THE ORIGINS AND SPREAD OF *ASPERGILLUS SYDOWII*, AN OPPORTUNISTIC PATHOGEN OF CARIBBEAN GORGONIAN CORALS

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THE ORIGINS AND SPREAD OF *ASPERGILLUS SYDOWII*, AN OPPORTUNISTIC PATHOGEN OF CARIBBEAN GORGONIAN CORALS

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Coral reefs are increasingly suffering outbreaks of disease, causing dramatic declines in population abundance and diversity. One of the best-characterized coral diseases is aspergillosis, caused by the fungus *Aspergillus sydowii*. My dissertation investigates the origins and spread of aspergillosis in Caribbean gorgonian coral communities.

The role of host resistance in aspergillosis is well established, however we know little about variation in resistance through time or the role of pathogen virulence. Using geographically distinct pathogen isolates in a clonally replicated design, I found equivocal evidence for variation in host response to pathogen isolates, with most fungal treatments showing no difference from the control. Interestingly, the two isolates that did induce a host response represent a pathogenic and an environmental isolate, suggesting that *Aspergillus sydowii* is a true opportunist.

Aspergillus sydowii is a globally distributed saprophyte commonly found in soil, so its presence in marine systems raises questions about its origin. Using microsatellite markers, I analyzed the population structure of *A. sydowii* from diseased sea fans, diseased humans, and environmental sources worldwide. The results indicate a single global population. Moderate differentiation between isolates from sea fans and those from environmental sources, along with higher growth rates at 37°C by sea fan isolates, suggests that selection within the marine environment could be driving

population subdivision.

Past researchers have suggested that *Aspergillus sydowii* originates from African dust blown into the Caribbean, and have identified *Aspergillus* from dust samples, although only to the genus level. To test this hypothesis, I isolated fungi from dust samples collected in Mali and St. Croix. Although I identified seven species of *Aspergillus* from airborne dust samples, none were found to contain *A. sydowii*.

Finally, the role of vectors in facilitating disease spread is poorly studied in marine systems. A specialist predator of gorgonian corals, *Cyphoma gibbosum*, is a likely vector for aspergillosis. I found viable fungus in the feces of snails fed spores and hyphae, and a preference by snails for diseased sea fans. Stable isotope studies confirm the role of *C. gibbosum* in between-host transmission, supporting the hypothesis that this snail is acting as a vector for aspergillosis.

BIOGRAPHICAL SKETCH

Krystal Leeanne Rypien was born on the 19th of February 1979 in Edmonton, Alberta, Canada. Krystal grew up a child of the prairies and the forest, surrounded by loving family and friends. Krystal first encountered nature in her own backyard, which was used not only for raising pet caterpillars and eating sugar snap peas off the vine, but was also where she learned to climb trees and ice skate. Krystal discovered the ocean visiting family on Vancouver Island. There she learned the lifelong skills of tidepooling and beach combing. Her passion for marine science was fostered by her grade seven math teacher, Mr. McKay, who led her class on a field trip to Bamfield, an isolated town on the west coast of Vancouver Island. Krystal continued to foster her love of science through high school, and began her undergraduate studies at the University of Alberta in 1996. During her bachelor's degree in environmental science, she took several more trips to the Bamfield Marine Sciences Center, which culminated in a semester-long residence. While there, she conducted honors thesis research with Dr. A. R. Palmer on aggressive interactions in porcelain crabs.

Despite her passion for marine invertebrates, Krystal was tempted by the underworld of fungus, taking several courses with Dr. Randy Currah and working at the University of Alberta Microfungus Collection and Herbarium with Dr. Lynne Sigler. In applying to graduate school, Krystal decided to merge her two loves, and found her way to Dr. C. Drew Harvell at Cornell University where she worked on a fungal pathogen of Caribbean corals.

With the guidance of Dr. Harvell and the other members of her doctoral committee, Drs. Monica Geber, Ann Hajek, and Kelly Zamudio, Krystal developed an independent research program on the origins and spread of a fungal pathogen in marine ecosystems. Krystal appreciated the stimulating intellectual and social environment of the Departments of Ecology and Evolutionary Biology and

Neurobiology and Behavior. Krystal will continue her research on coral reefs with a postdoctoral position at the Scripps Institute of Oceanography working with Dr. Farooq Azam.

For my parents and grandparents

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I was fortunate to share offices with Rulon Clark, Sam Flaxman, Lynn Fletcher, Gretchen Gerrish, Vik Iyengar, Jamie Mandel, Luana Maroja, Marie Nydam, and Becca Safran. I will cherish the friendships and memories formed, mostly after 4pm, in W343 Mudd Hall.

My research benefited greatly from interactions with other graduate students studying evolution and ecology, who not only formed an excellent academic environment, but are also great friends. In particular, I wish to thank Jason Andras, Dave Baker, Alex Bezzerides, Lauren Chan, Becky Doyle, Gretchen Gerrish, Jackie Grant, Katie Flinn, Robert Harris, Vik Iyengar, Ericka Iyengar, Karen Laughlin, Morgan Mouchka, Shannon Murphy, Marie Nydam, Dan Riskin, Jeanne Robertson, Becca Safran, Jessica Ward, and Matt Weeg. The support staff in the department have been invaluable, and I would especially like to thank Rosie Brainard, Carol Damm, Alberta Jackson, Janeen Orr, Patty Jordan, Linda Harrington, LuAnne Kenjerska, DeeDee Albertsman, Brian Mlodzinski, John Howell, Gary Oltz, Tim Larkin and Al Hand.

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CHAPTER 1

INFECTION OF SEA FANS BY *ASPERGILLUS SYDOWII*: HOST RESPONSE TO GEOGRAPHICALLY DISTINCT PATHOGEN ISOLATES

ABSTRACT

The fungus *Aspergillus sydowii* infects sea fan corals throughout the Caribbean, and its prevalence varies both spatially and temporally. This may be the result of variation in the host, the pathogen, or host-pathogen interactions. This study examined whether sea fans are locally adapted to pathogens by asking whether geographically varying isolates of *A. sydowii* induce differential responses in sea fan hosts from a single location. We performed a clonally replicated infection experiment using *Gorgonia ventalina* (common sea fan) from Florida, and nine isolates of *A. sydowii*. The response to infection was quantified by examining the efficacy of sea fan antifungal compounds in inhibiting fungal growth. There was considerable variation in the magnitude of host response among sea fan genotypes, emphasizing a strong role of host resistance in this system. There was an effect of fungal strain on host antifungal activity, but only two isolates differed from the control treatment: a pathogenic strain and an environmental strain, supporting the opportunistic nature of *A. sydowii*.

INTRODUCTION

Characteristics of the host (resistance, dispersal), the pathogen (virulence, transmission), and the interaction between the two (local adaptation, coevolution) are responsible for much of the spatial and temporal variation in disease prevalence. For most coral disease systems, compromised host resistance is cited as a major driver of disease dynamics, with many infectious diseases showing ties to known coral stressors

such as increased temperature and nutrient levels (Harvell et al. 1999, Cerrano et al. 2000, Harvell et al. 2001, Harvell et al. 2002, Kim and Harvell 2002, Kuta and Richardson 2002, Patterson et al. 2002, Bruno et al. 2003, Rosenberg and Falkovitz 2004, Voss and Richardson 2006a, b, Ward et al. 2007). The role of variation in pathogen virulence is poorly understood, and remains challenging to address, due to the lack of identified pathogens of corals (Sutherland et al. 2004). In addition, almost no research has been conducted on coevolutionary dynamics between host and pathogen, despite the emphasis placed on these interactions in terrestrial and freshwater disease systems (Ebert 1994, Lively and Dybdahl 2000, Thrall and Burdon 2003). Given the strong role of host resistance and environmental stressors in many coral disease systems, and the paucity of information on most pathogens, an examination of the role of local adaptation in driving disease outbreaks is a critical area of future research.

I used sea fan aspergillosis as a model system to test hypotheses about the role of local adaptation in host resistance on patterns of disease in a marine system. *Aspergillus sydowii*, the causative agent of aspergillosis (Geiser et al. 1998), is a terrestrial fungus not known to reproduce in the ocean (Smith et al. 1996). The current aspergillosis epizootic originated in the mid 1990s and is now Caribbean-wide, and causes partial and complete coral mortality (Nagelkerken et al. 1997a, Nagelkerken et al. 1997b). This disease also has population-level effects, such as selective mortality of large sea fans (Kim and Harvell 2004), and suppression of reproduction in infected individuals (Petes et al. 2003). Abiotic factors known to influence host-pathogen dynamics include nutrient levels (Kim and Harvell 2002, Bruno et al. 2003, Baker et al. in press), water clarity (Kim and Harvell 2002), and temperature (Alker et al. 2001, Ward et al. 2007).

Infection with *A. sydowii* induces a suite of generalized immune responses in the sea fan *Gorgonia ventalina* (Alker 2000, Kim et al. 2000a, Kim et al. 2000b, Dube et al. 2002, Alker et al. 2004, Mullen et al. 2004, Douglas et al. 2007, Mydlarz and Harvell 2007). Structural defenses are primarily the production of melanin, a common invertebrate immune response (Petes et al. 2003, Mullen et al. 2004, Mydlarz et al. 2006). Chemical defenses of sea fans include a broad range of antifungal and antimicrobial metabolites (Kim 1994, Kim et al. 2000a, Kim et al. 2000b, Alker et al. 2001, Dube et al. 2002, Alker et al. 2004), as well as more targeted molecules such as chitinase (Douglas et al. 2007) and peroxidase (Mydlarz and Harvell 2007). Cellular defenses are also observed, with amoebocytes aggregating at the site of infection (Mydlarz et al. in review).

Both the prevalence and severity of aspergillosis vary over spatial (meters to hundreds of kilometers) and temporal scales (months to years) (Nagelkerken et al. 1997a, Jolles et al. 2002, Kim and Harvell 2002, Kim and Harvell 2004). This variation may be the result of selective removal of susceptible individuals (Kim and Harvell 2004), variation in abiotic stressors (Kim and Harvell 2002), or vector and/or water-borne disease transmission (Jolles et al. 2002). Another hypothesis to explain this variation is the presence of multiple pathogen strains, with local adaptation of hosts to the most abundant pathogen genotype driving patterns of disease outbreak. Previous studies have suggested that distinct strains of *A. sydowii* exist, as demonstrated by variation in growth rate (Alker et al. 2001), carbon utilization patterns (Alker et al. 2001), and secondary metabolites (Malmstrøm et al. 2001). In addition, the presence of multiple strains of *A. sydowii* in marine systems is implicit in the three hypothesized origins of the pathogen in coral reef communities: terrestrial runoff (Smith et al. 1996, Geiser et al. 1998), African dust deposits (Shinn et al. 2000), or marine sources (Sparrow Jr. 1937, Roth Jr. et al. 1964). Each source could contain

several strains of *A. sydowii*, subjecting coral reefs to multiple isolates of the pathogen. Thus, there is the potential for sea fans to be locally adapting to the most common pathogen isolates.

To investigate the role of local adaptation of sea fan resistance to geographically distinct isolates of *A. sydowii*, I conducted a laboratory inoculation experiment. Sea fans from Florida were experimentally infected with pathogen strains isolated from locations throughout the range of the disease to assay how host response varied with exposure to different isolates of *A. sydowii*. If sea fans are locally adapted to pathogen isolates, I would expect to see higher host resistance to local pathogens (those from Florida) relative to pathogens isolated from other regions in the Caribbean. In contrast, if sea fans have a similar defense response to all pathogenic isolates of *A. sydowii*, this suggests that local adaptation is not occurring, potentially in response to a widely dispersed generalist pathogen. Non-pathogenic isolates of *A. sydowii* should induce the lowest host resistance in either scenario, given that they are not known to successfully infect sea fans (Geiser et al. 1998).

MATERIALS AND METHODS

Samples of healthy sea fans (N = 18) of intermediate size (40 to 60 cm in height) were collected from Eastern Sambo Reef, Florida Keys (24˚29.737′N, 81˚38.916′W) in May 2002. Each sample was subdivided into seven 48 cm^2 fragments, with one fragment processed immediately after collection to establish initial baseline conditions. The remaining six genetically identical fragments were acclimated for 24 hours prior to infection, after which each received one of nine randomly chosen fungal treatments. Due to the large number of treatments, the experiment was split into two experimental runs, with each sea fan colony receiving five of the nine fungal treatments and a control treatment in a single run. The two

experimental runs were performed in succession, with sea fans collected and acclimated for 24 hours immediately prior to each experiment.

The sea fan fragments were maintained outdoors in 20 l aquaria at the Mote Marine Tropical Research Laboratory on Summerland Key, FL, with water temperatures maintained at ambient levels by a flow-through sea water system, and natural light through shade cloth to simulate the light intensity experienced at a depth of 15 to 20 feet. All aquaria were closed systems to ensure that the pathogen was not released into the environment.

The nine fungal treatments differed in the source of *A. sydowii*: eight isolates were cultured from diseased sea fans around the Caribbean, and one isolate (NRRL244) was from a non-marine source, and is assumed to be non-pathogenic (Table 1.1). *Aspergillus sydowii* was grown in the dark at 25°C on peptone yeast glucose (PYG) agar (1 L distilled water, 1.25 g peptone, 1.25 g yeast extract, 3.0 g glucose, 30 g instant ocean, 20 g agar) with sterile gauze strips embedded just below the surface of the media. After seven days of growth, the gauze strips coated with agar and actively growing fungus were removed, and pushed through 1 cm long slits cut through the mesh of the sea fan; control treatments received gauze strips with no fungus.

Seven days after infection, the sea fan fragments were processed to assess host response to infection by examining the potency of crude antifungal extracts. Fungal inhibition by crude sea fan extracts was tested using methods modified from previous studies (Alker et al. 2001, Dube et al. 2002, Ward et al. 2007). Antifungal compounds were extracted from 16 cm^2 tissue samples twice overnight at -20˚C in dichloromethane, which was then evaporated under continuous N_2 flow. Extracts were weighed and re-suspended in acetone to a final concentration of 20 mg/ml. The growth assay was performed on 35 mm diameter Petri plates containing peptone yeast

Table 1.1. *Aspergillus sydowii* isolates used in the inoculation experiment. Source and date refer to the original isolation of the fungus.

Isolate	Abbrev.	Source and Date
Conch Reef	Conc	Diseased *G. ventalina*; Florida, 1999
Dump Reef	Dump	Diseased *G. ventalina*; Bahamas, 1998
Florida Keys 1	FK1	Diseased *G. ventalina*; Key West, Florida
GFP	GFP	Genetically modified; diseased *G. ventalina*, Florida
Grecian Rocks	Grocks	Diseased *G. ventalina*; Florida, 1999
NRRL244	NRRL	Dried fish in Japan; non-pathogenic to sea fans
Saba	Saba	Diseased *G. ventalina*; Netherland Antilles, 1995
San Salvador	SanSal	Diseased *G. ventalina*; Bahamas, 1995
Sombrero	Somb	Diseased *G. ventalina*; Florida, 1999

glucose (PYG) agar with 50 μg/mL tetracycline. On each of three replicate plates, 75 μL of antifungal extract was added, spread with a glass rod, and the acetone allowed to evaporate. Un-amended agar plates and acetone-amended plates served as controls. Fungus (FK11; isolated from a diseased sea fan in Florida) was inoculated onto the center of each plate using 2 μL of spore solution (2,250 spores/μL). Plates were incubated for three days at 27°C in the dark.

Digital images of circular fungal colonies were taken three days post-inoculation. Fungal colony area was calculated from two perpendicular measurements of colony diameter with digital imaging software (NIH Image version 1.6, National Institutes of Health). The antifungal activity of the crude extracts was calculated as the inhibition (I) of fungal growth on extract-amended plates relative to un-amended and acetone-amended control plates (Ward et al. 2007):

$$I = 1 - \left(\frac{\text{Area}_{\text{extracts}}}{\text{Area}_{\text{controls}}} \right)$$

Data were tested for normality (Shapiro-Wilk) and homogeneity of variance (Levene) prior to analysis, and were transformed as necessary. The inhibitory activity of sea fan extracts was analyzed using SAS 9.1 (SAS Institute, Inc.) as an incomplete blocked design ANOVA, with fungal treatment as a fixed factor, and sea fan colony as a random factor. As the experiment was divided in two separate experimental runs, the effect of run was examined by including it as a fixed factor in the model. Due to limitations of experimental design (insufficient degrees of freedom), it was not possible to test for a clone by treatment effect.

RESULTS

The inhibitory activity of host-derived antifungal extracts was affected by fungal treatment (Figure 1.1; $F = 2.48$, $p = 0.0085$). Tukey-Kramer post-hoc tests revealed that four pairs of treatments differed significantly: Control and NRRL, Control and SanSal, NRRL and Saba, Saba and SanSal. Experimental run had no significant effect on the activity of sea fan extracts ($F = 2.14$, $p = 0.1451$).

Since the experiment was run with each sea fan genotype replicated across fungal treatments, individual variation in antifungal activity as a response to fungal treatment can be described in norm of reaction plots (Figure 1.2, 1.3). The magnitude of sea fan response to different fungal treatments varied significantly (Figure 1.2). For example, corals infected with SanSal and NRRL showed a consistent induction of antifungal defenses, with 85.7% and 92.9% of colonies producing a higher response in infected than controls treatments (Figure 1.2). In contrast, responses of sea fans infected with Saba were more variable: only 44.4% showed an induction (Figure 1.2). Individual sea fan colonies also varied in the consistency of their response to fungal infection (Figure 1.3). 37.5% of sea fan colonies could be characterized as strong responders, or highly resistant, as they demonstrated an induced antifungal response to all fungal treatments. A lesser proportion of individuals (12.5%) can be considered highly susceptible, as they had no induction in antifungal activity in response to any fungal treatment (Figure 1.3). The majority of sea fan colonies however had mixed responses, with strong inductions of antifungal compounds in response to some fungal treatments, but no response to others (Figure 1.3). Interestingly, there was no single fungal treatment to which sea fans either had strong inductions or lacked inductions to: the variation in individual responses to a given fungal treatment is extremely high.

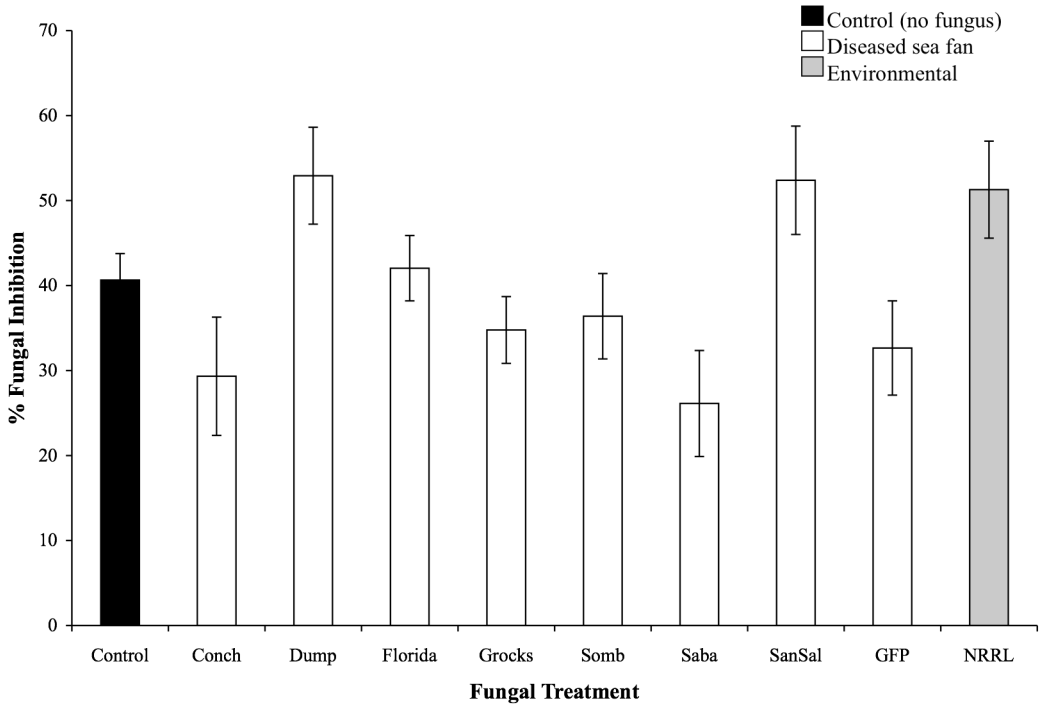


Figure 1.1. Inhibitory activity of sea fan antifungal extracts (N = 9) following infection with various isolates of the fungus *Aspergillus sydowii* (± standard error). Shading indicates source of fungal isolates. Fungal treatment abbreviations as in Table 1.1.

