the findings (see e.g., the comparison of disease dynamics between North American and Asian hibernacula, Hoyt et al., 2016a; Zukal et al., 2016).

Now that we know about the presence of both *Pd* clades, it will be important to differentiate between them in future work (particularly when working within the native range of *Pd*). Morphologically, I was unable to reliably separate the two species, and both produce the kidney-shaped spores described as characteristic of *Pd* (see Figure 2B). Some of the microsatellite loci have fixed alleles in *Pd*-2 or have significantly different fragment lengths (see Manuscript 1) which could be used to genetically test for clade identity (e.g., using PCR amplification and gel electrophoresis). Alternatively, species-specific qPCR/LAMP markers could be designed to rapidly differentiate between species, without requiring the culturing of isolates or even DNA extraction (see e.g., Lorch et al., 2010; Muller et al., 2013; Niessen et al., 2022 for diagnostic methods currently used in *Pd* research – yet currently without species-specificity). Ultimately, there is a strong need to improve/develop tools for easy (and cheap) differentiation between *Pd*-1 and *Pd*-2 which, while being a technical issue, will be crucial for the future research of White-Nose disease.

Potential drivers of speciation

My work describes the presence of two *Pseudogymnoascus* species pathogenic to bats though the conditions under which both species evolved (i.e., speciation) are currently unknown. Host-species shifts have been described as potential drivers of sympatric speciation, which occurs while both species inhabit the same geographic range (Giraud et al., 2010; Giraud et al., 2008). Speciation generally occurs when there are barriers to gene flow that lead to reproductive isolation, allowing the segregation of locally advantageous genes between two or more populations (e.g., Coyne & Orr, 2004). In the absence of geographical barriers (i.e., in sympatry), this can be caused by assortative mating (mate choice or habitat choice), for example if some genotypes only reproduced on/in a specific host, and other genotypes could not grow on/in the same host or were outcompeted (Giraud, 2006; Giraud et al., 2010; Nosil et al., 2005). This was demonstrated for the fungal pathogen *Venturia inaequalis*, whereby populations infecting apple and pycantha plants were found to be strongly differentiated, and cross-inoculations between both host plants were unsuccessful even though populations were interfertile in laboratory experiments (Gladieux et al., 2010; Le Cam et al., 2002). Considering the sympatric host preferences seen in *Pd*-1 and *Pd*-2, it may be that their speciation was driven by some genotypes' ability to grow and reproduce on a bat species that other genotypes were unable to infect and/or successfully reproduce on.

Given the current data, however, this is just one possibility. A historical period of allopatry (geographical isolation with associated barriers to gene flow) could also account for *Pd* speciation. So far, only *Pd*-2 has been detected in eastern Asia, with the easternmost observation of *Pd*-1 being the Ural Mountains in Russia (additional *Pd* samples from Russia, including from hibernacula further east, were collected by Kovacova et al., 2018 but without identification of the *Pd* clade). With the low number of isolates currently available from eastern Asia, *Pd*-1 may have simply been missed and may be rarer there. This would be intriguing considering that, in Europe, *Pd*-1 is by far the dominant clade (95% of isolates). It is therefore conceivable that *Pd*-1 was historically native to Europe while *Pd*-2 was historically native to (eastern) Asia, and both species only recently became sympatric through the range expansion of one or both of the clades. It is also worth noting that among the *Pd*-2 isolates from Europe, Gd614 from Iberia falls basally and seems clearly differentiated from the other European *Pd*-2 isolates. It is currently unknown whether this finding is an artefact or whether it implies an early presence of Gd614's ancestors in Europe or recent introduction from elsewhere (e.g., eastern Asia). While not an easy task, understanding the evolutionary histories of pathogens can provide insights into ecological adaptations (including the evolution of pathogenicity) as well as into the coevolutionary histories between bats and both pathogens (*Pd*-1 and *Pd*-2). Extended sampling in eastern Asia could reveal whether *Pd*-1 is truly absent there. If so, inoculation experiments on eastern Asian bat species (e.g., *Myotis petax* and *Plecotus ognevi*) could reveal whether these bats are immune to infection by *Pd*-1 or if other factors (e.g., environmental) are responsible for its absence in this geographic region. A further step could utilise comparative genomics to investigate areas of high differentiation using genome-wide scans, which could indicate species-specific selective sweeps and adaptations against gene flow (so called "genomic islands of speciation", e.g., Nadeau et al., 2012; Stukenbrock, 2013; Turner et al., 2005). Comparative genomics could also be used to estimate the timing of species divergence, which could provide further information on potential drivers of this genetic differentiation (e.g., Dutheil & Hobolth, 2012).

