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FIRST MYCOLOGICAL INVESTIGATION ON ITALIAN BATS

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ABSTRACT - To ascertain the occurrence of White-nose syndrome or similar mycotic diseases in Italian bats, fifteen bat carcasses (*Myotis capaccini, Miniopterus schreibersii, Myotis* sp., *Pipistrellus* sp.) found in a cave in southern Italy, two dead bats (*Rhinolophus hipposideros*) collected in a cave in Piedmont, and three living bats (*Tadarida teniotis*, *Hypsugo savii* and *Pipistrellus nathusii*) sampled in Turin (NW Italy) were analysed. Forty-six fungal strains, belonging to 15 species, were isolated in pure culture from different carcasses. Five other taxa were identified by direct microscopical analysis of small pieces of skin or hair. Since neither *Geomyces destructans* nor any other *Geomyces* species were found, we concluded that these fungi probably invaded bat hair and tissues only after the death of the animals. *Trichosporum chiropterorum* was reported for the first time in Italy.

Key words: WNS, White-nose Syndrome, Chiroptera, fungi, mycoses

RIASSUNTO - ***Prima indagine micologica dei pipistrelli italiani***. Al fine di verificare la presenza della "Sindrome del naso bianco" o altre malattie di origine fungina, 15 carcasse di pipistrello (*Myotis capaccini, Miniopterus schreibersii, Myotis* sp., *Pipistrellus* sp.), rinvenute in una grotta dell'Italia meridionale, due pipistrelli (*Rhinolophus hipposideros*) rinvenuti morti nella Grotta delle Vene in Piemonte e tre pipistrelli (*Tadarida teniotis*, *Hypsugo savii* and *Pipistrellus nathusii*), campionati in Torino sono stati analizzati per l'isolamento e l'identificazione delle diverse specie fungine presenti. In totale sono stati isolati 46 ceppi fungini appartenenti a 15 specie. Altri 5 *taxa*, sono stati identificati direttamente tramite analisi microscopica di frammenti di pelle o peli. Dal momento che né *Geomyces destructans* né altre specie di *Geomyces* sono state isolate, è altamente probabile che i funghi abbiano invaso i tessuti e il pelo dei pipistrelli solo dopo la morte degli animali. Degno di nota è il ritrovamento di *Trichosporum chiropterorum*, segnalato per la prima volta in Italia.

Parole chiave: WNS, sindrome del naso bianco, Chiroptera, funghi, micosi

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INTRODUCTION

Emerging wildlife diseases constitute a new and potentially devastating threat to animal populations worldwide. In 2006, at Howes Cave, NY, USA, biologists discovered a new disease affecting bats, which was named White-

nose Syndrome (WNS), because of the visually strikingly white fungal growth on muzzles, ears, and/or wing membranes of affected bats (Blehert et al. 2009). So far, affected species include *Perimyotis subflavus, Eptesicus fuscus, Myotis lucifugus, M. septentrionalis, M. leibii, M. sodalis, M. grisescens, M. velifer* and *M. austroriparius* (Courtin et al. 2010; Bat Conservation International 2010; Frick et al. 2010). The fungus associated with WNS has been described as the new species *Geomyces destructans* (Gargas et al. 2009).

Four different fungi species of the genus *Geomyces* were found in the soil mycoflora of two natural caves ("Mottera" and "Caudano") in Piedmont, NW Italy (Mosca and Campanino 1962); three species of bats (*Rhinolophus ferrumequinum, R. hipposideros, Barbastella barbatellus*) live inside the two caves but no anomalous mortality has been reported thus far. *Geomyces* spp. are keratinophilic, psychrotolerant fungi, often associated with Arctic permafrost soils (Möller and Dreyfuss 1996).

