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Full Length Research Paper

Mycoflora identified from loggerhead turtle (*Caretta caretta*) egg shells and nest sand at Fethiye beach, Turkey

Özgür Güçlü*, Halil Bıyık and Aslı Şahiner

[1]Adnan Menderes University, Faculty of Science and Arts, Department of Biology, 09010 Aydın, Turkey.

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The aim of this study was to investigate the mycoflora of the loggerhead turtle, *Caretta caretta*, from its nests and eggshells at Fethiye Beach, Turkey. During the 2004 breeding season, following the complete emergence of the hatchlings, sand samples were collected from 15 nests and eggs from these nests were swabbed. Rose Bengal chloramphenicol agar was used for isolation, and then mycoflora were subcultured onto suitable media. Fungi were counted and identified at genus level. Ten genera were identified within the nests and eggshells. *Aspergillus* sp., *Chrysosporium* sp., *Fusarium* sp. and *Penicillum* sp. were identified both in nests and eggshells. Furthermore, while *Absidia* sp., *Cylindrocarpon* sp., *Emericella* sp. and *Mucor* sp. were only identified in the nests, *Cladosporium* sp. *and Thielavia* sp. were only identified in eggshells. In addition, there was a positive correlation between total number of isolated fungus and embryonic death in nests ($r = 0.53$, $p < 0.05$). Hatching success was negatively correlated with total number of isolated fungus ($r = -0.54$, $p < 0.05$). Our study indicates a relation between hatching success and fungal flora of the nests. Presence of fungi may also be important for the researchers who digs nest to avoid infections or allergies due to these fungi.

Key words: Mycoflora, fungus, *Caretta caretta*, Fethiye beach.

INTRODUCTION

Unhatched eggs in sea turtle nests may occur due to anomalies in the eggs' development caused by environmental influences such as the nest's distance from the sea, the vegetation, nest temperature or humidity, the amount of rain, the ebb and flow of the tide during the eggs' developmental period, and predation by various microorganisms or invertebrates (Wyneken et al., 1988; Baran et al., 2001; Phillot et al., 2001). Limpus et al. (1983) suggest fungal presence within sea turtle nests may contribute to egg failure. Nevertheless, excavation of emerged nests at various sea turtle rookeries often results in the detection of fungal growth on the exterior shell and in the contents of unhatched eggs (Phillot et al., 2001). Microorganisms have been described from the exterior and/or embryonic tissue of eggs of several species of sea turtle. Wyneken (1988) determined that any transfer of bacteria from the adult individual to the egg decreases the rate of hatching success. Mo et al. (1990) found fungus and bacteria in the sand of the nest, egg, and cloaca of adult individuals of *Lepidochelys olivacea*. Phillott (2001) isolated the soil mycobiota *Fusarium solani* and *Pseudallescheria boydii* from the external surface of unfertilized eggs at natural and artificial green and loggerhead turtle nests. Phillott and Parmenter (2001) found that the random deaths of eggs in the nests of *Caretta caretta* and *Chelonia mydas* create the fundamental source of nutrients for *F. solani* and *P. boydii* and these fungi progressively move to the neighboring eggs. Phillott et al. (2004) detected mycobiota in the nests of *C. mydas*, *C. caretta*, *Eretmochelys imbricata* and *Natator depressus* all along coastal localities in eastern Australia. Phillot (2004) found that *F. oxysphorum*, *F. solani* and *P. boydii* that are detected in turtle nests use numerous enzymes to attack the embryonic tissue. On the other hand, Phillot (2002) determined the necessary conditions to minimize fungal attack on artificially incubated sea

*Corresponding author. E-mail: ozgguclu@yahoo.com, #oguclu@adu.edu.tr. Tel: 0090-256-2128498 / 2220. Fax: 0090-256-2135379.

turtle eggs used for laboratory studies. In addition, Baran et al. (2001) found invertebrate species infesting nests of loggerhead turtles on Fethiye Beach. Nevertheless, it was not determined whether these species are sea turtle nest predators, or saprophytes.

There have been no detailed studies to determine the identity of fungi from failed turtle eggs and nest sand of marine turtle rookeries along any Mediterranean coastline. Only Peters et al. (1994) reported *Mucor sp.* from a loggerhead turtle nest at Göksu Delta, Turkey.

