

Aspergillus fumigatus and Other Thermophilic Fungi in Nests of Wetland Birds

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Abstract A study was performed on the numbers and species diversity of thermophilic fungi (growing at 45 °C in vitro) in 38 nests of 9 species of wetland birds, taking into account the physicochemical properties of the nests and the bird species. It was found that in nests with the maximum weight (nests of Mute Swan), the number and diversity of thermophilic fungi were significantly greater than in other nests, with lower weight. The diversity of the thermophilic biota was positively correlated with the individual mass of bird and with the level of phosphorus in the nests. The dominant species within the mycobiota under study was *Aspergillus fumigatus* which inhabited 95 % of the nests under study, with average frequency of ca. 650 cfu g⁻¹ of dry mass of the nest material. In a majority of the nests studied (nests of 7 bird species), the share of *A. fumigatus* exceeded 50 % of the total fungi growing at 45 °C. Significantly higher frequencies of the fungal species were characteristic of the nests of small and medium-sized piscivorous species, compared with the other bird species. The number of *A. fumigatus* increased with increase in the moisture

level of the nests, whereas the frequency of occurrence of that opportunistic pathogen, opposite to the general frequency of thermophilic mycobiota, was negatively correlated with the level of phosphorus in the nest material, and with the body mass and length of the birds. The authors indicate the causes of varied growth of thermophilic fungi in nests of wetland birds and, in particular, present a discussion of the causes of accumulation of *A. fumigatus*, the related threats to the birds, and its role as a source of transmission in the epidemiological chain of aspergillosis.

Keywords Thermophilic fungi · *A. fumigatus* · Nests · Wetland birds

Introduction

Aspergillus fumigatus is one of the most dangerous opportunistic pathogens causing mainly pulmonary mycosis (pulmonary aspergillosis) in animals and humans with hyp immunity [1]. From the viewpoint of biosafety, *A. fumigatus* has been classified the group BSL 2 (biosafety level 2) which comprises species that cause massive opportunistic infections in people with serious disturbances of the immune system. That species is also classified among allergenic factors and may be the cause of allergic pulmonary alveolitis (*alveolitis alergica*) [2, 3]. Birds are especially susceptible to infection with that fungus. Tell [4]

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reports that *A. fumigatus* is the cause of 95 % of cases of aspergillosis in birds. Another species that causes bird aspergillosis, though less frequently, is *Aspergillus flavus* [4, 5]. However, *A. flavus* is primarily responsible for toxicoses (aflatoxicoses) in birds. These are caused primarily by the occurrence of mycotoxins (aflatoxins) in food contaminated with the fungus [5]. The infection with *A. fumigatus* occurs most frequently through the inhalation of spores of the fungus [3, 6]. In the case of birds, a frequent source of infection with *A. fumigatus* may be food contaminated with the fungus or contact with the soil during feeding [5]. *A. fumigatus* belongs to the ubiquitous fungi (polyphages), capable of degrading cellulose and hemicellulose, occurring in large amounts in plant material, as well as proteins, including keratin contained in hair, feathers and other animal remnants [7–9]. The fungus is a thermotolerant species, with growth optimum at 37 °C and good growth within the temperature range of 30–45 °C [10]. For these reasons, it is widespread especially in environments characterised, at least periodically, with increasing temperature, such as decomposing organic wastes or composts [7]. Similar thermal conditions prevail also in bird nests. During breeding, the nest temperature increases from 35 to 37 °C, with the bird body temperature being from 38 to 44 °C [4, 11, 12]. The fungus has also been frequently isolated from the plumage and from the ontocoenoses of the beak and the cloaca of healthy birds, including those inhabiting aquatic-wetland biotopes [5, 13, 14]. Cases of aspergillosis have been frequently noted among domestic birds, captive birds, as well as free-living birds from many groups: *Anseriformes*, *Lariformes*, *Galliformes*, *Columbiformes*, *Falconiformes* and, less frequently, *Passeriformes* [5, 6, 15–18]. Among free-living birds, the most susceptible to infections with *A. fumigatus* and also to other opportunistic pathogens from the genus *Aspergillus* are birds of prey and water and water–wetland birds [5, 13, 16, 18, 19]. Stress is of fundamental importance in the development of fungal infections in birds [16]. Predisposing factors include undernourishment, change of diet, incorrect air temperature and relative humidity, wounds, weakening caused by bacterial and virus infections, environmental pollution with chemical compounds, including pesticides [4–6, 16]. The most susceptible to infections are young and weak birds [17, 18]. Greater susceptibility to aspergillosis is also displayed by birds

performing long journeys to wintering areas [6, 15]. *A. fumigatus* can also penetrate eggs and kill embryos [5]. Cases of aspergillosis with the character of an epizootic have been noted, causing high mortality among birds, especially among water–wetland birds: geese and *Anatidae* [20, 21]. Those were caused mainly by *A. fumigatus*, less frequently by other species of *Aspergillus*. There have also been reports, though less frequent, about fatal poisoning with aflatoxins of *Aspergillus flavus* and the death of birds with symptoms of acute hepatic necrosis [22].

Nests of wetland birds are usually concentrations of a large mass of plant material, with an admixture of animal material (feathers and hair in nest lining, the fluff of chicks, remnants of prey and pellets of fledgling raptors and droppings). The substrate diversity of nest material causes that the nests of wetland birds, with their notable moisture, are an attractive substrate for various ecological groups: ubiquitous, cellulolytic and keratinolytic [23–25], including also the appearance of *A. fumigatus* and other species of thermotolerant fungi [23–25]. The moisture content and pH of the nest material are among those factors that determine the selection of fungal communities in bird nests [26, 27]. Hubalek [26], in relation to pH of nests, classified fungi as acidophilic, neutrophilic and alkalophilic. Studies by Hubalek [27] indicate also that both very low and high moisture of the nest material may have an inhibiting effect on the occurrence of fungi in bird nests [27].