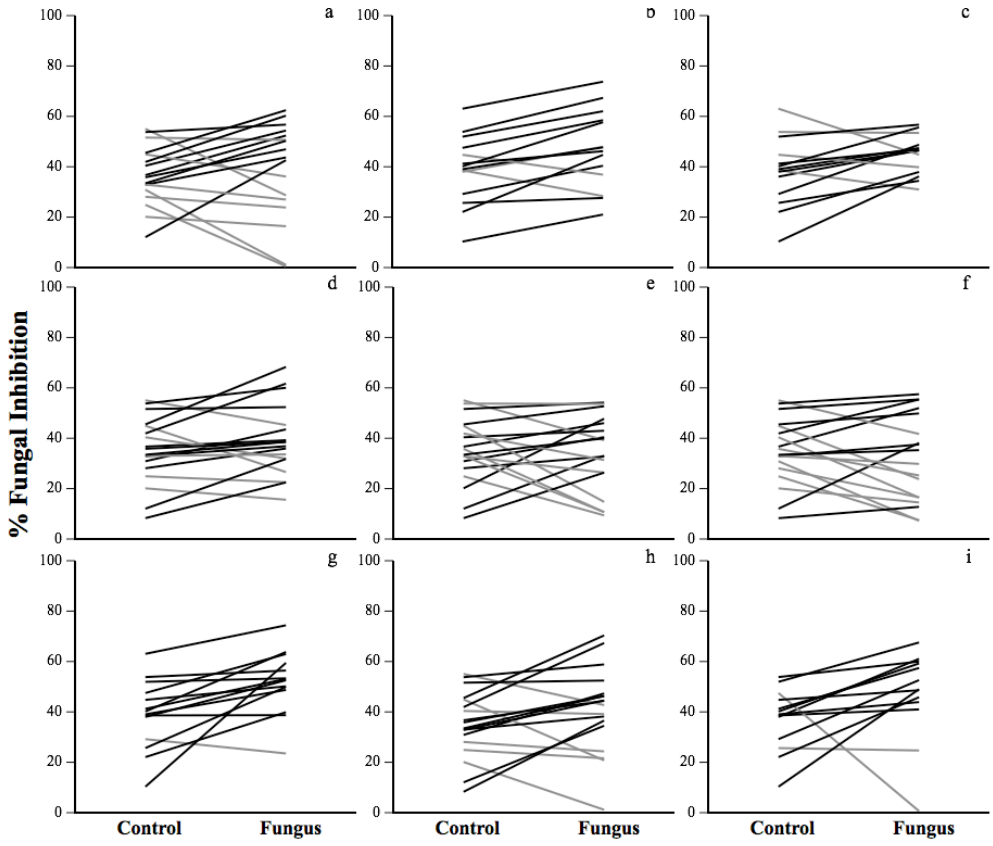


Figure 1.2. Antifungal activity of individual sea fan colonies exposed to fungal treatments relative to control (no fungus) treatments. Each panel represents a single fungal treatment: a) Conc, b) Dump, c) FK1, d) Grocks, e) Somb, f) Saba, g) SanSal, h) GFP, i) NRRL. Each line represents a single sea fan colony. Black lines denote colonies with an induced antifungal response to infection with *Aspergillus sydowii* (fungus > control), and grey lines denote colonies with a negative reaction to infection (control ≥ fungus).

Figure 1.3. Antifungal activity of thirty-two individual sea fan colonies infected with *Aspergillus sydowii* relative to control (no fungus) treatments. Each panel represents an individual sea fan clone, and each line represents a response to one of nine fungal treatments. Black lines denote colonies interactions resulting in an induced antifungal response to infection with *A. sydowii* (fungus > control), and grey lines denote sea fans that did not respond to infection (control ≥ fungus).

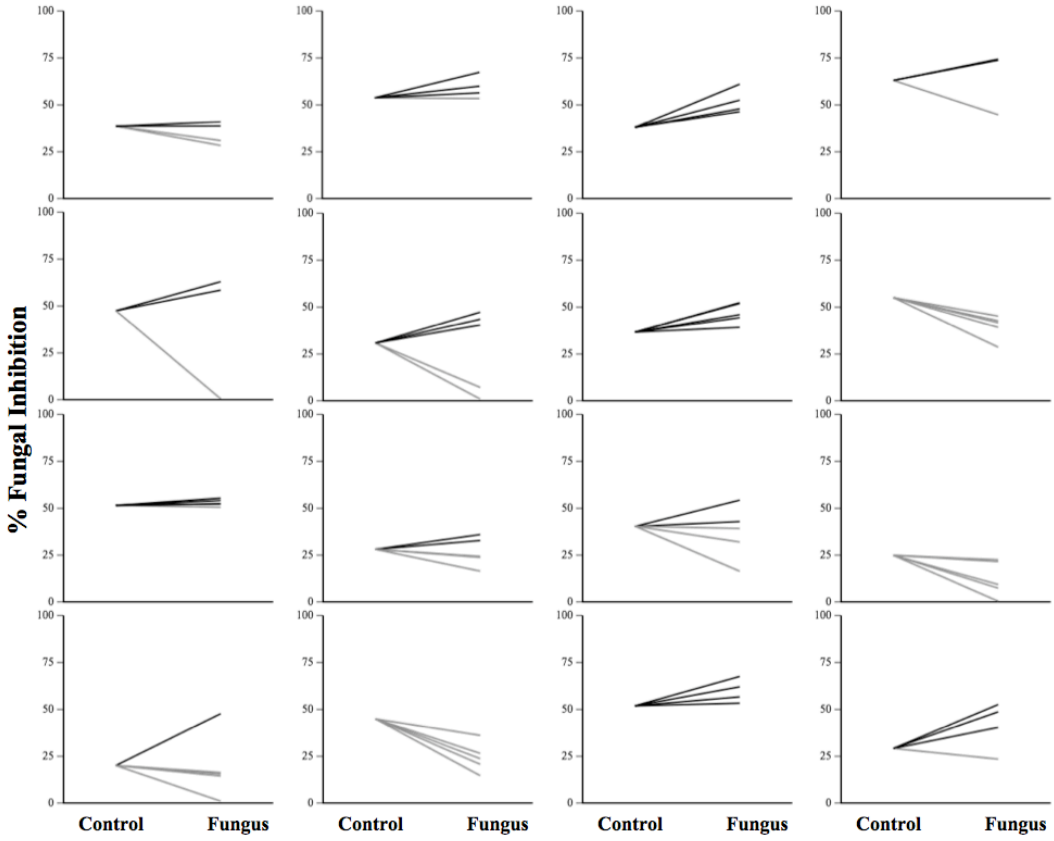
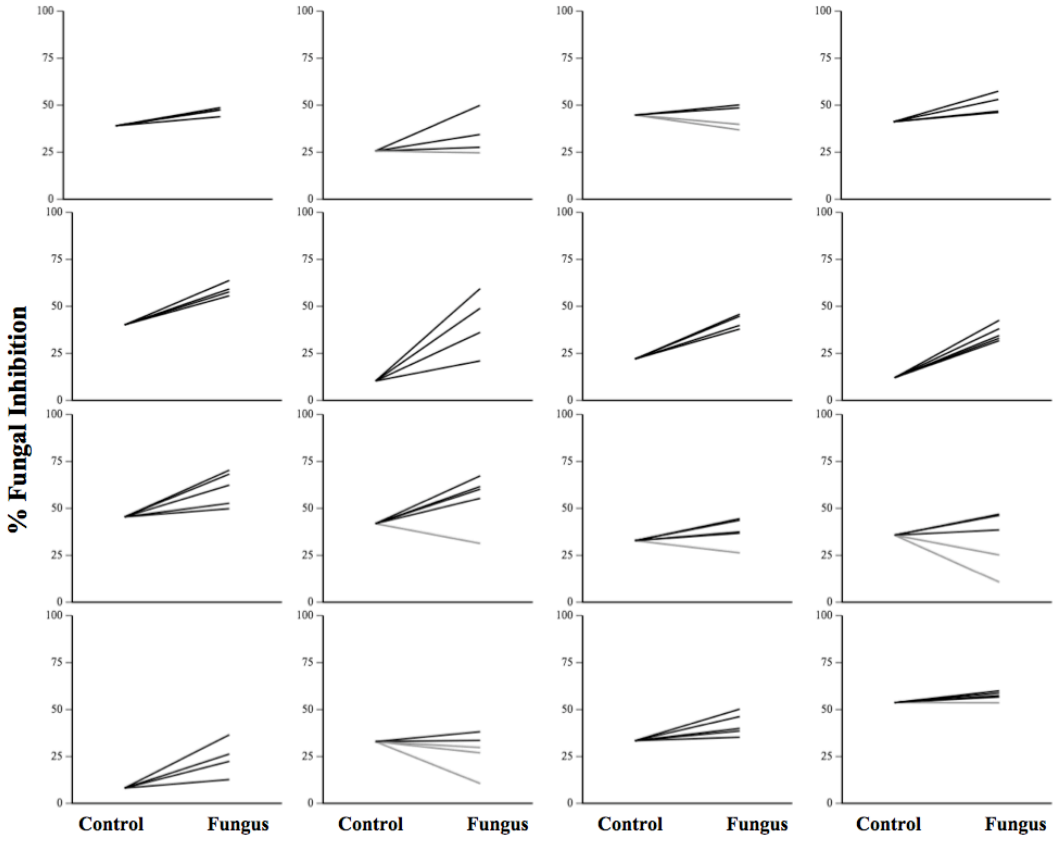

% Fungal Inhibition
100
75
50
25
0
Control
Fungus

Figure 1.3 (Continued)



DISCUSSION

A comparison of the magnitude of sea fan response following infection revealed that hosts responded differentially to geographically distinct isolates of *A. sydowii* (Figure 1.1). Two fungal treatments differed from the control (SanSal and NRRL), and represent isolates from a diseased sea fan and from dried fish, suggesting that non-pathogenic isolates can elicit similar responses in corals as pathogenic isolates. The degree to which pathogenic and non-pathogenic isolates of *A. sydowii* differ is unclear. Studies of fungal growth rates and HPLC profiles have detected differences between pathogenic and non-pathogenic isolates (Alker et al. 2001, Malmstrøm et al. 2001), but molecular studies find no evidence of differentiation (Chapter 2, Geiser et al. 1998). Other disease-causing aspergilli are exclusively opportunistic pathogens (Sweeney et al. 1976, Olufemi et al. 1983, Pier and Richard 1992, Latgé 1999, Munkvold 2003), and the ability of an environmental isolate to induce a sea fan defense response of similar magnitude as a pathogenic isolate suggests that *A. sydowii* is may also be opportunistic, indicative of a general pattern within the genus.

Variation in the host response to fungal treatments could also result from host rather than pathogen properties. Previous studies have documented high variation in sea fan resistance to infection among both individuals and populations (Kim et al. 2000b, Dube et al. 2002, Mullen et al. 2006). Similarly, this experiment found considerable variation among individual sea fan colonies in the induction of antifungal activity (Figure 1.2, 1.3). The majority of sea fans had a strong antifungal response to SanSal and NRRL (Figure 1.2). However, no single fungal treatment elicited a universally high or low response by sea fans, suggesting that individual-level host variation may be driving the observed pattern. When the responses of sea fans are examined according to clone, it is apparent that this is the case: some sea fans are

strong responders, showing a large induction of activity in response to all fungal treatments, while others did not respond to any fungal treatments (Figure 1.3). These results underscore the high variation in sea fan resistance among similar-sized individuals at a single geographic location, which has previously been suggested to be a major driver of disease prevalence (Kim et al. 2000a, Dube et al. 2002, Kim and Harvell 2004, Kim et al. 2006).

The results of this study underscore the high variation in defense responses present in a single population of similar-sized sea fans. In addition, there does not seem to be evidence of local adaptation, with sea fans responding equally strongly to *A. sydowii* isolated from a diseased sea fan in Florida and a dried fish in Japan. These results underscore the opportunistic nature of aspergillosis in corals. This is also evident from a recent molecular study of *A. sydowii* that found no genetic differentiation between pathogenic and non-pathogenic isolates (Chapter 2). Thus, similar to other members of its genus, *A. sydowii* is primarily an opportunistic invader, and host resistance and environmental stressors are the primary drivers of patterns of disease outbreak and emergence in gorgonian coral communities.

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REFERENCES

Alker, A. P. 2000. Localized Induction of a Multivariate Structural Response in Sea Fans to Predators, Competitors, and Pathogens. M.Sc. Cornell University, Ithaca, NY.

Alker, A. P., K. Kim, D. H. Dube, and C. D. Harvell. 2004. Localized induction of a generalized response against multiple biotic agents in Caribbean sea fans. Coral Reefs **23**:397-405.

Alker, A. P., G. W. Smith, and K. Kim. 2001. Characterization of *Aspergillus sydowii* (Thom et Church), a fungal pathogen of Caribbean sea fan corals. Hydrobiologia **460**:105-111.

Baker, D. M., S. E. MacAvoy, and K. Kim. in press. Relationship between water quality, 15N, and aspergillosis of Caribbean sea fan corals. Marine Ecology Progress Series.

Bruno, J. F., L. E. Petes, C. D. Harvell, and A. Hettinger. 2003. Nutrient enrichment can increase the severity of coral diseases. Ecology Letters **6**:1056-1061.

Cerrano, C., G. Bavestrello, C. N. Bianchi, R. Cattaneo-vietti, S. Bava, C. Morganti, C. Morri, P. Picco, G. Sara, S. Schiaparelli, A. Siccardi, and F. Sponga. 2000. A catastrophic mass-mortality episode of gorgonians and other organisms in the Ligurian Sea (Northwestern Mediterranean), summer 1999. Ecology Letters **3**:284-293.

Douglas, N. L., K. M. Mullen, S. C. Talmage, and C. D. Harvell. 2007. Exploring the role of chitinolytic enzymes in the sea fan coral, *Gorgonia ventalina*. Marine Biology **150**:1137-1144.

Dube, D., K. Kim, A. P. Alker, and C. D. Harvell. 2002. Size structure and geographic variation in chemical resistance of sea fan corals *Gorgonia ventalina* to a fungal pathogen. Marine Ecology Progress Series **231**:139-150.

Ebert, D. 1994. Virulence and local adaptation of a horizontally transmitted parasite. Science **265**:1084-1086.

Geiser, D. M., J. W. Taylor, K. B. Ritchie, and G. W. Smith. 1998. Cause of sea fan death in the West Indies. Nature **394**:137-138.

Harvell, C. D., K. Kim, J. M. Burkholder, R. R. Colwell, P. R. Epstein, D. J. Grimes, E. E. Hofmann, E. K. Lipp, A. D. M. E. Osterhaus, R. M. Overstreet, J. W. Porter, G. W. Smith, and G. R. Vasta. 1999. Emerging marine diseases: Climate links and anthropogenic factors. Science **285**:1505-1510.

Harvell, C. D., C. E. Mitchell, J. R. Ward, S. Altizer, A. P. Dobson, R. S. Ostfeld, and M. D. Samuel. 2002. Climate warming and disease risks for terrestrial and marine biota. Science **296**:2158-2162.

Harvell, D., K. Kim, C. Quirolo, J. Weir, and G. Smith. 2001. Coral bleaching and disease: Contributors to 1998 mass mortality in *Briareum asbestinum* (Octocorallia, Gorgonacea). Hydrobiologia **460**:97-104.

Jolles, A. E., P. Sullivan, A. P. Alker, and C. D. Harvell. 2002. Disease transmission of aspergillosis in sea fans: Inferring process from spatial pattern. Ecology **83**:2373-2378.

Kim, K. 1994. Antimicrobial activity in gorgonian corals (Coelenterata, Octocorallia). Coral Reefs **13**:75-80.

Kim, K., A. P. Alker, K. Shuster, C. Quirolo, and C. D. Harvell. 2006. Longitudinal study of aspergillosis in sea fan corals. Diseases of Aquatic Organisms **69**:95-99.

Kim, K., and C. D. Harvell. 2002. Aspergillosis of sea fan corals: Dynamics in the Florida Keys. Pages 813-824 *in* J. W. Porter and K. G. Porter, editors. The everglades, Florida Bay, and coral reefs of the Florida Keys: An ecosystem sourcebook. CRC Press, Boca Raton, FL.

Kim, K., and C. D. Harvell. 2004. The rise and fall of a six-year coral-fungal epizootic. The American Naturalist **164**:S52-S63.

Kim, K., C. D. Harvell, P. D. Kim, G. W. Smith, and S. M. Merkel. 2000a. Fungal disease resistance of Caribbean sea fan corals (*Gorgonia* spp.). Marine Biology **136**:259-267.

Kim, K., P. D. Kim, A. P. Alker, and C. D. Harvell. 2000b. Chemical resistance of gorgonian corals against fungal infections. Marine Biology **137**:393-401.

Kuta, K. G., and L. L. Richardson. 2002. Ecological aspects of black band disease of corals: Relationships between disease incidence and environmental factors. Coral Reefs **21**:393-398.

Latgé, J.-P. 1999. *Aspergillus fumigatus* and aspergillosis. Clinical Microbiology Reviews **12**:310-350.

Lively, C. M., and M. F. Dybdahl. 2000. Parasite adaptation to locally common host genotypes. Nature **405**:679-681.

Malmstrøm, J., S. C. Polson, S. W. Polson, G. W. Smith, and J. C. Frisvad. 2001. Study of secondary metabolites associated with virulent and non-virulent strains of *Aspergillus sydowii*: Sea fan pathogen. Pages 48-52 *in* Proceedings of the Symposium on the Natural History of the Bahamas. Gerace Research Center, San Salvador, Bahamas.

Mullen, K. M., C. D. Harvell, A. P. Alker, D. Dube, E. Jordán-Dahlgren, J. R. Ward, and L. E. Petes. 2006. Host range and antifungal resistance to aspergillosis in three sea fan species from the Yucatan. Marine Biology **149**:1355-1364.

Mullen, K. M., E. C. Peters, and C. D. Harvell. 2004. Coral resistance to disease. Pages 377-400 *in* E. Rosenberg and Y. Loya, editors. Coral Health and Disease. Springer-Verlag New York, Incorporated, New York.

Munkvold, G. P. 2003. Cultural and genetic approaches to managing mycotoxins in maize. Annual Review of Phytopathology **41**:99-116.

Mydlarz, L. D., and C. D. Harvell. 2007. Peroxidase activity and inducibility in the sea fan coral exposed to a fungal pathogen. Comparative Biochemistry and Physiology, Part A **146**:54-62.

Mydlarz, L. D., S. F. Holthouse, E. C. Peters, and C. D. Harvell. in review. Sea fan coral resistance to disease II: Amoebocyte aggregation as a cellular response to fungal invasion.

Mydlarz, L. D., L. E. Jones, and C. D. Harvell. 2006. Innate immunity, environmental drivers, and disease ecology of marine and freshwater invertebrates. Annual Review of Ecology, Evolution, and Systematics **37**:251-288.

Nagelkerken, I., K. Buchan, G. W. Smith, K. Bonair, P. Bush, J. Garzon-Ferreira, L. Botero, P. Gayle, C. D. Harvell, C. Heberer, K. Kim, C. Petrovic, L. Pors, and P. Yoshioka. 1997a. Widespread disease in Caribbean sea fans: II. Patterns of infection and tissue loss. Marine Ecology Progress Series **160**:255-263.

Nagelkerken, I., K. Buchan, G. W. Smith, K. Bonair, P. Bush, J. Garzon-Ferreira, L. Botero, P. Gayle, C. Heberer, C. Petrovic, L. Pors, and P. Yoshioka. 1997b. Widespread disease in Caribbean sea fans: I. Spreading and general characteristics. Proceedings of the 8th International Coral Reef Symposium **1**:679-682.