WNS is a deadly epidemic in the north eastern parts of the United States and Canada, where over the past four years it has caused the death of about one million bats, with some hibernation sites losing 90-100% of their animals (AAVV 2009). This new disease is a potential serious threat to mammalian diversity as there are about 1200 bat species in all representing about 20% of all known mammal species (Mickleburgh et al. 2002; Simmons 2005).

In the past, bats with fungal growths on the muzzle have been reported in two European countries (Feldmann 1984; Masing 1984). More recently, on 12 March 2009, in a cave 130 km north east of Bordeaux (France), a mouse-eared bat (*Myotis myotis*) with a powdery, white fungal growth on its nose was found. Genetic analyses of specific genes identified the fungus as *G. destructans*, but interestingly, the bat was healthy, not underweight and without any erosive epidermal lesions typical of WNS. Moreover, no associated mortality in the colony was reported (Puechmaille et al. 2010). At current time, at least seven further countries - Czech Republic, Estonia, Germany, Hungary, Netherlands, Romania and Switzerland -, have confirmed or suspect the presence of *G. destructans* in bat populations, but as reported in France, all bats remain healthy (Wibbelt et al. 2010).

During summer 2009, the Speleological Group “Le Grave” (Verzino, Crotone, Italy) found several dead bats (10% of an estimated population of 500-600 individuals) extensively colonised by fungi in a cave (“Grotta del Palummaro”; Fig. 1) located in southern Italy (Caccuri, Calabria region). Moreover in winter 2010, three healthy live bats (*Tadarida teniotis*, *Hypsugo savii* and *Pipistrellus nathusii*) showing white fungal growth were found in Turin (Piedmont, NW Italy) and two dead bats (*Rhinolophus hipposideros*) were collected in a cave (“Grotta delle Vene”, Viozene, Piedmont). To investigate if WNS or a similar mycotic disease is present in Italian bats, twenty bat carcasses from Calabria region, two from Piedmont and swab samples from the three live bats were analysed to isolate and identify the different fungal species on their bodies.