The objective of the study presented herein was the estimation of the frequency and diversity of thermophilic fungi (growing at 45 °C in vitro), with particular emphasis on *A. fumigatus*, in the nests of various wetland bird species in the area of central-eastern Poland that so far have not been studied in this aspect. In the course of the study, the authors also searched for correlations between the population of that fungus and the total numbers of thermophilic fungi (growing at 45 °C in vitro), and certain physical and chemical properties of the nests and the bird species.

Materials and Methods

Nests

The study was conducted on 38 nests of 9 bird species: Great Crested Grebe (*Podiceps cristatus* L.)—5; Grey Heron (*Ardea cinerea* L.)—6; Mute Swan (*Cygnus*

olor Gmel.)—5; Marsh Harrier (*Circus aeruginosus* L.)—3; Coot (*Fulica atra* L.)—5; Black-headed Gull (*Larus ridibundus* L.)—7; Common Gull (*Larus canus* L.)—1; Common Tern (*Sterna hirundo* L.)—5; Black Tern (*Chlidonias niger* L.)—1.

The nests were collected in July or in the first half of August, 2006–2008, at rainless weather, typical of that period in the central-eastern part of Poland. Depending on the species, bird nests were collected after 7–14 days, counting from the first flight (e.g. Marsh Harrier) or from the first leaving of the nests by young birds (e.g. Great Crested Grebe, Coot), as we wanted to be certain that the nests do not perform any function in relation to the juvenile birds any longer. It is a known fact that young birds of the species under study return to the nests for the night period. Earlier removal of the nests would be in violation of the Polish Environmental Law. In this context, nests of Coot and Common Gull were collected after 8 days, and that of Black-headed Gull, Great Crested Grebe, Black Tern and Common Tern after 7 days. Nest material of Marsh Harrier was collected after 10 days, and that of Mute Swan after 14 days. With the exception of nests of Grey Heron, built on tall trees, the nests of the other bird species were situated in reeds on water reservoirs, less frequently as floating nests or nests built on aquatic vegetation. For most of the species, the breeding biotopes were ponds, only Common Tern and Common Gull nested on lakes. The nests were built of plant material and contained various amounts of material of animal origin, partially incorporated in the structure of the nest (lining), and partially, it was secondary material. Detailed description of the nests: their location, sizes, structure and the composition of the building material (twigs, stems, stalks, roots, rhizomes and other parts of specific plant species) and non-plant material (feathers, hair, remnants of food, pellets containing undigested parts of prey, droppings, etc.), as well as the dates of their collection, are given in an earlier work by the authors [25]. The body weight and length values used in analyses are given after Busse [28] and Sokolowski [29]. For the calculations, the following bird body weight and length values were adopted: Great Crested Grebe (900 g, 54 cm), Grey Heron (16,000 g, 90 cm), Mute Swan (11,000 g, 160 cm), Marsh Harrier (600 g, 58 cm), Coot (680 g, 39 cm), Black-headed Gull (300 g, 39 cm), Common Gull (450 g, 48 cm), Common Tern (70 g, 25 cm), Black Tern (70 g, 25 cm) [29].

Isolation of *Aspergillus fumigatus*

The nests (with the exception of those of Marsh Harrier and Mute Swan) were collected whole. From the nests of Marsh Harrier and Mute Swan, only the above-water parts were taken. Each nest was placed in a sterile polyethylene bag and transported to the laboratory so as to preserve the nest structure intact. The nest material was taken at random from at least 10 different uniformly distributed places on each nest, in amounts of 100–200 g in total per nest. Samples were taken from the central part of the nests. Greater numbers of individual samples (15–20) were taken from the large nests (nests of Great Crested Grebe, Grey Heron, Mute Swan and Marsh Harrier). From 10 to 15, individual samples were taken from nests with smaller dimensions (Coot, gull and tern). At each sampling point, nest material was taken both from the surface and from a deeper layer. In the case of large nests (Mute Swan, Marsh Harrier), the maximum depth of nest material sampling did not exceed 20 cm. The samples were taken in a sterile manner, using sterile tweezers and scissors. Each time (for every sampling point and place separately), the tweezers and scissors were sterilised in the flame of a gas burner after prior immersion in ethyl alcohol. The nest material sampled was placed in sterile plastic string-sealed bags. The samples were averaged and fragmented through cutting and homogenisation. The nest material was cut, with sterilised scissors, into sections (0.5–1 cm). Those operations were performed close to the flame of a gas burner and in sterile atmosphere (microbiological box sterilised with UV radiation and disinfected). A total of 10 g of sample prepared in this manner was suspended in 90 cm³ of sterile distilled water with pH 6.0 (dilution of 10⁻¹) and homogenised under sterile conditions at 10,000 rpm for 5 min. All dilutions were shaken using a Vortex: mother dilutions (10⁻¹)—10 min, other dilutions for 5 min. *A. fumigatus* was isolated with the use of a selective technique applied for thermophilic fungi. The isolation was conducted with the plate dilution method, on the Martin medium, which is recommended for the isolation of fungi related to plant material [30], and the Sabouraud medium, preferable for fungi of animal origin (with an admixture of antibacterial antibiotics (streptomycin and chlortetracycline) in the same amounts as for the Martin medium). The cultures were incubated at 45 °C for 3–5 days.

The species identification of the fungi was conducted by inoculating all the colonies grown onto plates with PDA (potato-dextrose agar). The pure cultures obtained were identified by means of microscope observations on agar circles and macroscopic observations on plates. The final classification was made with the use of systematic works by Raper and Fannell [31], Domsch et al. [32] and Oorschot [33].

Determination of Physical and Chemical Properties of Nest Material

The content of water in the nest material was determined with the gravimetric method at 105 °C, while pH was assayed in H₂O and 0.1 M KCl. The content of organic C was determined with the Tiurin method, total S—with the method of elementary analysis through high-temperature incineration in oxygen and assays in thermal conductivity detector. The contents of N-total, P-total, K, Ca and Mg were determined after the wet mineralisation of the samples in a mixture of concentrated H₂SO₄ and hydrogen peroxide, with the method of flow spectrophotometry (N-total, P-total) and with the method of atomic absorption spectrometry (K, Ca, Mg). The data concerning the chemical properties and the moisture levels of the nests, given in the form of ranges of values, are presented in Table 1. The full characterisation of the physical and chemical parameters of the nests can be found in the work by Kornilowicz-Kowalska et al. [25].