Olufemi, B. E., C. Agius, and R. J. Roberts. 1983. Aspergillomycosis in intensively cultured tilapia from Kenya. The Veterinary Record **112**:203-204.

Patterson, K. L., J. W. Porter, K. B. Ritchie, S. W. Polson, E. Mueller, E. C. Peters, D. L. Santavy, and G. W. Smith. 2002. The etiology of white pox, a lethal disease of the Caribbean elkhorn coral, *Acropora palmata*. Proceedings of the National Academy of Sciences **99**:8725-8730.

Petes, L. E., C. D. Harvell, E. C. Peters, M. A. H. Webb, and K. M. Mullen. 2003. Pathogens compromise reproduction and induce melanization in Caribbean sea fans. Marine Ecology Progress Series **264**:167-171.

Pier, A. C., and J. L. Richard. 1992. Mycoses and mycotoxicoses of animals caused by Aspergilli. Pages 233-248 *in* J. W. Bennett and M. A. Klich, editors. *Aspergillus*: Biology and Industrial Applications. Butterworth-Heinemann, Boston, MA.

Rosenberg, E., and L. Falkovitz. 2004. The *Vibrio shiloi*/*Oculina patagonica* model system of coral bleaching. Annual Review of Microbiology **58**:143-159.

Roth Jr., F. J., P. A. Orpurt, and D. G. Ahearn. 1964. Occurrence and distribution of fungi in a subtropical marine environment. Canadian Journal of Botany **42**:375-383.

Shinn, E. A., G. W. Smith, J. M. Prospero, P. Betzer, M. L. Hayes, V. Garrison, and R. T. Barber. 2000. African dust and the demise of Caribbean coral reefs. Geophysical Research Letters **27**:3029-3032.

Smith, G. W., L. D. Ives, I. A. Nagelkerken, and K. B. Ritchie. 1996. Caribbean sea-fan mortalities. Nature **383**:487.

Sparrow Jr., F. K. 1937. The occurrence of saprophytic fungi in marine muds. Biological Bulletin **73**:242-248.

Sutherland, K. P., J. W. Porter, and C. Torres. 2004. Disease and immunity in Caribbean and Indo-Pacific zooxanthellate corals. Marine Ecology Progress Series **266**:273-302.

Sweeney, J. C., G. Migaki, P. M. Vainik, and R. H. Conklin. 1976. Systemic mycoses in marine mammals. Journal of the American Veterinary Medical Association **169**:946-948.

Thrall, P. H., and J. J. Burdon. 2003. Evolution of virulence in a plant host-pathogen metapopulation. Science **299**:1735-1737.

Voss, J. D., and L. L. Richardson. 2006a. Coral diseases near Lee Stocking Island, Bahamas: Patterns and potential drivers. Diseases of Aquatic Organisms **69**:33-40.

Voss, J. D., and L. L. Richardson. 2006b. Nutrient enrichment enhances black band disease progression in corals. Coral Reefs **25**:569-576.

Ward, J. R., K. Kim, and C. D. Harvell. 2007. Temperature affects coral disease resistance and pathogen growth. Marine Ecology Progress Series **329**:115-121.

CHAPTER 2

GLOBALLY PANMICTIC POPULATION STRUCTURE IN THE OPPORTUNISTIC FUNGAL PATHOGEN *ASPERGILLUS SYDOWII*

ABSTRACT

Recent outbreaks of new diseases in many ecosystems are caused by novel pathogens, impaired host immunity, or changing environmental conditions. Identifying the source of emergent pathogens is critical for mitigating the impacts of diseases, and understanding the causes of their recent appearances. One ecosystem suffering recent outbreaks of disease is coral reefs, where pathogens such as the fungus *Aspergillus sydowii* have caused catastrophic population declines. Aspergillosis is one of the best-characterized coral diseases, yet the origin of this normally terrestrial fungal decomposer in marine systems remains unknown. We examined the genetic structure of a global sample of *A. sydowii*, including isolates from diseased corals, diseased humans, and environmental sources. Twelve microsatellite markers reveal a pattern of global panmixia among the fungal isolates. Low to moderate differentiation between sea fan pathogens and environmental isolates is likely due to differential success or failure of isolates within the marine environment. The land-sea barrier does not provide a substantial barrier against gene flow based on the high allelic richness and genotypic diversity among sea fan isolates, and the lack of evidence for a recent bottleneck. A neighbor-joining phylogeny shows that sea fan isolates are interspersed with environmental isolates, suggesting there have been multiple introductions from land into the ocean. Overall, our results underscore that *A. sydowii* is a true opportunist, with a diversity of non-related isolates able to cause disease in corals.

INTRODUCTION

Coral reefs have been decimated worldwide over recent decades as a result of climate change, overfishing, pollution, and disease outbreaks (Hughes 1994, Wilkinson 2002, Gardner et al. 2003, Hughes et al. 2003, Sutherland et al. 2004). In the Caribbean, gorgonian corals, the dominant taxa on these reefs (Chiappone and Sullivan 1994), have suffered declines due to a disease caused by the fungus *Aspergillus sydowii* (Geiser et al. 1998b, Kim and Harvell 2004). Despite being one of the best-studied coral diseases, the pathogen origin, mode of transmission, and mechanisms of pathogenicity remain unknown, hampering remediation and prevention of future disease outbreaks in these fragile ecosystems.

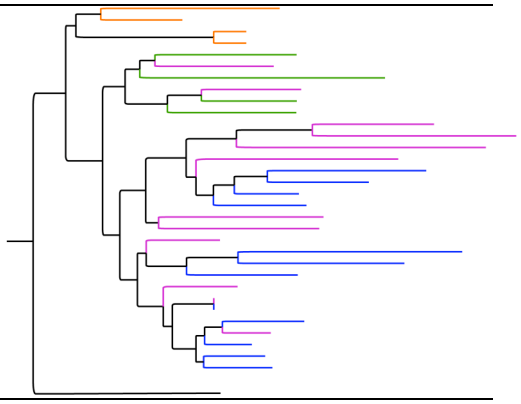
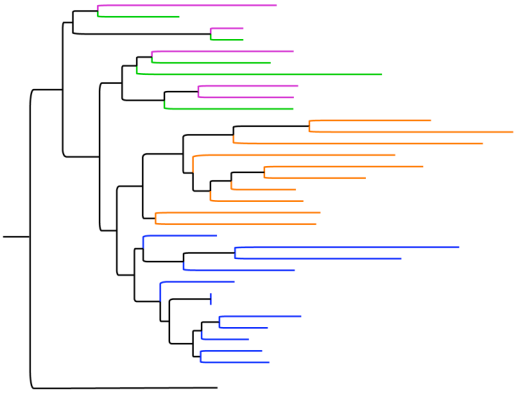
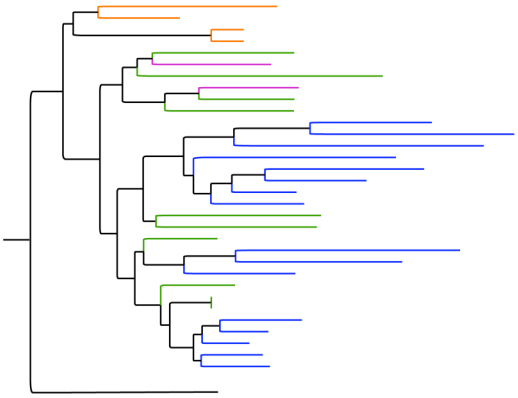
Aspergillosis of sea fan corals was first described in 1995 (Nagelkerken et al. 1997a, Nagelkerken et al. 1997b), although anecdotal reports date to the 1980s (Guzmán and Cortés 1984). The pathogen, *A. sydowii*, was identified and Koch's postulates were fulfilled in 1998 (Geiser et al. 1998b). *Aspergillus sydowii* is a globally distributed decomposer most commonly found in soil (Raper and Fennell 1965, Klich 2002). There are several hypotheses to explain the origins of this normally terrestrial fungus in marine systems. The Endemic Marine hypothesis suggests that this fungus has always existed for an indefinite time in the ocean, and has only recently been able to cause disease due to changes in the environment or host immunity. Support for this hypothesis includes evidence of *A. sydowii* in water samples prior to the epidemic (Sparrow Jr. 1937, Roth Jr. et al. 1964, Steele 1967), and a well-documented role of host immunity and environmental stress in mediating aspergillosis (Kim et al. 2000, Alker et al. 2001, Bruno et al. 2003, Ward et al. 2007). The African Dust hypothesis suggests that *A. sydowii* recently entered marine ecosystems from Saharan dust (Shinn et al. 2000, Garrison et al. 2003). Evidence for this hypothesis includes the culturing of *Aspergillus* from Saharan dust samples

(Kellogg et al. 2004), and the recent increase in deposition of Saharan dust in the Caribbean (Prospero and Nees 1986, Prospero and Lamb 2003). The Terrestrial Runoff hypothesis suggests that *A. sydowii*, a common terrestrial fungus, is entering marine ecosystems with local terrestrial runoff (Smith et al. 1996), and is also supported by the increase in terrestrial sediments entering the Caribbean due to coastal development (Burke and Maidens 2004).

Disease is an increasing concern in many ecosystems due to a rising number of outbreaks caused by apparently novel pathogens in the past few decades (Schrag and Wiener 1995, Harvell et al. 1999, Daszak et al. 2000, Dobson and Foufopoulos 2001, Anderson et al. 2004, Harvell et al. 2004). Similar to aspergillosis in Caribbean sea fans, the origins of these emerging pathogens are varied, and include endemic microorganisms facilitated by changing in host resistance or environmental conditions, or novel pathogens whose entry is mediated by human transport, climate change, host switches, or host movement (Lafferty et al. 2004). Identifying the source of emerging pathogens is difficult due to the lack of baseline data. Molecular techniques provide a means to work around an absence of samples from early in an epidemic, and patterns of genetic diversity can help narrow pathogen origins. For example, a recent study using microsatellite markers of the chytrid fungus *Batrachochytrium dendrobatidis*, responsible in part for global amphibian declines, revealed low genetic diversity and no relationship between genotype and geography, suggesting that this pathogen was recently introduced to North America (Morgan et al. 2007).

Here, we use a population genetic approach to identify the putative source of disease-causing isolates of *A. sydowii*. The three hypothesized origins (Endemic Marine, African Dust, or Terrestrial Runoff) generate a series of predicted genetic patterns (Table 2.1). The African Dust hypothesis generates the most distinct population genetic patterns, as it posits a single origin (or small number of entries)

Table 2.1. Expected patterns of genetic diversity (allelic richness & gene diversity), isolation by distance, and population structure for the three hypothesized sources of coral disease-causing *Aspergillus sydowii*: Endemic Marine, African Dust, and Terrestrial Runoff. For the predicted phylogenetic trees, colors indicate the source of fungal isolates: green = terrestrial isolates, blue = coral disease isolates, purple = endemic marine isolates, orange = African dust isolates.

Hypothesized Pathogen Source	Genetic Diversity	Isolation by Distance	Population Structure
Endemic Marine	- High richness & diversity - No bottleneck	- No	
African Dust (Single Origin)	- Low richness & diversity - Bottleneck	- Yes	
Terrestrial Runoff	- High richness & diversity - No bottleneck	- Maybe	

from a presumably geographically distinct population. Given the extreme conditions of the high altitudes at which dust clouds move, the deposition of *A. sydowii* isolates capable of infecting corals in the Caribbean would be a rare event. This would result in reduced genetic diversity and allelic richness, with evidence of a recent bottleneck in coral disease causing isolates (Table 2.1). In addition, isolation by distance would be expected among disease-causing *A. sydowii*, as these fungi would share a common evolutionary history. Differentiating between the Endemic Marine and Terrestrial Runoff hypotheses is more challenging, as their predicted population genetic patterns are not mutually exclusive (Table 2.1). Both sources would result in high genetic diversity and allelic richness in sea fan isolates, and depending on the evolutionary history of terrestrial and marine isolates, may also show isolation by distance. One pattern that may allow us to differentiate between the Endemic Marine and Terrestrial Runoff hypotheses is population structure with regards to geographic location. As there are many potential entry points for terrestrial sediment in the Caribbean, we would expect that sea fan isolates from a given geographic location would be closely related to environmental isolates from the same location. In contrast, the Endemic Marine hypothesis would generate less geographic structure. Ultimately, our ability to conclusively differentiate between these hypotheses will require isolates from all putative source pools, on land and in the sea.

Here, we describe the population genetic structure of *A. sydowii*, using a global sample of nearly all available isolates from diseased corals, infected humans, and environmental sources. Isolates were characterized using recently developed microsatellite markers (Rypien and Andras 2008) and polymorphic markers originally designed for *A. fumigatus* (Bart-Delabesse et al. 1998). Morphological measurements of colony growth and microscopic structures were taken as additional characters to distinguish isolates. Individual isolates were analyzed as a single population, and by

source to determine whether infectious isolates are different from environmental isolates. This detailed analysis using both morphological and molecular characters will distinguish among the three hypotheses about the origins of the pathogen responsible for sea fan aspergillosis in the Caribbean, and help to inform broader patterns of emergent disease in a diversity of ecosystems.

MATERIALS AND METHODS

Fungal Strains

Isolates of *A. sydowii* from five continents were gathered from environmental sources (N=16) and isolated from both diseased sea fans (N=14) and humans (N=4). An additional seven isolates lack information about either their origins or source, as this information was absent in culture collection records (Table 2.2). This collection of *A. sydowii* represents most of the known isolates of this species, including all isolates cultured from diseased sea fans, and all isolates housed in the USDA Agricultural Research Service Culture Collection. All isolates were stored in 10x phosphate buffered saline with 10% glycerol at -80°C. Isolates were grown on potato dextrose agar at 25°C for 7 days prior to DNA extraction.

DNA Extraction, Amplification, and Genotyping

Fungal tissue was lysed by vortexing with 425 - 600 μm glass beads (Sigma) for 30 seconds, followed by 30 seconds on ice, with this procedure repeated three times. Whole genomic DNA was extracted using phenol-chloroform (Sambrook and Russell 2001). Twelve microsatellite loci were amplified using polymerase chain reaction (PCR): three markers originally designed for *A. fumigatus* (Bart-Delabesse et al. 1998), and nine designed for *A. sydowii* (Appendix; Rypien and Andras 2008). PCR reactions were 10 μL total volume, containing approximately 10 ng genomic

Table 2.2. Isolates of *Aspergillus sydowii* used for molecular and morphological analysis. Superscript indicates source of fungal isolate: a) Dr. Garriet Smith, University of South Carolina Aiken, USA, b) Dr. C. Drew Harvell, Cornell University, USA, c) NRRL (Northern Regional Research Laboratory), currently the Agricultural Research Service Culture Collection, United States, Department of Agriculture, Peoria, IL, U.S.A., d) SRRC (Southern Regional Research Center), United States Department of Agriculture, New Orleans, LA, U.S.A.

Culture Collection Number & Source	Geographic Origin
INFECTIOUS – SEA FAN	
FK2A[a]	Key West, FL
FKrefIII[b]	Key West, FL
A2B[b]	Key West, FL
FK11 Florida[b]	Key West, FL
SS7[b]	San Salvador, Bahamas
SA-25 Saba[b]	Saba, Netherland Antilles
DumpD[b]	Dump Reef, San Salvador, Bahamas
SombA[b]	Sombrero Reef, FL
FK1[b]	Key West, FL
15B1[b]	Tennessee Reef, FL
16B1[b]	Tennessee Reef, FL
18WA1[b]	Tennessee Reef, FL
19B[b]	Tennessee Reef, FL
Mex 08/06[b]	Akumal, Mexico
INFECTIOUS - HUMAN	
NRRL254[c]	Georgia, USA
297072[c]	St. Paul, Minnesota
NRRL 32297[c]	Edmonton, Alberta, Canada
NRRL 253[c]	

Table 2.2 (Continued)

ENVIRONMENTAL	
Kir382A[a]	Orinoco River
SRRC2540[d]	Durban, South Africa
SRRC1112[d]	Australia
SRRC342[d]	
NRRL 5913[c]	
NRRL 4790[c]	Japan
NRRL1732[c]	Washington, DC
NRRL 249 [c]	Philadelphia
NRRL 245 [c]	Jamaica
NRRL 247 [c]	Florida, USA
NRRL 251 [c]	Sri Lanka
NRRL 663 [c]	
NRRL 242 [c]	Austria
NRRL 244 [c]	Japan
NRRL 520 [c]	
NRRL 4792 [c]	
UNKNOWN SOURCE	
NRRL 250 [c]	
NRRL 246 [c]	
NRRL 248 [c]	
NRRL 252 [c]	
NRRL 719 [c]	
NRRL 1268 [c]	
NRRL 4878 [c]	

DNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 μM dNTPs, 0.2 μM fluorescently labeled primer (Applied Biosystems), 0.2 μM unlabelled primer, and 0.25 U *Taq* polymerase (Roche). Locus AS93 amplified best at 2.0 mM $MgCl_2$. The PCR profile was 95°C for 5 min, 35 cycles of 95°C for 1 min, a specified annealing temperature for 1 min (as described in Rypien and Andras (2008) and Bart-Delabesse et al. (1998)), 72°C for 1 min, and a final extension at 72°C for 30 min. Fragment sizes were analyzed on an ABI 3100 automated capillary DNA sequencer using the GeneScan-500 LIZ size standard (Applied Biosystems). Allele sizes were determined using Genemapper version 3.5 (Applied Biosystems) and were verified by eye.

Genetic Diversity

We tested for evidence of linkage disequilibrium with FSTAT 2.9.3.2 (Goudet 1995) and GenePop on the web (Raymond and Rousset 1995), using a Markov chain method with 5,000 dememorization steps, 1,000 batches of 5,000 iterations, and a Bonferroni correction for multiple comparisons. We used two different methods to assess linkage disequilibrium because *A. sydowii* is a haploid fungus that reproduces predominantly asexually (Raper and Fennell 1965), making it likely that loci may be linked due to a lack of recombination. As the Bayesian analysis methods assume linkage equilibrium, we wanted to be sure that the loci did not violate this assumption.

FSTAT 2.9.3.2 was used to calculate expected heterozygosity (gene diversity, computed according to Nei (1987)), and allelic richness. Allelic richness was corrected for unequal sample sizes by rarefaction as described by El Mousadik and Petit (1996). Genotypic diversity (based on the number and frequency of multilocus genotypes), computed according to Nei and Tajima (1981), was calculated with MultiLocus 1.3b (Agapow and Burt 2001). Rarefied allelic richness and gene diversity were compared among source groups with ANOVA (JMP 6.0, Cary NC).

Population Differentiation

Population differentiation was assessed for *A. sydowii* isolates grouped by source (infectious - sea fan; infectious - human; environmental). Isolates could not be analyzed based on geographic origin due to the low number of independent replicates available within each region. Differentiation was estimated using Weir and Cockerham's (1984) θ (F_{ST}) and Rousset's (1996) ρ_{ST} (R_{ST}), with the standardization approach of Goodman (1997). Using FSTAT 2.9.3.2, population differentiation parameters were calculated, not assuming Hardy-Weinberg equilibrium, and significance was determined using permutation tests with 10,000 replicates following a Bonferroni adjustment.

Analysis of patterns of genetic and geographic variation was performed using Alleles in Space 1.0 (Miller 2005). We used three tests to examine the correlation between genetic and geographic distance: a Mantel test, spatial autocorrelation, and allelic aggregation. Mantel tests determine correlation between genetic and geographic distances. Spatial autocorrelation compares the average genetic distance between pairs of individuals over distance classes, and is summarized with the global statistic V. Allelic aggregation analysis tests for nonrandom patterns of genetic diversity across a landscape, and the index (R_j) ranges from -1 to 1, where $R_j < 1$ indicates aggregation, and $R_j > 1$ indicates spatial uniformity. For all analyses, significance was tested using 1,000 permutations.

The relationship between individual isolates was visualized using a neighbor-joining tree. We calculated the individual pairwise genetic distance based on the number of loci that differed using GenAlEx 6 (Peakall and Smouse 2006). We used Phylip 3.67 (Felsenstein 1989) to construct an un-rooted neighbor-joining tree using

the method of Saitou and Nei (1987) with a random input order of taxa. We repeated this process ten times to ensure that the addition order was not affecting tree topology.