Pathogenicity in *Pd*-1 and *Pd*-2

Besides host-species specificity, there were also differences in pathogenicity (i.e., expected host damage, Casadevall & Pirofski, 1999) between *Pd*-1 and *Pd*-2. *Pd*-1 yielded a higher visual *Pd*-score (see Fritze et al., 2021), indicating that infected areas were more extensive and that *Pd-1* generally

exhibited more pronounced growth on the bats. The maximum *Pd*-score [4] was obtained for nearly 10% of samples containing *Pd*-1 yet was not present at all among samples containing *Pd*-2, while roughly 45% of *Pd*-1 samples but over 85% of *Pd*-2 showed the lowest level of growth [1] (very weak visual growth). To ensure the reliability of the *Pd*-scores, the data only included samples from one region in north-eastern Germany where most samples were collected by the same person. Going forward, *Pd*-scores should be obtained from additional regions in Eurasia (for *Pd*-1 and *Pd*-2 samples) to determine whether pathogenicity is uniform across the native range, or dependent on bat species, environmental conditions or even variable among genotypes. In fact, differences in pathogenicity among different genotypes or groups of genotypes, which may then be termed 'pathotypes', are not rare among fungi (e.g., Goodwin et al., 1995; Jenner & Henry, 2022; Murithi et al., 2021). These differences in pathogenicity can also mean that hosts that have found ways to deal with one pathotype (through phenotypical or behavioural resistance, e.g., Butler & Behringer, 2021) or are immune to infection may be susceptible to novel introductions of the same species but different genotype (see e.g., the regular re-introductions of novel genotypes of *Phytophtora infestans* and their impact on hosts; Wang et al., 2017). I did not investigate the presence of such pathotypes in detail, though this should be addressed in future and could have major implications for the long-term dynamics of disease.

The role of co-infections in White-Nose disease dynamics

The co-occurrence of the same or differing genotypes on hosts is common among pathogens and further complicates questions surrounding pathogenicity (see e.g., Fortuna et al., 2018; Susi et al., 2015; Tollenaere et al., 2016). The outcomes of such diverse infections for the hosts (in terms of pathogenicity) vary among disease systems, and in many cases also depend on the relatedness between co-infecting genotypes with higher relatedness often resulting in lower mutual inhibition (kin selection; Buckling & Brockhurst, 2008; López-Villavicencio et al., 2011). In bacterial isolates of *Xenorhabdus bovienii*, genetically diverse infections led to the production of a growth inhibitor (bacteriocin) causing spiteful interactions (i.e., harming both themselves and others) which resulted in a reduction of pathogenicity against its nematode hosts (Buckling & Brockhurst, 2008; Inglis et al., 2009). In other host-pathogen systems however, for example human HIV (Lawn, 2004) and the fungal plant pathogens *Podosphaera plantaginis* (Laine & Mäkinen, 2018) and *Microbotryum lychnidis*-dioicae (Buono et al., 2014; López-Villavicencio et al., 2011), pathogenicity

increased when infections were diverse. These findings highlight the varying impacts that multiple (genotypically diverse) infections can have in different disease systems, and some authors have suggested that multiple infections may also increase a pathogen's epidemic potential (see e.g., Tollenaere et al., 2016).