Evaluation of Results

The total numbers of thermophilic fungi (growing at 45 °C in vitro) are given in colony forming units per 1 g of dry matter of the nest material (cfu g⁻¹ d.m.). The results are given as means from 3 replications. The significance of differences among the mean values was tested by means of Tukey's intervals of confidence at significance level of $\alpha = 0.05$. The test applied permits the division of the objects compared (nests of particular bird species) into statistically homogeneous groups and to determine the least significant difference of the means (LSD). The share of *A. fumigatus* in the total of thermophilic micromycetes is given in cfu g⁻¹ d.m. and as percentage values. The estimation of the species diversity of thermophilic fungi was made with the use of the

Shannon–Wiener index [34] which was calculated with the following formula:

$$H = - \sum [P_i \log (P_i)]$$

where P is quotient of the number of isolates of species “ i ” and the number of isolates of all species. The species diversity measured with the Shannon–Wiener index increases with increase in the number of species and also with the equalisation of the number of species in the sample [34]. In relation to the distribution of data, non-parametric or parametric tests were used. Two classes of data were compared with Mann–Whitney U test or t Student's tests. More than two classes of data were compared with using the Kruskal–Wallis test or one-way ANOVA. Frequencies of species were compared with the chi-square test with Yates corrections. For the identification of trends, the Pearson correlations were ascertained [35].

Results

Physical and Chemical Properties of Nests

Significant interspecies differences were found in the moisture levels of the nests under study (Kruskal–Wallis test: $H = 20.73$, $df = 8$, $n = 38$, $p = 0.008$). In particular, that concerned the nests of Marsh Harrier and Grey Heron which were notably drier than the nests of the other species (Mann–Whitney test: $Z = -4.20$, $n_1 = 9$, $n_2 = 29$, $p = 0.0001$). The moisture levels of the nests of those species were 49.5–76.9 % and 18.8–64.8 %, respectively, while for the other species, they varied within the range of 76.2–90.6 % (Table 1), with the sole exception of the Black-headed Gull nest No. 20, whose moisture was 44.1 % [25].

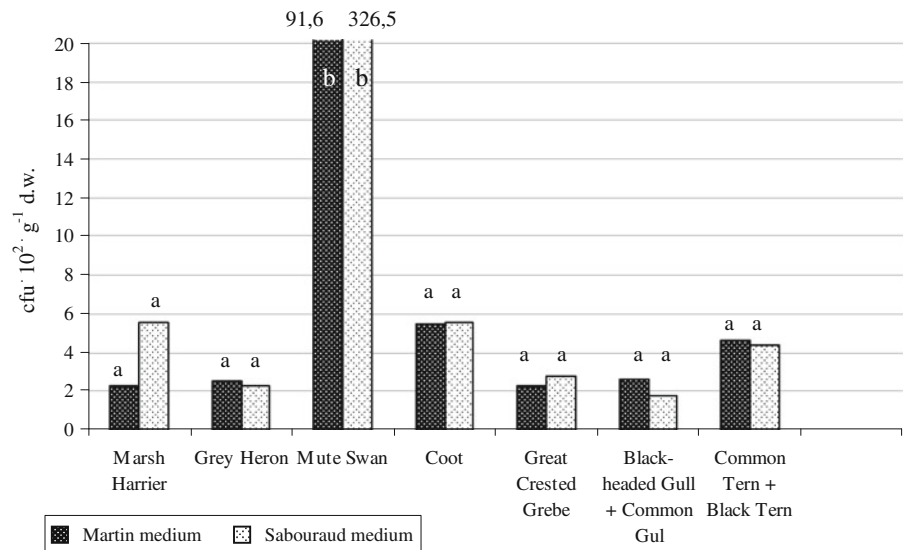
The reaction of the nests was close to neutral or slightly alkaline (pHH₂O 6.55–7.76) (Table 1). Only a single Grey Heron nest (No. 7) was characterised by a weakly acid reaction, pHH₂O = 5.99 [25]. No statistically significant differences were found in the pH of the nests among the bird species (Kruskal–Wallis tests: $H = 12.10$, $df = 8$, $n = 38$, $p = 0.147$ for pHH₂O; $H = 8.69$, $df = 8$, $n = 38$, $p = 0.369$ for pHKCl).

The content of organic C in the nest material was from 24.1 to 47.9 % of dry matter (Table 1). No statistically significant differences were found in the level of that component among the particular nests.

Table 1 Content of macroelements (% of dry matter), pH and moisture (% of dry matter) in nests of wetland birds

No.	Bird species	C-total/C-org.	N-total	S-total	P-total	K	Ca	Mg	pH H ₂ O/KCl	Moisture
1	Marsh Harrier	45.68–47.24 42.27–43.95	2.17–2.27	0.28–0.40	0.09–0.23	0.10–0.40	0.47–0.70	0.030–0.129	5.89–7.23 6.24–7.44	49.56–70.54
2	Grey Heron	36.84–49.10 32.30–40.74	2.29–3.47	0.34–0.61	0.78–6.07	0.29–1.19	1.49–6.10	0.106–0.309	5.99–7.76 5.26–6.90	18.78–64.76
3	Mute Swan	37.62–48.23 33.18–45.16	1.79–2.72	0.25–0.47	0.11–0.21	0.11–0.53	0.43–6.92	0.040–0.216	6.55–7.10 5.64–7.08	78.44–90.61
4	Coot	44.82–48.69 39.92–45.28	1.60–2.57	0.28–0.82	0.12–0.26	0.10–1.46	1.23–2.13	0.119–0.291	6.70–7.14 5.95–7.34	84.17–88.31
5	Black-headed Gull	25.71–46.69 24.05–40.88	1.39–2.83	0.23–0.96	0.15–0.63	0.05–0.52	0.74–2.80	0.169–0.305	6.72–7.57 6.63–7.32	44.09–87.33
6	Common Gull	48.88 47.88	2.02	0.29	0.18	0.28	1.99	0.052	6.84 6.76	85.26
7	Great Crested Grebe	37.47–42.39 33.18–38.25	1.83–2.67	0.29–0.62	0.10–0.43	0.11–0.56	1.08–6.42	0.111–0.436	6.90–7.47 6.85–7.36	79.64–88.06
8	Common Tern	46.87 42.52	1.28	0.22	0.15	0.68	1.85	0.185	6.47 7.17	82.16
9	Black Tern	41.90–44.78 40.52–42.40	2.62–3.04	1.05–1.36	0.11–0.16	0.04–0.08	2.54–3.38	0.072–0.092	6.90–7.34 6.63–7.08	85.02–86.08

