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ORIGINAL ARTICLE

First Isolation of *Pseudogymnoascus destructans*, the Fungal Causative Agent of White-Nose Disease, in Bats from Italy

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Abstract White-nose disease, caused by the dermatophyte *Pseudogymnoascus destructans,* is a devastating pathology that has caused a massive decline in the US bat populations. In Europe, this fungus and the related infection in bats have been recorded in several countries and for many bat species, although no mass mortality has been detected. This study reports for the first time the presence of *P. destructans* in Italy. The fungus was isolated in the Rio Martino cave, a site located in the Western Alps and included in the Natura 2000 network. Twenty bats, belonging to five different species, were analysed. The fungus was retrieved on eight individuals of *Myotis emarginatus*. The allied keratolytic species *P. pannorum* was observed on two other individuals, also belonging to *M. emarginatus*. Strains were isolated in pure culture and characterized morphologically. Results were validated through molecular analyses. Future work should be dedicated to understand the distribution and the effects of the two *Pseudogymnoascus* species on Italian bats.

Keywords *Geomyces destructans* · *Myotis emarginatus* · *Pseudogymnoascus pannorum* · White-nose syndrome

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Introduction

White-nose disease (WND), also known as white-nose syndrome (WNS), is a devastating pathology that has recently caused the death of millions of bats across the USA [1]. It has been described as a condition of hibernating bats, so named because of the striking white fungal growth on muzzles, ears, and/or flight membranes that often characterizes affected bats [2]. Although the disease was firstly documented in 2006 [2], its causative agent, the fungus *Pseudogymnoascus destructans,* (Blehert & Gargas) Minnis & D.L. Lindner (formerly *Geomyces destructans*), was described only in 2009 [3].

Pseudogymnoascus destructans is a slow growing, psychrophilic fungus with an optimum growth at 12.5–15.8 °C (upper limit 19.0–19.8 °C) under laboratory conditions [4]. The distinctive characteristic of the species is the curved shape of conidia, borne singly at the tips or in short chains in verticillate branched conidiophores [3]. The species was taxonomically validated in 2013 [5], together with other allied species within the family of Pseudoeurotiaceae. Among these, *Pseudogymnoascus pannorum,* (Link) Minnis & D.L. Lindner, is closely related to *P. destructans*. This is also a ubiquitous psychrophilic, keratinolytic fungus that has been isolated from a variety of sources and from different biogeographic regions. The species is known to cause dermatophyte infections in humans and domestic animals [6].

Bats undergo the infection during their annual hibernation period. Martínková and co-authors [7] observed the highest intensity peaks of infection when the body surface temperature of hibernating bats is between 5 and 6 °C. Fungal hyphae of the pathogen penetrate the skin, causing characteristic epidermal erosions and ulcers, and can progress invading the underlying connective tissue [8–10].

In Europe, the occurrence of *P. destructans* was first reported in France [11]. Afterwards, the presence of white-nose disease was also confirmed (starting from the Czech Republic) [12], even if, to date, no mass mortality has been recorded in the affected countries. The presence of the fungus has then been confirmed through molecular analyses in 17 bat species (Table 1), but has never been recorded in Italy [13].

At the beginning of June 2018, a large *Myotis* (*M. blythii* vel *myotis*) showing a pale area on its muzzle, similar to that observed on bats affected by WND, was noticed during a bat survey in the Rio Martino cave (Piedmont, NW Italy) (Fig. 1). The bat hung high on the cave ceiling, and it was not possible to capture it to ascertain whether it was actually exhibiting fungal growth or it was just covered in limestone powder. Following this observation, in order to verify the presence/absence of *P. destructans* in the cave, we collected and analysed skin swabs from bats captured at the site.

Materials and Methods

The Study Site

The Rio Martino cave (Grotta di Rio Martino) is located in the upper Po Valley (inner sector of the Western Alps, Italy), within the Unesco Biosphere Reserve Monviso. It is included in the Natura 2000 network (Special Area of Conservation IT1160037) and managed by the Ente di gestione delle aree protette del Monviso.

The cave entrance (WGS84 44.699054N, 7.146427E) opens at 1530 m a.s.l. The cave is 2905 m long, with a 191 m of elevation difference. A small river flows inside, giving origin to a 42 m high waterfall between the upper and the lower branches of the cave [22, 23].

