Filamentous Fungi Transported by Birds During Migration Across the Mediterranean Sea

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Abstract The potential for the transport and diffusion of some pathogenic microorganisms by migratory birds is of concern. Migratory birds may be involved in the dispersal of microorganisms and may play a role of mechanical and biological vectors. The efficiency of dispersal of pathogenic microorganisms depends on a wide range of biotic and abiotic factors that influence the survival or disappearance of a given agent in a geographical area. In the present study, 349 migratory birds were captured in four sites (Mazara del Vallo, Lampedusa, Ustica and Linosa), representing the main stop-over points during spring and autumnal migration, and analyzed for the presence of filamentous fungi. A total of 2,337 filamentous fungi were isolated from 216 birds and identified by a combined phenotypic-genotypic approach to species level. Twelve species were identified in the study, with *Cladosporium cladosporioides*, *Alternaria alternata*, and *Aspergillus niger* as the most abundant. The transport of these fungal species isolated in this study is of considerable importance because some of these species can create dangers to human health.

Introduction

The migration of birds depends on the seasonality of food resources; it takes place in autumn and spring with a round trip to the resting areas and a return to the territories of nesting, respectively [18, 19]. The islands of the Mediterranean Sea represent important resting sites (stop-over), where birds increase the fat lost during their flights between North Africa and Europe and vice versa [10]. The monitoring of bird movements is important not only to investigate on the demography of the different species [3], but also to evaluate the microbial populations transported by birds [7].

The recent studies carried out on the microbial populations transported by birds have been mainly related to safety aspects [24, 27], since birds contribute to disseminate various microorganisms in the environment [22]. Birds may play a defining role in long distance movement of microorganisms; for example, in the case of yeasts, it has been shown that insects may transport cells within approximately 10 km [9], while migratory birds are effective at longer distances [7]. So far, a few studies analyzed filamentous fungi associated with migratory birds [4, 14], while bacteria and yeasts have been better investigated [4, 6, 7]. Thus, on the above considerations, the main objective of the present study was to analyze the filamentous fungi carried by birds at the moment of landing at their stop-over during spring (at Lampedusa and Ustica) and winter (at Linosa and Mazara del Vallo) migration in the Mediterranean.

Materials and Methods

Sampling Sites and Analysis of Birds

Migrating birds were monitored during their movements within the "Progetto Piccole Isole" of the Sicily region (southern Italy). Birds to be ringed were caught in four experimental sites located within Sicily region: (1) Lampedusa island (35°51′N 12°58′E; 25th–27th April 2009); (2) Ustica island (38°7′N 13°18′E; 2nd–4th May 2009); (3) Linosa island (35°86′N 12°86′E; 27th–29th

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September 2009); (4) Riserva Naturale Integrale Lago Preola and Gorghi Tondi (Mazara del Vallo, TP; 37°39′N 12°35′E; 4th–6th September 2009). Sites 1–3 are small islands surrounding Sicily (Fig. 1). All sites represent important stop-over points for migrating birds [25]. The birds were captured and identified as previously reported by Francesca et al. [7]. The analysis included the measurement of subcutaneous fat amount (SFA) of the abdominal region [15] that was evaluated by visual biometric measurement. The main energy source during flight is the body fat. The birds with low fat reserves (0 or 1) have the necessity of stopping in some resting sites (stop-over) along the route. Birds were released in the same areas of capturing soon after analysis.

Isolation and Identification of Filamentous Fungi

In order to evaluate the contribution of birds to filamentous fungi dissemination through feces, only cloacae of birds were analyzed [4, 7]. Cloacae were plugged with sterile cotton swabs and streaked onto malt extract agar (MEA) (Oxoid, Milan, Italy) supplemented with streptomycin sulfate (0.05 g/l) (Sigma-Aldrich, Milan, Italy). Petri dishes were incubated at ambient temperature during transport and, once in laboratory, at 25 ± 1 °C for 3 weeks; Petri dishes were observed at 24-h intervals and each fungal colony (Ø about 2 mm) was immediately transferred to a new Petri dish containing the same agar substrate. All samples were analyzed in duplicate.