Fig. 1 Mean numbers of thermophilic fungi (growing at 45 °C in vitro) in the nests of the particular species of wetland birds. $LSD_{0,05}$ for Martin medium 63,8; $LSD_{0,05}$ for Sabouraud medium 255,0. The same letters were used to denote means that do not differ significantly; different letters denote means that differ significantly



Also, no such differences were revealed in relation to the content of N-total, in spite of the notable variation of that component in the nests: 1.28–5.38 % d.m, whereas significant differences were observed in relation to the content of C-total (Kruskal–Wallis Test: $H = 16.47$, $df = 8$, $n = 38$, $p = 0.036$) which varied from 25.71 to 49.10 %. The levels of the remaining macroelements (except for potassium), S, P, Mg and Ca, in the nests of the particular bird species were also significantly differentiated. In the Kruskal–Wallis test (at $df = 8$ and $n = 38$), the values of H and p were as follows: S-total ($H = 22.09$, $p = 0.0048$), P-total ($H = 22.30$, $p = 0.0064$), Mg ($H = 21.02$, $p = 0.007$) and Ca ($H = 6.05$, $p = 0.048$), respectively.

Total Numbers of Thermophilic Fungi (Growing at 45 °C In Vitro)

The highest total numbers of thermophilic fungi, significantly higher than in the other nests, were observed in the nests of Mute Swan (Fig. 1). In terms of mean values (from 5 nests), they were 9,160 cfu g^{-1} d.m. (Martin medium) and 33,000 cfu g^{-1} d.m. (Sabouraud medium), respectively. The number of thermophilic fungi in the nests of the remaining bird species did not differ significantly and amounted to, on average, from 154 to 506 cfu g^{-1} d.m. (Martin medium) or from 189 to 698 cfu g^{-1} d.m. (Sabouraud medium) (Fig. 1).

The Richness and Frequency of Species of Thermophilic Fungi

The identification of 1,374 isolates of fungi isolated at 45 °C from 38 nests studied (767 on Martin medium, 617 on Sabouraud medium) revealed 12 genera and 18 species (Table 2). The greatest species diversity of the fungi was observed in the nests of Mute Swan and Eurasian Coot (10 species), and the smallest (2 species) in nests of Black-headed Gull (Table 2). The number of species of thermophilic fungi found in the nests of the other bird species varied from 4 (Marsh Harrier, Great Crested Grebe) to 6 (Grey Heron). The assays did not include the nests of Common Gull and Black Tern, for the simple reason that only 1 nest each was available (Table 2).

It was demonstrated that an increase in the individual mass of birds using the nests is conducive to an increase in the diversity of the biota of thermophilic fungi (growing on the Martin medium), expressed by the values of the Shannon–Wiener index (H) ($r = 0.393$, $n = 37$, $p = 0.016$). The value of index H for the populations of those fungi was also positively correlated with the content of phosphorus in the nest material ($r = 0.368$, $n = 37$, $p = 0.027$). The single nest of Common Gull was omitted from the analyses. The remaining chemical parameters (C-total, C-org., N-total, S-total, Ca, Mg, K) had no significant effect on the value of the index.

Table 2 Species composition and frequency of thermophilic fungi (growing at 45 °C in vitro) in nests of wetland birds (dil. 10⁻¹)

No.	Fungus species	Marsh Harrier (3)*		Grey Heron (6)		Mute Swan (5)		Coot (5)		Great Crested Grebe (5)		Black-headed Gull (7)		Common Gull (1)		Common Tern (5)		Black Tern (1)	
		M	S	M	S	M	S	M	S	M	S	M	S	M	S	M	S	M	S
1	<i>A. corymbifera</i> (Cohn) Sacc.&Trotter	-	-	-	-	-	16	-	4	-	15	-	-	-	-	7	-	-	-
2	<i>Acronium</i> sp.	-	-	-	-	1-	1	19	-	-	-	-	-	-	-	-	-	-	-
3	<i>A. flavus</i> Link ex Gray	-	-	19	18	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4	<i>A. fumigatus</i> Fres	59**	36	88	38	18	33	78	36	39	63	84	116	20	13	100	101	2	2
5	<i>A. niger</i> van Thieghem	-	5	1	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-
6	<i>A. terreus</i> Thom	-	-	-	-	-	20	-	-	-	-	-	-	-	-	-	-	-	-
7	<i>Chaetomium thermophile</i> La Touche	-	-	-	-	14	-	2	-	-	-	-	-	-	-	-	-	-	-
8	<i>Ch. keratinophilum</i> Frey ex Carmichael	-	-	-	-	2	-	-	-	-	-	-	-	-	-	-	-	-	-
9	<i>Ch. tropicum</i> Carmichael	-	-	36	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
10	<i>C. sepedonium</i> (Emmons) v. Arx	-	-	-	-	-	20	-	1	-	-	-	-	-	-	-	-	-	-
11	<i>P. variotii</i> Bain.	-	15	-	-	-	5	-	21	2	2	-	-	-	-	-	-	-	-
12	<i>P. purpurogenum</i> Stoll	-	-	-	-	-	-	31	-	23	-	7	-	-	-	-	-	-	-
13	<i>P. expansum</i> Link ex Grey	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
14	<i>R. pusillus</i> (Lindt) Schipper	-	-	-	-	11	-	5	-	-	-	-	-	-	-	8	-	-	-
15	<i>T. emersonii</i> Stolk	-	-	-	-	-	-	-	-	-	-	-	-	-	-	6	7	-	-
16	<i>Talaromyces</i> sp.	-	-	2	-	-	-	-	6	-	-	-	-	-	-	-	-	-	-
17	<i>T. aurantiacus</i> Miele	42	-	-	-	17	18	-	-	-	-	-	-	-	-	-	-	-	-
18	<i>T. lanuginosus</i> Tsiklinsky	-	-	-	-	-	-	2	4	-	-	-	-	6	-	-	-	-	-
19	Dark-pigmenting non-sporifying moulds	-	-	-	-	-	-	-	-	-	-	-	-	-	-	15	-	-	-
Total		101	56	147	57	63	113	137	72	64	80	91	116	26	13	136	108	2	2
Grand total		767 (M) + 617 (S) = 1,374																	