In this study, we investigated of mycoflora identified from Loggerhead (*C. caretta*) sea turtle egg shells and nest sand at Yanıklar, Fethiye, Turkey, in the 2004 hatchling season.

MATERIALS AND METHODS

Collecting of samples

During the 2004 hatchling season, sand samples were collected from 15 randomly chosen nests, and were put into sterile bags aseptically. Eggshells from these nests were swabbed using a sterile cotton stick. Physiological saline solution (0.85%) was used as a diluent. Samples were refrigerated at +4°C until the analysis at laboratory.

Isolation and counting

Certain amounts of sand samples were diluted by using sterile saline solution (0.85 %). Eggshell samples were weighted and transferred into sterile saline solution. Serial dilutions of each samples was done and1 ml solution of each sample was inoculated on Rose Bengal Chloramphenicol Agar and incubated at 25°C for 5 days. The plate with 30-300 colonies was chosen to calculate colony forming units in 1 ml. After incubation, growing fungi colonies were counted and then stock cultured onto slant Malt Extract Agar media until identification. Czapek Dox Agar (*Aspergillus*, *Emericella*) and Malt Extract Agar (*Aspergillus*) were also used for identification.

Identification

Mycoflora were grown on potato dextrose agar for identification. A few spores or a tuft of mycelium was picked off by needle and used to inoculate a single point on the plate, and incubated at 25°C during 5 days. Cultural and microscopic characteristics of fungi were determined and the genera were identified on the basis of morphological characters (Domsch et al., 1980; Barnett and Hunter, 1987; Watanabe, 2002). The determination of the morphological structures was carried out on material in a modified mounting medium, Lacto-Cotton Blue as proposed by Sime and Abbott (2002). Slides of the mould growth for microscopic examination were prepared in the following way: A portion of the growth was picked off with a needle and teased out in a drop of lactophenol-cotton blue placed on a microscope slide. It was covered with a clean cover slide, with care to exclude air bubbles. The prepared slide was examined under the light microscope using the x40 and x100 objectives for vegetative mycelium; presence or absence of cross-walls, and diameter of hyphae, and the asexual and sexual reproductive structures: sporangia, conidial heads, zygospores, arthrospores, ascospores etc.

Fungal colonies were examined under the ×10 (low power) objective of the microscope. The colonial characteristics of size, surface, appearance, texture and colour of the colony were recorded.

Statistical analyses

For statistical analysis, Statistica 6.0 (Statsoft Inc, 2001) was used. Correlation test was performed to determine the relationship among counts of total fungus, death embryo and hatching success.

RESULTS AND DISCUSSION

Fungal densities of nests were determined from the counted colonies of the nest sand. Number of total isolated fungus and hatching success were calculated (Table 1). Phillott and Parmenter (2001) found that fungal attack takes place especially on the upper side and the edges of the nests. The eggs contaminated by fungi were usually found in the parts of the nests in touch with the sand. Egg shells with colorations due to fungal pigment formations were chosen and swab examples were taken. In eggs where fungal colonization is intense, thinning and a loss of rigidity of the shell were seen due to enzymatic activity and the acidic products created by metabolic activity. A total of ten fungal genera were identified (Table 2).

All fungal cultures were examined for their colonial and morphological properties. The fungi were also analysed for their microscopic appearance by light microscope. The *Absidia*, *Aspergillus*, *Chrysosporium*, *Cladosporium*, *Cylindrocarpon*, *Emericella*, *Fusarium*, *Mucor*, *Penicillium* and *Thielavia* species were identified as described below.

