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Is Bat Guano a Reservoir of *Geomyces destructans*?

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ABSTRACT

Bat guano from six different karst caves in Slovenia was screened by PCR for the presence of *Geomyces destructans* [1]. Two identical DNA sequences from guano heaps in two of the caves with recent bat droppings were retrieved. These sequences were closely related to several unidentified *Geomyces* clones and to pathogenic *G. destructans*, but definitive identification remained elusive. Despite the presence of *Geomyces* species, thus far there has been no report of White-nose syndrome (WNS) symptoms in bats in Slovenia. Overall physical and chemical conditions in guano, particularly in large guano heaps, offer a suitable environment which might serve as a reservoir for pathogenic *Geomyces* strains and could represent a permanent reservoir for *in situ* infection of bats. Screening of guano and analysis of more data related to guano ecology could lend clues to control WNS outbreaks and mortality linked to *G. destructans*.

Keywords: *Geomyces destructans*; Bat Guano; Cave; Slovenia

1. Introduction

White-nose syndrome (WNS) is a widespread, epizootic disease in bats associated with the fungus *Geomyces destructans* (Ascomycota, Helotiales) [2]. This psychrophilic fungus exhibits pronounced proteolytic activities and invades living tissue of cave-roosting bats during hibernation. *G. destructans* collected from the environment shows slow recovery rates under laboratory conditions (4 - 8 weeks), with the highest growth rate around 12˚C - 15˚C [3,4]. WNS causes unprecedented morbidity and mortality (often >90%) in bats in North America. The fungus is always found on bats with WNS where large numbers of hibernating bats have died [5]. *G. destructans* appears to have been already present in Europe before the disease outbreak in the USA [6,7]. While the naïve bat populations in North America die easily from infection [6,8], in contrast, in Europe no mass mortality in bats linked to WNS has been reported, although *G. destructans* is widespread. Many bat species from the genus *Myotis* are infected on both continents. In different European countries eight *Myotis* species, particularly *M. myotis*, have been observed with visible fungal growth usually colonizing wings, tail, ears and muscles [7,9-12]. It is suspected that shorter winter periods in warmer regions in Europe are associated with lower incidence of *G. destructans* in bats. In Europe, bats with fungal mycelia are first seen in January; in February the number increases and reaches a peak in March, then drops again in April, when bats emerge from hibernation [10].

Because bats play major roles in the ecosystem, such as plant pollination, seed dissemination, forest regeneration and insect control [13], their health and endangerment should be of high concern. Cave walls and hibernacula could serve as passive vectors and/or reservoirs for *G. destructans* [10,14-16]. Bat guano is known to harbor several bats and human pathogens, such as the fungus *Histoplasma capsulatum* [17,18]. The guano of insectivorous bats is rich in organic matter, especially keratin [19]. Therefore, bat guano is a potential habitat or reservoir for *G. destructans*. The idea of detecting *G. destructans* in guano is appealing for several reasons. First, guano is found in close proximity to bat roosts and is likely to have exposure to bat pathogens. Sampling of guano is less invasive than direct sampling of bats and may be conducted at times when bats are absent from hibernacula. Finally, the chemical and physical characteristics of guano make it appear to be a suitable substrate for *G. destructans* growth or maintenance. However, although there are several studies from Europe reporting the presence of *Geomyces* in bat guano, for example *G. pannorum* var. *pannorum* from Domica Cave in Slovakia [20], very few investigations of *G. destruc-*

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tans in guano have been reported [21]. Perhaps the particular soil and debris material of guano are not optimal for isolation of *G. destructans*, or guano is unsuitable for sustaining growth of *G. destructans*.