Isolates from diseased sea fans were tested for evidence of a bottleneck using BOTTLENECK version 1.2.02 (Piry et al. 1999). Populations that have undergone a recent bottleneck show a reduction in allelic richness and heterozygosity, with richness being reduced faster. Thus, the observed heterozygosity in these populations is greater than expected given the number of alleles present. We compared observed heterozygosity (gene diversity) to expected heterozygosity simulated using a coalescent process under both a stepwise mutation model and a two-phased model with 1,000 iterations. A Wilcoxon sign-rank test was used to determine significance.

Population Structure

As we had no *a priori* indication of the number of populations in the dataset, we used two Bayesian methods to analyze population structure: STRUCTURE 2.2 (Pritchard et al. 2000) and STRUCTURAMA 1.0 (Huelsenbeck and Andolfatto 2007, Huelsenbeck et al. submitted). Both programs use Bayesian clustering to estimate population structure without prior information about the source of sampled individuals. STRUCTURE estimates the posterior probability that individuals belong to K clusters, where K is given. STRUCTURAMA treats K as a random variable, and hence estimates the number of populations in a sample. In STRUCTURE, we determined the posterior probability of K for K = 1 to 5 using Markov chain Monte Carlo (MCMC) sampling methods, with 10 independent MCMC runs of 3,000,000 steps following a 500,000 step burn-in. We assumed correlated allele frequencies among populations, did not use information on the population of origin, and followed an admixture model with a single value of lambda inferred for all populations. We estimated K based on the log likelihood score and posterior probability of K (Pritchard

et al. 2007). In STRUCTURAMA 1.0, we inferred the number of genetic clusters (K), with the prior value of K set as a random variable, using two independent MCMC runs of 2,000,000 steps, with samples taken every 1,000 generations, and the first third of samples removed as burn-in. The MCMC analysis was summarized using a mean partition that minimizes the squared distance to all of the sampled partitions, and the posterior probability of each K (Huelsenbeck et al. submitted).

Morphology

We collected morphological data for a series of characters commonly used to identify *Aspergillus* species (Klich 2002). These included colony growth rate on three different media (Czapek Yeast Agar (CYA), Malt Extract Agar (MEA), Czapek Yeast Agar 20% Sucrose (CY20S)) at two different temperatures (25°C, 37°C), with colonies measured following seven days of growth in the dark. Colony color and the production of exudates and pigment exuded into the media were also noted. Microscopic measurements were made at 100x, and included the diameter of conidia, stipes, and vesicles, as well as conidia color and surface texture. Measurements were made on fungal tissue sampled from the growing edge of a colony on MEA media following seven days of growth. A minimum of three replicate measurements were taken for each morphological character.

For statistical analysis, quantitative microscopic morphological characters (conidia, stipe and vesicle diameter) and growth rate (CYA at 25°C, CYA at 37°C, MEA at 25°C, CY20S at 25°C) were analyzed in separate MANOVAs (JMP 6.0, Cary NC), with isolates grouped by source. Prior to analysis, data were checked for both univariate and multivariate normality (Mardia 1974), and standardized as Z-scores for the multivariate analysis (SAS 9.1, SAS Institute, Inc.).

RESULTS

Genetic Diversity

After Bonferroni corrections, three pairs of loci showed significant linkage disequilibrium in GenePop (AS203 + AS214, AS206 + AS251, AS210 + AS262; adj alpha = 0.0007578). However, according to FSTAT, none of the loci were linked. To determine if the linkage identified by GenePop would affect the results of Bayesian analysis of population structure, we performed an additional analysis in STRUCTURE using the nine unlinked loci, and found that the results were unchanged. Thus, for all remaining analyses, we used all twelve loci.

Overall, allelic richness varied from 2 to 11 alleles per locus (average 5.8), and gene diversity (expected heterozygosity) ranged from 0.148 to 0.799. Null alleles were rare, with total amplification for all individuals at eight of the loci, and low frequencies of null alleles in the remaining four loci (AfumD 5%; AS93 5%; AS206 2%; AS251 5%).

Genotypic diversity of source-based populations was extremely high due to the large number of unique clonal haplotypes (Table 2.3). Gene diversity and rarefied allelic richness were not significantly different among source groups (diversity ANOVA p = 0.890; richness Kruskal-Wallis p = 0.387; Table 2.3).

Population Differentiation

Population differentiation, as measured by F_{ST} and R_{ST}, revealed low to moderate levels of differentiation among isolates from different sources (overall R_{ST} = 0.0536, F_{ST} = 0.083), with significant differentiation between isolates from diseased sea fans and those from environmental sources (F_{ST} = 0.1064, p = 0.0167).

There is no evidence of isolation by distance for isolates with recorded geographic locations (N = 27) based on both the Mantel test (r = 0.1013, p = 0.138)

Table 2.3. Genotypic and gene diversity for *Aspergillus sydowii* grouped according to source. Genotypic diversity calculated according to Nei and Tajima (1981), and gene diversity calculated according to Nei (1987). N = number of isolates. Neither gene diversity ($p = 0.890$) nor allelic richness ($p = 0.387$) were significantly different among groups.

Source	**N**	**Rarefied Allelic Richness**	**Genotypic Diversity**	**Gene Diversity**
Infectious - Sea fan	14	1.85	0.989	0.479
Infectious - human	4	1.64	1.000	0.500
Environmental	16	2.08	0.992	0.470
Unknown	7	3.33	1.000	0.518
Overall	41	2.23	0.998	0.507

and spatial autocorrelation (V = 0.04501, p = 0.619). The allelic aggregation index suggests a random distribution of alleles across space (R_j = 1.012, p = 0.21). Within isolates causing disease in corals (N=14), there is also no evidence of isolation by distance (Mantel r = 0.080, p = 0.307; autocorrelation V = 0.065, p = 0.779; allelic aggregation R_j = 0.985, p = 0.213).

An un-rooted neighbor-joining tree based on pairwise individual genetic distance showed no significant population structure with regard to the source of fungal isolates (Figure 2.1). When examining the neighbor-joining tree coded by the geographic location where isolates were collected, a similar lack of structure is evident (Figure 2.2).

There was no evidence of a bottleneck in isolates from diseased sea fans. The Wilcoxon sign-rank test found no significant difference between the observed and expected gene diversity under either mutation model (SMM p = 0.151; TPN p = 0.339).

Population Structure

Bayesian analysis of population struture revealed a single genetic cluster for *A. sydowii* (STRUCTURE Pr(K = 1) = 1.0; STRUCTURAMA Pr[K = 1 | X] = 0.8202), suggesting that sufficient gene flow exists among the 41 individual isolates to maintain a single panmictic population.

Morphology

Most of the morphological characters were extremely consistent among isolates regardless of source (Table 2.4, 2.5). *Aspergillus sydowii* from all source groups produced a striking red-orange pigment. The chemical identity of these exuded

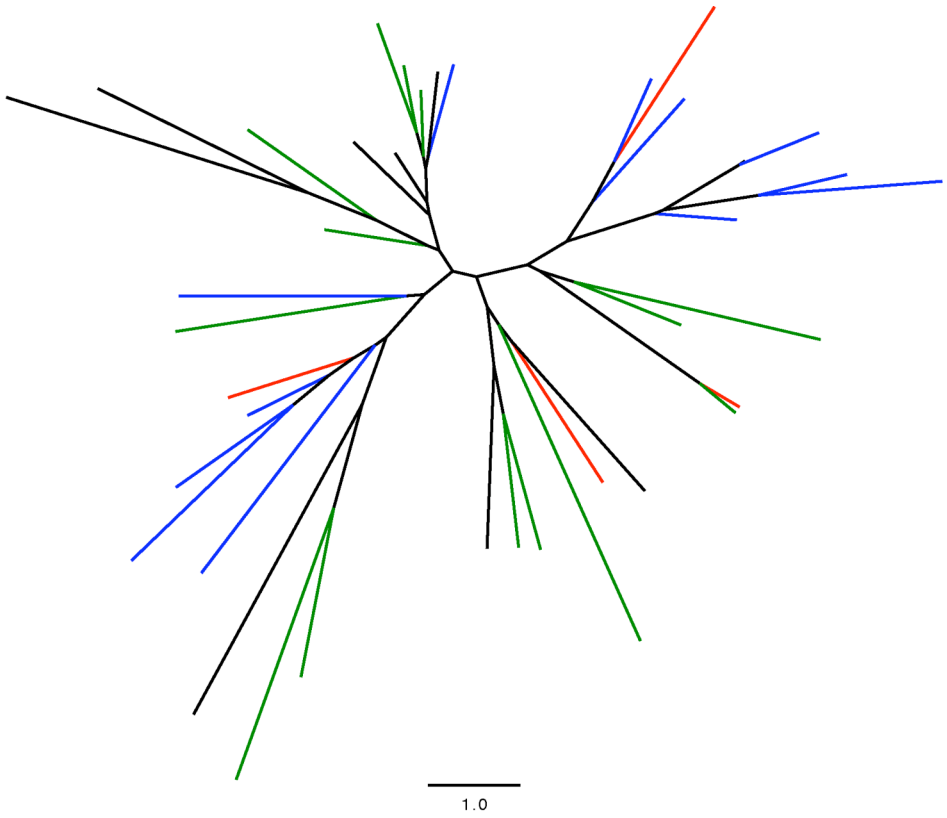


Figure 2.1. An un-rooted neighbor-joining tree of *Aspergillus sydowii* isolates based on pairwise individual genetic distance. Branches are colored according to source (blue = infectious - sea fan; red = infectious – human; green = environmental; black = unknown).

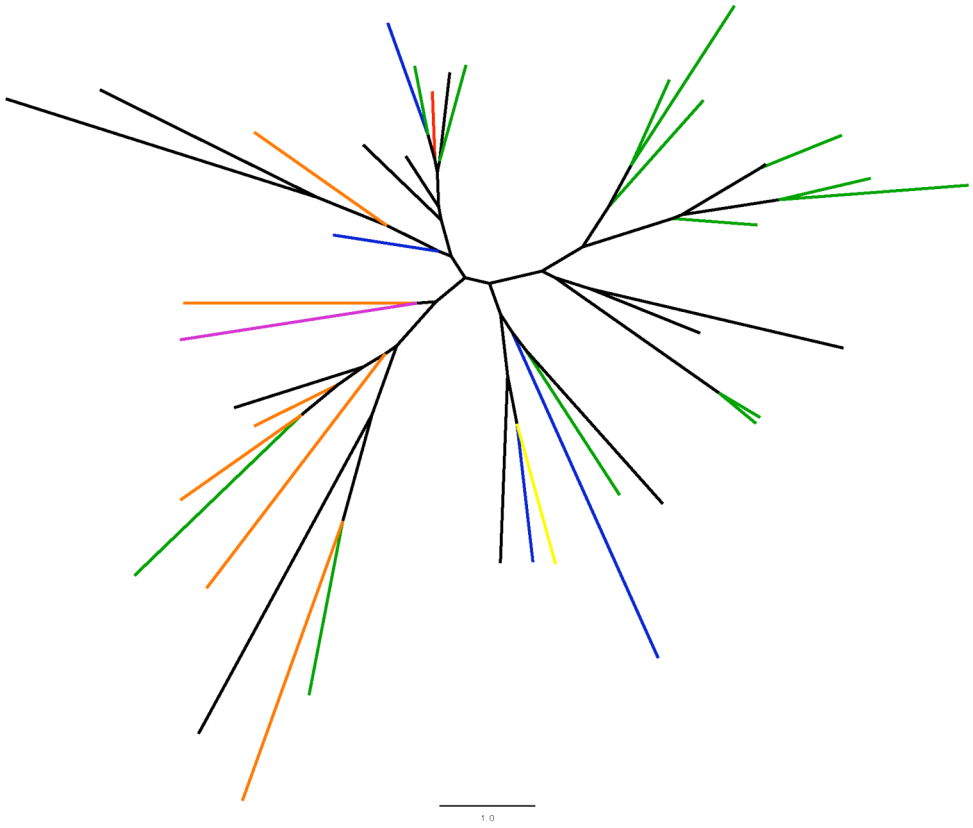


Figure 2.2. An un-rooted neighbor-joining tree of *Aspergillus sydowii* isolates based on pairwise individual genetic distance. Branches are colored according to geographic location (green = North America; red = Europe; blue = Asia; purple = Australia; yellow = Africa; orange = Caribbean; black = unknown).

Table 2.4. Comparison of microscopic morphology (100x) of *Aspergillus sydowii* isolates grouped by source. Mean (± standard error) based on triplicate measurements of fungi grown for seven days at 25°C on Malt Extract Agar (MEA).

Source	**Conidia Shape**	**Conidia Diameter (μm)**	**Conidia Texture**	**Vesicle Diameter (μm)**	**Stipe Diameter (μm)**
Infection - sea fan (N = 14)	Round	3.17 ± 0.08	Rough	9.34 ± 0.60	4.28 ± 0.18
Infection - human (N = 4)	Round	3.29 ± 0.13	Rough	9.88 ± 1.11	4.58 ± 0.11
Environmental (N = 16)	Round	3.15 ± 0.06	Rough	8.72 ± 0.53	3.91 ± 0.20

Table 2.5. Comparison of colony growth rates of *Aspergillus sydowii* isolates grouped by source. Mean growth rate per day (mm/day) (± standard error) following growth for seven days on three types of media (Czapek Yeast Agar (CYA), Malt Extract Agar (MEA), Czapek Yeast Agar 20% Sucrose (CY20S)) at two different temperatures (25°C, 37°C). The production of an extracellular pigment was also recorded on colonies grown on CYA at 25°C for seven days.

	Colony Growth Rate (mm/day)				**Extracellular Pigment**
Source	**CYA 25°C**	**MEA 25°C**	**CY20S 25°C**	**CYA 37°C**	
Infection - sea fan (N = 14)	3.18 ± 0.12	3.22 ± 0.12	3.43 ± 0.15	1.00 ± 0.14	Red/orange
Infection - human (N = 4)	3.40 ± 0.24	3.62 ± 0.18	3.88 ± 0.32	0.62 ± 0.36	Red/orange
Environmental (N = 16)	3.37 ± 0.11	3.28 ± 0.15	3.87 ± 0.20	0.52 ± 0.13	Red/orange

compounds is unknown, however the exudates did not contain the common *Aspergillus* toxin, sterigmatocystin (Rypien, unpublished data).

Three variables were not normally distributed (growth rate on MEA, growth rate on CYA at 37°C, conidia diameter). Growth rate on MEA and conidia diameter were normalized using a Box-Cox transformation, however transformation for growth rate on CYA at 37°C was not successful. Both the microscopic morphological variables and colony growth rates were multivariately normal.

Results of the MANOVA using an Identity Response Design with individuals grouped by source (infectious - sea fan, infectious - human, environmental) for microscopic characters showed no significant difference in the multivariate response variables among groups (Wilk's lambda p = 0.6785; Pillai's Trace p = 0.6662; Hoetelling-Lawley p = 0.6912; Roy's Max root p = 0.3690). Similarly, the results of the MANOVA for colony growth rate also showed no difference in multivariate response variables among source groups (Wilk's lambda p = 0.2445; Pillai's Trace p = 0.2535; Hoetelling-Lawley p = 0.2378; Roy's Max root p = 0.0608).

DISCUSSION

The isolates of *A. sydowii* analyzed in this study reveal a single global population, with sufficient gene flow to prevent substantial differentiation across geographic locations or sources. Analysis of morphological characters also showed strikingly low variation between isolates (Table 2.4, 2.5). These results are surprising because molecular analysis of other widely distributed fungi have found multiple phylogenetic species with restricted geographic distributions (Koufopanou et al. 1997, Geiser et al. 1998a, Kasuga et al. 2003). However, *A. fumigatus*, an opportunistic pathogen of humans, is also a globally panmictic species (Pringle et al. 2005),

suggesting that perhaps commonalities of life history and modes of infection have led to similar patterns of population genetics in these two species.

Despite sufficient gene flow among isolates preventing the formation of distinct genetic demes, F_{ST} values indicate modest differentiation between *A. sydowii* isolated from diseased sea fans and from environmental sources. This pattern could be the result of a variety of processes, including current or historic barriers to dispersal. The most obvious barrier is the land-sea interface; however, the repeated isolation of *A. sydowii* from ocean water samples (Sparrow Jr. 1937, Roth Jr. et al. 1964, Steele 1967) suggests that there is good connectivity between terrestrial and aquatic systems. In addition, there was no evidence of a bottleneck in sea fan isolates, and individuals showed similar allelic richness and genetic diversity to environmental isolates (Table 2.3). The neighbor-joining phylogeny shows that sea fan isolates are interspersed with environmental isolates (Figure 2.1), suggesting that there have been multiple introductions into the ocean.

Another process that could result in the observed differentiation between sea fan and environmental isolates is local adaptation to the marine environment despite ongoing gene flow. This could include adaptation to local conditions (ie. salinity, temperature), as well as adaptation to the barrage of defenses produced by the coral host. Previous studies have demonstrated that *A. sydowii* grows optimally at 30°C (Alker et al. 2001), a temperature near the thermal limits of most corals (Hoegh-Guldberg 1999), which is known to impair constitutive defenses against fungal pathogens in sea fans (Alker et al. 2001). Thus, differential success of fungi that can tolerate conditions in the marine environment could drive the moderate differentiation indicated by F_{ST} values.

The population genetic patterns of *A. sydowii* allow some conclusions to be drawn about the origin of this emergent pathogen. Evidence of panmixia and lack of

isolation by distance suggest that a single origin of the pathogen is unlikely. However, these patterns could also result from a relatively recent invasion of this pathogen into the ocean. Given the high allelic richness and genetic diversity and lack of evidence of a recent bottleneck in the sea fan populations, a recent introduction into the ocean seems unlikely. Therefore, the data do not support the African Dust hypothesis. An examination of neighbor-joining phylogenies (Figures 2.1, 2.2) reveals that *A. sydowii* causing disease in corals do not form a distinct clade, and their closest relatives vary by both their source and geographic location. The evidence of a single global panmictic population in this species implies that any isolate has the potential of reaching marine ecosystems and causing disease there. Not all isolates may be equally successful in establishing in the marine environment, but evidence of high gene flow and repeated historic isolations of *A. sydowii* from marine sources suggests that fungi causing disease in corals are most likely from Endemic Marine or Terrestrial Runoff source pools. Unfortunately, to conclusively differentiate between the three hypothesized sources of pathogenic *A. sydowii*, we require fungal isolates cultured from all source pools. We are currently lacking isolates from both African dust (Chapter 3, Griffin et al. 2003, Shinn et al. 2003, Kellogg et al. 2004, Weir-Bush et al. 2004) and marine sources prior to the epidemic. Overall, these results do not identify a single source, but instead suggest that *A. sydowii* is an opportunistic pathogen with ongoing gene flow between widely dispersed geographic regions.

Patterns of population differentiation and genetic diversity observed in this study are strikingly similar to that of another opportunistic pathogen, *A. fumigatus*. This is the most common airborne fungal pathogen of humans in recent decades, largely due to the increase in the number of immunosuppressed patients (Latgé 1999). Considerable effort has been directed towards identifying virulence factors in the isolates of this fungus, with little success (Latgé 1999). One explanation for the lack

of specific pathogenicity genes is the absence of genetic differentiation between infectious and environmental isolates, such that any isolate is capable of infecting a human with a sufficiently weakened immune system (Debeaupuis et al. 1997, Pringle et al. 2005). In fact, several other *Aspergillus* species are capable of infecting immune-compromised hosts, including humans (Latgé 1999), fish (Olufemi et al. 1983), marine mammals (Sweeney et al. 1976), insects (Pier and Richard 1992), and plants (Munkvold 2003). Thus, a more general trend emerges, whereby fungi such as *Aspergillus* are capable of causing emergent infectious diseases in hosts (especially those with compromised immune systems) due to their high phenotypic plasticity and evolutionary potential, and it is these traits, rather than any specific virulence factor, that confer the ability to cause disease.

That *A. sydowii* appears to be an opportunistic pathogen of corals should not deter continued research on its epidemiology. In fact, if its congener *A. fumigatus* is any indication, extensive information can be gained on the functioning of invertebrate immune systems regardless of whether pathogens are opportunistic or obligate. What should be avoided are time-intensive searches for co-evolved immune and virulence traits as general immune responses are likely to be more important in this disease system.