Absidia sp: Potato dextrose agar was used for isolation of these fungi. Colonies were floccose, white-pale grey; with a diameter of approximately 6 cm. Sporangiophores were hyaline. *Aspergillus* sp: Czapek Dox Agar and Malt Extract Agar were used for identification. The bases of the colonies were white or yellow. Conidial heads were large, globose, dark brown, radiate. Conidiophores were smooth-walled, hyaline or pale brown (Figure 1). *Chrysosporium* sp: Potato dextrose agar was used for identification. Colonies were grown rapidly and they were woolly with white cream or tan colour. The reverse was white to brown. Conidiophores were very short or lacking. Conidia were aleuriosporous, ellipsoidal, apiculate at one end (Figure 2). *Cladosporium* sp: Potato dextrose agar was used for identification. Colonies were slow growing at 25°C and the texture was velvety to powdery, olivaceous green from the front and black from the reverse. Conidia were irregular in shape, ovate and cylindrical. *Cylindrocarpon* sp: Potato dextrose agar was used for identification. Colonies were yellowish brown. Conidiophores were hyaline. There were two kinds of conidia. Macroconidia were cylindrical, 4 - 5 septate. Microconidia were smaller, ellipsoidal, apiculate at one end. *Emericella* sp: Czapek Dox agar was used for identification. Colonies were dark green with orange to

Table 1. Fungi counting from sand samples of nests along with number of total isolate and hatching success in nests.

Nest no	Counting results (cfu/g)	Number of total isolate	Hatching success (%)
1	3.35×10^3	197	16.1
2	2.30×10^3	81	89.5
3	1.11×10^3	31	60.0
4	3.11×10^3	72	84.0
8	3.05×10^2	6	36.1
9	7.87×10^2	12	72.1
Y1	6.34×10^2	138	55.6
Y2	9.89×10^2	86	66.3
Y3	2.98×10^3	170	47.7
Y4	3.29×10^3	90	61.4
Y7	1.53×10^3	144	53.7
Y8	4.11×10^1	3	77.5
A1	2.75×10^2	30	89.2
A2	1.73×10^3	40	83.8
A3	1.54×10^1	3	66.7

Table 2. Percentage of isolated fungi genera.

Fungus	Number of isolate in sand	(%)	Number of isolate in eggshell	(%)	Number of total isolate	(%)
Absidia	14	3.48	-	-	14	1.27
Aspergillus	11	2.74	61	8.71	72	6.53
Chrysosporium	159	39.55	199	28.43	358	32.48
Cladosporium	-	-	8	1.14	8	0.72
Cylindrocarpon	5	1.24	-	-	5	0.45
Emericella	8	1.99	-	-	8	0.72
Fusarium	174	43.28	376	53.71	550	49.9
Mucor	12	2.99	-	-	12	1.08
Penicillium	17	4.23	40	5.71	57	5.17
Thielavia	-	-	11	1.57	11	0.99
Unidentified	2	0.50	5	0.71	7	0.63
Total	402	100.00	700	100.00	1102	100

yellow at 25°C. Reverses were purplish to olive. Exudate was usually present and brown. Conidial heads were columnar. Conidiophores were brown and smooth-walled. Vesicles were hemispherical. Conidia were globose and rough. Ascospores were reddish brown; Hulle cells were very abundant, globose to sub-globose (Figure 3). *Fusarium* sp: Potato dextrose agar was used for identification. Colonies were growing rapidly, woolly to cottony at 25°C. From the front, the colour of the colony was white, cream, salmon or pink. From the reverse, it was tan, or brown. Conidiophores were hyaline, short. There are two kinds of conidia. Macroconidia are boat-shaped, 4 celled. Microconidia were ellipsoidal and 1 celled (Figure 4). *Mucor* sp: Potato dextrose agar was used for identification. Colonies were floccose, white-greyish brown, cover the surface of PDA. Columella was hyaline. Sporangia were round grey to black in colour, and were filled with sporangiospores. The sporangio-spores were ellipsoidal. *Penicillium* sp: Potato dextrose agar was used for identification. The colonies were rapid growing, velvety in texture. The colonies were initially white and became greyish green. Conidiophores were hyaline with verticillate metula. Conidia were green, subglobose, smooth (Figure 5). *Thielavia* sp: Potato dextrose agar was used for identification. Colonies were dark brown on. Cleistothecia were black, globose. Ascospores were olive-coloured, ellipsoidal.