Detection methods for *G. destructans* remain in development. Definitive identification may be done by culturing, but requires appropriate culture facilities as well as attention to pathogen containment protocols. In contrast to culturing techniques, molecular methods offer greater sensitivity and the advantage of detecting the fungus from samples in which the fungus is nonviable. Specific real-time PCR assays have recently been developed [22,23], but real-time PCR requires substantial monetary resources for reagents and access to specialized equipment. Conventional PCR, though less specific, allows more cost-effective screening of environmental samples for *G. destructans* genomic DNA, and may be followed with sequencing for species verification. In a recent study, a *G. destructans*-specific polymerase chain reaction (PCR) using primers developed by Lorch and co-workers [1] had 100% diagnostic specificity and 96% diagnostic sensitivity in bat tissue. On the other hand these primers lacked the specificity to distinguish between *G. destructans* and closely related *Geomyces* spp. in environmental samples [16]. Nonetheless, the advantages of a PCR-based approach remain apparent for allowing rapid screening when culturing facilities are unavailable.

G. destructans has been detected in many European countries, but no reports exist from Slovenia on either its presence in caves or association with geomycosis in bats. The objective of this study was to screen bat guano as a potential habitat for the presence of *G. destructans* and to unravel its ecology and distribution in Slovenian karst caves. Bat guano samples were screened for *G. destructans* by conventional PCR in combination with DNA sequencing. Two out of six guano samples from Slovene caves contained sequences closely related to several unidentified *Geomyces* clones and to pathogenic *G. destructans*. This is one of the first published investigations of the presence of *Geomyces* in bat excrement.

2. Material and Methods

2.1. Caves and Bats

In Slovenia many caves host bats, but large quantities of bat guano are preserved only in some of them. For this study five caves with bats and significant amounts of guano were selected. Air temperature was measured using a portable Kestrel 4500 Pocket Weather Tracker (USA). Huda luknja Cave (Huda luknja, no. 413 in the cave register of the Karst Research Institute ZRC SAZU and Speleological Association of Slovenia) has a total length of 2339 m, a maximum depth of 119 m, and the cave entrance is at an altitude of 508 m above sea level (date of sampling 19 January 2010, air temperature 7.2˚C). The chamber with bat guano is regularly populated by bats. Predjama Cave (Predjama, cave register no. 734, sampled 20 June 2011, air temperature 13.4˚C) is the third largest Slovenian cave, totalling 13.1 km long and 143 m deep. The entrance to the cave is at an altitude of 490 m a.s.l. The section with guano, named Netopirjev rov (Bat's passage), is historically known to be densely populated by bats, but unfortunately guano heaps were almost completely removed from the cave several decades ago by local people. Spodnja klevevška Cave (Spodnja klevevška jama, cave register no. 410, sampled 18 July 2011, air temperature 18.1˚C) has a thermal spring (~20˚C), is 318 m long and 14 m deep, and the cave entrance is at an altitude of 177 m a.s.l. Due to water oscillations guano heaps in the cave are usually no older than one season; nevertheless scattered bat droppings can be found throughout the entire cave. Škocjan Caves (Škocjanske jame, cave register no. 735) have a total length of 5.8 km, and the entrance is at an altitude of 425 m. Škocjan Caves are listed in the UNESCO World Heritage list and are the first underground karst wetland under the Ramsar Wetland Classification System. In these caves two guano heaps were sampled on 21 June 2011. The first sample (ŠK1) was taken from guano which was abandoned by a bat colony in the years 1975/76 after the installation of a lighting system at this site; however some individuals are sporadically seen at the site. The second guano sample (ŠK2) was taken from guano in a chamber where bats roost during the winter. Temperature conditions in a guano heap, correlated to the surrounding cave atmosphere, were monitored at site ŠK2 in Škocjan Caves. Temperature data loggers (Anset StowAway TidbiT Temp Logger-TBI32-20 + 50, USA) were placed 5 cm deep in the guano and 20 cm above the guano heap, exposed to the cave atmosphere. Turjeva Cave (Turjeva jama, cave register no. 821, sampled 2 February 2011, air temperature 10.4˚C) is 375 m long and 5 m deep with the entrance at an altitude of 253 m a.s.l. In recent years, no bats have been observed above this guano heap.

Fresh guano samples were characterized by fresh rod-shaped excrement indicating the roosting colony of bats at the site. An attribution of "recent" was given to those samples which contained some relatively recent rod-shaped excrement over old guano base, and samples were considered "old" if guano had no characteristics of fresh and/or recent rod-shaped excrement, and if within a recent time period, usually a few decades, no bat colony was recorded above the guano heap (**Table 1**).