MATERIALS AND METHODS

Due to high levels of decomposition, not all

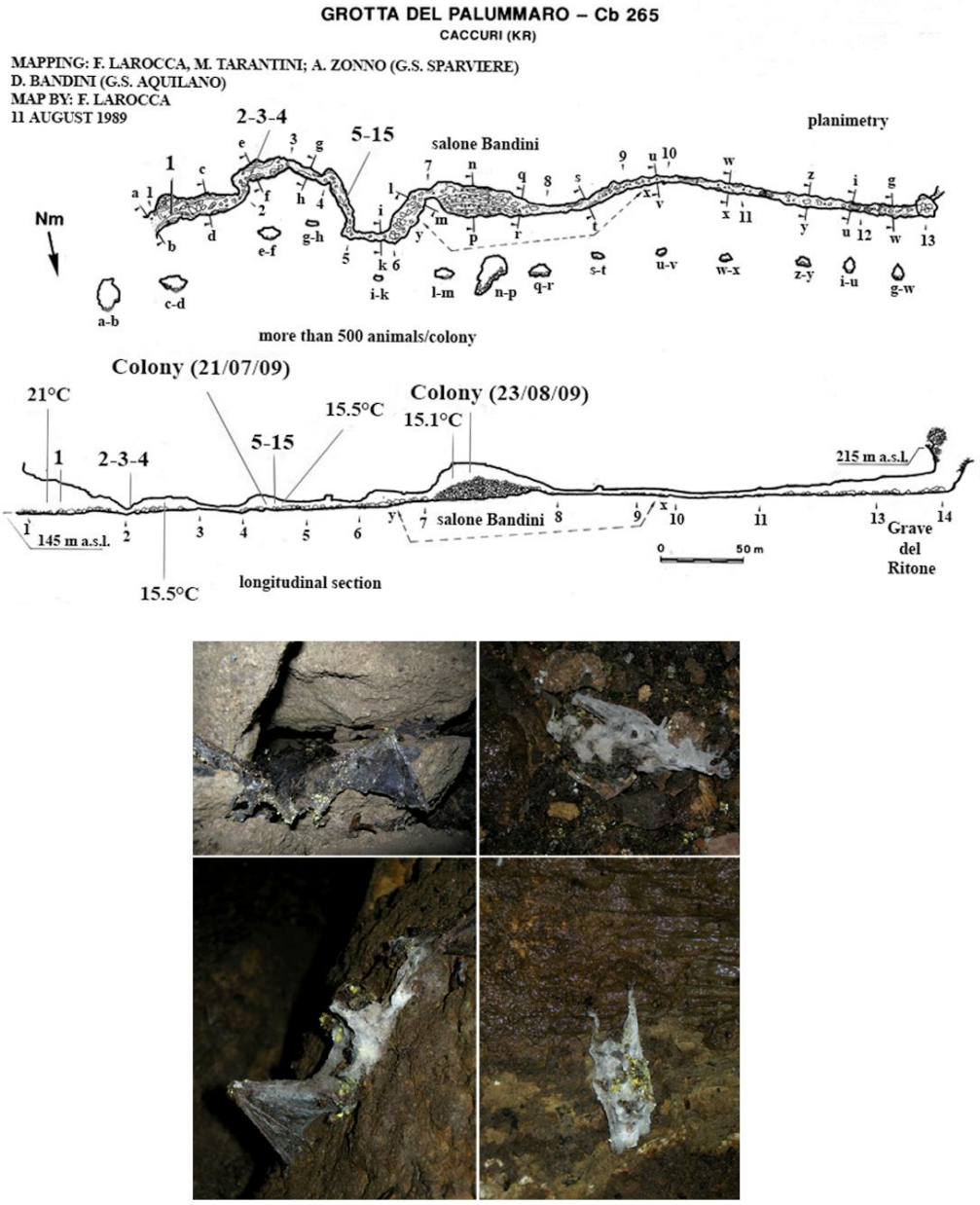

Figure 1 - Palummaro cave (Caccuri, Crotone, Italy) and bat carcasses.

bat carcasses could be identified to species level and five were discarded. The remaining 17 specimens belonged to *Myotis capaccini, Miniopterus schreibersii, Myotis* sp., *Pipistrellus* sp. and *Rhinolophus hipposideros*.

Small pieces of skin were placed onto the surface of Sabouraud-Dextrose agar plates

treated with antibiotics (gentamycin 40mg/l; chloramphenicol 50mg/l). Plates were incubated at 10°C and examined daily for fungal growth for a period of two weeks. After this period, all plates were incubated for two additional weeks at 24°C. Growing fungi were isolated in pure cultures. Five taxa, for which isolation in pure culture was not possible, were identified by direct microscopical analysis.

Swab samples from the wings, tails, ears and muzzles of the three live specimens from Turin were collected, inoculated onto Sabouraud-Dextrose agar plates and incubated as described above.

Fungi were identified conventionally according to their macroscopic and microscopic features. After determination of their genera, they were transferred to recommended media for species identification (Domsch *et al*., 1980; Von Arx, 1981; Kiffer and Morelet, 1997).

Fungal entities for which the identification, based on morphology, was found to be questionable were therefore identified by the amplification of the ITS region of the nuclear rDNA coding region using ITS1F and ITS4 primers (White *et al*., 1990).

The DNA sequences obtained were compared to the GenBank (NCBI) database by BlastN algorithm (http://www.ncbi.nlm.nih.gov/). Finally, BlastN-based identifications were checked by microscopic observations.