All fungal colonies were picked up from agar plates and purified to homogeneity after several sub-culturing steps onto MEA. The isolates were first differentiated by morphological characterization including color, texture, diffusible pigments, exudates, growth zones, aerial, and submerged hyphae, growth rate, and topography. All colonies were subjected to microscopic analysis [2, 5] through a light microscope (Carl Zeiss Ltd). Subsequently, up to 10 isolates or less for the less numerous morphological groups were subjected to genetic analysis. Genomic DNA was extracted from single-spore cultures following a standard cetyl-trimethyl-ammonium-bromide-based protocol [23]. Filamentous fungi were analyzed by restriction fragment length polymorphism of the region spanning the internal transcribed spacers (ITS1 and ITS2) and the 5.8S rRNA gene. The DNA fragments were amplified with the primer pair ITS1F [8] and ITS4 [29] by means of T1 Thermocycler (Biometra, Göttingen, Germany). The amplicons were then digested with the endonucleases *Cfo*I and *Hae*III (MBI Fermentas, St. Leon-Rot, Germany) at 37 °C for 8 h. ITS products, as well as restriction fragments were analyzed on agarose gels using 2 % (w/v) agarose in $1 \times$ TBE (89 mM Tris–borate, 2 mM EDTA pH 8) buffer. Gels were stained with SYBR safe DNA gel stain (Invitrogen, Milan, Italy), visualized by UV transillumination and recorded with Gel Doc 1000 Video Gel Documentation System (BioRad, Richmond, USA). GeneRuler 100 bp (Fermentas, Milan, Italy)—standard DNA ladders were used as molecular markers. One isolate per group was processed by sequencing of the 5.8S-ITS rRNA region to confirm the morphological identification. PCR product of 5.8S-ITS rRNA region was amplified and visualized as above. DNA cycle sequencing reactions were performed at Primm-Biotech (Milan, Italy). The identities of the sequences were determined by BlastN [1] search against the NCBI non-redundant sequence database located at http://www.ncbi.nlm.nih.gov.

Statistical Analysis

Data obtained by evaluation of bird SFA were subjected to statistical analysis by Chi-square test. All the analyses were conducted in PSPP v.0.7.8 (Copyright (C) 2007 Free Software Foundation, Inc.).

Fig. 1 Sites of bird ringing
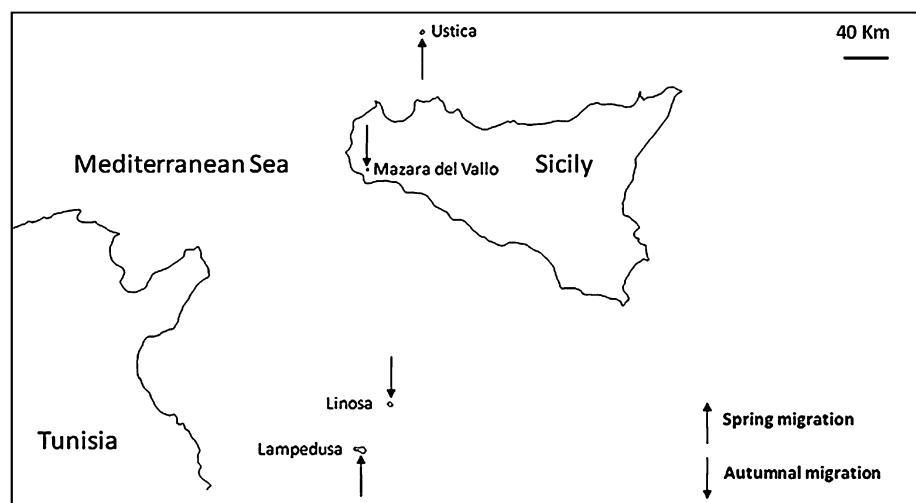
Results

Isolation of Filamentous Fungi

The identification of the 349 birds captured was previously showed by Francesca et al. [7]. Two hundred and sixteen individuals, representing 61.89 % of the birds analyzed, were positive for filamentous fungi isolation (Table 1). The bird families showing the highest frequencies of filamentous fungi were Sylviidae (145 individuals), Muscicapidae (37), and Turdidae (25). A total of 2,337 filamentous fungal isolates were collected from bird cloacae and stored in glycerol stocks at −20 °C until further determination.

Grouping and Identification of Fungi

After microscopic inspection and morphological differentiation, the isolates were divided into 12 groups (Table 2).