Twelve different bat species have been identified in Huda luknja Cave, eleven in Predjama Cave, five in Spodnja klevevška Cave, twelve in Škocjan Caves, and five in Turjeva Cave [24,25]. The most numerous bat species

Table 1. Prevalent bat species [24] and characteristics of guano.

Cave	Bat species	Guano description	Sample	Volume (l)	pH	Bacteria (CFU/g)	Fungi (CFU/g)
Huda luknja Cave	*Miniopterus schreibersii*[*] *Rhinolophus ferrumequinum* *R. hipposideros*	Recent	HUD	630	4.7	3.41×10^4	2.01×10^4
Predjama Cave	*Miniopterus schreibersii*[*] *Rhinolophus ferrumequinum* *R. hipposideros*	Recent	PRE	50	6.1	1.90×10^7	3.86×10^3
Spodnja klevevška Cave	*Miniopterus schreibersii* *Myotis myotis* *Rhinolophus euryale* *R. hipposideros* *R. ferrumequinum*	Fresh	SPO	30	8.0	$2.72 \times 10^{6++}$	$1.96 \times 10^{5++}$
Škocjan Caves	*Miniopterus schreibersii*[*] *Myotis capaccini* *M. blytii oxygnathus* *M. myotis* *Rhinolophus ferrumequinum*	Old	ŠK1	450	4.5	0	1.27×10^4
Škocjan Caves	*Miniopterus schreibersii*[+*] *Myotis capaccini*[+] *M. blytii oxygnathus*[+] *M. myotis*[+] *Rhinolophus ferrumequinum*[+]	Recent	ŠK2	400	3.5	1.19×10^8	2.51×10^5
Turjeva Cave	*Miniopterus schreibersii*[+*] *M. myotis/blytii oxygnathus*[+] *Rhinolophus hipposideros*	Old	TUR	800	4.4	1.28×10^2	0

[*]main origin of guano; [+]probable historical presence; [++]underestimate number due to frozen sample.

and some characteristics of the sampled guano heaps are summarized in **Table 1**. For analyses, guano heaps were aseptically sampled with a spoon in a range from zero to five cm depth. Each sample was a combination of the top five centimetres. The pH was measured using pH indicator strips (EMD Chemicals, Germany) after homogenization of guano with an equal part of deionised water. In the laboratory guano samples were screened for culturable microbiota. For molecular analyses guano was stored at −20˚C until DNA was isolated.

2.2. Estimation of Culturable Microbes

To estimate the quantity of culturable microbiota, guano samples were screened for bacteria and fungi using RIDA®COUNT test plates. This system has already been used successfully on various cave samples [26]. All guano samples were screened after sampling, except the sample from Spodnja klevevška Cave, which was processed after freezing (**Table 1**). Fifteen millilitres of sterile physiological saline were added to approximately 2 g of guano and vigorously vortexed. This mixture was serially diluted up to 10^{-5}. One millilitre of these serial dilutions was applied onto RIDA®COUNT test plates. RIDA®COUNT Total Aerobic Count test plates were used for total bacterial counts and RIDA®COUNT Yeast & Mold Rapid for total counts of yeasts and moulds. Counts of bacterial colonies were made after 48 hours of incubation at 35˚C, and of yeasts and moulds after 72 hours at 25˚C [27].

2.3. DNA Isolation, PCR and DNA Sequencing

High molecular weight genomic DNA was isolated from bat guano using the NucleoSpin® Soil kit with SL2 lysis buffer (Macherey-Nagel, Germany). DNA purity and concentration were determined spectrophotometrically using standard absorbance at 260 nm and 280 nm on a NanoDrop ND-1000 UV/Vis spectrophotometer (NanoDrop Technologies, USA). 260/280 absorbance ratio ranged from 1.82 to 1.92.