M—Isolation on Martin medium, S—Isolation on Sabouraud medium, * number of nests, ** number of isolates

Characterisation of Species of Thermophilic Fungi (Growing at 45 °C In Vitro) Isolated from Nests of Wetland Birds

The descriptions take into account the systematic classification, physiological capabilities, environmental requirements (temperature, pH and substrate moisture) and pathogenicity.

- *Absidia corymbifera* (Cohn) Sacc. & Trotter: *Zygomycota–Mucorales*, chitinolytic fungus, colonises native keratin, classified among the psychrotolerant thermophilic fungi with optimum growth temperature of 35–37 °C, optimum pH 3.8–6.8, potentially pathogenic (mucormycosis of lungs, nasal sinuses and other organs) [32].
- *Acremonium* sp., anamorph (teleomorph: *Hypocreales*, *Sordariomycetes*, *Ascomycota*), genus comprising ubiquitous fungi, preferring decomposing organic material, optimum temperature for growth 20–30 °C (certain species grow at 37 °C), mesophilic or hydrophilic, certain species are pathogenic for humans and animals [32].
- *Aspergillus flavus* Link ex Gray, anamorph (teleomorph: *Emericella*; *Ascomycota–Eurotiales*), ubiquitous fungus, optimum temperature for growth is 25–42 °C and the maximum is 47–48 °C, optimum pH 6.5–7.5, causes infections of the respiratory tract (less frequently) and aflatoxicoses (more often) especially in domestic and caged birds [13, 32].
- *Aspergillus fumigatus* Fres., anamorph (teleomorph: *Emericella*; *Ascomycota–Eurotiales*), colonises mostly environments with elevated temperature (composts, bird nests and plumage, droppings and pellets), ubiquitous fungus, weakly keratinolytic, thermotolerant, grow at pH 3.8–7.8, high requirements concerning water conditions (a_w 0.9–0.95), zoopathogenic (aspergillosis of lungs, especially in birds), toxinogenic species (less strongly than *A. flavus*) and allergenic [10, 13, 32, 47].
- *Aspergillus niger* van Tieghem, anamorph (teleomorph: *Emericella*; *Ascomycota–Eurotiales*), colonises the soil, composts, seeds and other parts of plants, bird nests, plumage and droppings, ubiquitous fungus, thermotolerant (optimum 17–42 °C), grows in a broad range of pH (2.0–8.0) and may cause pneumomycoses in domestic birds [13, 32].
- *Aspergillus terreus* Thom, anamorph (teleomorph: *Emericella*; *Ascomycota–Eurotiales*), appears in the soil, composts, seeds of plants, nests of free-living birds, ubiquitous species, thermotolerant (optimum temp. 35–40 °C), range of pH ≤ 2.0 , ≥ 8.0 , toxinogenic, potentially zoopathogenic [13, 32].
- *Chaetomium thermophilum* La Touche, teleomorph: *Ascomycota–Sordariales*, appears in the soil, composts, bird nests and plumage, cellulolytic and coprophilic fungus, obligatory thermophile (optimum 50 °C, grows within the range of 27–58 °C), saprotrophic [32].
- *Chrysosporium keratinophilum* Frey and Carmichael, anamorph, (teleomorph: *Aphanoascus keratinophilus* Punsola & Cano, *Ascomycota–Onygenales*), species occurring in the soil, bird plumage, nests and pellets, composts containing keratin wastes, outstandingly keratinolytic, thermotolerant (optimum growth at 25 °C, maximum 30–40 °C), alkalophilic (range of pH 4.52–8.74), hydrophilic, potentially zoopathogenic [13, 25–27, 32, 33].
- *Chrysosporium tropicum* Carmichael, anamorph (teleomorph: *Onygenaceae*, *Ascomycota–Onygenales*), appears in bird plumage, nests and pellets; strongly keratinolytic species, thermotolerant (optimum temperature 25–30 °C, maximum 30–40 °C), acidotolerant fungus (range of pH 5.08–8.20), tolerant to elevated humidity >30 %, potentially pathogenic [13, 23, 25–27].
- *Corynascus sepedonium* (Emmons) v. Arx, teleomorph: *Ascomycota–Sordariales*. Occurs in the soil, bird nests, manure; cellulolytic species, thermotolerant (optimum growth at 25–35 °C, maximum 46 °C) [32].
- *Paecilomyces variotii* Bain., anamorph (teleomorph: *Byssochlamys*, *Ascomycota–Eurotiales*), colonises the soil, composts, bird nests, plumage and pellets; ubiquitous and cellulolytic species, causes soft rot of wood, relatively thermophilic (optimum growth at 25–35 °C, certain isolates up to 40 °C). Causal agent of paecilomycosis in animals, also causes toxicoses [13, 32].
- *Penicillium purpurogenum* (Stoll), anamorph (teleomorph: *Talaromyces purpurogenus* (Stoll) Samson, Yilmaz, Erisvad & Seifert, *Ascomycota–Eurotiales*), soil fungus, also isolated from bird nests and plumage, ubiquitous and cellulolytic

Table 3 Frequency of thermophilic (growing at 45 °C in vitro) strains of *A. fumigatus* (dil. 10^{−1}) in particular nests of bird species under study