Table 1 Bird captured in four Sicilian ringing sites

Bird family	Bird species	No. of birds	No. of birds carrying fungi	Sampling sites (number of birds)
Fringillidae	*Carduelis cannabina*, Linnaeus 1758 (linnet)	2	0	
Hirundinidae	*Hirundo rustica*, Linnaeus 1758 (swallow)	12	2	Ln (1); U (1)
Laniidae	*Lanius senator*, Linnaeus 1758 (woodchat shrike)	2	1	L (1)
Motacillidae	*Anthus trivialis*, Linnaeus 1758 (tree pipit)	2	2	L (1); U (1)
Muscicapidae	*Ficedula albicollis*, Temminck 1815 (collared flycatcher)	1	1	U (1)
	F. hypoleuca, Pallas 1764 (pied flycatcher)	6	3	U (3)
	Muscicapa striata, Pallas 1764 (spotted flycatcher)	51	33	L (2); U (31)
Oriolidae	*Oriolus oriolus*, Linnaeus 1758 (golden oriole)	3	0	
Paridae	*Parus major*, Linnaeus 1758 (great tit)	2	2	MdV (2)
Passeridae	*Passer hispaniolensis*, Temminck 1820 (Spanish sparrow)	8	2	MdV (2)
	P. montanus, Linnaeus 1758 (tree sparrow)	1	0	
Sylviidae	*Acrocephalus arundinaceus*, Linnaeus 1758 (great-reed warbler)	3	0	
	A. schoenobaenus, Linnaeus 1758 (sedge warbler)	6	3	L (1); U (2)
	Acrocephalus scirpaceus, Hermann 1804 (reed warbler)	12	6	MdV (4); U (2)
	Cettia cetti, Temminck 1820 (cetti's warbler)	2	2	MdV (2)
	Hippolais icterina, Vieillot 1817 (icterine warbler)	24	15	L (4); U (11)
	H. polyglotta, Vieillot 1817 (melodious warbler)	1	1	U (1)
	Phylloscopus collybita, Vieillot 1817 (chiffchaff)	1	1	L (1)
	P. sibilatrix, Bechstein 1793 (wood warbler)	5	4	U (4)
	P. trochilus, Linnaeus 1758 (willow warbler)	7	3	L (2); Ln (1)
	Sylvia atricapilla, Linnaeus 1758 (blackcap)	2	0	
	S. borin, Boddaert 1783 (garden warbler)	90	64	L (1); Ln (5); MdV (9); U (49)
	S. cantillans, Pallas 1764 (subalpine warbler)	40	32	Ln (21); MdV (11)
	S. communis, Latham 1787 (whitethroat)	8	5	L (5)
	S. curruca, Linnaeus 1758 (lesser whitethroat)	1	1	MdV (1)
	S. melanocephala, Gmelin 1789 (sardinian warbler)	15	8	L (1); MdV (7)
Turdidae	*Erithacus rubecula*, Linnaeus 1758 (robin)	1	1	Ln (1)
	Luscinia megarhynchos, Brehm 1831 (nightingale)	4	3	L (1); MdV (1); U (1)
	Monticola solitaries, Linnaeus 1758 (blue rock-thrush)	1	1	Ln (1)
	Phoenicurus phoenicurus, Linnaeus 1758 (redstart)	12	7	L (3); Ln (4)
	Saxicola rubetra, Linnaeus 1758 (winchat)	23	13	L (9); U (4)
	Turdus philomelos, Brehm 1831 (song thrush)	1	0	
Total		349	216 (61.89 %)	L (33); Ln (33); MdV (39); U (111)

L Lampedusa, *Ln* Linosa, *MdV* Mazara del Vallo, *U* Ustica

The results of restriction analysis of 5.8S-ITS region confirmed the subdivision into 12 groups. One strain for each group was identified at species level as follows: *Alternaria alternata* (Fr.) Keissl 1912 (strain Uf142-1), *Aspergillus tubingensis* Mosseray 1934 (strain Uf125-1), *Aspergillus niger* Tiegh 1867 (strain Uf221-1), *Aspergillus ochraceus* K. Wilh 1877 (strain Uf76-1), *Botrytis cinerea* Pers 1794 (strain Uf144-1), *Cladosporium cladosporioides* (Fresen.) G.A. de Vries 1952 (strain Uf247-2), *Fusarium oxysporum* Schlecht Emend Snyder AND Hansen 1940 (strain Uf96-1), *Fusarium lateritium* Nees 1817 (strain Uf94-2), *Fusarium acuminatum* Ellis and Everh 1895 (strain Uf130-1), *Penicillium chrysogenum* Thom 1910 (strain Uf147-1), *Talaromyces verruculosus* Peyronel (strain Uf225-3), and *Rhizopus arrhizus* var. *arrhizus* A. Fisch (strain Uf122-1).