A *Geomyces destructans*-specific diagnostic PCR [1] was performed with slight modifications. Amplifications were carried out in 50 µl reaction mixtures containing 1 µl of DNA extract (at 15 - 30 ng/µl), 10 pmol/µl of each primer, nu-SSI(1506)-184-5'-Gd (GGGGACGTCCTAAAGCCT) and nu-5.8S-144-3'-Gd (TTGTAATGACGCTCGGAC), and 0.5 µl of the HOT FIREPol® DNA Polymerase (Solis BioDyne) with a standard PCR programme: initial denaturation at 95˚C for 15 min, followed by 45 cycles of denaturation at 95˚C for 1 minute, annealing at 50˚C for 2 min, and extension at 72˚C for 3 min, followed by a final extension step of 72˚C for 10 min. The amplicons were electrophoresed on 2% agarose in TAE buffer, visualized by Midori Green Advance DNA Stain (Biozyme), and compared to a Step ladder 50 - 3000 bp marker (Sigma-Aldrich). Amplified PCR products of proper size (~620 bp) were purified using the QIAquick Gel Extraction Kit (USA).The purified amplified fragments were sequenced using the same primers to confirm the specificity of the PCR by direct

sequencing of the PCR products using the ABI PRISM® BigDye sequencing kit (PE Applied Biosystems, Langen, Germany). Sequencing was carried out in a 310 ABI PRISM® automated sequencer; sequences were obtained from both strands in two independent setups. The nucleotide sequences were assembled in GeneDoc. Multiple sequence alignment was performed by pairwise alignments using the CLUSTAL algorithm.

Sequence data were deposited in the GenBank database under the following accession numbers: JX467177 (sample ŠK2 designated as SK2011) and JX467178 (sample HUD designated as HUD2010).

3. Results and Discussion

3.1. Analyses of *Geomyces* Sequences

PCR with primers reported to be specific for *G. destructans* [1] was performed on total DNA isolated from guano samples collected from the upper five centimetres of guano heaps. Several independent PCR reactions were carried out and all gave the same results. Altogether, two samples were positive for the expected PCR product size; these were a recent guano sample from Škocjan Caves, sample ŠK2, and a recent guano sample from Huda luknja Cave, sample HUD (**Figure 1**).

The PCR primers used in the study amplify a 624-bp segment of the ribosomal RNA gene region that consists of the 1506 intron for which no unambiguous sequence could be obtained, a small portion of SSU (small 18S ribosomal subunit), ITS 1, 5.8 S and part of ITS 2 [1]. Two bands of appropriate size (~620 bp) were bidirectionally sequenced. Sequences were obtained from both strands; from both positive PCR products only a 364 bp fragment could be sequenced. The sequences from the two *Geomyces*-positive samples, SK2011 and HUD2010, are both 364 bp long and 100% identical to one another. These two sequences showed highest sequence identity, 100% (364/364 bp), to several unidentified *Geomyces* clones detected in soil from bat hibernacula in the USA (GenBank accession numbers: HM848968, HM848971, HM848981, HM848983, HM848985, HM848988; [16]). The next highest sequence identity (363/364 bp) was to two strains of *G. pannorum*, one isolated from arctic cryopegs and marine deposits (DQ189224; [28] and one from wheat rhizosphere in the UK (AJ938165), and, among others, to several clones/strains of *G. destructans* isolated from bats in Germany (e.g. JF502400-JF502402 [10], HM222616 [7]) and in the Czech Republic (e.g. HM584949-HM584975; [12]). Because PCR products were directly sequenced rather than being first cloned, if *G. destructans* were present in low abundance it could easily be masked by other related strains.

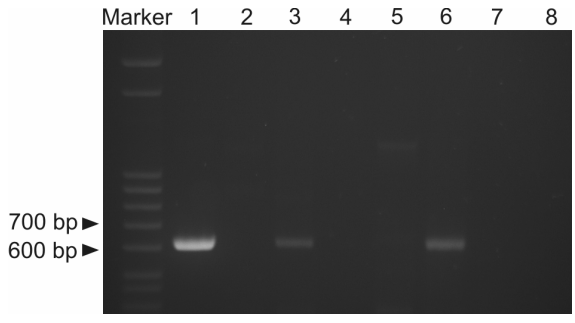

Figure 1. PCR-based detection of *Geomyces destructans* in guano (Marker-Step ladder, Sigma-Aldrich, 1-positive control using genomic DNA of *G. destructans* as a template, 2-ŠK1, 3-ŠK2, 4-PRE, 5-TUR, 6-HUD, 7-SPO, 8-negative control). A 624-bp band matches the expected size for *G. destructans* for samples 3 and 6.