Of these, *Fusarium* sp. and *Chrysosporium* sp. were the most frequent ones. *Chrysosporium* is a keratinophilic filamentous fungus commonly isolated from soil where it

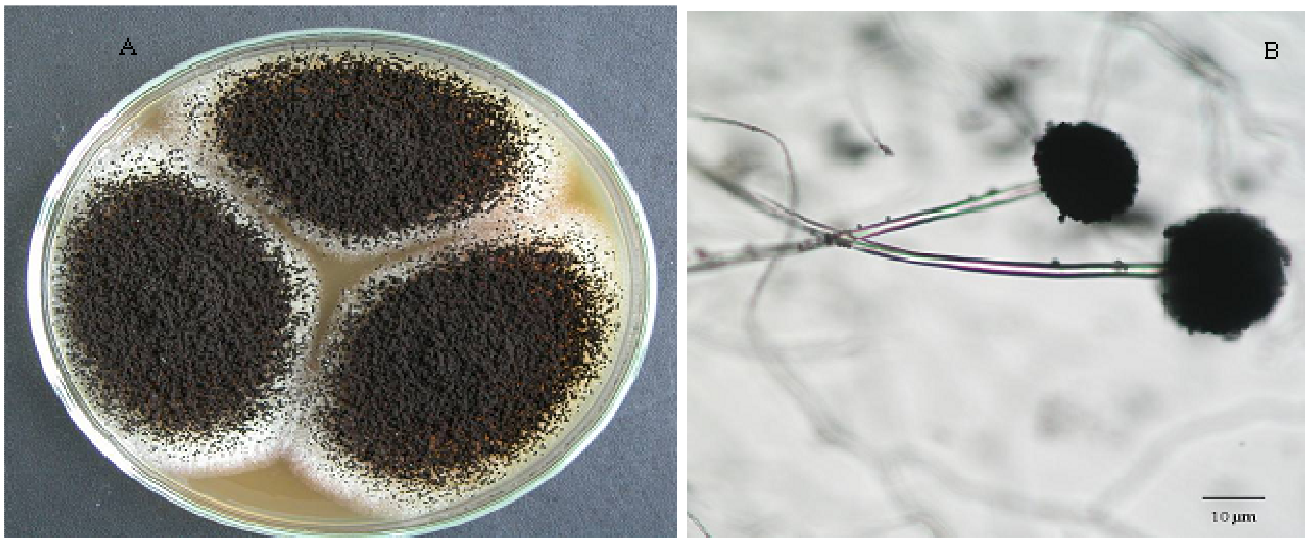

Figure 1. *Aspergillus* sp A) Colonial appearance (7 days); Light microscopic appearance of B) conidial head.

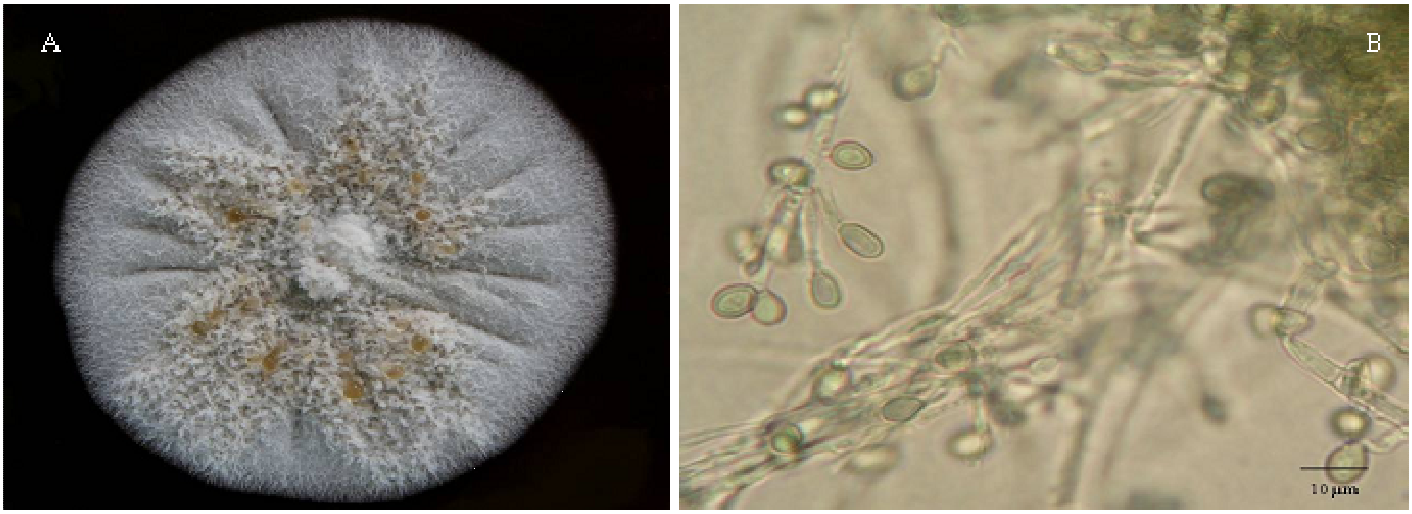

Figure 2. *Chrysosporium* sp. A) Colonial appearance (7 days); Light microscopic appearance of B) fertile hyphae and conidi.