The humidity is always close to 100%. Daily temperature fluctuations are very slight, around 0.02 °C [23]. During spring, while the external temperature increases, causing snow melting, the inflow of snowmelt underground keeps the cave temperature low [24]. In the upper parts of the cave, it remains around 5 °C throughout the year, while in the lower parts, it is more variable, being around 5 °C in summer and varying in the range of 2–5 °C in the other seasons [25].

During the last thirty years, the presence of at least thirteen bat species have been ascertained in the cave: *R. hipposideros*, *M. bechsteinii*, *M. blythii*, *M. daubentonii*, *M. emarginatus*, *M. mystacinus*, *M. nattereri* (group), *Pipistrellus* sp., *H. savii*, *E. serotinus*, *Plecotus* sp., *B. barbastellus*, and *M. schreibersii*. Bats can be found throughout the whole year, but their numbers peak during swarming and hibernation. In the winter that preceded the survey (2017/18), 231 individuals were counted, mainly belonging to *B.*

Table 1 Occurrence of *P. destructans* in Europe

Bat species	Country
Barbastella barbastellus	Czech Republic [21][a], [30]
Eptesicus nilssonii	Czech Republic [21][a], [30], Latvia [30], Russia [30]
Miniopterus schreibersii	Slovenia [30]
Myotis alcathoe	Czech Republic [30]
M. bechsteinii	Czech Republic [21][a], [30]
M. blythii/oxygnathus	Hungary[14], Portugal [17]
M. brandtii	Czech Republic [21][a], [30], Estonia [15], Latvia [30], Germany [14], Russia [30]
M. dasycneme	Czech Republic [21][a], [30], Germany [14], Latvia [30], Netherlands [15], Russia [30]
M. daubentonii	Czech Republic [21][a], [30], Germany [19], Latvia [30], Netherlands [15], Russia [30], United Kingdom [20]
M. emarginatus	Czech Republic [21], Slovenia [30][a]
M. myotis	Belgium [15, 19], Croatia [18], Czech Republic[21][a], [30], France[11, 15, 19], Germany[14, 15, 19], Hungary[14, 15], Luxemburg [19], Poland [15, 19], Slovakia [16], Slovenia[30], Switzerland[14], Ukraine [15, 19]
M. mystacinus	Belgium [15], France [19], Germany [15]
M. nattereri	Czech Republic [21][a], [30]
Plecotus auritus	Czech Republic [21][a], [30], Russia [21]
P. austriacus	Czech Republic [30]
Rhinolophus euryale	Slovenia [30]
R. hipposideros	Czech Republic [21][a], [30]

[a]Data confirmed only through histopathology, no PCR confirmation

Fig. 1 Large *Myotis* (*M. blythii* vel *myotis*) observed on the 4 June 2018 in the Rio Martino cave

barbastellus (84%), distantly followed by *M. emarginatus* (8%)[26].

The lower branch of the cave, which hosts the majority of the bats, has a horizontal development and can be easily walked; moreover, the site has been equipped with gangways and handrails which further facilitate visits. On the contrary, the upper parts of the cave require technical equipment and speleological skills to be visited. As a result, the cave is attractive to both inexperienced public and speleologists, and it has been intensely visited for many years. In 2010, a gate was placed at the entrance of the cave in order to control human access. At present, with the exception of a few visits for study purposes, the access is forbidden from 1 November to 31 March and the prohibition can be extended throughout April if required to protect hibernating bats.

Sample Collection and Fungal Isolation

On 10 June and 15 July 2018, a mist net was positioned at a narrow passage where bats transit to exit/enter the study site. The net was kept open until midnight. All the bats caught were identified, weighed and observed under direct light and by transillumination for patagium inspection. Features such as visible fungal growth or evident wing lesions possibly related to fungal presence, including holes, necrotic edges, pale areas and other irregular pigmentation in the wing membranes, tacky and dry wing membrane were recorded.