3.2. Bats, Guano and *Geomyces*

To date there has been no report of fungal growth characteristic of *G. destructans* on bats in these two caves. This is somewhat surprising given the prevalence of the fungus in other European countries, but could be attributed to a rather low abundance of *Myotis* (Primož Presetnik, personal communication) and/or to poor surveillance of bats in Slovenian caves. Our results neither rule out nor confirm the presence of *G. destructans* in Slovenia, but do indicate that very closely related strains are detectable in bat guano of Slovene caves.

It is reasonable that guano could be suited for the growth or propagation of *G. destructans* because in guano a variety of keratin substrata are available, such as bat hairs and partly digested insect fragments including wings, wing parts and leg scales [19,29]. Other *Geomyces* species are known to be keratinophilic, and thus *G. destructans* is presumed to be as well. Once *G. destructans* has been introduced to a guano heap, this could represent a permanent source of infection for hibernacula, particularly if these heaps are close to a bat colony. Movement of air masses caused by bats is not negligible, and air draught and water splashing are two important factors for spreading aerosolized microbial spores in caves [30].

Interestingly, two guano heaps only 450 m apart in Škocjan Caves were analysed, but we obtained positive PCR only for the heap containing recent bat droppings. The guano that had been abandoned by the bat colony in 1975/76, presumably derived from the same bat species roosting at the other site today (Primož Presetnik, personal communication), was negative by PCR. This finding could suggest a recent introduction of these *Geomyces* species to Slovenia (less than ~40 years) by migratory bats, but, more likely, indicates that the detected *Geomyces* have a limited life in guano and will only be detected in relatively fresh samples. If conditions in aging guano do not support its growth, guano could not be a long-term reservoir of this fungus. Recent analysis of

sediments from bat hibernacula in the United States confirms that *G. destructans* can persist in these substrates for at least several months [14]. The average sedimentation rate of bat excrement in guano heaps from Domica Cave in Slovakia is 0.99 mm per year [19]. By analysing guano in a range from zero to five cm depth, and if we assume a similar deposition rate in Slovenian caves due to similar size of guano and number of bats, the age of screened guano in the 5-cm deep sample ranged from 0 up to 50 years, much longer than the time period tested in US sediments.

More studies are needed to better understand the ecology and distribution of *Geomyces* in caves and other underground cavities. Besides *G. destructans*, additional lineages of psychrophilic and saprophytic fungi exist, and some of these are distributed rather widely in temperate climates [31,32]. Screening of potential habitats of *G. destructans* with PCR could give us valuable information on its distribution in Slovenia and more widely in Europe. Because geomycosis has been shown to exist in Europe [9] such information may help prevent and/or control WNS outbreak and transmission in bats.

3.3. Microbial Estimators and Conditions in Guano

Guano contains very diverse microbiota [33]. We tested whether the guano sampled for this study contained viable fungi and bacteria. Among the sampled guanos, culturable bacterial and fungal abundances varied by up to four orders of magnitude. The overall culturable fungal densities in the two guano piles with positive PCR results were in the same range as the other tested guano samples from the sampled caves (**Table 1**). In Huda luknja Cave (HUD) 2.01×10^4 CFU/g and in Škocjan Caves (ŠK2) 2.51×10^5 CFU/g of fungi were found. Changes in composition and abundance of microbiota over time is known to occur in guano, which could also explain several unsuccessful attempts to retrieve culturable bacteria from old guano in Škocjan Caves (ŠK1), although 1.27×10^4 fungal CFU/g were detected on RIDA®COUNT test plates. Due to cultivation conditions for fungal plates (25˚C, 72 hours) and nature of the fungal medium we did not retrieve psychrophilic strains, but we did retrieve an estimate of the abundance of nutrient non-demanding fast growing culturable fungi. Although this guano is clearly able to support fungal growth, we infer from our sequencing results and from the fact that PCR results were positive only with prolonged amplification that *G. destructans* is probably not very abundant in bat guano. Indeed, even in soil samples Lindner and co-workers reported that *G. destructans* does not dominate the pool of fungal DNA [16].