The low level of differentiation among isolates of *A. sydowii* from both infectious and environmental sources has important disease management implications. Because this fungus is a globally distributed saprophyte, limiting its entry into marine ecosystems would be extremely difficult. Its presence in a diversity of substrates (terrestrial sediments, oceanic, Saharan dust) suggests that it is probably present and has been for many years in most marine ecosystems. As disease-causing isolates are not genetically distinct from environmental isolates, the existence of specific virulence factors seems unlikely, and it should be assumed that any isolate of *A. sydowii* can

cause aspergillosis. Thus, variation in disease prevalence is more likely due to site-dependent factors such as host density (Jolles et al. 2002), temperature (Ward et al. 2007), nutrient levels (Bruno et al. 2003, Baker et al. in press), and water movement (Kim and Harvell 2002), as well as variation in the ability of hosts to resist infection (Dube et al. 2002, Kim and Harvell 2004). Coral reefs are an ecosystem under threat from numerous stressors, making them susceptible to infection by any number of potential pathogens. Active management of these external stressors, such as nutrient inputs and sedimentation, as well as climate management are the only logical solutions to mitigate the effects of this coral disease.

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REFERENCES

Agapow, P.-M., and A. Burt. 2001. Indices of multilocus linkage disequilibrium. Molecular Ecology Notes **1**:101-102.

Alker, A. P., G. W. Smith, and K. Kim. 2001. Characterization of *Aspergillus sydowii* (Thom et Church), a fungal pathogen of Caribbean sea fan corals. Hydrobiologia **460**:105-111.

Anderson, P. K., A. A. Cunningham, N. G. Patel, F. J. Morales, P. R. Epstein, and P. Daszak. 2004. Emerging infectious diseases of plants: pathogen pollution, climate change and agrotechnology drivers. Trends in Ecology & Evolution **19**:535-554.

Baker, D. M., S. E. MacAvoy, and K. Kim. in press. Relationship between water quality, 15N, and aspergillosis of Caribbean sea fan corals. Marine Ecology Progress Series.

Bart-Delabesse, E., J. F. Humbert, E. Delabesse, and S. Bretagne. 1998. Microsatellite markers for typing *Aspergillus fumigatus* isolates. Journal of Clinical Microbiology **36**:2413-2418.

Bruno, J. F., L. E. Petes, C. D. Harvell, and A. Hettinger. 2003. Nutrient enrichment can increase the severity of coral diseases. Ecology Letters **6**:1056-1061.

Burke, L., and J. Maidens. 2004. Reefs at Risk in the Caribbean. World Resources Institute, Washington, DC.

Chiappone, M., and K. M. Sullivan. 1994. Ecological structure and dynamics of nearshore hard-bottom communities in the Florida Keys. Bulletin of Marine Science **54**:747-756.

Daszak, P., A. A. Cunningham, and A. D. Hyatt. 2000. Emerging infectious diseases of wildlife - Threats to biodiversity and human health. Science **287**:443-449.

Debeaupuis, J.-P., J. Sarfati, V. Chazalet, and J.-P. Latagé. 1997. Genetic diversity among clinical and environmental isolates of *Aspergillus fumigatus*. Infection and Immunity **65**:3080-3085.

Dobson, A., and J. Foufopoulos. 2001. Emerging infectious pathogens of wildlife. Philosophical Transactions of the Royal Society of London B Biological Sciences **356**:1001-1012.

Dube, D., K. Kim, A. P. Alker, and C. D. Harvell. 2002. Size structure and geographic variation in chemical resistance of sea fan corals *Gorgonia ventalina* to a fungal pathogen. Marine Ecology Progress Series **231**:139-150.

El Mousadik, A., and R. J. Petit. 1996. High level of genetic differentiation for allelic richness among populations of the argan tree (*Argania spinosa* (L.) Skeels) endemic to Morocco. Theoretical and Applied Climatology **92**:832-839.

Felsenstein, J. 1989. PHYLIP - Phylogeny inference package (version 3.2). Cladistics **5**:164-166.

Gardner, T. A., I. M. Côte, J. A. Gill, A. Grant, and A. W. Watkinson. 2003. Long-term region-wide declines in Caribbean corals. Science **301**:958-960.

Garrison, V. H., E. A. Shinn, W. T. Foreman, D. W. Griffin, C. W. Holmes, C. A. Kellogg, M. S. Majewski, L. L. Richardson, K. B. Ritchie, and G. W. Smith. 2003. African and Asian dust: from desert soils to coral reefs. BioScience **53**:469-480.

Geiser, D. M., J. I. Pitt, and J. W. Taylor. 1998a. Cryptic speciation and recombination in the aflatoxin-producing fungus *Aspergillus flavus*. Proceedings of the National Academy of Sciences **95**:388-393.

Geiser, D. M., J. W. Taylor, K. B. Ritchie, and G. W. Smith. 1998b. Cause of sea fan death in the West Indies. Nature **394**:137-138.

Goodman, S. J. 1997. Rst Calc: A collection of computer programs for calculating estimates of genetic differentiation from microsatellite data and determining their significance. Molecular Ecology **6**:881-885.

Goudet, J. 1995. FSTAT (Version 1.2): A computer program to calculate F-statistics. Journal of Heredity **86**:485-486.

Griffin, D. W., C. A. Kellogg, V. H. Garrison, J. T. Lisle, T. C. Borden, and E. A. Shinn. 2003. Atmospheric microbiology in the northern Caribbean during African dust events. Aerobiologia **19**:143-157.

Guzmán, H. M., and J. Cortés. 1984. Mortandad de *Gorgonia flabellum* innaeus (Octocorallia: Gorgoniidae) en las Costa Caribe de Costa Rica. Revista de Biologia Tropical **32**:305-308.

Harvell, C. D., K. Kim, J. M. Burkholder, R. R. Colwell, P. R. Epstein, D. J. Grimes, E. E. Hofmann, E. K. Lipp, A. D. M. E. Osterhaus, R. M. Overstreet, J. W. Porter, G. W. Smith, and G. R. Vasta. 1999. Emerging marine diseases: Climate links and anthropogenic factors. Science **285**:1505-1510.

Harvell, D., R. Aronson, N. Baron, J. Connell, A. Dobson, S. Ellner, L. Gerber, K. Kim, A. Kuris, H. McCallum, K. Lafferty, B. McKay, J. Porter, M. Pascual, G. Smith, K. Sutherland, and J. Ward. 2004. The rising tide of ocean diseases: unsolved problems and research priorities. Frontiers in Ecology and Evolution **2**:375-382.

Hoegh-Guldberg, O. 1999. Climate change, coral bleaching and the future of the world's coral reefs. Marine and Freshwater Research **50**:839-866.

Huelsenbeck, J. P., and P. Andolfatto. 2007. Inference of population structure under a Dirichlet Process model. Genetics **175**:1787-1802.

Huelsenbeck, J. P., E. T. Huelsenbeck, and P. Andolfatto. submitted. Structurama: Bayesian inference of population structure. Bioinformatics.

Hughes, T. P. 1994. Catastrophes, phase shifts, and large-scale degradation of a Caribbean coral reef. Science **265**:1547-1551.

Hughes, T. P., A. H. Baird, D. R. Bellwood, M. Card, S. R. Connolly, C. Folke, R. Grosberg, O. Hoegh-Guldberg, J. B. C. Jackson, J. Kleypas, J. M. Lough, P. Marshall, M. Nyström, S. R. Palumbi, J. M. Pandolfi, B. Rosen, and J. Roughgarden. 2003. Climate change, human impacts, and the resilience of coral reefs. Science **301**:929-933.

Jolles, A. E., P. Sullivan, A. P. Alker, and C. D. Harvell. 2002. Disease transmission of aspergillosis in sea fans: Inferring process from spatial pattern. Ecology **83**:2373-2378.

Kasuga, T., T. J. White, G. Koenig, J. McEwen, A. Restrepo, E. Castaneda, C. Da Silva Lacaz, E. Heins-Vaccari, R. S. De Freitas, R. M. Zancope-Oliveria, Z. Qin, R. Negroni, D. A. Carter, Y. Mikami, M. Tamura, M. Lucia Taylor, G. F. Miller, N. Poonwan, and J. W. Taylor. 2003. Phylogeography of the fungal pathogen *Histoplasma capsulatum*. Molecular Ecology **12**:3383-3401.

Kellogg, C. A., D. W. Griffin, V. H. Garrison, K. K. Peak, N. Royall, R. R. Smith, and E. A. Shinn. 2004. Characterization of aerosolized bacteria and fungi from desert dust events in Mali, West Africa. Aerobiologia **20**:99-110.

Kim, K., and C. D. Harvell. 2002. Aspergillosis of sea fan corals: Dynamics in the Florida Keys. Pages 813-824 *in* J. W. Porter and K. G. Porter, editors. The everglades, Florida Bay, and coral reefs of the Florida Keys: An ecosystem sourcebook. CRC Press, Boca Raton, FL.

Kim, K., and C. D. Harvell. 2004. The rise and fall of a six-year coral-fungal epizootic. The American Naturalist **164**:S52-S63.

Kim, K., C. D. Harvell, P. D. Kim, G. W. Smith, and S. M. Merkel. 2000. Fungal disease resistance of Caribbean sea fan corals (*Gorgonia* spp.). Marine Biology **136**:259-267.

Klich, M. A. 2002. Identification of common *Aspergillus* species. Centraalbureau voor Schimmelcultures, Utrecht, Netherlands.

Koufopanou, V., A. Burt, and J. W. Taylor. 1997. Concordance of gene genealogies reveals reproductive isolation in the pathogenic fungus *Coccidioides immitis*. Proceedings of the National Academy of Sciences **94**:5478-5482.

Lafferty, K. D., J. W. Porter, and S. E. Ford. 2004. Are diseases increasing in the ocean? Annual Review of Ecology and Systematics **35**:31-54.

Latgé, J.-P. 1999. *Aspergillus fumigatus* and aspergillosis. Clinical Microbiology Reviews **12**:310-350.

Mardia, K. V. 1974. Applications of some measures of multivariate skewness and kurtosis in testing normality and robustness studies. Sankhyā B **36**.

Miller, M. P. 2005. Alleles in space: Computer software for the joint analysis of interindividual spatial and genetic information. Journal of Heredity **96**:722-724.

Morgan, J. A. T., V. T. Vredenburg, L. J. Rachowicz, R. A. Knapp, M. J. Stice, T. Tunstall, R. E. Bingham, J. M. Parker, J. E. Longcore, C. Moritz, C. J. Briggs, and J. W. Taylor. 2007. Population genetics of the frog-killing fungus *Batrachochytrium dendrobatidis*. Proceedings of the National Academy of Science **104**:13845-13850.

Munkvold, G. P. 2003. Cultural and genetic approaches to managing mycotoxins in maize. Annual Review of Phytopathology **41**:99-116.

Nagelkerken, I., K. Buchan, G. W. Smith, K. Bonair, P. Bush, J. Garzon-Ferreira, L. Botero, P. Gayle, C. D. Harvell, C. Heberer, K. Kim, C. Petrovic, L. Pors, and

P. Yoshioka. 1997a. Widespread disease in Caribbean sea fans: II. Patterns of infection and tissue loss. Marine Ecology Progress Series **160**:255-263.

Nagelkerken, I., K. Buchan, G. W. Smith, K. Bonair, P. Bush, J. Garzon-Ferreira, L. Botero, P. Gayle, C. Heberer, C. Petrovic, L. Pors, and P. Yoshioka. 1997b. Widespread disease in Caribbean sea fans: I. Spreading and general characteristics. Proceedings of the 8th International Coral Reef Symposium **1**:679-682.

Nei, M. 1987. Molecular Evolutionary Genetics. Columbia University Press, New York.

Nei, M., and F. Tajima. 1981. DNA polymorphism detectable by restriction endonucleases. Genetics **97**:145-163.

Olufemi, B. E., C. Agius, and R. J. Roberts. 1983. Aspergillomycosis in intensively cultured tilapia from Kenya. The Veterinary Record **112**:203-204.

Peakall, R., and P. E. Smouse. 2006. Genalex 6: Genetic analysis in Excel. Population genetic software for teaching and research. Molecular Ecology Notes **6**:288-295.

Pier, A. C., and J. L. Richard. 1992. Mycoses and mycotoxicoses of animals caused by Aspergilli. Pages 233-248 *in* J. W. Bennett and M. A. Klich, editors. *Aspergillus*: Biology and Industrial Applications. Butterworth-Heinemann, Boston, MA.

Piry, S., G. Luikart, and J.-M. Cornuet. 1999. BOTTLENECK: A computer program for detecting recent reductions in the effective population size using allele frequency data. Journal of Heredity **90**:502-503.

Pringle, A., D. M. Baker, J. L. Platt, J. P. Wares, J. P. Latgé, and J. W. Taylor. 2005. Cryptic speciation in the cosmopolitan and clonal human pathogenic fungus *Aspergillus fumigatus*. Evolution **59**:1886-1899.

Pritchard, J. K., M. Stephens, and P. Donnelly. 2000. Inference of population structure using multilocus genotype data. Genetics **155**:945-959.

Pritchard, J. K., X. Wen, and D. Falush. 2007. Documentation for *structure* software: Version 2.2.

Prospero, J. M., and P. J. Lamb. 2003. African droughts and dust transport to the Caribbean: climate change implications. Science **302**:1024-1027.

Prospero, J. M., and R. T. Nees. 1986. Impact of the North African drought and El Niño on mineral dust in the Barbados trade winds. Nature **320**:735-738.

Raper, K. B., and D. I. Fennell. 1965. The genus *Aspergillus*. The Williams & Wilkins Company, Baltimore.

Raymond, M., and F. Rousset. 1995. GENEPOP (version 1.2): Population genetics software for exact tests and ecumenicism. Journal of Heredity **86**:248-249.

Roth Jr., F. J., P. A. Orpurt, and D. G. Ahearn. 1964. Occurrence and distribution of fungi in a subtropical marine environment. Canadian Journal of Botany **42**:375-383.

Rousset, F. 1996. Equilibrium values of measures of poplation subdivision for stepwise mutation processes. Genetics **142**:1357-1362.

Rypien, K. L., and J. P. Andras. 2008. Isolation and characterization of microsatellite loci in *Aspergillus sydowii*, an opportunistic pathogen of Caribbean gorgonian corals. Molecular Ecology Resources **8**:230-232.

Saitou, N., and M. Nei. 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. Molecular Biology and Evolution **4**:406-425.

Sambrook, J., and D. W. Russell. 2001. Molecular Cloning: A Laboratory Manual. 3rd edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Schrag, S. J., and P. Wiener. 1995. Emerging infectious disease: what are the relative roles of ecology and evolution? Trends in Ecology and Evolution **10**:319-324.

Shinn, E. A., D. W. Griffin, and D. B. Seba. 2003. Atmospheric transport of mold spores in clouds of desert dust. Archives of Environmental Health **58**:498-504.

Shinn, E. A., G. W. Smith, J. M. Prospero, P. Betzer, M. L. Hayes, V. Garrison, and R. T. Barber. 2000. African dust and the demise of Caribbean coral reefs. Geophysical Research Letters **27**:3029-3032.

Smith, G. W., L. D. Ives, I. A. Nagelkerken, and K. B. Ritchie. 1996. Caribbean sea-fan mortalities. Nature **383**:487.

Sparrow Jr., F. K. 1937. The occurrence of saprophytic fungi in marine muds. Biological Bulletin **73**:242-248.

Steele, C. W. 1967. Fungus populations in marine waters and coastal sands of Hawaiian, Line, and Phoenix Islands. Pacific Science **21**:317-331.

Sutherland, K. P., J. W. Porter, and C. Torres. 2004. Disease and immunity in Caribbean and Indo-Pacific zooxanthellate corals. Marine Ecology Progress Series **266**:273-302.

Sweeney, J. C., G. Migaki, P. M. Vainik, and R. H. Conklin. 1976. Systemic mycoses in marine mammals. Journal of the American Veterinary Medical Association **169**:946-948.

Ward, J. R., K. Kim, and C. D. Harvell. 2007. Temperature affects coral disease resistance and pathogen growth. Marine Ecology Progress Series **329**:115-121.

Weir, B. S., and C. C. Cockerham. 1984. Estimating F-Statistics for the analysis of population structure. Evolution **38**:1358-1370.

Weir-Bush, J. R., V. H. Garrison, G. W. Smith, and E. A. Shinn. 2004. The relationship between gorgonian coral (Cnidaria: Gorgonacea) diseases and African dust storms. Aerobiologia **20**:119-126.

Wilkinson, C. 2002. Status of coral reefs of the world: 2002. Australian Institute of Marine Science, Townsville, Australia.

CHAPTER 3

A TARGETED SEARCH FOR *ASPERGILLUS SYDOWII*, THE CAUSATIVE AGENT OF SEA FAN DISEASE, IN AFRICAN DUST

ABSTRACT

Infection of sea fans by the fungal pathogen *Aspergillus sydowii* is one of the most widespread coral diseases in the Caribbean. The source of this normally terrestrial fungus in marine ecosystems is perplexing, and yet tracking sources of pathogens provides one of the few avenues to limit pathogen spread. Hypothesized inputs of *A. sydowii* include terrestrial deposits, marine sources, or African dust. Windborne dust deposits from Africa amount to nearly one billion tons per year, much of which is deposited over the Caribbean region. Several studies have examined the microbiota of African dust and detected the presence of *Aspergillus*, although identifications were only to the genus level. I used specific culture conditions to determine if this coral pathogen is present in four samples of airborne dust from Mali (Africa) and St. Croix, and three samples of deposited dust from Mali. A diversity of fungi were documented, including seven species of *Aspergillus*. However, none of the samples contained *A. sydowii*. The lack of *A. sydowii* in dust samples supports the rejection of the African Dust hypothesis as a source of this coral pathogen. Although there is high spatial and temporal variation in fungal abundance and diversity, data from this study taken in conjunction with recent molecular evidence suggest that African dust as a source of the marine pathogen *A. sydowii* should be considered unlikely.

INTRODUCTION

Coral reefs are an ecosystem increasingly suffering the synergistic effects of a variety of stressors, including temperature (Hoegh-Guldberg 1999, Harvell et al. 2002), over-fishing (Hughes 1994, Hughes et al. 2003, Pandolfi et al. 2003, Lotze et al. 2006), eutrophication (Fabricius and De'ath 2004, Fabricius 2005), and disease (Epstein 1998, Harvell et al. 1999, Harvell et al. 2004, Harvell et al. 2007). The dramatic rise in coral disease since the 1970s (Epstein 1998, Harvell et al. 1999, Ward and Lafferty 2004) is an area of extensive focus, as the causes behind the origin and spread of marine disease remain largely unknown. One factor that has been suggested to affect both the host and the pathogen is the input of wind-borne dust into Caribbean ecosystems (Shinn et al. 2000, Hayes et al. 2001, Garrison et al. 2003). The transport of dust from Africa to the Atlantic and Caribbean via trade winds was first identified in the late 1960s (Prospero 1968, Prospero et al. 1970). The amount of deposited dust is significant (about one billion tons per year, D'Almeida 1986), and has been increasing steadily since the 1970s (Prospero and Nees 1986, Prospero and Lamb 2003). Recent climate modeling indicates that this increase in dust transport is due the North Atlantic Oscillation (NAO), mediated by decreased rainfall in Africa (Moulin et al. 1997, Prospero and Lamb 2003).

The pattern of increased African dust since the 1970s correlates strongly with the observed increase in disease outbreaks in Caribbean coral reef communities (Hayes et al. 2001). There are several hypothesized mechanisms by which an increase in African dust could result in an increase in coral disease. African dust is known to enhance deposition of nutrients to the Atlantic (Prospero et al. 1996, Arimoto et al. 2003), including iron, a limiting micronutrient in many marine systems (Duce and Tindale 1991, Jickells et al. 2005). Local enrichment of iron as a result of dust deposits may facilitate pathogen growth and spread (Hayes et al. 2001) or cause

declines in coral health, since corals normally live in oligotrophic waters. Besides nutrients, African dust also deposits organic pollutants (van Dijk and Guicherit 1999, Erel et al. 2006), which could impair host immunity and lead to an increase in disease (Garrison et al. 2003). Finally, African dust has been posited as a direct source of pathogenic organisms (Shinn et al. 2000). This hypothesis is consistent with numerous examples of long distance dispersal (via wind) for a range of plant (Brown and Hovmoller 2002, Aylor 2003) and animal (Garrison et al. 2003) pathogens.