While multiple infections of the same genotype (i.e., clones) are difficult to investigate in the wild, I frequently detected multiple genotypes on the same bat both in *Pd*-1 and *Pd*-2 (and mixed between both clades, Manuscripts 1, 3 & 4). Interestingly, competition among *Pd* isolates was previously hypothesised following a study by Johnson et al. (2014) who investigated the effect of different *Pd* spore loads (500, 5,000, 50,000 and 500,000 per bat) on the survival odds of a North American bat species (*Myotis lucifugus*). Only the lowest infection load of 500 spores per bat significantly reduced survival odds, while greater loads showed no difference in mortality from control groups. The authors suspected self-inhibition of *Pd* on the bats when many spores are present (i.e., through competition) and hypothesised that mortality should generally be higher when bats are only exposed to a relatively low number of spores early in hibernation. These findings are highly consistent with characteristics of the environmental reservoir (Manuscript 3), whereby the number of spores transferred from walls to bats (causing the initial infection) was estimated to amount to a few hundred. It should be noted here that in the artificial inoculation of bats by Johnson et al. (2014), only spores from a single isolate were used (collected in North America, i.e., belonging to *Pd*-1) hence competition among different genotypes was not addressed. Diverse co-infections are generally common in White Nose disease but their effect on hosts (i.e., in terms of pathogenicity), and the morphological or chemical tools *Pd* isolates might use during resource competition, are currently unknown. *Pd* provides an excellent system for studying the interaction of co-infecting genotypes with reduced variation (in the invasive range) and natural variation (in the native range), as well as between closely related pathogen species (*Pd*-1 and *Pd*-2) which can also infect bats in interspecific co-infections.

The risk posed by genetic exchange between pathogens

Genetic change within populations/species can arise through de-novo mutations or through the exchange of genetic material between genotypes (which can involve recombination). While the rate of de-novo mutations can enable us to estimate the risk posed by a pathogen in terms of genetic diversity and adaptive potential (higher mutation rates generally increase the danger

posed by pathogens, see e.g., Alexander & Day, 2010; McDonald & Linde, 2002), we cannot specifically prevent them from occurring. However, we can prevent (or reduce the risk of) the anthropogenically-aided genetic exchanges that may cause jumps in virulence and/or changes to tolerated ecological niches including host-species shifts – and which can ultimately lead to the emergence of novel pathogens or exacerbate existing diseases.

In fungi, genetic exchanges can occur via multiple pathways, though the most common ones are likely to be horizontal gene transfer (Feurtey & Stukenbrock, 2018) and sexual reproduction (e.g., Voelz et al., 2013). It is currently unknown whether and to what extent either of these pathways occur in *Pd*. That being said, there are some indicators of facultative sexual reproduction in *Pd* (although only clonal reproduction via spores has hitherto been observed). Firstly, it has been predicted that most (if not all) fungal pathogens should be able to sexually reproduce as this allows pathogens to coevolve with changing environments and their hosts (see Box 2, Drenth et al., 2019). Secondly, the genetic structure of *Pd* in its native range shows that the distribution of mating types is not monophyletic in microsatellite trees (even within each hibernaculum; genotypic data and corresponding mating types available with Manuscripts 3 & 4). In the complete absence of sexual reproduction, each of the mating types should form monophyletic clades with genotypic diversity accumulating from de-novo mutations. In other words, the high relatedness observed between genotypes of different mating types implies sexual reproduction that has resulted in closely related offspring of both mating types during the recent evolutionary history of current genotypes. Thirdly, both mating types were regularly found on the same bat body parts (i.e., obtained from the same swab sample) suggesting that close physical proximity is frequent (for *Pd*-1, *Pd*-2, and between both clades). Consequently, it seems likely that facultative sexual reproduction occurs in *Pd*, though its frequency, timing and location are currently unknown and remain to be addressed.

When genetic exchanges occur between two genetically distant individuals (historically isolated genotypes within the same species or hybridisation between two closely related species) there is an increased risk of novel phenotypes with specific ecological or epidemiological adaptations not found in their progenitors (see e.g., Drenth et al., 2019; Stukenbrock, 2016; Stukenbrock et al., 2012). I detected three areas of low gene flow for *Pd*-1 and one larger area of low gene flow for *Pd*-2 reducing the genetic connectivity between geographic regions of Eurasia. The

(anthropogenic) movement of isolates between any of these regions (where natural genetic exchanges seem rare) should be avoided to prevent the potential emergence of novel phenotypes (see e.g., Farrer et al., 2011; Voelz et al., 2013).