Fungal Frequency and Distribution

Table 2 also shows the distribution of the 12 fungal species among birds. The fungal species most frequently isolated from migratory birds were *C. cladosporioides* (70.90 %), *A. alternata* (13.74 %), *A. niger* (5.39 %), and *F. oxysporum* (4.02 %). The highest number of isolates and species were found in the most numerous bird groups. Thus, garden warblers and spotted flycatchers were the richest sources of fungal diversity; on the contrary, golden orioles showed the less diversity in terms of filamentous fungi, since only one isolate was collected from this bird species. All bird species hosting a consistent number of filamentous fungi were characterized by a "trans-saharan" migration. Although 57.34 % of filamentous fungi were isolated from birds with a SFA value comprised between zero and one (Table 3), no significant statistical differences were found among birds with 0–1 and 2–7 SFA.

Discussion

The main aim of the present study was to evaluate the environmental dissemination of filamentous fungi by migrating birds. For this purpose, the cloacae of 349 birds captured soon after landing in four strategic stop-over points during spring and autumnal migration, located in the Mediterranean Sea, were examined. The isolation and identification of these microbial agents gained interest in recent years for safety issues related with birds covering long distances. Hubalek et al. [14] reported a general check-list of human pathogenic filamentous fungi carried by birds, thus, the analysis of filamentous fungi carried by birds and released in small islands like those object of this study is of interest. The isolation of microbial agents from cloacae is particularly important, since it represents the last tract reached by microorganisms after gastric transit.

By means of morphological differentiation 12 fungal groups were collected from cloacae and, for this reason, the primary source of fungal contamination has to be imputable to the food ingested.

By restriction analysis of 5.8S-ITS rRNA region and its sequencing all selected isolates were differentiated into 12 groups in accordance to morphological analysis. On the other hand, for some species such as *A. tubingensis*, *P. chrysogenum*, and *B. cinerea*, the microscopic inspection was essential in the recognition of species.

Nowadays, the morphological clustering of the isolates based on dichotomous key analysis [5] associated to the restriction analysis of 5.8S-ITS rRNA region and its sequencing is a reliable technique for the analysis of filamentous fungi till species level [13] showing that a polyphasic procedure is the correct approach to obtain the proper identification of filamentous fungi.

Our results showed that 61.89 % of birds analyzed were positive for the presence of filamentous fungi, showing a higher percentage than that (10.93 %) reported by Cafarchia et al. [4] who analyzed a similar number of birds (421). These results are probably due to different species of birds captured as well as the sampling sites. To our knowledge, this research represents the first report on the isolation and molecular identification of filamentous fungi carried by living migratory birds at long distances. From this perspective, this is also the first report on filamentous fungi transported by birds in the main migratory stop-over points in the Sicilian area. Since birds were analyzed soon after landing, the contamination of bird cloacae with local filamentous fungi has to be excluded. In a previous study, Francesca et al. [7], by means of persistence tests performed on robins, demonstrated that yeast maybe disseminated approximately 12 h after ingestion. A similar timing maybe supposed for the other filamentous fungi. This interval is enough to cover a distance within 300–350 km [18, 19] and it is compatible with a no-stop flight commonly performed by birds with low SFA (comprised between 0 and 1) [10]. The body fat is the first energy source dissipated by birds during migration and when SFA reaches a value of 0 or 1, a stop is necessary [10]. Filamentous fungi isolated in the present study are ubiquitous in several areas and may cause different problems. In particular conditions, only some strains of the fungal species identified play positive roles: *B. cinerea* can positively affect the grape and wine quality (noble rot) [26]; some strains of *A. alternata* are antagonist of *Plasmopara viticola* responsible for grapevine diseases [21]; *Rhizopus arrhizus* var. *arrhizus* is used in the production of alcoholic beverages in Asia and Africa, since some strains of this species are able to degrade amylase compound of food promoting the alcoholic fermentation [28]. The species with the highest frequency of isolation (*C. cladosporioides*,