For African dust deposits to be directly increasing pathogen inputs, microorganisms must be able to survive the harsh conditions of the high atmosphere (temperature, UV) for about a week, the average length of time for a dust cloud to move from Africa to the Caribbean (Prospero and Nees 1986, Prospero et al. 2005). Previously, it was thought that very few organisms could survive under these conditions, but a diversity of microorganisms have been detected in dust samples (Griffin et al. 2001, Griffin et al. 2003, Shinn et al. 2003, Kellogg et al. 2004, Weir-Bush et al. 2004). The survival of these organisms is supported by models indicating that higher levels of dust clouds can act as a highly efficient UV screen, protecting microorganisms found in dust clouds at lower altitudes (Herman et al. 1999).

African dust deposits have been suggested to be the source of the Caribbean gorgonian coral pathogen responsible for aspergillosis, *Aspergillus sydowii* (Shinn et al. 2000). This disease was first observed in the 1980s (Garzón-Ferreira and Zea 1992), and has caused large mortality throughout the Caribbean (Nagelkerken et al. 1997a, Nagelkerken et al. 1997b, Kim and Harvell 2004). This disease system has been a model for experimental studies as both the host and pathogen can be successfully grown in the laboratory. Despite the high prevalence and large effect of *A. sydowii* on gorgonian-dominated communities, the origin of the pathogen is not known and is hotly debated (Shinn et al. 2000). Some authors have suggested

terrestrial sediments (Smith et al. 1996, Geiser et al. 1998) and marine sources (Sparrow Jr. 1937, Roth Jr. et al. 1964, Steele 1967) as the most likely pathogen inputs. Shinn et al. (2000) cite the presence of the genus *Aspergillus* in dust samples as evidence that African dust is the primary source for the coral pathogen *A. sydowii.*

Long-term dust collections have provided an excellent set of samples with which to test the African Dust hypothesis. Several previous studies examined the fungal biota of African dust (Griffin et al. 2001, Griffin et al. 2003, Shinn et al. 2003, Kellogg et al. 2004, Weir-Bush et al. 2004), and although several isolated *Aspergillus* spp. (Griffin et al. 2003, Shinn et al. 2003, Kellogg et al. 2004, Weir-Bush et al. 2004), none detected the presence of *A. sydowii*. Given that there are at least 180 recognized species within this genus (Pitt et al. 2000), and the high occurrence of *Aspergillus* in soil samples (Domsch et al. 1980), odds are low that any given *Aspergillus* species would be *A. sydowii*. Despite this, numerous authors continue to cite support for the hypothesis that African dust deposits are the source of this coral pathogen (Shinn et al. 2000, Garrison et al. 2003, Weir-Bush et al. 2004). The lack of *A. sydowii* identified from dust samples may either reflect the genuine absence of this microbe in African dust, or may be the result of insufficiently precise culturing techniques. Growth media and temperature can strongly influence the microbes cultured from environmental samples. In addition, culture-based techniques are themselves biased in that they only identify a subset of organisms (Barer et al. 1993). Given that the spores of *Aspergillus* are durable to temperature and UV stress (Abdalla 1988, Tong and Lighthart 1998, Klich 2002a), specific methods should be used to investigate whether *A. sydowii* is transported to the Caribbean in wind-blown dust deposits.

To determine if African dust is a source of *A. sydowii* in Caribbean coral reefs, we identified fungi cultured from airborne dust collected in the Caribbean and Africa, and deposited dust in Africa. This study, using techniques specific to the isolation and

identification of *Aspergillus* spp., is the first definitive test of the African Dust hypothesis.

METHODS

Air samples were collected from two locations (Table 3.1): Emetteur Kati, Bamako, Mali (12°41′17″N, 8°01′09″E), at an elevation of 555m during three dust events (March 2006), and Pt. Udall, St. Croix at sea level during a dust event (September 2006). In both locations, samples were taken using a portable vacuum filter sampling device as described by Kellogg et al. (2004). Briefly, air is filtered through sterile cellulose nitrate membranes with a pore size of 0.2 μm, at 10 L/min for 10 to 12 minutes. Following sampling, the filters were sealed, placed in Ziploc bags, and mailed to the United States. To account for potential contamination during handling, shipping, and processing of the filters, a control was included whereby the filtration apparatus was removed from its sterile packaging, but the cellulose membrane was not directly exposed to air. These samples were otherwise handled in an identical manner to the membranes which had air filtered across them.

Upon arrival in Ithaca, NY, the filters were cut in half with sterile scissors, and placed sample side up on malt extract agar (MEA) and dichloran-glycerol agar (DG18), both amended with 50 μg/mL tetracycline. These media were chosen specifically with the aim of culturing the coral pathogen *A. sydowii*. MEA is a commonly used media for *Aspergillus* spp. (Klich 2002b), and best visualizes the diagnostic color of *A. sydowii*. DG18 is used to culture xerophilic fungi, such as *Aspergillus* spp. (Hocking and Pitt 1980). Plates were incubated at 25°C for 14 days. Colonies were counted and examined under a dissecting microscope daily, and resulting fungal cultures were sub-cultured onto MEA. Samples of deposited dust were taken from three sites in Africa, two on Sal Island, Cape Verde (Pedre de Lume

Table 3.1. Dust sampling locations and conditions, and mean colony forming units per liter (CFU/L) of air sampled following 14 days of growth at 25°C. SLPM = standard liters per minute.

Sampling Location and Date	Air Temp. (°C)	Sampling Time (min)	Flow Rate (SLPM)	Dust Event?	Wind Speed (km/h)	CFU/L
Emetteur Kati, Bamako, Mali 23 March 2006	38	10	12.5	Yes	12	0.076
Emetteur Kati, Bamako, Mali 26 March 2006	40	10	12.5	Little dust	1 - 15	0.019
Emetteur Kati, Bamako, Mali 28 March 2006	> 40	10	12.25	Smoke / dust	5 - 10	0.043
Pt. Udall, St. Croix 17 Sept 2006	40.5	12	17.5	Yes	18 - 25	0.019

and Punta Fiura) and one in Bamako, Mali (Emetteur Kati) in March 2006. Samples were taken from the top 0.5 cm of sediment over a surface area of approximately 55 cm^2, and were collected and packaged using sterile technique. Upon arrival in the United States, the deposited dust was serially diluted (10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}) and plated onto both MEA and DG18 amended with tetracycline. Plates were incubated at 25°C for 14 days. Colonies were counted and examined under a dissecting microscope daily, and resulting fungal colonies were sub-cultured daily.

Fungi were identified to genus using Domsch et al. (1980) and St. Germain and Summerbell (1996). All *Aspergillus* spp. were identified to species using Klich (2002b) which uses colony-level morphological characteristics (color, growth rate) and microscopic characteristics observed under standard light microscopy (100x) (shape and color of conidia, sporulating structures (vesicles) and hyphae) to identify species. All fungi were preserved in both 70% ethanol and phosphate buffered saline with 10% glycerol, and stored at -80°C. The number of colony forming units per liter of air (CFU/L) are reported for airborne dust, and the number of colony forming units (CFU) are reported for deposited dust.

RESULTS

Both growth media were effective at isolating a diversity of fungi from the airborne dust samples (Table 3.2). After two weeks of growth, the number of fungi in the samples from Mali ranged from 0 to 0.104 CFU/L, and those from St. Croix ranged from 0.005 to 0.033 CFU/L (Table 3.2). Overall, samples from Mali had higher fungal abundance than those from St. Croix, although the three sampling dates in Mali were quite variable. Fungal abundance appears to be related to the magnitude

Table 3.2. Fungal species from air sampled during dust events in Mali and St. Croix. Abundance is the proportion of colony forming units (CFUs) on both types of media for all replicate filters following 14 days of growth at 25°C.

Fungal Species	Abundance (% of total CFU)			
	Mali 23 March	**Mali 26 March**	**Mali 28 March**	**St. Croix 17 Sept**
Cladosporium	49.1		28.1	6.3
Yeast	6.8	62.5		9.4
All *Aspergillus* spp.	10.2	6.3	15.6	3.1
A. fumigatus			3.1	
A. niger	5.1		6.3	3.1
A. niveus		6.3		
A. sydowii	0	0	0	0
A. terreus	5.1			
A. ustus			3.1	
Eurotium sp.	1.7			
Emericella sp.			3.1	
Penicillium	1.7		3.1	3.1
Curvularia	1.7		6.3	
Acremonium			3.1	15.6
Fusarium				9.4
Stachybotrys		6.3		
Paecilomyces				3.1
Sporothrix				3.1
Nigrosporium				3.1
Unidentified filamentous	30.5	25	43.8	43.8

of the dust event: the highest fungal counts were sampled during obvious dust events (ie. Mali, 23 March), and the lowest when little dust was in the air (Mali, 26 March). No fungus grew in the handling controls, indicating that the manipulation, shipping, and storage of samples was effective in preventing contamination.

The most common fungi in airborne dust samples from Mali was *Cladosporium*, comprising 35.5% of all colonies (Table 3.2). In St. Croix, the most common fungus was *Acremonium*. Seven species of *Aspergillus* (and related taxa) were observed overall, and ranged from 3 to 15% of the total colonies (Table 3.2). Airborne dust from Mali contained all seven *Aspergillus* species, and dust from St. Croix contained only *A. niger*. None of the recorded *Aspergillus* were *A. sydowii*. Although *Aspergillus* can be a taxonomically challenging genus, we have high confidence in the species identifications as we used an established set of morphological characters based on growth on four types of media at two temperatures (Klich 2002b). Of the species we observed, none were easily confused with *A. sydowii*, which has a characteristic turquoise colony color, relatively small colony growth rates, and extremely rough-walled conidia (Klich 2002b).

Deposited dust samples had much higher fungal abundance than air samples, and a dilution of at least 10^{-4} was necessary to prevent complete overgrowth within 48 hours (Table 3.3). Samples from one of the sites on Cape Verde (Punta Fiura) had low abundance of fungi; dilutions greater than 10^{-3} showed no growth. Overall, it was difficult to assess fungal diversity in the deposited dust samples due to the high density of colonies. Similar species were observed as in the airborne dust samples (*Cladosporium*, *Penicillium*, yeast, *Aspergillus* spp.). Three species of *Aspergillus* were observed (*A. niger*, *A. terreus*, *A. ustus*), but *A. sydowii* was not detected (Table 3.3).

Table 3.3. Fungal species from deposited dust sampled in Mali. Abundance is the number of colony forming units (CFUs) averaged over both types of media for all replicate filters following 48 hours of growth at 25°C.

	Abundance (CFU)		
Dilution	**Mali**	**Pedra de Lume**	**Punta Fiura**
	26 March 2006	**16 March 2006**	**16 March 2006**
10^{-2}	58.5	25.5	5.5
10^{-3}	30	7.5	0
10^{-4}	9	1	0
10^{-5}	4.5	overgrown	0
Common Fungi	*A. niger*, *Cladosporium*, zygomycete, *Penicillium*, yeast	*Cladosporium* *A. ustus*, *A. terreus*, yeast, zygomycete	*Cladosporium*

DISCUSSION

We found no evidence of the coral pathogen *A. sydowii* in airborne dust samples from Africa and the Caribbean, or deposited dust samples from Africa (Table 3.2, 3.3). Our targeted approach, using specific growth media and detailed identification methods, was successful, yielding seven different species of *Aspergillus*. The most common species was *Cladosporium*, which agrees with Lacey (1991) that this is the most abundant fungus in temperate and tropical air samples.

A comparison of these results with previous examinations of the fungal biota of African dust revealed some interesting trends (Table 3.4). The abundance of fungi in Mali air samples from this study are lower than those collected in 2001 and 2002 in the same region (Kellogg et al. 2004). This could be the result of factors such as large-scale climate patterns (ie. NAO) or local conditions (humidity, UV, temperature). Temporal variation in microbial abundance in air samples is well documented, and is related to the amount of deposited dust, which varies seasonally, with maximum dust deposition in June/July, and inter-annually, with dust deposition being strongly tied to low rainfall in Africa and large scale climate processes (Prospero and Lamb 2003, Prospero et al. 2005).

From an examination of previous studies, it was not possible to assess the role of different growth media on fungal abundance, as this was confounded with both sampling location and time (Table 3.4). However, several authors have found no significant difference in CFUs using nutrient-rich (ie. Sabouraud) and nutrient-poor (ie. R2A) media (Kellogg et al. 2004, Prospero et al. 2005). The results from this study were similar, with little difference in either fungal abundance or diversity between the two types of media used. Some of the lowest fungal abundances observed were from Barbados (Prospero et al. 2005), the study that used the highest incubation

Table 3.4. A comparison of the methodology and results of previous studies examining the fungal biota of African dust. Only samples taken during documented dust events are included. CFU = colony forming units.

Source	Sampling Location	Sampling Date	Media	Incubation Temperature (°C)	Incubation Time	CFU/L	Presence of *Aspergillus*	Presence of *A. sydowii*
Griffin et al. 2001	St. John	July 2000	R2A	Room temperature	2 weeks	0.048	N	N
Griffin et al. 2003	USVI	July 2001	R2A	23	48 h	0.024	N	N
Griffin et al. 2003	USVI	August 2001	R2A	23	48 h	0.065	Y	Unknown (ID to genus)
Weir-Brush et al. 2004	USVI	July 1999	YEG, MEG	28	Unknown	-	Y	Unknown (ID to genus)
Weir-Brush et al. 2004	USVI	September 1999	YEG, MEG	28	Unknown	-	Y	Unknown (ID to genus)
Kellogg et al. 2004	Mali	2001, 2002	R2A	26	48 h	0.225	Y	Unknown (ID to genus)

Table 3.4 (Continued)

Prospero et al. 2005	Barbados	April 1996	Sabouraud	37 for 48h, 30 for 2 weeks	2 weeks	0.003	-	-
Prospero et al. 2005	Barbados	June 1997	Sabouraud	37 for 48h, 30 for 2 weeks	2 weeks	0.015	-	-
This study	Mali	March 2006	MEA, DG18	25	2 weeks	0.035	Y	N
This study	St. Croix	September 2006	MEA, DG18	25	2 weeks	0.015	Y	N

temperature (30°C). This could be the result of either the temperature under which fungi were grown, or geographic variation.

Several previous studies have documented the presence of *Aspergillus* spp. in African dust (Griffin et al. 2003, Kellogg et al. 2004, Weir-Bush et al. 2004). However, none of these studies identified fungi to the species level. Methods of identifying fungi from dust samples include morphology (Weir-Bush et al. 2004) and 16S/18S rDNA sequencing (Griffin et al. 2001, Griffin et al. 2003, Kellogg et al. 2004). The presence of *Aspergillus* spp. does not necessarily indicate that the coral pathogen *A. sydowii* is present; this is a diverse genus and species identification can be difficult. For example, *A. versicolor*, a close relative and morphologically similar species to *A. sydowii*, is extremely widespread in soils and air samples (Domsch et al. 1980, Samson et al. 2001, Klich 2002a). We used detailed morphological analysis of fungi based on growth on four media types at two temperatures to confirm the identity of all *Aspergillus* species (Klich 2002b). This method has high specificity to the species level, in contrast to many of the molecular methods used by previous studies that can only identify fungi to genus. Regardless of the method, if the goal is to identify specific fungi (such as the coral pathogen *A. sydowii*), more detailed analysis of the morphological and molecular traits is required to identify to the species level.

A lack of *A. sydowii* in dust samples from both Africa and the Caribbean in this study makes it tempting to conclude that African dust is not a viable source of this coral pathogen. However, given the large spatial and temporal variation in fungal diversity and abundance, we cannot conclusively rule out the African Dust hypothesis, or the possibility that *A. sydowii* was present in dust from earlier years. Comparisons with previous studies emphasize that inputs from African dust into marine systems are likely to be spatially and temporally variable, and specific culturing and identification methods must be used to conclusively determine the presence of *A. sydowii* in African

dust. Future tests of the African Dust hypothesis are important and should use targeted searches with potential pathogens identified to as fine a taxonomic level as possible. It is not enough to identify to the genus level, as aspergillosis of corals is caused by a single species. *Aspergillus* is a diverse genus, and the evidence of several other species of *Aspergillus* within our dust samples suggests that taxonomic resolution to the species-level is necessary.

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REFERENCES

Abdalla, M. H. 1988. Prevalence of airborne *Aspergillus flavus* in Khartoum (Sudan) Airspora with reference to dusty weather and inoculum survival in simulated summer conditions. Mycopathologia **104**:137-141.

Arimoto, R., R. A. Duce, B. J. Ray, and U. Tomza. 2003. Dry deposition of trace elements to the western North Atlantic. Global Biogeochemical Cycles **17**:1010.

Aylor, D. E. 2003. Spread of plant disease on a continental scale: Role of aerial dispersal of pathogens. Ecology **84**:1989-1997.

Barer, M. R., L. T. Gribbon, C. R. Harwood, and C. E. Nwoguh. 1993. The viable but non-culturable hypothesis and medical bacteriology. Reviews in Medical Microbiology **4**:183-191.

Brown, J. K. M., and M. S. Hovmoller. 2002. Aerial dispersal of pathogens on the global and continental scales and its impact on plant disease. Science **297**:537-541.

D'Almeida, G. A. 1986. A model for Saharan dust transport. Journal of Climate and Applied Meterology **25**:903-916.

Domsch, K. H., W. Gams, and T. H. Anderson. 1980. Compendium of Soil Fungi. Academic Press, London.

Duce, R. A., and N. W. Tindale. 1991. Atmospheric transport of iron and its deposition in the ocean. Limnology and Oceanography **36**:1715-1726.

Epstein, P. R. 1998. Marine ecosystems, emerging diseases as indicators of change: Health of the oceans from Labrador to Venezuela. The Center for Health and the Global Environment, Harvard Medical School, Oliver Wendell Holmes Society, Boston, MA.

Erel, Y., U. Dayan, R. Rabi, Y. Rudich, and M. Stein. 2006. Trans boundary transport of pollutants by atmospheric mineral dust. Environmental Science & Technology **40**:2996-3005.

Fabricius, K. E. 2005. Effects of terrestrial runoff on the ecology of corals and coral reefs: review and synthesis. Marine Pollution Bulletin **50**:125-146.

Fabricius, K. E., and G. De'ath. 2004. Identifying ecological change and its causes: A case study on coral reefs. Ecological Applications **14**:1448-1465.

Garrison, V. H., E. A. Shinn, W. T. Foreman, D. W. Griffin, C. W. Holmes, C. A. Kellogg, M. S. Majewski, L. L. Richardson, K. B. Ritchie, and G. W. Smith. 2003. African and Asian dust: from desert soils to coral reefs. BioScience **53**:469-480.

Garzón-Ferreira, J., and S. Zea. 1992. A mass mortality of *Gorgonia ventalina* (Cnidaria: Gorgoniidae) in the Santa Marta area, Caribbean coast of Columbia. Bulletin of Marine Science **50**:522-526.

Geiser, D. M., J. W. Taylor, K. B. Ritchie, and G. W. Smith. 1998. Cause of sea fan death in the West Indies. Nature **394**:137-138.

Griffin, D. W., V. H. Garrison, J. R. Herman, and E. A. Shinn. 2001. African desert dust in the Caribbean atmosphere: Microbiology and public health. Aerobiologia **17**:203-213.

Griffin, D. W., C. A. Kellogg, V. H. Garrison, J. T. Lisle, T. C. Borden, and E. A. Shinn. 2003. Atmospheric microbiology in the northern Caribbean during African dust events. Aerobiologia **19**:143-157.

Harvell, C. D., K. Kim, J. M. Burkholder, R. R. Colwell, P. R. Epstein, D. J. Grimes, E. E. Hofmann, E. K. Lipp, A. D. M. E. Osterhaus, R. M. Overstreet, J. W. Porter, G. W. Smith, and G. R. Vasta. 1999. Emerging marine diseases: Climate links and anthropogenic factors. Science **285**:1505-1510.