lives on remains of hairs and feathers. They are common soil saprophytes they are usually considered as contaminants (Verweij and Brand, 2007). Keratinophilic fungi have been found colonizing marine and terrestrial saline habitats (Al-Musallam, 1990).

Fusarium species are common soil saprophytes and plant pathogens. *F. solani* is a common soil mycobiota that has previously been isolated from the exterior of failed eggs in natural loggerhead sea turtle nests (Phillot et al., 2001). Among the fungus types isolated from *C. caretta* nests on the Yanıklar coast, the most frequently seen group is *Fusarium* sp. (43.28% for sand, 53.71% for eggs). Cabanes et al. (1997) reported *F. solani* as the agent of a cutaneous infection of loggerhead sea turtle in the Mediterranean.

Most of the identified genera from the failed eggshells were also found in the nest sand (Table 2) meaning that fungi coming from the nest sand might have contaminated turtle eggs. In fact, *F. oxysporum*, *F. solani* and *Pseudallescheria boydii* have regularly been isolated as soil borne pathogens on the exterior of failed eggs (Phillott et al., 2001). In addition, *Chrysosporium* sp., a soil saprophyte like *Fusarium* sp. hitherto not mentioned in previous studies, was detected at a ratio of 32.48% in 12 nests. Mo et al. (1990) reported that *Aspergillus* sp. was consistently found in successful nests. In fact, *Aspergillus spp*. has been contracted by airborne contamination (Phillott, 2002). *Aspergillus* sp. has been also identified in lesions of dermal and superficial mycoses of young loggerheads (Mallo et al., 2002).

Figure 3. *Emericella* sp. A hulle cell.

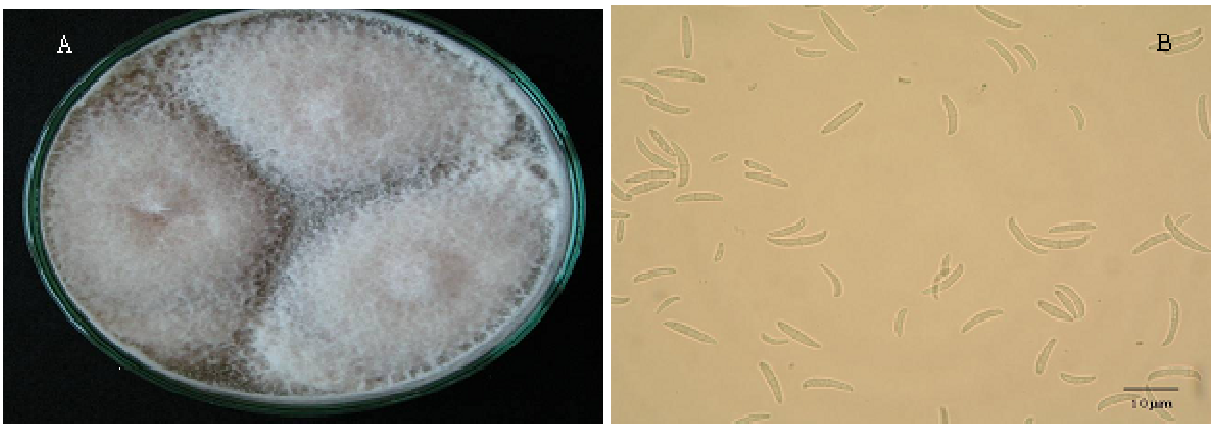

Figure 4. *Fusarium* sp. A) Colonial appearance (7 days); Light microscopic appearance of B) macroconidia.

Aspergillus sp. compared to other types of fungi, was also seen very frequently (in 8 nests out 15, 6.53%).

