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Marine isolates of *Aspergillus flavus*: Denizens of the deep or lost at sea?

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ABSTRACT

Most fungal species from marine environments also live on land. It is not clear whether these fungi reach the sea from terrestrial sources as spores or other propagules, or if there are separate ecotypes that live and reproduce in the sea. The emergence of marine diseases has created an urgency to understand the distribution of these fungi. *Aspergillus flavus* is ubiquitous in both terrestrial and marine environments. This species is an opportunistic pathogen in many hosts, making it a good model to study the relationship between genetic diversity and specificity of marine fungi. In this study, an intraspecific phylogeny of *A. flavus* isolates based on Amplified Fragment Length Polymorphisms (AFLPs) was used to determine if terrestrial and marine isolates form discrete populations, and to determine if phylogeny predicts substratum specificity. Results suggest lack of population structure in *A. flavus*. All isolates may compose a single population, with no clade particular to marine environments.

Introduction

Fungal species in the sea are defined as either obligately marine or facultative: "*Obligate marine fungi* are those that grow and sporulate exclusively in a marine or estuarine habitat; *facultative marine fungi* are those from freshwater or terrestrial milieus able to grow (and possibly also to sporulate) in the marine environment" (Kohlmeyer & Volkmann-Kohlmeyer 2003; Shearer *et al.* 2007). Facultative marine fungi may be carried to the sea by wind, rain or runoff. Given that some are very common in the sea, they may include populations that have evolved adaptations to grow in marine environments, eventually becoming obligately marine. There are certainly precedents for this: colonization of the sea by fungi has happened many times independently (Hibbett & Binder 2001).

The emergence of marine diseases has created an urgency to understand the role and origin of the microbiota associated with marine organisms (Harvell *et al.* 2007; Rosenberg *et al.* 2007). Studies of fungi associated with corals have mainly focused on aspergillosis disease of sea fans (*Gorgonia ventalina*). This disease has been attributed to *Aspergillus sydowii*, a fungus common in soils (Geiser *et al.* 1998b), though this finding has recently been questioned (Toledo-Hernández *et al.* 2007, 2008; Zuluaga-Montero *et al.* 2010). There is a debate about how inoculum of *A. sydowii* reaches the Caribbean; the leading theory is that the inoculum is terrestrial, comes from the Sahel, and crosses the Atlantic in dust clouds (Weir-Brush *et al.* 2004). However, there is evidence for distinct marine and terrestrial populations: marine strains caused aspergillosis when inoculated into sea

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fans whereas terrestrial strains could not (Geiser *et al.* 1998b), and carbon utilization profiles showed differences between marine and terrestrial strains (Alker *et al.* 2001). On the other hand, DNA fingerprints based on microsatellites failed to distinguish between marine and terrestrial strains of *A. sydowii* (Rypien *et al.* 2008).

In the present study, we used *Aspergillus flavus* as a model species to address this marine *vs.* terrestrial debate. *A. flavus* is ubiquitous in terrestrial environments and it is increasingly clear that it is ubiquitous in marine environments as well (Koh *et al.* 2000; Morrison-Gardiner 2002; Zuluaga-Montero *et al.* 2010). Its high salt tolerance and wide range of substrata make it a logical candidate to adapt to life in the sea. *A. flavus* has been extensively studied as an opportunistic pathogen in chronic and invasive pulmonary and systemic infections, especially in immune-compromised patients (Hedayati *et al.* 2007). It can also cause disease in a broad range of organisms other than humans, including birds, insects and plants (Raper *et al.* 1965; Leger *et al.* 2000). In addition, *A. flavus* produces aflatoxins – secondary metabolites that are potent carcinogenic and immunosuppressive toxins in animals when ingested, and pose a significant threat to human health (Pitt 2000; Yu *et al.* 2005). This fungus frequently invades susceptible crops such as corn, cotton, peanuts and tree nuts before or after harvest, causing aflatoxin contamination (Cotty *et al.* 1994).