Harvell, C. D., C. E. Mitchell, J. R. Ward, S. Altizer, A. P. Dobson, R. S. Ostfeld, and M. D. Samuel. 2002. Climate warming and disease risks for terrestrial and marine biota. Science **296**:2158-2162.

Harvell, D., R. Aronson, N. Baron, J. Connell, A. Dobson, S. Ellner, L. Gerber, K. Kim, A. Kuris, H. McCallum, K. Lafferty, B. McKay, J. Porter, M. Pascual, G. Smith, K. Sutherland, and J. Ward. 2004. The rising tide of ocean diseases: unsolved problems and research priorities. Frontiers in Ecology and Evolution **2**:375-382.

Harvell, D., E. Jordán-Dahlgren, S. Merkel, E. Rosenberg, L. Raymundo, G. Smith, E. Weil, and B. Willis. 2007. Coral disease, environmental drivers, and the balance between coral and microbial associates. Oceanography **20**:172-195.

Hayes, M. L., J. Bonaventura, T. P. Mitchell, J. M. Prospero, E. A. Shinn, F. Van Dolah, and R. T. Barber. 2001. How are climate and marine biological outbreaks functionally linked? Hydrobiologia **460**:213-220.

Herman, J. R., N. Krotkov, E. Celarier, D. Larko, and G. Labow. 1999. Distribution of UV radiation at the Earth's surface from TOMS-measured UV-backscattered radiances. Journal of Geophysical Research **104**:12059-12076.

Hocking, A. D., and J. I. Pitt. 1980. Dichloran-glycerol medium for enumeration of xerophilic fungi from low-moisture foods. Applied and Environmental Microbiology **39**:488-492.

Hoegh-Guldberg, O. 1999. Climate change, coral bleaching and the future of the world's coral reefs. Marine and Freshwater Research **50**:839-866.

Hughes, T. P. 1994. Catastrophes, phase shifts, and large-scale degradation of a Caribbean coral reef. Science **265**:1547-1551.

Hughes, T. P., A. H. Baird, D. R. Bellwood, M. Card, S. R. Connolly, C. Folke, R. Grosberg, O. Hoegh-Guldberg, J. B. C. Jackson, J. Kleypas, J. M. Lough, P.

Marshall, M. Nyström, S. R. Palumbi, J. M. Pandolfi, B. Rosen, and J. Roughgarden. 2003. Climate change, human impacts, and the resilience of coral reefs. Science **301**:929-933.

Jickells, T. D., Z. S. An, K. K. Andersen, A. R. Baker, G. Bergametti, N. Brooks, J. J. Cao, P. W. Boyd, R. A. Duce, K. A. Hunter, H. Kawahata, N. Kubilay, J. laRoche, P. S. Liss, N. Mahowald, J. M. Prospero, A. J. Ridgewell, I. Tegen, and R. Torres. 2005. Global iron connections between desert dust, ocean biogeochemistry, and climate. Science **308**:67-71.

Kellogg, C. A., D. W. Griffin, V. H. Garrison, K. K. Peak, N. Royall, R. R. Smith, and E. A. Shinn. 2004. Characterization of aerosolized bacteria and fungi from desert dust events in Mali, West Africa. Aerobiologia **20**:99-110.

Kim, K., and C. D. Harvell. 2004. The rise and fall of a six-year coral-fungal epizootic. The American Naturalist **164**:S52-S63.

Klich, M. A. 2002a. Biogeography of *Aspergillus* species in soil and litter. Mycologia **94**:21-27.

Klich, M. A. 2002b. Identification of common *Aspergillus* species. Centraalbureau voor Schimmelcultures, Utrecht, Netherlands.

Lacey, J. 1991. Aerobiology and health: The role of airborne fungal spores in respiratory disease.*in* D. L. Hawksworth, editor. Frontiers in Mycology. C.A.B. International, Wallingford, Oxon, UK.

Lotze, H. K., H. S. Lenihan, B. J. Bourque, R. H. Bradbury, R. G. Cooke, M. C. Kay, S. M. Kidwell, M. X. Kirby, C. H. Peterson, and J. B. C. Jackson. 2006. Depletion, degradation, and recovery potential of estuaries and coastal seas. Science **302**:1806-1809.

Moulin, C., C. E. Lambert, F. Dulac, and U. Dayan. 1997. Control of atmospheric export of dust from North Africa by the North Atlantic Oscillation. Nature **387**:691-694.

Nagelkerken, I., K. Buchan, G. W. Smith, K. Bonair, P. Bush, J. Garzon-Ferreira, L. Botero, P. Gayle, C. D. Harvell, C. Heberer, K. Kim, C. Petrovic, L. Pors, and P. Yoshioka. 1997a. Widespread disease in Caribbean sea fans: II. Patterns of infection and tissue loss. Marine Ecology Progress Series **160**:255-263.

Nagelkerken, I., K. Buchan, G. W. Smith, K. Bonair, P. Bush, J. Garzon-Ferreira, L. Botero, P. Gayle, C. Heberer, C. Petrovic, L. Pors, and P. Yoshioka. 1997b. Widespread disease in Caribbean sea fans: I. Spreading and general characteristics. Proceedings of the 8th International Coral Reef Symposium **1**:679-682.

Pandolfi, J. M., R. H. Bradbury, E. Sala, T. P. Hughes, K. A. Bjorndal, R. G. Cooke, D. McArdle, L. McClenachan, M. J. H. Newman, G. Paredes, R. R. Warner, and J. B. C. Jackson. 2003. Global trajectories of the long-term decline of coral reef ecosystems. Science **301**:955-958.

Pitt, J. I., R. A. Samson, and J. C. Frisvad. 2000. List of accepted species and their synonyms in the family Trichocomaceae. Pages 9-49 *in* R. A. Samson and J. I. Pitt, editors. Integration of modern taxonomic methods for *Penicillium* and *Aspergillus* classification. Harwood Academic Publishers, Reading, UK.

Prospero, J. M. 1968. Atmospheric dust studies on Barbados. Bulletin American Meterological Society **49**:645-652.

Prospero, J. M., K. Barrett, T. Church, F. Dentener, R. A. Duce, J. N. Galloway, H. Levy, J. Moody, and P. Quinn. 1996. Atmospheric deposition of nutrients to the North Atlantic Basin. Biogeochemistry **35**:27-73.

Prospero, J. M., E. Blades, G. Mathison, and R. Naidu. 2005. Interhemispheric transport of viable fungi and bacteria from Africa to the Caribbean with soil dust. Aerobiologia **21**:1-19.

Prospero, J. M., E. Bonatti, C. Schubert, and T. N. Carlson. 1970. Dust in the Caribbean atmosphere traced to an African dust storm. Earth and Planetary Science Letters **9**:287-293.

Prospero, J. M., and P. J. Lamb. 2003. African droughts and dust transport to the Caribbean: climate change implications. Science **302**:1024-1027.

Prospero, J. M., and R. T. Nees. 1986. Impact of the North African drought and El Niño on mineral dust in the Barbados trade winds. Nature **320**:735-738.

Roth Jr., F. J., P. A. Orpurt, and D. G. Ahearn. 1964. Occurrence and distribution of fungi in a subtropical marine environment. Canadian Journal of Botany **42**:375-383.

Samson, R. A., J. Houbraken, R. C. Summerbell, B. Flannigan, and J. D. Miller. 2001. Common and important species of fungi and actinomycetes in indoor environments. Pages 287-292 *in* B. Flannigan, R. A. Samson, and J. D. Miller, editors. Microorganisms in Home and Indoor Work Environments. Taylor and Francis, New York.

Shinn, E. A., D. W. Griffin, and D. B. Seba. 2003. Atmospheric transport of mold spores in clouds of desert dust. Archives of Environmental Health **58**:498-504.

Shinn, E. A., G. W. Smith, J. M. Prospero, P. Betzer, M. L. Hayes, V. Garrison, and R. T. Barber. 2000. African dust and the demise of Caribbean coral reefs. Geophysical Research Letters **27**:3029-3032.

Smith, G. W., L. D. Ives, I. A. Nagelkerken, and K. B. Ritchie. 1996. Caribbean sea-fan mortalities. Nature **383**:487.

Sparrow Jr., F. K. 1937. The occurrence of saprophytic fungi in marine muds. Biological Bulletin **73**:242-248.

St. Germain, G., and R. Summerbell. 1996. Identifying filamentous fungi: A clinical laboratory handbook. Star Publishing, Belmont, USA.

Steele, C. W. 1967. Fungus populations in marine waters and coastal sands of Hawaiian, Line, and Phoenix Islands. Pacific Science **21**:317-331.

Tong, Y. Y., and B. Lighthart. 1998. Effect of simulated solar radiation on mixed outdoor atmospheric bacterial populations. FEMS Microbiology Ecology **26**:311-316.

van Dijk, H. F. G., and R. Guicherit. 1999. Atmospheric dispersion of current-use pesticides: A review of the evidence from monitoring studies. Water, Air, and Soil Pollution **115**:21-70.

Ward, J. R., and K. D. Lafferty. 2004. The elusive baseline of marine disease: Are diseases in ocean ecosystems increasing? PLoS Biology **2**:542-547.

Weir-Bush, J. R., V. H. Garrison, G. W. Smith, and E. A. Shinn. 2004. The relationship between gorgonian coral (Cnidaria: Gorgonacea) diseases and African dust storms. Aerobiologia **20**:119-126.

CHAPTER 4

CYPHOMA GIBBOSUM AS A POTENTIAL VECTOR OF THE EMERGING SEA FAN DISEASE ASPERGILLOSIS

ABSTRACT

Vectors play a critical role in the ecology of infectious disease by facilitating between-host disease spread, and emphasize the multi-species nature of disease. Corals are suffering an onslaught of infectious diseases, yet we know little about the role of vector species in the ecology of these emergent diseases. The infection of gorgonian corals by the fungus *Aspergillus sydowii* is a widespread Caribbean coral disease. The snail *Cyphoma gibbosum* is a likely vector species because it is a specialist predator of gorgonian corals, moves regularly among coral colonies, and aggregates on diseased corals. We demonstrate in lab experiments one snail fed *A. sydowii* passed viable spores and hyphae in its feces. Further, the use of isotopically-labeled fungus allowed us to definitively trace *A. sydowii* through the guts of *C. gibbosum* from ingestion to feces. Results from a choice-feeding experiment demonstrate that snails prefer food amended with diseased sea fan extracts. Overall, this supports the hypothesis that *C. gibbosum* vectors aspergillosis between hosts.

INTRODUCTION

Infectious disease rarely involves just pathogen and host. Rather, networks of species interact with the pathogen either directly through vector-based transmission and multiple host species, or indirectly through disease-mediated effects on community structure and function (Antonovics et al. 1995, Dobson 2004, Hatcher et al. 2006, Keesing et al. 2006). As such, it is important to consider the community of

species interacting with pathogens to gain a holistic view of the effects of disease on ecosystems.

Vectors are a critical component of the network of species interacting with infectious diseases due to their influence on the epidemiological cycle. Vector-borne diseases are some of the best examples of terrestrial pathogens with large impacts on humans (for example, malaria and Lyme disease), and the most lethal, due to the increase in transmission efficiency and subsequent increase in virulence they afford (Ewald 1983). Community-level effects of disease are also influenced by the presence of vectors: vector-borne diseases tend to have frequency-dependent transmission (Antonovics et al. 1995), which alters the impact of species diversity on disease dynamics (Dobson 2004, Keesing et al. 2006). Vector biology can also interact with environmental changes to influence disease dynamics, for example climate change and globalization can alter the distribution and life cycle duration of vectors (Harvell et al. 2002, Anderson et al. 2004)

In contrast to well-known vector dynamics on land, vectors of marine diseases are comparatively rare (McCallum et al. 2004). However, this could be the result of insufficient study rather than a genuine pattern. With the recent increase in the number and severity of marine epidemics (Harvell et al. 1999, Harvell et al. 2004, Ward and Lafferty 2004), identifying marine diseases where vectors play a role is critical to management. This is especially true for diseases infecting a sessile host, where the presence of vectors could dramatically increase the rate of disease spread (McCallum et al. 2003).

Corals are particularly vulnerable to emergent disease due to the numerous environmental stressors placed upon the coral holobiont (Hughes 1994, Hoegh-Guldberg 1999, Wilkinson 2002, Gardner et al. 2003, Hughes et al. 2003, Harvell et al. 2007), as well as their proximity to coastal pathogen sources (Patterson et al. 2002).

Several coral diseases have vectors that contribute to, but are not entirely responsible for, disease spread. These vectors are almost exclusively coral predators that inadvertently ingest pathogens and spread them during feeding movements. Corallivorous snails have been identified as vectors of an unknown disease in Caribbean acroporid corals (Williams and Miller 2005), and have been correlated with coral disease outbreaks in the Red Sea (Antonius and Riegl 1997). In the Mediterranean, the predatory polychaete *Hermodice carunculata* is a vector and reservoir of the coral pathogen *Vibrio shiloi* (Sussman et al. 2003). Although these vectors are not the sole mechanism of transmission, positive feedback due to injury from predation and aggregative feeding behavior suggest that they play a critical role in the ecology of these coral diseases.

Aspergillosis is a fungal disease of Caribbean gorgonian corals caused by *Aspergillus sydowii*, and is a prime example of a disease involving a network of species. This disease was first observed in the early 1990s (Nagelkerken et al. 1997a, Nagelkerken et al. 1997b), and infects at least eight different species of gorgonian corals (Kim and Harvell 2004, Smith and Weil 2004, Ward et al. 2006), the dominant group on many Caribbean reefs (Opresko 1973, Yoshioka and Yoshioka 1989). Specialist predators, such as the snail *Cyphoma gibbosum*, that are dependent on gorgonian corals for habitat, food, and oviposition sites, are a group that will likely suffer the effects of aspergillosis. *C. gibbosum* makes regular mate- and food-searching movements (Nowlis 1993), with snails moving among corals on average every 3 days (Rypien, unpublished). Observations of increased density of *C. gibbosum* on diseased sea fans (Nagelkerken et al. 1997a, Slattery 1999) suggest that aspergillosis may be impacting non-host species. Although the mode of transmission of aspergillosis is unknown, an analysis of spatial patterns of disease and observations of aggregation of diseased individuals at the 1 – 3 m scale, suggest that vectors may

facilitate spread at locations with high disease prevalence (Jolles et al. 2002). Thus, *C. gibbosum* is a key link in the disease ecology of aspergillosis, as it is likely influenced by disease through the removal of its gorgonian coral prey, and may in turn influence the between-host transmission of aspergillosis by acting as a vector.

Here we investigate the putative role of *C. gibbosum* as a vector for aspergillosis in Caribbean gorgonian corals. Using a novel application of stable isotope techniques, we examined the successful passage of ingested fungus through the gut of snails. The ecological likelihood of snails feeding on diseased corals was assessed using a choice-feeding assay, where snails were offered artificial food amended with crude organic extracts from diseased and healthy corals. The results of these studies demonstrate *C. gibbosum* is a likely vector of aspergillosis.

METHODS

Spore Passage Experiment

Twenty-two adult (> 2.5 cm) *C. gibbosum* were collected from reefs near Key Largo, FL in June 2007 and were shipped to Ithaca, NY. There, the snails were acclimated without feeding in an artificial sea water system (12:12 light, temperature 26.5°C) for 48 hours. Snails were housed in individual cages (14 cm x 14 cm x 14 cm) constructed from plastic containers with mesh panels to provide flow.

Snails were fed artificial food consisting of 3.5% carrageenan (Type I), 16% feeding attractant (20 cm fragment of *Plexaura homomalla*, a preferred gorgonian coral, homogenized in 10 mL deionized water), and either ^{15}N enriched or un-enriched *A. sydowii*. The fungus was originally cultured from a diseased sea fan in Saba, Netherland Antilles (Smith et al. 1996), and was grown on potato dextrose agar amended with either 98%^{15}N-labeled sodium nitrate (16 mg/L) or un-enriched sodium nitrate. The fungus was grown at 25°C for 7 days, after which the hyphae and spores

were scraped from the surface using a sterile wooden dowel, re-suspended in a solution of phosphate buffered saline (10x) with 10% glycerol and 0.1% Tween 80. This stock was stored at -80°C. The fungal mixture contained both spores and hyphae (approximately 10:1 ratio), and fungus was added to the artificial snail food to reach a final concentration of two million spores/mL. We confirmed that *A. sydowii* remained viable within the artificial food by analyzing the resulting fungal growth from food placed on potato dextrose agar at 25°C.

To make the artificial food, the carrageenan was dissolved in distilled water, and cooled while stirring. At a temperature just above solidifying, the feeding attractant and fungus were added, and the food was immediately poured into an acrylic mold (30 cm x 2 cm x 0.3 cm) lined with plastic window screen for support. Once solid, the food was cut into 2 cm x 1 cm x 0.3 cm blocks. To ensure that contamination between the enriched and un-enriched food did not occur, separate equipment and lab space was used for each treatment.

At the start of the experiment, each snail was given one block of artificial food. Every 48 hours the food was removed, the amount of grazing recorded (feeding intensity), fecal pellets collected, and another block of artificial food was added to the cage. This was repeated a total of four times. Fecal pellets were stored at 4°C until the end of the experiment, when all the feces from a single snail were pooled and prepared for isotope analysis.

The amount of artificial food consumed by each snail was quantified based on the amount of observed grazing over the course of the entire experiment. Following 48 hours of grazing, each block of artificial food was assigned a value of 2 (intense; > 90% of the food was removed), 1 (moderate grazing; some of the food was removed) or 0 (none of the food was removed). The sum of the feeding intensity values over the course of the experiment was used in subsequent analysis.

To assess spore viability following gut passage, we fed 12 additional snails either artificial food containing un-enriched *A. sydowii* spores and hyphae or control (no fungus) food. Feces were collected, rinsed, and plated on potato dextrose agar with 0.005% tetracycline. Samples of seawater (1 mL) and other biotic substrates (filamentous algae) were plated as controls. The samples were kept at 25°C, and fungi were identified daily using morphological features (Klich 2002).

Stable Isotope Analysis

Feces were pooled by individual to obtain sufficient mass for isotope analysis, and were rinsed with deionized water and dried at 60°C for 24 hours. Samples of initial snail feces (produced from feeding on wild food sources), enriched and un-enriched fungus, and enriched and un-enriched artificial food were also dried at 60°C. Three snails from each treatment group were sacrificed for tissue isotope analysis to detect signs of digestion and assimilation of fungal-derived nitrogen, and were removed from their shells and dissected to remove the digestive tract. The remaining tissue was oven dried at 60°C for 24 hours, frozen in liquid nitrogen and homogenized using a mortar and pestle. All samples were stored in a desiccation chamber prior to analysis.

Samples were weighed (up to 3.0 mg) into 4 x 6 mm tin capsules. Isotope values were determined by the Cornell University Stable Isotope Lab (COIL) using a Finnegan MAT Delta Plus isotope-ratio mass spectrometer (IRMS) coupled to a Carlo-Erba elemental analyzer via a Conflo II open-split interface. ^{15}N labeled and natural abundance samples were analyzed in separate runs. Precision of an in-house standard (± SD) for $\delta^{15}N$ was ± 0.046 ‰ and ± 0.068 ‰, respectively.

The ^{13}C content of fecal samples was also analyzed to determine if the snails were ingesting artificial food equally well in both treatments. Precision of $\delta^{13}C$ was ± 0.031 ‰ and ± 0.041 ‰, respectively.

Data Analysis

$\delta^{15}N$ and $\delta^{13}C$ values of feces and tissue from initial, enriched fungus-fed, and un-enriched treatments were compared using ANOVA. Student's t-test was used to conduct pair-wise comparisons of means. The relationship between $\delta^{15}N$ enrichment and feeding intensity was assessed using regression analysis. All statistical tests were performed using JMP 6.0 (Cary, NC).

Food Choice Experiment

Sixteen adult (> 2.5 cm) *C. gibbosum* were collected from North Norman's Reef (23°47′386′′N, 76°08′273′′W), Bahamas. Snails were maintained in individual cages (14 cm x 14 cm x 14 cm) in a flow-through natural seawater system at the Perry Institute for Marine Science, Caribbean Marine Research Center. Snails were acclimated and starved for four days prior to experimentation.