Table 2 Filamentous fungi carried by migratory birds

Birds species (no. of birds)		Species filamentous fungi (% similarity; GenBank ID)												Total no.
		Alternaria alternata (99; JQ693408)	*Aspergillus tubingensis* (99; JQ693399)	*A. niger* (99; JQ693403)	*A. ochraceus* (99; JQ693406)	*Botrytis cinerea* (99; JQ693407)	*Cladosporium cladosporioides* (99; JQ693401)	*Fusarium oxysporum* (99; JQ693405)	*F. lateritium* (99; JQ693397)	*F. acuminatum* (99; JQ693398)	*Penicillium chrysogenum* (99; JQ693404)	*Talaromyces verruculosus* (99; JQ693402)	*Rhizopus arrhizus* var. *arrhizus* (99; JQ693400)	
Blackcap (2)	•[a]	2					16	1						17
Blue rock-thrush (1)	■	4					1	1				1	1	6
Cetti's warbler (2)	•	4					37	8			4	4		57
Chiffchaff (1)	•	9					15	1						20
Collared flycatcher (1)	•													9
Garden warbler (90)	•	72		33	1	10	581	18	2		18	16	3	754
Great tit (2)	■	4		4	1		6	6			11	2		30
Icterine warbler (24)	•	21		2			150							173
Lesser whitethroat (1)	•	7		8			14							29
Melodious warbler (1)	•						6							6
Nightingale (4)	•	3		1		2	4	1						10
Pied flycatcher (6)	•	4		1			21							27
Redstart (12)	•	4					8	1			8	4		25
Reed warbler (12)	•	7	3		2	1	86	12		1				112
Robin (1)	•			1			5							6
Sedge warbler (6)	•	13					102	1						116
Spanish sparrow (8)	■			7		2	7	1				3		20
Spotted flycatcher (51)	•	73		4			227	19	2		8	8		341
Winchat (23)	•	27	1	18	1		111	3			3	1		165
Subalpine warbler (40)	•	36		25	5		141	17				1	2	227
Swallow (12)	•	1		17			4	3						25
Tree pipit (2)	•	23	1				77	1						101
Whitethroat (8)	•	2		1	1		2					1		10
Willow warbler (7)	•	1		2			23					1	2	27
Wood warbler (5)	•	7		1			13				1			21
Woodchat shrike (2)	•	1		1										3
Total no. of isolates		321	5	126	11	15	1657	94	4	1	53	42	8	2337

[a] Symbols indicate the migration strategy: • trans-saharan migratory bird, ■ partial migratory bird

Table 3 Filamentous fungi isolated from birds grouped on the basis of subcutaneous fat amount

Species	SFA	
	0–1	2–7
Total isolates ($n = 2,337$)	1,340 (57.34 %)	997 (42.66 %)
Alternaria alternata ($n = 321$)	171	150
Aspergillus niger ($n = 126$)	77	46
Aspergillus ochraceus ($n = 11$)	7	4
Aspergillus tubingensis ($n = 5$)	3	2
Botrytis cinerea ($n = 15$)	9	6
Cladosporium cladosporioides ($n = 1,657$)	955	702
Fusarium acuminatum ($n = 1$)	0	1
Fusarium lateritium ($n = 4$)	3	1
Fusarium oxysporum ($n = 94$)	63	31
Penicillium chrysogenum ($n = 53$)	29	24
Talaromyces verruculosus ($n = 42$)	23	19
Rhizopus arrhizuz var. *arrhizus* ($n = 8$)	6	2

SFA subcutaneous fat amount

A. alternata as well as members of the genera *Aspergillus*, *Penicillium*, and *Fusarium*) are generally defined as plant pathogens and soil-borne but also allergens for humans and animals [12, 16, 17, 20]. Moreover, most of these species found are well known producers of mycotoxins, common contaminants of foods and feeds [11].

In contrast to other studies [24, 27] that showed birds exclusively as vectors of human or animal fungal (yeasts and filamentous fungi) pathogens, most of our isolates represent plant pathogens and/or food contaminants. On the other hand, in accordance to data reported by Tsiodras et al. [27], birds sampled in the present study were found to be carriers of different species of yeast (such as *Cryptococcus* spp.) as reported in the first part of this study published by Francesca et al. [7].

Migratory birds play an active role in the dissemination of filamentous fungi during their movements from North Africa to Europe and viceversa. The species identified in this study are important because they may represent safety issues for human health. Except for a few species, most of the filamentous fungi identified are responsible for human diseases such as allergies and foodborne intoxication due to their secondary metabolites (mycotoxins) [11, 12, 20].

These findings are important for the small islands (Lampedusa, Ustica and Linosa) that are not characterized by massive exchanges of people and commodities, except in summer season when tourist activities become relevant or in case of illegal immigration, which may contribute to transport microorganisms; in this context, migratory birds represent the main vectors of fungal biodiversity.

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