Factors expected to influence gene flow in the native and invasive ranges

While I did not specifically investigate any differences in ecological or epidemiological parameters between the invasive and native ranges of *Pd*-1, it is noticeable that the low rates of gene flow among hibernacula in the native range (Publications 1 & 3) at first seem inconsistent with the rapid geographical expansion of the disease in North America (between 200 and 900 km per year, U.S. Fish and Wildlife Service, 2022). A major difference between *Pd* in its invasive and native ranges may be the amount of competition experienced by *Pd*. As mentioned above, competition among isolates, and particularly different genotypes and possibly between *Pd*-1 and *Pd*-2, on the bat hosts may play a significant role in the infection dynamics (likely exacerbated in Eurasia, where infections are more genetically diverse). Gene flow between hibernacula in the native range, which already harbour *Pd*, is therefore a separate process from the novel colonisation of hibernacula in the invasive range (e.g., Kerth & Petit, 2005; Wade & McCauley, 1988).

In addition, *Pd* likely experiences some form of intra- or inter-specific competition in hibernacula environments (e.g., on the walls), which contain diverse microbiota (e.g., Kosznik-Kwaśnicka et al., 2022). Indeed, some cave-dwelling microorganisms can produce secondary metabolites (e.g., antibiotics or antifungals – which can be volatile) to inhibit the growth of other nearby microorganisms (see e.g. Cornelison et al., 2014; Zada et al., 2021). While *Pd* can be assumed to have coevolved with the cave-dwelling microbiota typically encountered in Eurasia, it is possible that the lack of coevolutionary history in North America gives *Pd* a competitive advantage. Lack of competition and/or predators, resulting in an increased competitive ability in the invasive range, is frequently a predictor of a species' invasiveness (e.g., Blossey & Notzold, 1995; Crawley et al., 1987; Van Kleunen et al., 2010), yet its role in the emergence of *Pd* in North America remains to be investigated.

Managing White-Nose disease in North America

A large proportion of problematic pathogens infecting humans and wildlife possess durable spores which remain viable in the environment and can be transmitted to hosts from environmental reservoirs (Fisher et al., 2012; Hopkins et al., 2022). Such environmental reservoirs, as described herein for *Pd*, create challenges for disease management because methods that target the hosts (in an aim to reduce host-to-host transmission) are often ineffective when most individuals become infected not from other hosts but from the environment. In addition, traditional management strategies for pathogens carried by wild animals, such as the culling of affected populations, are heavily criticised due to their impact on biodiversity (including genetic diversity) and for ethical reasons (Lambert et al., 2021; Miguel et al., 2020). The seasonal re-occurrence of White-Nose disease in hibernating bats is driven by the environmental reservoir which infects healthy bats when they return to hibernacula after summer. Therefore instead of employing management strategies that focus on the infected hosts (e.g., culling infected bats in an attempt to reduce host-to-host infections, discussed in Langwig et al., 2015b), it might be better to reduce/remove *Pd* from hibernacula environments to break the cycle of re-infections. In the long-term, a reduction of *Pd* on hibernacula walls could be achieved through inhibition of growth on hibernating bats – assuming that far fewer spores would be moved from less heavily infected bats to walls – and several inhibitors of *Pd* have already been found (e.g., Boire et al., 2016; Cheng et al., 2017; Micalizzi & Smith, 2020). Alternatively, environmental *Pd* could also be targeted directly, for example using heat (Verant et al., 2012) or ultraviolet radiation (UV-C; Palmer et al., 2018). The latter has been shown to significantly reduce *Pd* from walls, with a 97.3% reduction of viable environmental *Pd* after whole-room UV-C treatment (Kwait et al., 2022; Palmer et al., 2018). However, this method's efficacy in real hibernacula, which typically have many small cracks and crevasses, remains to be tested. In general, it is difficult to find methods that are feasible (i.e., do not need to be constantly repeated or involve high technical complexity) and relatively inexpensive. There is currently no method which can prevent the bat-mediated return of *Pd* to a hibernaculum after its eradication there, meaning that any management strategy (whether based on the hosts or the reservoir) would need to be repeated regularly. Methods targeting the environmental reservoir would probably need to be repeated every two to three years given that the numbers of infected bats, mortality rates, and the amount of *Pd* in the environment following its introduction to a hibernaculum (i.e., first detection) show a lag one year after introduction but

strongly increase in subsequent years (e.g., Frick et al., 2017; Verant et al., 2018). It should be mentioned here that I do not consider the complete eradication of *Pd* from North America, or even from the majority of hibernacula, realistic (at least not with currently available tools). Instead, data on endangered bat populations and their movements could be used to select refugia for disinfection, enabling a proportion of bats to survive.