RESULTS

Forty-six fungus strains, belonging to 15 species, were isolated in pure culture from bat carcasses (Tab. 1, Fig. 2). Among the isolated fungal entities, 59% belong to Zygomycetes, 35% to Ascomycetes and 6% to Basidiomycetes. The most abundant species were *Mucor hiemalis* f. *hiemalis* (13 isolates), *M. racemosus* (9 isolates), *Fusarium equiseti* (4 isolates) and *Trichosporon chiropterorum* (3 isolates). As far as we know, this is the first report of *Trichosporon chiropterorum* in Italy. A further 5 taxa (*Chrysosporium merdarium*, *Alternaria* sp., *Aspergillus* sp., *Cladosporium cladosporioides* and an Ophiostomataceous fungus) were identified by direct microscopical analysis of small pieces of skin or hairs.

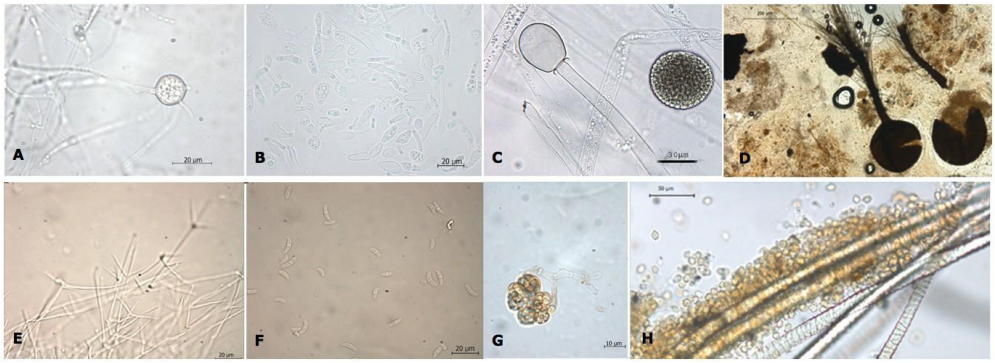

Figure 2 - A: *Mortierella polycephala*; B: *Trichosporon chiropterorum*; C: *Mucor hiemalis f. hiemalis*; D: Ophiostomatacea; E: *Verticillium lecanii*; F: *Fusarium dimerum*; G: Gymnoascacea; H: bat fur and conidia of *Chrysosporium merdarium*.

Table 1 - Fungal entities identified from bat carcasses (*fungi able to use keratinous materials as a nutrient source).

Class	Fungal taxa	Bat species
Ascomycetes	*Alternaria* sp.	*Myotis capaccini*
	Aspergillus sp.	*Miniopterus schreibersii*
	Candida palmioleophila	*Myotis capaccini*
	Chrysosporium/Gymnoascus	*Myotis capaccini*
	*Chrysosporium merdarium**	*Myotis capaccini, Myotis* sp.*, Miniopterus schreibersii, Pipistrellus* sp.
	Cladosporium cladosporioides	*Myotis capaccini*
	Fusarium dimerum	Undetermined bat
	*Fusarium equiseti**	*Myotis* sp.; *Miniopterus schreibersii*
	Gimnoascacea	*Myotis capaccini*
	Ophiostomatacea	*Miniopterus schreibersii,* undetermined bat
	*Penicillium griseofulvum**	*Pipistrellus* sp.
	Penicillium sp.	undetermined bat
	Thielavia sp.	*Myotis* sp.*, Miniopterus schreibersii, Pipistrellus* sp.
	*Verticillium lecanii**	*Myotis capaccini*
Basidiomycetes	*Trichosporon chiropterorum*	*Myotis capaccini, Myotis* sp.
Zygomycetes	*Mortierella polycephala*	*Pipistrellus* sp. and undetermined bat
	Mortierella gamsii	*Rhinolophus hipposideros*
	*Mucor hiemalis f. hiemalis**	*Myotis capaccini, Myotis* sp.*, Miniopterus schreibersii, Pipistrellus* sp. and undetermined bat
	*Mucor plumbeus**	*Pipistrellus* sp.
	*Mucor racemosus**	*Myotis capaccini, Myotis* sp.*,* and undetermined bat

Table 2 - Fungal entities isolated from live bats (*fungi able to use keratinous materials as a nutrient source).