Nest No.	Bird species	Number of isolated strains		cfu 10 ¹ g ^{−1} d.m.		Index H (Martin medium)	
		Martin medium	Sabouraud medium	Martin medium	Sabouraud medium	For nests	For bird species
1	Marsh Harrier	15	2	50.92	6.79	0	0.976
2		14	2	37.63	5.38	0.353	
3		30	32	59.50	63.47	0.987	
4	Grey Heron	4	1	9.57	2.39	1.719	1.500
5		69	15	183.02	39.79	0.108	
6		8	3	16.32	6.12	1.272	
7		0	3	0	8.51	–	
8		7	16	12.88	29.45	0.902	
9	Mute Swan	0	0	0	0	0.148	
10		4	8	32.57	65.15	0	2.200
11		4	5	22.95	28.69	0	
12		0	0	0	0	0	
13		4	8	20.74	41.47	0.722	
14	Coot	6	12	27.83	55.66	1.843	
15		7	0	59.88	0	1.44	1.700
16		49	14	368.70	105.34	0.715	
17		14	16	90.73	103.69	0.966	
18		1	2	6.32	12.63	0	
19	Black-headed Gull	7	4	60.71	34.69	0	
20		4	4	7.15	7.15	0	0.391
26		13	17	90.72	118.63	0	
27		11	6	86.82	47.36	0	
28		7	6	48.41	41.49	0	
29	Common Gull	8	7	59.48	52.05	0	
30		29	56	136.02	262.66	0	
31		13	20	54.65	84.07	0.934	
36		20	13	135.69	88.20	0	0.779
21	Great Crested Grebe	4	4	24.01	24.01	0.918	1.220
22		3	1	22.71	7.57	0.811	
23		3	0	25.13	0	0.811	
24	Black Tern	0	3	0	30.33	0	
32		29	55	139.69	264.93	0.99	
25		2	2	11.21	11.21	0	0
33	Common Tern	31	38	218.93	268.36	0	1.336
34		15	9	107.76	64.66	0.34	
35		33	21	220.29	140.19	0.432	
37		16	7	111.11	48.61	0	
38		5	26	35.34	183.75	0	

species, thermotolerant (optimum growth at 30 °C, grows also at 37 °C), acidophilic and hydrophilic fungus, toxinogenic [13, 32].

- *Penicillium expansum* Link ex Gray, anamorph (teleomorph: *Eupenicillium*, Ascomycota–Euroti-ales), species occurring in the soil, on seeds, cereal

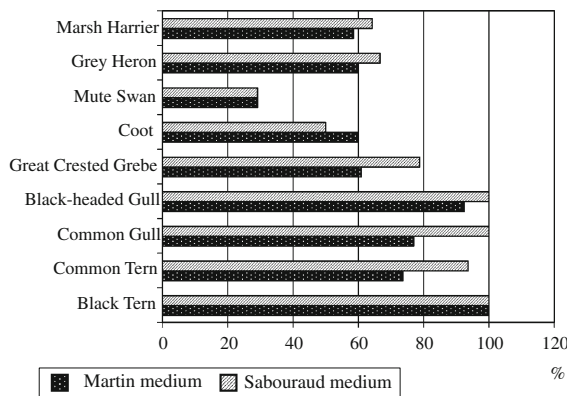


Fig. 2 Share (%) of populations of *A. fumigatus* in the total numbers of thermophilic fungi (growing at 45 °C in vitro) colonising the nests of wetland birds

grain, fruits, also isolated from bird nests, ubiquitous and cellulolytic fungus, optimum temperature for growth 23–30 °C, toxinogenic [32].

- *Rhizomucor pusillus* (Lindt) Schipper, *Zygomycota–Mucorales*, fungus isolated from the soil, composts, manure, bird nests, feathers and pellets, degrades carbohydrates but not cellulose, thermophilic fungus (optimum temperature for growth 50 °C, slow growth at 20 °C), potentially pathogenic for animals, produces mycotoxins [32].
- *Talaromyces emersonii* Stolk, teleomorph: *Ascomycota–Eurotiales*, species observed in the soil, composts and manure, ubiquitous and cellulolytic fungus, thermophilic (minimum temperature for growth <30 °C, optimum 45 °C, maximum 55–65 °C) [32].
- *Thermoascus aurantiacus* Miehe, teleomorph: *Ascomycota–Eurotiales*, fungus isolated from the soil, composts, nest material and bird feathers, ubiquitous and cellulolytic species, obligatory thermophile (grown within temperature range of 22–58 °C, optimum 40–46 °C (51 °C), maximum 55–62 °C) [32].
- *Thermomyces lanuginosus* Tsiklinsky, anamorph (teleomorph: *Chaetomiaceae*, *Ascomycota–Eurotiales*), isolated from the soil, composts, bird nests, ubiquitous and thermophilic fungus (optimum growth temperature 47.5–52.5 °C, minimum ca. 30 °C) [32].

Frequency of *Aspergillus fumigatus*

Aspergillus fumigatus (growing at 45 °C in vitro) inhabited 36 among the 38 nests under study (95 %)—

Table 3. In total, 927 isolates of *A. fumigatus* were isolated from the 36 nests: 489 on the Martin medium and 438 on the Sabouraud medium (Tables 2, 3), which constitutes 63.75 and 70.99 %, respectively, of the total of the isolated thermophilic fungi. Taking into account the dilution of the samples (10^{-1}), it was calculated that the estimated mean number of propagation units of *A. fumigatus* in 1 nest was from 620 (Sabouraud medium) to 683 (Martin medium) per 1 g of dry matter of nest (the analyses were made on averaged samples of 10 g each). The share of the population of that fungus in the thermophilic mycobiota of nests of the particular bird species varied from 28.6 to 92.3 % (isolation on the Martin medium) and from 29.2 to 100 % (isolation on the Sabouraud medium). It increased with decrease in the species diversity, up to and including monoculture (100 %). The lowest share of *A. fumigatus* (28.6 and 29.2 %, respectively) was observed in the thermophilic mycobiota of the nests of Mute Swan. In the nests of the remaining 7 bird species, it was very high and exceeded 50 % (Fig. 2). The highest percentage share of populations of *A. fumigatus* was recorded in nests of Black-headed Gull (92.3 and 100 %, respectively) and Common Tern (73.5, 93.5 %). Lower values, though still high, were noted in the nests of Marsh Harrier, Grey Heron, Coot and Great Crested Grebe: 56.9–60.9 % (Martin medium) and 54.0–78.7 % (Sabouraud medium) (Fig. 2).