Sterile cotton swabs were used to take a superficial sample from the skin of wings, ears and muzzles of a sample of bats, representative of all the captured species. Individuals either showing or lacking features possibly related to the fungal infection were selected. Samples were placed in sterile containers and kept at 4 °C, in the dark, until laboratory analyses, which started the morning after sampling. Fungal isolation was performed according to the procedure described by Gargas et al. [3]. Sample preparation procedures were carried out under laminar flow hood, to prevent external contamination. Swabs were gently streaked onto the surface of 9 cm Petri dishes containing Sabouraud Dextrose Agar medium (Biolife). Plates were incubated in the dark at 4 °C and checked weekly up to two months.

Fungal Identification

Initial detection of fungal structures was carried out by means of a stereomicroscope (× 10–50) and on the basis of their micro-morphological (100–1000 ×) features. Growth of colony at 4 °C, shape and dimension of conidia and conidiophores were considered diagnostic characters for strains ascribable to *P. destructans* [3]. Strains were isolated in pure culture and preserved at the fungal collection of the Laboratory of Mycology and Plant Pathology, Department of Earth and Environmental Sciences, University of Pavia.

Subsequently, genomic DNA was extracted from all the collected strains morphologically ascribable to *P. destructans*, following the protocol described by Garzoli et al. [27]. Briefly, mycelium was gently scraped from Petri dish, placed in a 2 mL microtube and disrupted by means of sterile pestles. DNeasy Plant Mini Kit (Qiagen, Inc.) was employed for DNA extraction. Internal Transcribed Spacer region ITS rDNA was amplified using universal primers ITS1/ITS4 [28]. PCR was performed in a T100 Thermocycler (Biorad). Purified PCR samples were sequenced using Sanger sequencing techniques at Macrogen Europe. Consensus sequences were obtained by using Sequencer 5.0 (Gene Code Corporation, http://www.genecodes.com). Taxonomic assignments were inferred by querying with the Blast n algorithm (default setting), hosted at NCBI (National Center for Biotechnology Information—http://www.ncbi.nlm.nih.gov), the newly generated sequences against the nucleotide database of NCBI (GenBank). Pairwise alignments were also performed at http://www.mycobank.org/ against the Mycobank database (hold by the International Mycological Association). Similarity values equal or higher than 98% (e-value > e^{-100}) were considered credible, and the results were confirmed morphologically. Newly generated sequences were deposited at NCBI database (GenBank Accession numbers from MK421359 to MK421368).

Results

In June, 18 bats were captured while exiting from the cave and two (belonging to *B. barbastellus*) while entering the site; in July, only one individual (a *M. daubentonii*) was caught while emerging, and six other bats while they were entering the cave. Their body mass did not differ from the values reported in the literature [29]. None of the bats showed visible fungal growth, but some had traces of limestone powder on their ears, wings and forearms, which had to be examined by a magnifying glass to exclude they were hyphae.

Eleven individuals of the first trapping session (8 *M. emarginatus*, 1 *M. bechsteinii*, and 2 *B. barbastellus*) and two of the second (1 *M. nattereri* and 1 *B. barbastellus*) showed wing lesions (holes, irregular pigmentation, necrotic edges, scars), although never severe. No signs of damage were noticed on the other bats (8 *M. emarginatus* and 1 *M. bechsteinii* captured in June; 1 *M. emarginatus*, 1 *M. daubentonii*, 1 *M. nattereri*, and 2 *B. barbastellus* captured in July).

Thirteen of the bats captured in June (seven with wing damage and six without) and all those captured in July (two with wing damage and five without) were analysed for the presence of fungi (Table 2).

Eight (62%) individuals of June resulted positive to the presence of *P. destructans* (Fig. 2).

All the positive individuals belonged to *M. emarginatus*; three of them had wing damage, but the other five had not. Two other *M. emarginatus* one of which showing wing damage, resulted positive to the allied keratolytic species *P. pannorum*. No colony was isolated in July samples, including the only *M. emarginatus* mist-netted.

Isolated strains ascribable to *P. destructans* were slow growing at 4 °C, with no growth at 25 °C: colonies on SAB reached 3–4 mm in 2 weeks, appearing white marginally with distinctive conidial masses at colony centres powdery grey; conidiophores were erect, bearing verticils of 2–4 branches; conidia were predominantly curved, 5–8 × 2–3 µm, truncated, with an evident scar at the base. Molecular analyses confirmed for all strains morphological findings.