A. flavus, together with other congenerics, is commonly isolated from marine substrata, including sponges (Holler *et al.* 2000), sclerectinian corals (Kendrick *et al.* 1982), and soft corals (Koh *et al.* 2000). We found *A. flavus* is common in diseased tissue of the sea fan *G. ventalina* in Puerto Rico, suggesting a possible role in sea fan aspergillosis (Toledo-Hernández *et al.* 2008; Zuluaga-Montero *et al.* 2010). However, the biology of marine isolates of *A. flavus* has scarcely been explored.

In the present study, we used Amplified Fragment Length Polymorphisms (AFLPs) to identify intraspecific relationships among *A. flavus* isolates from terrestrial and marine sources. We tested the hypothesis that marine isolates will be more closely related to other marine isolates than to terrestrial isolates, suggesting that some clades have adaptations for life in the sea. On the other hand, if the source of marine isolates is terrestrial input, marine isolates are not expected to form distinct clades. In addition, we tested the hypothesis that isolates from diseased tissue of sea fans form a clade apart from isolates from healthy tissue, which would suggest that certain genotypes are associated with disease.

Table 1 – Isolates of *Aspergillus flavus* used in this study with substratum, site of isolation and GenBank accession number of ITS sequence

# ID isolate	Substratum	Site of isolation	GenBank accession number
A1	Coffee	PR	HM167490
A2	Algae	PR	EU645653
A3	Air, walnut orchard	Wolfskill, Winters, CA	HM167491
A4	Soil, walnut orchard	Wolfskill, Winters, CA	HM167492
B1	Soil	PR	HM167494
B2	Soil	PR	EU645681
B3	Soil	Nigeria	HM167488
B4	Soil	Nigeria	HM167489
B5	Soil	Nigeria	HM167495
DT1	Diseased sea fan tissue	PR	HM178946
DT2	Diseased sea fan tissue	PR	EU554579
DT3	Diseased sea fan tissue	PR	EU554578
DT4	Diseased sea fan tissue	PR	
DT5	Diseased sea fan tissue	PR	EU554582
DT6	Diseased sea fan tissue	PR	EU554577
HT1	Healthy sea fan tissue	PR	EU554586
HT2	Healthy sea fan tissue	PR	HM167496
HT3	Healthy sea fan tissue	PR	HM167497
HT4	Healthy sea fan tissue	PR	HM178947
HT5	Healthy sea fan tissue	PR	HM167499
HT6	Healthy sea fan tissue	PR	EU554573
HT7	Healthy sea fan tissue	PR	HM167500
HT8	Healthy sea fan tissue	PR	HM167501
HT9	Healthy sea fan tissue	PR	HM167502
SW1	Seawater	PR	EU645713
SW2	Seawater	PR	EU645706
SW3	Seawater	PR	EU645692
SW4	Seawater	PR	EU645703
SW5	Seawater	PR	EU645702
SW6	Seawater	PR	HM167493

Materials and methods

Fungal isolates and DNA sequencing

Thirty isolates of *A. flavus* were obtained from different environmental sources (Table 1). Isolates from seawater and from healthy and diseased sea fan (*G. ventalina*) tissue were collected from different reefs around Puerto Rico (Zuluaga-Montero *et al.* 2010). Other isolates from soil, dried, green coffee beans and air were included for comparison. All isolates were cultured on Glucose Peptone Yeast Agar (GPYA, Difco Labs) incubated at 25 °C and transferred to liquid medium (potato-dextrose broth) for DNA extraction.

DNA was extracted using a Plant Mini Extraction Kit (Qiagen Sciences). To ensure that all isolates were *A. flavus*, the nuclear ribosomal ITS region was amplified using primers ITS 1F and ITS 4 (White *et al.* 1990; Gardes & Bruns 1993), and sequenced in the University of Puerto Rico Sequencing and Genotyping Facility (UPR SGF). Sequences were assembled and manually examined for errors using Sequencher software (version 3.1), and aligned using CLUSTALX (Version 1.8,