It was found that significantly greater (Student's *t* test: $t = 2.20$, $df = 36$, $p = 0.034$) numbers (0.842 ± 0.258) of *A. fumigatus* occurred in small- and medium-sized nests ($n = 20$) of piscivorous species (Black-headed Gull, Common Gull, Common Tern, Great Crested Grebe) compared with the nests of the remaining ($n = 18$) bird species (0.598 ± 0.401).

Among the piscivorous species studied, Grey Heron, Black-headed Gull, Common Tern and Great Crested Grebe, there were also significant differences in the frequency of *A. fumigatus* (ANOVA: $F_{3,19} = 8.75$, $p = 0.0007$).

Comparison of the frequency of *A. fumigatus* (Martin medium) between the nests (Tables 2, 3) of the particular bird species revealed highly significant differences in the number of the fungus for Marsh Harrier and Mute Swan ($\chi^2 = 9.60$, $df = 1$, $p = 0.0002$), and for Coot and Mute Swan ($\chi^2 = 9.60$, $df = 1$, $p = 0.002$), whereas no differences in the frequency of *A. fumigatus* between the

nests of Coot and Marsh Harrier were observed ($\chi^2 = 2.73$, $df = 1$, $p = 0.10$).

Effect of Ecological Factors on Frequency of *Aspergillus fumigatus*

It was demonstrated that the number of *A. fumigatus* increases with increasing moisture of the nests (Pearson $r = 0.373$, $n = 38$, $p = 0.021$) whereas a negative correlation was obtained between the frequency of *A. fumigatus* and the content of phosphorus (Pearson $r = -0.310$, $n = 38$, $p = 0.05$). The remaining chemical parameters did not correlate significantly with the frequency of that fungus in the nests of the birds under study.

It was found that with increase in the body mass and length of the birds, the frequency of occurrence of *A. fumigatus* (opposite to the overall frequency of thermophilic fungi) in the nest material decreased: Pearson $r = -0.597$, $n = 38$, $p = 0.0001$ and Pearson $r = -0.396$, $n = 38$, $p = 0.014$, respectively. A stronger relation was observed between the frequency of the fungus and the body length of the birds.

Discussion

Thermophilic fungi comprise two groups: obligatory thermophiles (optimum ≥ 50 °C, minimum ≥ 20 °C) and thermotolerant fungi, that is, facultative thermophiles (maximum 50 °C, minimum ≤ 20 °C) [36, 37]. In this study, due to the fact that only one temperature was applied in the isolation of thermophilic fungi (45 °C), that division was not taken into account.

The total numbers of thermophilic fungi in the nests of a majority (7) of the bird species studied varied from 150 to 700 cfu g⁻¹ d.m. of nest material. Due to the lack of data concerning the populations of those fungi in bird nests, the results obtained were compared with their numbers in soil. It was found that the number of the thermophilic mycobiota in the nests of the bird species under study was generally greater than the number of thermophilic fungi (growing at 45 °C in vitro) in soils strongly heated by the sun [38]. Ellis and Keane [38], analysing the soils of 6 climatic regions of Australia, demonstrated that the populations of those fungi exceeded 200 cfu g⁻¹ of soil.

Among the nests studied, the exception was the nests of Mute Swan in which the number of thermophilic

mycobiota was significantly greater in relation to the number of those fungi in the nests of the remaining bird species. The average values were 9,160 cfu g⁻¹ d.m. (Martin medium) and 33,000 cfu g⁻¹ d.m. (Sabouraud medium). In that respect, the nests of Mute Swan were close to composts of plant material enriched with feather waste [39]. It can be assumed that nests of Mute Swan are a kind of natural “compost pile”, as they are concentrations of very large amounts of decomposing parts of plants, droppings, feathers and other residues of animal origin, which has been described in the work by Kornilowicz-Kowalska et al. [25]. Earlier, Sharma [40] took note of the phenomenon of composting in bird nests.

It should be assumed that the factors inducing the growth of thermophiles are not only the warming of nests by the birds (during breeding or when used for other purposes) and by the sun [36], but also the microbiological processes taking place during the composting of the nest material, primary among which is the biodegradation of carbohydrates. Model studies [39] demonstrated that in the course of composting of lignin-cellulose wastes (cereal straw, pine bark) and chicken feathers moistened to 70 %, there took place spontaneous heating of the biomass decomposed by microorganisms to the temperature of 41 °C. That effect was coupled with intensive degradation of carbohydrates, cellulose in particular and proteins. The composting of plant biomass containing more simple organic compounds causes a stronger increase in temperature [41]. Therefore, the greater the mass of a nest, at sufficient moisture levels, the more intensive the process of composting with participation of the thermophilic phase, and the other way round. In nests containing small amounts of organic materials (e.g. nests of Black-headed Gull), the process of composting could not take place. As a consequence, those nests were characterised by considerably smaller populations of thermophilic fungi compared with large nests (Mute Swan).

From the 38 nests of 9 bird species under study, a total of 19 species of thermophilic fungi were isolated. Greater diversity of the thermophilic mycobiota (27 species) in bird nests was observed by Apinis and Pugh [11]. Those authors, however, analysed nests of land birds, representing *Passeriformes* (54 nests, 12 bird species). Those are usually nests with low moisture levels, conducive to the growth of fungal xerophiles representing mainly numerous species of *Penicillium*

and *Aspergillus* and their teleomorphs, as well as dark-pigmenting fungi: *Cladosporium*, *Humicola* or *Torula*.