Laboratory studies on the temperature optimum for psychrophilic *G. destructans* showed good growth at 4˚C and a growth optimum around 12˚C - 15˚C, with no growth observed above approximately 20˚C [3,4]. Temperatures in hibernacula affected by WNS usually range between 2˚C and 14˚C [5]. In the current study, temperature conditions in the monitored guano, 10˚C - 14˚C, were favourable for *G. destructans*. The temperature in guano generally followed the temperature changes in the cave atmosphere. In the colder part of the year the temperature in guano was slightly higher than in the cave atmosphere, while in early summer guano temperature was slightly below cave atmosphere temperature (**Figure 2**). Besides being a source of nutrients for *G. destructans*, guano might also serve as an insulator that dampens large temperature changes, which could be especially important where guano is exposed to more extreme atmospheric conditions, for example at cave entrances and at sites with pronounced airflow.

The pH of fresh guano is neutral to alkaline, while older guano of insectivorous bats tends to be acidic [34] (**Table 1**). Because *G. destructans* could be isolated on either Sabouraud agar (pH~5.6) or Rose Bengal agar (pH~7.2) [3] it can be expected that the acidic pH of older guano is not a limiting factor for this organism; in fact, both *Geomyces*-positive samples were considerably more acidic than pH 5.6. These two samples were further characterized by a relatively high quantity of guano material deposited, and by the presence of some relatively fresh bat droppings at the sampling sites. In both chambers in Huda luknja Cave and Škocjan Caves (site ŠK2) where we took samples bats have been observed during bat surveying and sampling (**Table 1**).

In insectivorous mammals toxic metals bioaccumulate [35]; for this reason guano also contains a high quantity of heavy metals. In guano from Škocjan Caves the quantity of Cu exceeded values for agricultural soil in both samples, and in recent guano (ŠK2) Cd and Zn also exceeded these values [36]. Further studies are needed to

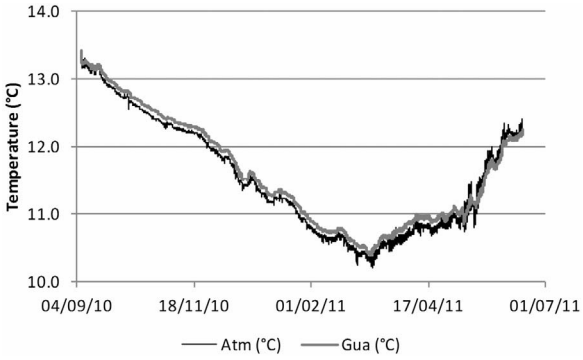

Figure 2. Temperature changes in the bat guano heap (Gua) where *G. destructans*-specific PCR was positive and in cave atmosphere (Atm) in Škocjan Caves between 7 September 2010 and 16 June 2011.

reveal if heavy metals affect the ecology of *G. destructans* in guano.

4. Conclusions

Bat guano in two caves from Slovenia hosted *Geomyces* strains which are related to the virulent *G. destructans* that causes WNS. If conditions in guano support viability of *G. destructans*, guano heaps could represent a permanent reservoir for *in situ* infection of bats. Screening of guano could help to control WNS outbreaks, and in the USA screening of guano profiles might provide additional information on the approximate period when the European version of *G. destructans* was introduced.

In sum, our results suggest that *G. destructans* could be present in Slovenia and that screening of fresh guano samples is a potential technique for identifying it in other caves or places where bats dwell. Molecular analysis of excrement provides an alternative method when direct sampling of bats is impossible.

5. Acknowledgements

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