Discussion

This is the first study to assess the presence of *Pseudogymnoascus destructans* in Italy. This fungal species was detected on eight bats captured in the Rio Martino cave, all belonging to *Myotis emarginatus*. This bat species was already reported as positive to the fungus, but records confirmed molecularly were only available for the Czech Republic [30]. Histopathological features matching WND diagnostic criteria were also observed in three *M. emarginatus* from Slovenia, but PCR did not confirm the presence of the pathogen [30].

Pseudogymnoascus destructans was not detected in any of the individuals belonging to *M. bechsteinii*, *M. daubentonii*, *M. nattereri* (group), and *B. barbastellus* captured in the Rio Martino cave, although these species were recognized to host the fungus in other European Countries [15, 19–21, 30, 31]. Several factors may explain the negative result: i) the low number of bats sampled ii) the fact that the majority of them were captured while entering the cave, possibly arriving from not infected sites iii) the sampling period, long after hibernation, which is not optimal to detect *P. destructans* because euthermia is known to negatively influence its prevalence in bats [32]. Concerning the latter aspect, it should be noted that hibernation period can be unusually long in *Myotis emarginatus* [29], and this was observed also in Rio

Table 2 Presence of *P. destructans* and *P. pannorum* on bats of the Rio Martino Cave

Code	Species	Sex	FA (mm)	BM (g)	Damage signs[a]	*P. destructans*	*P. pannorum*
10 June 2018							
ME1	*Myotis emarginatus*	M	38.9	6.6	–	MK421359	–
ME2	*Myotis emarginatus*	M	38.6	6.4	–	–	–
ME3	*Myotis emarginatus*	M	40.1	6.1	–	MK421360	–
ME4	*Myotis emarginatus*	M	39.9	6.0	ip	MK421361	–
ME5	*Myotis emarginatus*	M	39.8	6.8	–	MK421362	–
ME6	*Myotis emarginatus*	M	38.5	6.4	–	MK421363	–
ME7	*Myotis emarginatus*	M	40.0	6.6	n,h	MK421364	–
ME8	*Myotis emarginatus*	M	39.0	6.1	–	MK421365	–
ME9	*Myotis emarginatus*	M	38.4	6.2	–	–	MK42136
ME10	*Myotis emarginatus*	M	39.4	7.3	n	MK421367	–
ME15	*Myotis emarginatus*	M	38.0	5.5	ip,h	–	MK421368
MB1	*Myotis bechsteinii*	M	41.8	7.4	h	–	–
BB1	*Barbastella barbastellus*	M	40.4	7.4	s, ip, h	–	–
15 July 2018							
MD1	*Myotis daubentonii*	M	35.6	5.9	ip	–	–
MN2	*Myotis nattereri* (group)	M	41.2	6.8	h	–	–
MN3	*Myotis nattereri* (group)	M	40.3	6.3	–	–	–
BB4	*Barbastella barbastellus*	M	39.1	7.9	–	–	–
BB5	*Barbastella barbastellus*	M	40.2	8.8	–	–	–
BB7	*Barbastella barbastellus*	M	38.7	7.9	s, ip, h	–	–
M6	*Myotis emarginatus*	M	39.7	8.2	–	–	–

FA forearm length, *BM* body mass

[a]Damage signs observed on patagium: ip irregular pigmentation, with discoloured areas; n necrotic edges; h holes; s multiple small dark scars

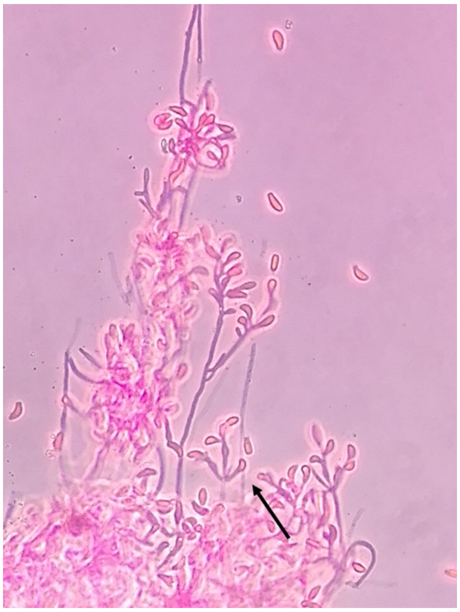

Fig. 2 *Pseudogymnoascus destructans* isolated from *M. emarginatus* ME1 (400×). Black arrow indicates distinctive curved-shaped conidia brought on short verticillate conidiophores