It was found that within the thermophilic mycobiota, the colonisers of the nests studied were mainly species classified among the thermotolerant fungi, and in particular *A. fumigatus*, *A. flavus*, *A. terreus*, *C. tropicum*, *Ch. keratinophilum*, *Paecilomyces variotii*, *P. purpurogenum* and *A. corymbifera* [32, 37]. The obligatory thermophiles included *Chaetomium thermophile*, *Coryneascus sepedonium*, *T. aurantiacus*, *T. lanuginosus*, *T. emersonii* and *R. pusillus* [32, 37]. Most of the fungal species enumerated were also isolated from nests of various species of *Passeriformes* [11], from sun-heated soils [42], and from composts of straw of cereals and legumes [43]. Species specific for the studied nests of wetland birds (due to their high moisture level) included *Ch. keratinophilum*, *Aphanoascus fulvescens* and *P. purpurogenum* classified among the hydrophilic fungi [23, 25]. The occurrence of those fungal species in nests of wetland and raptor birds was reported earlier by Hubalek [13].

The greatest richness of fungal genera and species was found in nests of Mute Swan and Coot, and the smallest in those of the gulls and terns. It appears — apart from differences in the substrate composition of the nest material, expressed in its greater diversity (Mute Swan)—that effect was a result of the mycological quality of the biotopes. Mute Swan and Coot most often inhabited and found their food (plants and small invertebrates) in the littoral zone of eutrophic ponds contaminated with fungi of land origin, whereas gulls and terns more frequently tend to feed on the open water zone of lakes and ponds, characterised with higher microbiological purity [44], and their food is composed primarily of small fish, caught in flight and other small water vertebrates.

In this study, it has been demonstrated that the size of birds is a factor that strongly affects the species diversity of thermophilic fungi in their nests. In the nests of large birds (Mute Swan) and medium-sized birds (Grey Heron, Marsh Harrier, Coot, Great Crested Grebe), that diversity was significantly higher than in the nests of smaller birds (gulls and terns), which remains in close relation with the size of the zone warmed by the birds.

Moreover, the species diversity of the thermophilic mycobiota was positively correlated with the level of phosphorus in the nest material. The significant

correlation between the level of phosphorus in the environment (soil) and the abundance and frequency of fungi decomposing organic material has been reported in another paper by these authors [45]. Phosphorus is a well-known factor stimulating catabolic transformations and processes of cellular synthesis, and consequently the growth and development of fungi [46].

The results presented here indicate a selection of populations of *Aspergillus fumigatus* within the thermophilic mycobiota of the nests of birds of wetland habitats. That species colonised 36 out of the 38 nests under study, which constituted 94.7 % degree of colonisation. Noteworthy is the fact that, with the exception of the nests of Mute Swan, the population of *A. fumigatus* represented over ≈ 50 % of the total populations of thermophilic fungi (growing at 45 °C in vitro). The mean numbers of *A. fumigatus* were 650 cfu g⁻¹ d.m. and varied from 300 cfu g⁻¹ d.m. in the nests of Mute Swan to 1,400 cfu g⁻¹ d.m. in those of Common Tern. These are numbers close to those observed in composts. Fischer et al. [7] found that in the composting of kitchen and garden wastes, the populations of *A. fumigatus* were from 10² to 10³ cfu g⁻¹ d.m.

It was demonstrated that the growth of *A. fumigatus* intensified in nests with poor sanitation (Marsh Harrier, Grey Heron), contaminated with residues of animal matter (feathers, remnants of food, pellets and droppings). That effect is related to *A. fumigatus* preference for animal substrates [13], whereas the high frequency of the fungus in nests of smaller birds (gulls and terns), characterised by lower level of contamination with animal matter residues, was probably a result of more uniform warming during breeding. The substrate for *A. fumigatus* in those nests was primarily plant material, also successfully utilised by that polyphage [7].

Apart from temperature, the growth of *A. fumigatus* is also stimulated by high moisture of nests. In terms of requirements concerning the moisture conditions, the species is classified among so-called tertiary colonisers. Those are fungi that have high indices of water activity: 0.9–0.95 [47]. As follows from our earlier studies [23, 25], high level of moisture of the nest material stimulates also the growth of other hydrophilic species, for example, *Trichoderma viride*, *Ch. keratinophilum* and *P. purpurogenum* in bird nests. These observations are in conformance with the

results of studies by Hubalek [27] who demonstrated that although fungi prefer bird nests with lower moisture levels (in the range from 5 to 20 %), they are also fairly frequently isolated from nests with higher moisture of ≥ 50 %. That author [26], on the basis of a study involving 84 nests of the European Tree Sparrow and 193 nests of other bird species appearing in the territory of the Czech Republic, demonstrated a relation between the pH of the nest material and the occurrence of fungi in the nests. He distinguished acidophilic and acidotolerant fungi, and alkalophilic and alkalotolerant fungi. Moreover, Hubalek [26] noted that numerous species of fungi appeared within a broad range of pH values of the nests. In this study, no correlation was detected between the frequency of *A. fumigatus* and pH of the nest material. However, the nests that we studied were characterised by relatively weakly diversified reaction.

The proliferation and high frequency of *A. fumigatus* in the nests of birds of aquatic and wetland habitats permits to believe, with a high level of probability, that in the vicinity of nests, there appear bioaerosols of that zoopathogen. During its saprophytic growth, *A. fumigatus* produces a great abundance of conidia that easily penetrate to the atmosphere. This suggestion is supported by studies by Fischer et al. [7] who observed air contamination with that fungus at a composting site, detectable even at a distance of 5 m from the compost surface. The emission of spores of *A. fumigatus* from environments characterised by its abundant growth (composts, waste dumps, poultry farms) has also been reported by Lugauskas et al. [48] and Krikštaponis et al. [49]. The aerosols of *A. fumigatus* that form in nests may constitute the greatest hazard to such altricial species as Marsh Harrier, or to precocial species which often use their nests also after the breeding period, for example, as roosting sites (Mute Swan) and to smaller bird species (gulls, terns), due to the very high accumulation of the pathogen in the nests. Since *A. fumigatus* is a fungal species that grows and produces spores within a broad range of temperatures, which was the basis for its classification among the so-called psychrotolerant thermophiles [11], it is to be assumed that the growth of the fungus does not stop after the birds have abandoned the nests. Therefore, abandoned nests constitute a specific reservoir of that opportunistic pathogen and a source of its

transmission, and thus a significant link in the epidemiological chain of aspergillosis.

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