One difficulty hereby will be to select the level of intervention regarding how many hibernacula/populations are chosen. Signatures of genetic adaptations to combat *Pd* have already been detected in some North American *Myotis lucifugus* survivors of *Pd* (Auteri & Knowles, 2020). It is currently unknown whether these positively selected genes could spread through sufficient numbers of individuals rapidly enough to protect species from extirpations and extinctions ("evolutionary rescue", see e.g., Gomulkiewicz & Holt, 1995; Gomulkiewicz & Shaw, 2013), particularly considering bats' slow life histories (e.g., Brunet-Rossinni & Austad, 2004; Keen & Hitchcock, 1980). In general, individuals must experience selection pressure for adaptive change to occur which, in this case, requires exposure to infection by *Pd*. It is therefore not only a question of which management strategy should be chosen, but also how many bats should be protected (e.g., through refugia) considering that in some cases management action is expected to have a negative impact on populations' and species' long-term survival compared to complete inaction (Maslo et al., 2017). More specifically, there will likely be a trade-off between managing the disease – to maintain bats' census sizes and associated genetic diversity (which is at the basis of adaptive potential) – and letting bats experience severe infections and associated selection pressure, even if some bats die. While there is often pressure to rapidly manage newly emerging diseases, we need to accept that White-Nose disease has become firmly established in North America. We should therefore take the time to evaluate different approaches and model their expected long-term outcomes while including the possibility that affected North American bats could accumulate genetic resistance/immunity (see also Bernard et al., 2020; Fletcher et al., 2020; Langwig et al., 2015b; Maslo et al., 2017).

4.2.8 Preventing further introductions of *Pd* to novel ranges

The design of effective management strategies for wildlife pathogens (which can include the above-mentioned deliberate lack of disease management) is a central aspect after a pathogen has emerged and is the only way to limit disastrous effects on populations, species and ecosystems.

However, 'prevention is better than cure' and preventing the emergence of novel pathogens can save a lot of resources and, more importantly, conserve biodiversity. In most cases, emerging infectious diseases are associated with a pathogen's arrival in a novel geographic range, where hosts have never experienced the pathogen before and are therefore evolutionarily not well equipped to combat it (Daszak et al., 2000; Jones et al., 2008). This can also be observed in White-Nose disease, where co-evolved Eurasian hosts do not succumb to the infection while some North American bat species exhibit high levels of mortality following the introduction of *Pd*. The spread of *Pd* across shorter distances and its associated geographical spread across North America is largely due to bat-mediated transport of viable fungal material (e.g., Maher et al., 2012; Wilder et al., 2011). However, human-mediated transport of *Pd* is currently the only explanation for the introduction of *Pd* from Eurasia to North America. In addition, the sudden occurrence of *Pd* in western North America in 2016, 2,100 km away from the nearest other observation of White-Nose disease at the time, is highly inconsistent with the speed and pattern of observed 'natural' expansion of *Pd* in North America (Lorch et al., 2016; Maher et al., 2012). This finding is most likely due to an anthropogenic introduction from eastern North America to the west, which is phylogenetically congruent (Lorch et al., 2016). It is currently unclear whether frequent anthropogenic movement of *Pd* also occurs in Eurasia, where it is more difficult to detect such movements due to the pan-continental distribution of *Pd*.