Class	Fungal taxa	Bat species
Ascomycetes	*Aspergillus fumigatus* var. *fumigatus**	*Tadarida teniotis; Pipistrellus nathusii*
	*Aspergillus sydowii**	*Hypsugo savii*
	Candida sp.	*Pipistrellus nathusii*
	*Cladosporium cladosporioides**	*Hypsugo savii*
	Trichosporon chiropterorum	*Tadarida teniotis*

The only two fungus species found on all of the analysed carcasses were *M. hiemalis* f. *hiemalis* and *C. merdarium*. Six fungal entities, belonging to five species, were isolated from live bats (Tab. 2); at least two species, *i.e. Trichosporon chiropterorum* and *Cladosporium cladosporioides*, were also found on some carcasses.

DISCUSSION

Although the advanced state of decomposition of the bat carcasses impaired any histopathologic analysis, our results show that the high rate of bat mortality observed in the Palummaro cave, and the death of the two bats found in the Vene cave, was not caused by WNS, since neither *G. destructans* nor any other *Geomyces* species were found. As far as we know, *G. desctructans* has never been reported in Italy, while other ubiquitous species of the same genus have been frequently isolated from soil and air samples and, may occasionally cause superficial infection of the skin and nails in humans and animals (Gianni *et al*., 2003).

The fungi identified are all saprotrophic and have already been reported to be associated to bats, spiders, sediments, dung, carcasses, air and other substrates commonly found in cave systems (Mok et al. 1982; Larcher et al. 2003; Sugita et al. 2005; Yoder et al. 2009; Nieves-Rivera et al. 2009; Nováková 2009; Jurado et al. 2010); most of them are also known to colonise and destroy keratinous material, such as feathers, hairs, horn, nails and wool (Filipello Marchisio 2000; Hubálek 2000; Blyskal 2009). The fungi retrieved from living bats belonged to the same species or genus as those found on dead bats. According to Muller et al. (2001), animals harbour many saprotrophic moulds and yeasts on their coat and skin (genera *Alternaria*, *Aspergillus*, *Aureobasidium*, *Chrysosporium*, *Cladosporium*, *Mucor*, *Penicillium* and *Rhizopus*), which in most cases probably represent a transient contamination by soil or air-borne fungi. Cave environments offer ideal physical and chemical parameters (darkness, humidity, and constant temperature), as well as ideal nutritive substrates, for the survival and growth of a diverse saprobic or pathogenic mycobiota, which contribute to the decomposition of organic matter, making it available to the whole cave community (Nieves- Rivera et al. 2009).

On the other hand, it is well known that some saprotrophic fungi can act as opportunistic pathogens. Although usually innocuous, several of the isolated fungi (*Aspergillus fumigatus* var. *fumigatus*, *A. sydowii*, *Cladosporium cladosporioides*, *Candida palmioleophyla, Chrysosporium spp., Fusarium dimerum, Mucor hiemalis f. hiemalis, M. plumbeus, M. racemosus, Penicillium griseofulvum;* de Hogg et al. 2000), have already been proven to become pathogenic if the host, both humans and other mammals, is abnormally susceptible to infections, e.g. because of general immunesuppression.

Considering the low pathogen potential of the species described here, we can argue that these fungi probably invaded bat hairs and tissues after the death of the animals and were not involved in the death of the bats from Palummaro and Vene caves.

Bats are 'keystone species' in many ecosystems, where they play a major role in plant pollination, forest regeneration and control of insect populations (Mickleburgh et al. 2002; Kunz and Fenton 2003; Lobova et al. 2009). As a consequence, the decline of bat populations would be likely to have far-reaching ecological consequences.
Recently, a method for the detection of *G. destructans* by rapid PCR has been set up (Lorch et al. 2010). New methods of analysis together with the establishment of a network of individuals with different expertise will help to monitor and control the possible occurrence of WNS or other bat diseases in Italy.

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