There was a positive correlation between total number of isolated fungus and death embryo in nests ($r = 0.53$, $p < 0.05$). Hatching success was negatively correlated with total number of isolated fungus ($r = -0.54$, $p < 0.05$). Wyneken et al. (1998) did not determine any correlation between hatching success and bacteria count in the nests. Phillott et al. (2001) reported that fungal invasion does not kill the newly laid egg. Fungus must penetrate the inorganic and organic layers of the eggshell to utilise embryonic tissue as a source of nutrients (Phillott, 2002). Phillott et al. (2002) suggested some precautions for minimizing fungal invasion during the artificial incubation of sea turtle eggs.

On the other hand, more than 80 genera of fungi are reported to be associated with symptoms of respiratory tract allergies (Horner et al., 1995). The genus *Penicillium* is among the most abundant mesophilic airborne fungi in nature and in the human environment. The increase of in relative humidity and the growth of vegetation with increasing the temperature and rainfall in the area contributed to increasing *Penicillium* spore counts in air. *Cladosporium*, *Aspergillus* and *Fusarium* were among the most common allergic genera (Fang et al., 2005). In addition, *Penicillium*, *Aspergillus* and *Fusarium* are associated with “bronchopneumonia”, a type of lung inflammation in sea turtles (Glazebrook et al., 1993). Therefore, marine turtle researchers should be careful while digging the nests for control openings and should use gloves and masks if possible in order to prevent the risk of respiratory tract allergies.

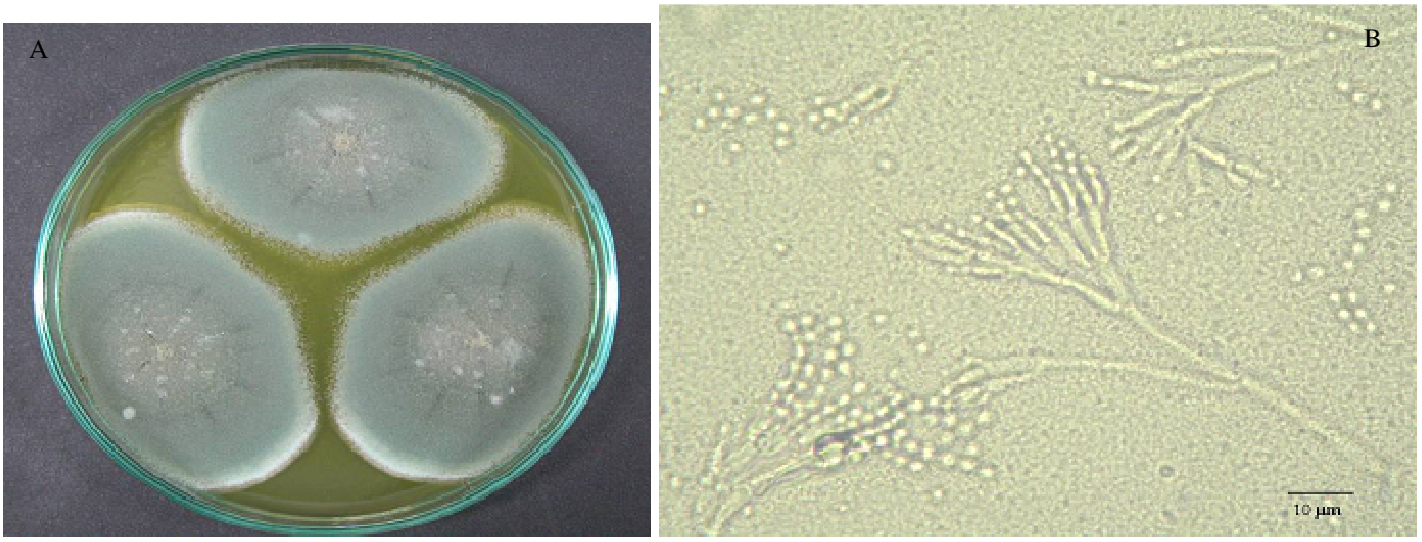

Figure 5. *Penicillium* sp. A) Colonial appearance (7 days); Light microscopic appearance of B) penicilli and phialides and conidia.

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