The extensive dataset of genotypes from Eurasia allowed me to ascertain the most likely region of *Pd*'s anthropogenic introduction from Eurasia to North America (Podilia, Ukraine). Determining the source population of invasive species is generally of great interest as it can provide more detailed information on the mode of species' introductions (e.g., Fofonoff et al., 2003; Kolar & Lodge, 2002; Rollins et al., 2006). Going forward, a more tight-knit sampling of hibernacula within the region of origin could elucidate the exact hibernaculum (or the two to three most likely ones) to provide information on the type (tourism, caving, other) and intensity of anthropogenic activities there. With this information, risk assessment analyses of further anthropogenic movements of *Pd* could be significantly improved, and particular risks (e.g., from tourism) might become apparent. In addition, the source population of an introduced organism can be used as a benchmark of genotypic diversity and local adaptation against which genetic changes in the invasive range may be measured. This might be of particular importance for *Pd* considering its large genetic diversity and could mean that changes of *Pd* in the North American range (e.g.,

potential adaptations to environmental conditions, to microbial competition, or to hosts) could be better determined in future. The identification of a pathogen's source population is very often hampered by the lack of knowledge on a novel pathogen's natural distribution and genotypic richness, as well as the need to sample a broad geographic range with sufficient numbers of individuals (see e.g., Muirhead et al., 2008). The contributions of many collaborators and scientists, particularly regarding sample collection but also laboratory work, were essential for guaranteeing the coverage required to address the origin of *Pd-1'*s North American introduction.

Currently, only *Pd*-1 has been introduced to North America, and there is a clear danger of *Pd*-2 also being anthropogenically introduced (e.g., in the same way that *Pd*-1 was introduced). In Eurasia, *Pd*-1 and *Pd*-2 show a clear host-species preference, with *M. daubentonii* not being susceptible to infection by *Pd*-1 but being the main host for *Pd*-2. Therefore it is likely that some North American bat species currently (largely) unaffected by the presence of *Pd*-1 (see e.g., Frank et al., 2014; Jackson et al., 2022; Langwig et al., 2012; Turner et al., 2011) could be susceptible to infection by *Pd*-2. It is also currently unknown what effect the co-infection of bats with *Pd*-1 and *Pd*-2 might have on hosts' outcomes, particularly on bats in the invasive range. Apart from North America, bats from the southern hemisphere could also be in danger if *Pd* (*Pd*-1, *Pd*-2 or both) is introduced there. This particularly affects bats in Australia and New Zealand (Holz et al., 2019; Turbill & Welbergen, 2020), with less risk posed to South American bats (e.g., *Myotis chiloensis*) due to their lower rates of bat-to-bat contact making *Pd* less likely to be spread and maintained in that range (Lilley et al., 2020). In Australia (and probably also New Zealand), environmental conditions are consistent with the potential establishment of *Pd* though it is unclear whether bat species are susceptible to infection by *Pd*-1 and/or *Pd*-2 and further work will be required to assess the hibernation biology of Australian and New Zealand bats (McNab & O'Donnell, 2018; Turbill & Welbergen, 2020).

To sum up, the movement of pathogens among different regions/continents should be avoided as it risks exposing naïve hosts to a pathogen they might not be well adapted to, risking population crashes and biodiversity loss. As a scientific community, we need to prioritise the detection and characterisation of fungal pathogens to limit their accidental transport and combat local outbreaks rapidly (see e.g., Cunningham et al., 2017; Gao, 2019). Regarding *Pd*, the danger of further pathogen introductions could be significantly reduced if all visitors to caves and bat

hibernacula (e.g., tourists, recreational cavers and researchers) strictly adhered to decontamination protocols between visits to different sites (Salleh et al., 2020; Zhelyazkova et al., 2020). Generally, intercontinental trade – and the associated transport of products (particularly of animal and plant products, as well as soils) – should be limited or, if unavoidable, strict decontamination protocols should be followed (through the establishment of 'prezootic'/'biosafety' protocols).