Martino cave; many individuals of the species were still lethargic at the beginning of May 2018, while almost all the barbastelles had already left the site by the end of March. The euthermic condition of bats could also explain the lack of *P. destructans* in July mist-netted bats, including the only *M. emarginatus* examined, although further surveys, including sampling sessions in March–April, are needed to confirm this hypothesis. However, in light of the generalist behaviour of the fungus described both for Nearctic and Palearctic bats [21], *P. destructans* might occur in all the species hibernating in the cave.

Interestingly, two *M. emarginatus* resulted positive for the allied species *P. pannorum*. Although ubiquitous, this fungus has been reported to be a rare pathogen causing skin lesions on human and animals (dog, cat, horse) [6, 33].

A careful evaluation of the possible impact of the two pathogenic *Pseudogymnoascus* species should be done, in particular, verifying whether the bats of the cave show the typical histopathological consequences of WND.

Concerning the disease, it seems appropriate to explain why we have used the term WND instead of the more commonly used *white-nose syndrome*. The term *white-nose syndrome* was coined by Al Hicks in 2007 (DeeAnn Reeder, *pers. comm.*). It was a good and spontaneous choice in front of a sudden and so traumatic event without clear explanations of its causes. "Syndrome"—whose meaning is "a number of symptoms occurring together"—was chosen to describe a set of symptoms with an unknown cause. Today, this term does not reflect properly the current state of knowledge on this infection; we know its aetiological agent, its symptoms, the progression of the disease, and many epidemiological details. Moreover, a grading system for evaluating the infection severity has been recently proposed by Pikula et al. [10]. From a pathological point of view, a disease is marked by some basic factors: an established biological cause behind the condition, a defined group of symptoms and clear changes in anatomy due to the condition [34, 35]. Therefore, we believe that the term "disease" is more appropriate than "syndrome" to indicate the pathological condition caused by *P. destructans* in bats. The term white-nose disease was already adopted by Paiva-Cardoso et al. [17] and, recently, it has also been supported by Frick et al. [36].

An alternative term—geomycosis—was proposed by Chaturvedi and Chaturvedi [37] for both *P. destructans* and *P. pannorum*, but it should be noticed that it was already used in the past for all the infections caused by the species of the genus *Geomyces* (now partially attributed to the genus *Pseudogymnoascus*). Therefore, in our opinion, it results too generic for an invasive and destructive disease specific of bats, as WND.

Many diseases can occur with different degrees of severity: from mild, followed by a complete recovery, to mortal. Fungal colonization may be limited to the skin surface, and bats do not show any effective sign of disease, or may penetrate epidermis and dermis, causing the disease (sensu Meteyer et al. [8]) and eventually resulting in sporadic deaths (as suggested by data collected in Europe and Asia [10, 12, 38]) or in mass mortality (as observed in North America [36]). Moreover, it cannot be excluded that the disease has some significant demographic consequences also in Europe, since mortality events might be more frequent just after the end of hibernation and go undetected because of the difficulty to monitor bats in that period.

In Italy, the fungus was found in the Rio Martino Cave, which presents ideal conditions (low temperatures and high humidity) for this psychrophilic species. Nevertheless, similar environmental conditions occur in other caves used by bats in the Alps and Apennines, which appear to be additional likely sites for the presence of *P. destructans.* Further work is needed to characterize the distribution of the fungus across the country, to identify host bat species and to understand if *P. destructans* causes significant consequences on them, from the individual to the population level. In the meanwhile, precautionary measures should be adopted to avoid the risk of man-mediated spread of the pathogen.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. Captures of bats were carried out under licence (Italian Environment Ministry m_amte.PNM.REGISTRO UFFICIALE.U.0007303.07-04-2017; Province of Cuneo prot. 2017/11.12/000009).

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