4.3 Conclusion

While often not central to initial investigations of invasive emerging pathogens, the native range (i.e., where a pathogen originated from) can provide valuable information on the biology and evolutionary history of the pathogen. *Pd* was introduced from Eurasia (most likely Ukraine) to North America, and by investigating its genetic differentiation and diversity in the native range I was able to provide evidence for the existence of a second causative agent of White-Nose disease (*Pd*-2). This second species had previously been 'cryptic' due to the fact that infections by *Pd*-1 (corresponding to *P. destructans sensu stricto*) and *Pd*-2 cannot be visually differentiated in the field. However, the two species differ in host-species preferences and exhibit differences in pathogenicity even though they occur in sympatry in Europe. Currently, only *Pd*-1 is present in North America and the additional introduction of *Pd*-2 could have drastic effects on North American bats. Similarly, bats in the Southern hemisphere are currently not experiencing White-Nose disease but might also be susceptible to, and threatened by, *Pd*-1 and *Pd*-2 in future (e.g., Turbill et al., 2011). The high level of intraspecific genetic differentiation observed within *Pd*-1 and *Pd*-2 implies the need to prevent the movement of genotypes even within the native range – considering that genetic exchange between distant genotypes (or species) can result in host-species shifts and/or jumps in pathogenicity. These findings highlight the need for 'prezootic'/biosecurity measures to be put in place to prevent the accidental movement of pathogens beyond their native range (on a genotype and species level).

The high genotypic diversity in Eurasia also allowed me to follow genotypes among hibernacula and between bats and walls to study the infection pathways of White-Nose disease. This method, which would have been impossible in North America given *Pd*'s low genotypic richness there (due to the invasion bottleneck) revealed the presence of a functional environmental reservoir consisting of viable *Pd* spores on the walls of hibernacula. As had previously been hypothesised

(e.g., Puechmaille et al., 2011), it is this reservoir which drives the yearly occurrence of White-Nose disease by infecting healthy bats when they return to hibernacula after summer. Furthermore, environmental reservoirs are associated with an increased risk of species extinctions, and impact the effectiveness of certain disease management strategies (Daszak et al., 2000).

Wildlife pathogens are a major threat to plants, animals and humans, as well as global biodiversity, yet they are severely understudied (Almeida et al., 2019; Fisher et al., 2012; Kainz et al., 2020; Rodrigues & Nosanchuk, 2020; Rokas, 2022). In this thesis I address key aspects regarding the phylogeny, ecology and epidemiology of a wildlife pathogen of bats, and highlight the dangers associated with the movement of pathogens. More work like this will be needed to create a catalogue of knowledge on the distributions and population structures of fungal pathogens so that, as a scientific community, we can better understand and combat their emergence.

5. References

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6. Eigenständigkeitserklärung

Hiermit erkläre ich, dass diese Arbeit bisher von mir weder an der Mathematisch-Naturwissenschaftlichen Fakultät der Universität Greifswald noch einer anderen wissenschaftlichen Einrichtung zum Zwecke der Promotion eingereicht wurde.

Ferner erkläre ich, dass ich diese Arbeit selbstständig verfasst und keine anderen als die darin angegebenen Hilfsmittel und Hilfen benutzt und keine Textabschnitte eines Dritten ohne Kennzeichnung übernommen habe.

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Nicola M. Fischer

7. Curriculum vitae

PERSONAL INFORMATION

Name: Nicola Mira Fischer

Contact: Fischer.Nicola@yahoo.com

EDUCATION

04/2018 – present: **University of Greifswald** and between 04/2019 and 07/2021 guest researcher at the **University of Montpellier**: PhD on the wildlife pathogen *Pseudogymnoascus destructans* under supervision by Prof. Dr. Gerald Kerth and Dr. Sébastien Puechmaille

10/2014 – 06/2017: **University of Greifswald**, Biodiversity and Ecology
Degree: Master of Science

10/2010 – 09/2013: **University of Konstanz**, Biological Sciences
Degree: Bachelor of Science

07/2010: **Albert-Schweitzer Gymnasium**
Receipt of Higher Education Entrance Qualification (Abitur)

PUBLICATIONS

FISCHER, N.M.; DUMVILLE, I.; ZHELYAZKOVA, V.; STECKER, R.-M.; BLOMBERG, A.; DOOL, S.E.; FRITZE, M.; TILAK, M.-K.; BASHTA, A.-T.; CHENAL, C.; FISTON-LAVIER, A.-S.; PUECHMAILLE, S. J. Uncovering cryptic fungal diversity reveals a second causative agent of bat White-Nose disease with distinct host specialisation. *Science* (in review)

ZHELYAZKOVA, V.; **FISCHER, N.M.**; PUECHMAILLE, S.J. Genetic diversity and population structure of *P. destructans*, the causative agent of White-nose disease in bats. From large scale differentiation to locally homogeneous populations. *Journal of Wildlife Diseases* (in revision)

FISCHER, N.M.; ALTEWISCHER, A.; RANPAL, S.; DOOL, S.E.; KERTH, G.; PUECHMAILLE, S. J. Population genetics as a tool to elucidate pathogen reservoirs: Lessons from Pseudogymnoascus destructans, the causative agent of White-Nose disease in bats. *Molecular Ecology* (2022)

FISCHER, N.M.; DOOL, S.E.; PUECHMAILLE, S. J. Seasonal patterns of *Pseudogymnoascus destructans* germination indicate host–pathogen coevolution. *Biology Letters* (2020)

DOOL, S.E.; ALTEWISCHER, A.; **FISCHER, N.M.**; DREES, K.P.; FOSTER, J. T.; FRITZE, M.; PUECHMAILLE, S. J. Mating type determination within a microsatellite multiplex for the fungal pathogen *Pseudogymnoascus destructans*, the causative agent of White-Nose disease in bats. *Conservation Genetics Resources* (2020)

CONTRIBUTIONS TO CONFERENCES

24 – 26/02/2017	**FISCHER, N.M.;** ALTEWISCHER, A.; PUECHMAILLE, S. J., Specificities of bat life cycle drive the need for an environmental reservoir for the pathogen *Pseudogymnoascus destructans*, the causative agent of White-Nose disease. International Berlin Bat Meeting in Berlin, Germany (**Talk**).
19 – 21/04/2017	**FISCHER, N.M.;** ALTEWISCHER, A.; PUECHMAILLE, S. J., Yearly outbreak of White-Nose disease in bats associated with an environmental reservoir for the pathogen *Pseudogymnoascus destructans*. German Zoological Society Graduate Meeting in Plön, Germany (**Talk**; 1st prize for student presentation)
01 – 05/08/2017	**FISCHER, N.M.;** ALTEWISCHER, A.; PUECHMAILLE, S. J., Investigating the role of environmental reservoirs in the persistence and transmission of P*seudogymnoascus destructans*, the causative agent of White-Nose disease. European Bat Research Symposium in Donostia, the Basque Country (**Talk**)
10 – 15/09/2018	**FISCHER, N.M.;** ALTEWISCHER, A.; DOOL, S. E.; PUECHMAILLE, S. J., The role of environmental reservoirs in the persistence and transmission of pathogens: Lessons from *Pseudogymnoascus destructans*, the causative agent of White-Nose disease. Annual Meeting of the German Zoological Society in Greifswald, Germany (**Talk**)
24 – 25/10/2019	**FISCHER, N.M.;** ALTEWISCHER, A.; DOOL, S. E.; PUECHMAILLE, S. J., Approximate Bayesian Computation (ABC) as a tool to determine the proportion of environmental infection in bat White-Nose disease. Conference on Models in Ecology and Evolution in Montpellier, France (**Talk**)
04 – 07/05/2021	**FISCHER, N.M.;** ALTEWISCHER, A.; DOOL, S. E.; PUECHMAILLE, S. J., Presence of an environmental reservoir for the pathogen *Pseudogymnoascus destructans* (White-Nose disease). European Bat Research Symposium in Turku, Finland, but held online (**Talk**)

WORKSHOPS

02 – 06/12/2019	**Adaptation Genomics** - Learning how to analyses genomic data (Quality control, population genetics, genome-wide summary statistics, adaptive genomic regions, genotype-environment associations). Course organised by Physalia and led by Dr. Philine Feulner and Dr. Jessica Stapley in Berlin, Germany.

NICOLA M. FISCHER

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For his assistance at every stage of my PhD, a great thank-you also goes to my co-supervisor, Professor Dr. Gerald Kerth. I genuinely appreciate that I could always come to him with any issue or need – be it scientific or personal – and I could always count on his support.

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