Artificial food consisted of 2.5% carrageenan, 12.5% feeding attractant (10 cm fragment of homogenized *Plexaura flexuosa*, a preferred gorgonian coral food source, in 10 mL distilled water), and organic extracts from either healthy or diseased *Gorgonia ventalina*. Organic extracts were prepared by collecting fragments from *G. ventalina* colonies (10 healthy, 10 diseased), extracting twice in dichloromethane, evaporating the dichloromethane, and re-suspending in a minimal amount of acetone. The extracts were added to the artificial food at naturally volumetric concentrations. The artificial food was poured into molds, and cut into 3 cm x 1 cm x 0.2 cm pieces and weighed. Snails were given a block of food from each treatment spaced

equidistantly in the cage. Once 50% of the food was eaten (approximately 20 hours), the food was removed, patted dry, and re-weighed. The proportion of food eaten was checked for normality, and analyzed using a paired t-test using JMP 6.0 (Cary, NC).

RESULTS

Spore Passage Experiment

The feces of one of six snails fed artificial food amended with fungus grew *A. sydowii*, confirmed using microscopic morphological features. None of the feces from six snails fed control (fungus-free) food grew *A. sydowii*. This suggests that it is possible for viable fungus to survive gut passage, however the relative frequency of this occurring is unknown.

Preliminary analysis of fungus grown on enriched media confirmed assimilation of $^{15}NO_3^-$ in contrast to that grown on un-enriched media (media $\delta^{15}N$ [labeled vs. non-labeled]: 1144.5 ‰ vs. 0.2 ‰; fungus $\delta^{15}N$: 1671.3 ‰ vs. 0.6 ‰), confirming the effectiveness of the isotopic enrichment. However, there was no apparent difference between enriched and un-enriched fungus with respect to $\delta^{13}C$ (media $\delta^{13}C$: -16.5 ‰ vs. -16.0 ‰; fungus $\delta^{13}C$: -14.7 ‰ vs. -14.8 ‰). The viability of fungus added to artificial food was also confirmed by the growth of *A. sydowii* on all samples of fungus-containing food, and the absence of *A. sydowii* on all samples of fungus-free food.

During the course of the experiment, three snails died: two in the un-enriched treatment, and one in the enriched treatment. In addition, not all snails ingested sufficient quantities of artificial food to provide ample feces for isotope analysis. Thus, the final number of snails that could be used for analysis was six fed un-enriched artificial food, and eight fed enriched food.

There was a significant effect of fungus treatment on fecal pellet $\delta^{15}N$ (Figure 4.1; $F = 55.08$, $df = 2$, $p < 0.0001$). Enriched fungus-fed snails produced feces with significantly higher $\delta^{15}N$ values than initial and un-enriched treatments (on average 7.9 ‰ and 8.0 ‰ higher, respectively). However, $\delta^{15}N$ of snail tissue was not different between treatments (Figure 4.1; $t = -1.43$, $df = 4$, $p = 0.224$) indicating that ingested fungus was not assimilated, and was being passed through the snail gut. A scored measure of feeding intensity over the entire course of the experiment was positively correlated with fecal pellet enrichment in enriched fungus-fed snails (Figure 4.2; $R^2 = 0.68$, $p = 0.011$).

There was a significant effect of treatment on fecal pellet $\delta^{13}C$ ($F = 113.75$, $df = 2$, $p < 0.0001$). However, this difference was driven by initial (wild) feces, which were on average 13 ‰ more depleted than $\delta^{13}C$ values from experimental feces. There was no significant difference between feces from enriched fungus-fed snails and un-enriched fungus-fed snails ($t = 0.54$, $df = 12$, $p = 0.59$), confirming that the snails were eating the artificial food equally in both treatments.

Food Choice Experiment

There was a significant difference in the amount of artificial food consumed by *C. gibbosum* ($t = 2.13$, $p = 0.0527$), with snails eating more of the food amended with diseased coral extracts (Figure 4.3).

DISCUSSION

These experiments support the hypothesis that *C. gibbosum* readily eat diseased sea fans and pass viable *A. sydowii* spores and hyphae in their fecal pellets, and therefore are a potential vector for aspergillosis of Caribbean gorgonian corals.

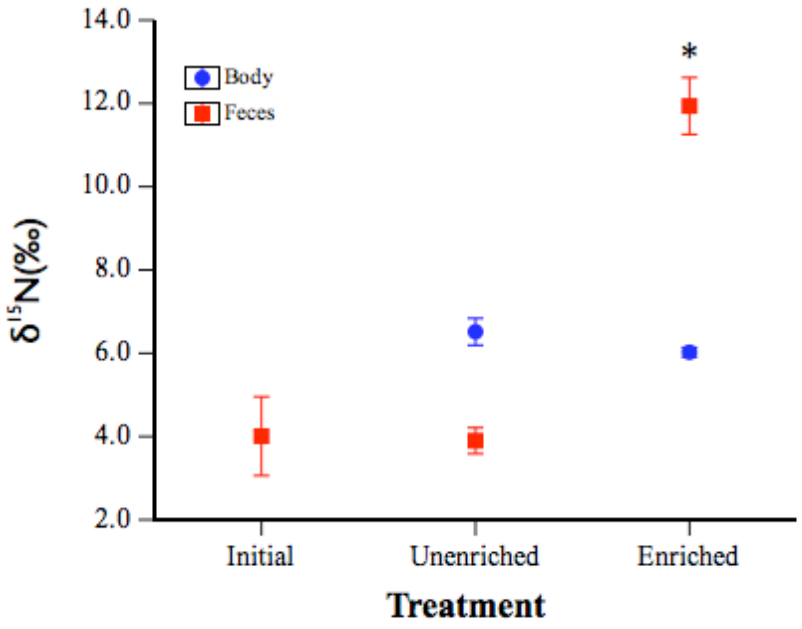

Figure 4.1. δ15N values (mean ± standard error) of *Cyphoma gibbosum* body tissue (blue circles) and feces (red squares) following feeding on artificial food amended with either un-enriched or enriched spores and hyphae of *Aspergillus sydowii*. Asterix indicates significance within tissue type at $\alpha = 0.05$.

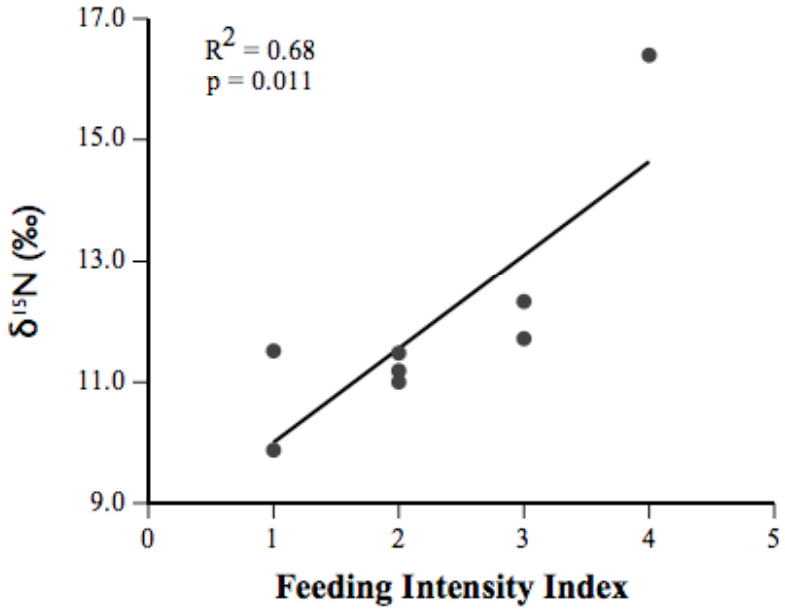

Figure 4.2. $\delta^{15}N$ in the feces of *Cyphoma gibbosum* fed artificial food containing enriched spores and hyphae of *Aspergillus sydowii* (N=8) as a function of feeding intensity index, a qualitative assessment of food consumed over the entire course of the experiment.

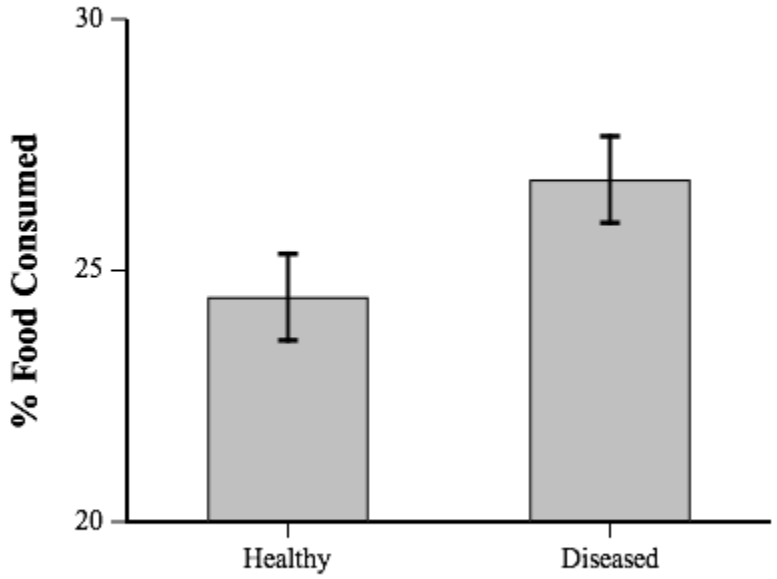

Figure 4.3. The proportion of food containing extracts from healthy and diseased sea fans that was eaten by *Cyphoma gibbosum* (N=16) in a choice feeding assay (mean ± standard error).

Evidence of viable *A. sydowii* in the feces of at least one snail fed fungus-amended artificial food, and in none of the feces from snails fed fungus-free food suggests that ingested fungus has the potential survive gut passage, although amount of fungal mortality due to ingestion remains unknown. Combined with the preference by *C. gibbosum* for diseased sea fan corals (Figure 4.3), these results suggest that snail predators can act as an important link in the disease ecology of aspergillosis.

Isotopically-labeled fungus allowed us to track the movement of ingested *A. sydowii* into the feces of snails; snails fed isotopically-enriched fungus had fecal pellets which were significantly enriched relative to feces from snails fed un-enriched fungi (Figure 4.1). The magnitude of isotopic enrichment of the feces was highly correlated with the amount of food consumed (Figure 4.2). Given that we pooled feces over the course of eight days, the amount of nitrogen from enriched fungus was diluted with other sources of fecal derived nitrogen, representing a mixed pool of previously consumed material, artificial food, as well as fungus. Therefore, this signal is highly conservative given the potential for background interferences. This demonstrates the utility of stable isotope techniques to trace the movement of pathogen propagules through the guts of hosts. This method is more sensitive than the traditional microscopic examination of feces in detecting the successful gut passage of fungus (Dromph 2001, Colgan III and Claridge 2002, Malaquias et al. 2004, Prom and Lopez Jr. 2004, Lilleskov and Bruns 2005), and if partial digestion occurs, can provide a quantitative measure of the amount of un-digested fungal tissue passing through the gut.

The results of the food choice experiment indicate that *C. gibbosum* prefer diseased sea fans (Figure 4.3), corroborating previous observations of an increased density of *C. gibbosum* on diseased sea fans (Nagelkerken et al. 1997a, Slattery 1999). This indicates that the fungal pathogen *A. sydowii* can affect non-host species by

altering the feeding preference of predators. One possible mechanism for the shift in host preference is a reduction in feeding deterrents in diseased sea fans, potentially resulting from a trade-off by corals between antifungal and anti-predator defenses. A reduction in the anti-predator defense metabolite julieannafuran in sea fans following infection by aspergillosis has been demonstrated to increase the palatability of corals to generalist omnivorous fish predators (Slattery 1999).

The shift in feeding behavior as a result of disease is likely to be compounded by the change in host availability due to infection-related mortality. Aspergillosis is known to infect up to eight species of gorgonian corals (Smith and Weil 2004). Although *C. gibbosum* has been observed on most gorgonian species, it does demonstrate distinct host preferences (Kinzie 1970, Harvell and Suchanek 1987, Lasker and Coffroth 1988, Lasker et al. 1988, Botero 1990, Nowlis 1993), and the removal of preferred food sources due to infection will undoubtedly result in altered feeding and oviposition behavior. Given the large effects of aspergillosis on just one of its host species (over 50% of sea fan tissue was lost in Florida between 1997 and 2002 (Kim and Harvell 2004)), aspergillosis can dramatically shift the available host community for specialist predators such as *C. gibbosum*.

If *C. gibbosum* is acting as a vector for aspergillosis, it is likely not the sole method of transmission, similar to other coral disease vectors. Evidence of multiple methods of transmission was suggested by an examination of spatial patterns of aspergillosis (Jolles et al. 2002). In fact, the patchy distribution of *C. gibbosum* (Birkeland and Gregory 1975, Hazlett and Bach 1982, Harvell and Suchanek 1987, Lasker and Coffroth 1988, Botero 1990, Chiappone et al. 2003) precludes it from being the sole mechanism of transmission for this disease. However, the regular feeding and mate-searching movements of this snail among a diversity of gorgonian coral hosts (Gerhart 1986, Lasker et al. 1988, Nowlis 1993) suggests that a positive

feedback loop may occur: once disease is present at a site, snails can spread it among corals, leading to a rapid increase in prevalence, which results in an increased likelihood of snails feeding on infected corals. In addition, the observed passage time of ingested food (approximately 2 days) is similar to the frequency of individual snail movements among corals (every 3 days; Rypien, unpublished). The role of injury due to grazing behavior may also facilitate infection by allowing the fungus to penetrate some of the external host defenses. Fungal hyphae are rarely observed in the tissue of sea fans, and are almost exclusively seen inside the inert (non-cellular) skeleton (Mullen et al. 2004). This suggests that host defenses may provide a substantial barrier to fungal proliferation in the sea fan tissue, and that injury due to grazing could allow for more successful fungal infections.

The role of *C. gibbosum* in increasing the rate of disease spread on local scales may be particularly important at sites with low external pathogen inputs or low host density, as a small number of pathogen propagules could have a larger effect than if they were spread by currents or secondary contact alone. Recent work suggests that the removal of top predators due to over-fishing may allow the release of *C. gibbosum*, leading to a large increase in gorgonian damage due to grazing (Burkepile and Hay 2007). This suggests that sites with low top predator densities may also be particularly susceptible to vectored disease transmission.

Infectious disease is a component of all natural ecosystems, and pathogens interact with numerous species beyond just their host. As this study demonstrates, the infection of Caribbean gorgonian corals with *A. sydowii* not only negatively affects the density and reproductive status of coral hosts, but also affects the distribution and behavior of specialist gastropod predators, such as *C. gibbosum*. The potential vectoring capability of *C. gibbosum* may help to explain spatial and temporal patterns of disease prevalence. As vectors have the potential to spread disease rapidly in local

areas, and to carry more virulent pathogen strains (Ewald 1983), an assessment of the actual importance of these snails as transmission vectors is a critical next step. Given what we know about the strong environmental drivers of aspergillosis (Alker et al. 2001, Bruno et al. 2003, Kim et al. 2006, Ward et al. 2007) and the role of climate change in altering the distribution and life cycle of terrestrial vectors (Harvell et al. 2002), a more holistic view of the interactions between pathogens such as *A. sydowii* and non-host species in the community is vital for predicting and mitigating future disease outbreaks.

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REFERENCES

Alker, A. P., G. W. Smith, and K. Kim. 2001. Characterization of *Aspergillus sydowii* (Thom et Church), a fungal pathogen of Caribbean sea fan corals. Hydrobiologia **460**:105-111.

Anderson, P. K., A. A. Cunningham, N. G. Patel, F. J. Morales, P. R. Epstein, and P. Daszak. 2004. Emerging infectious diseases of plants: pathogen pollution, climate change and agrotechnology drivers. Trends in Ecology & Evolution **19**:535-554.

Antonius, A., and B. Riegl. 1997. A possible link between coral diseases and a corallivorous snail (*Drupella cornus*) outbreak in the Red Sea. Atoll Research Bulletin **447**:2-9.

Antonovics, J., Y. Iwasa, and M. P. Hassell. 1995. A generalized model of parasitoid, venereal and vector-based transmission processes. American Naturalist **145**:661-675.

Birkeland, C., and B. Gregory. 1975. Foraging behavior and rates of feeding of the gastropod, *Cyphoma gibbosum*. Bulletin of the Natural History Museum of Los Angeles **20**:57-67.

Botero, L. 1990. Observations on the size, predators and tumor-like outgrowths of gorgonian octocoral colonies in the area of Santa Marta, Caribbean coast of Colombia. Northeast Gulf Science **11**:1-10.

Bruno, J. F., L. E. Petes, C. D. Harvell, and A. Hettinger. 2003. Nutrient enrichment can increase the severity of coral diseases. Ecology Letters **6**:1056-1061.

Burkepile, D. E., and M. E. Hay. 2007. Predator release of the gastropod *Cyphoma gibbosum* increases predation on gorgonian corals. Oecologia **154**:167-173.

Chiappone, M., H. Dienes, D. W. Swanson, and S. L. Miller. 2003. Density and gorgonian host-occupation patterns by flamingo tongue snails (*Cyphoma gibbosum*) in the Florida Keys. Caribbean Journal of Science **39**:116-127.

Colgan III, W., and A. W. Claridge. 2002. Mycorrhizal effectiveness of *Rhizopogon* spores recovered from faecal pellets of small forest-dwelling mammals. Mycological Research **106**:314-320.

Dobson, A. 2004. Population dynamics of pathogens with multiple host species. The American Naturalist **164**:S64-S78.

Dromph, K. M. 2001. Dispersal of entomopathogenic fungi by collembolans. Soil Biology and Biochemistry **33**:2047-2051.

Ewald, P. W. 1983. Host-Parasite Relations, Vectors, and the Evolution of Disease Severity. Annual Review of Ecology and Systematics **14**:465-485.

Gardner, T. A., I. M. Côte, J. A. Gill, A. Grant, and A. W. Watkinson. 2003. Long-term region-wide declines in Caribbean corals. Science **301**:958-960.

Gerhart, D. J. 1986. Gregariousness in the gorgonian-eating gastropod *Cyphoma gibbosum*: tests of several possible causes. Marine Ecology Progress Series **31**:255-263.

Harvell, C. D., K. Kim, J. M. Burkholder, R. R. Colwell, P. R. Epstein, D. J. Grimes, E. E. Hofmann, E. K. Lipp, A. D. M. E. Osterhaus, R. M. Overstreet, J. W. Porter, G. W. Smith, and G. R. Vasta. 1999. Emerging marine diseases: Climate links and anthropogenic factors. Science **285**:1505-1510.

Harvell, C. D., C. E. Mitchell, J. R. Ward, S. Altizer, A. P. Dobson, R. S. Ostfeld, and M. D. Samuel. 2002. Climate warming and disease risks for terrestrial and marine biota. Science **296**:2158-2162.

Harvell, C. D., and T. H. Suchanek. 1987. Partial predation on tropical gorgonians by *Cyphoma gibbosum* (Gastropoda). Marine Ecology Progress Series **38**:37-44.

Harvell, D., R. Aronson, N. Baron, J. Connell, A. Dobson, S. Ellner, L. Gerber, K. Kim, A. Kuris, H. McCallum, K. Lafferty, B. McKay, J. Porter, M. Pascual, G. Smith, K. Sutherland, and J. Ward. 2004. The rising tide of ocean diseases: unsolved problems and research priorities. Frontiers in Ecology and Evolution **2**:375-382.

Harvell, D., E. Jordán-Dahlgren, S. Merkel, E. Rosenberg, L. Raymundo, G. Smith, E. Weil, and B. Willis. 2007. Coral disease, environmental drivers, and the balance between coral and microbial associates. Oceanography **20**:172-195.

Hatcher, M. J., J. T. A. Dick, and A. M. Dunn. 2006. How parasites affect interactions between competitors and predators. Ecology Letters **9**:1253-1271.

Hazlett, B. A., and C. E. Bach. 1982. Distribution pattern of the Flamingo Tongue Shell (*Cyphoma gibbosum*) on its gorgonian prey (*Briareum asbestinum*). Marine Behavior and Physiology **8**:305-309.

Hoegh-Guldberg, O. 1999. Climate change, coral bleaching and the future of the world's coral reefs. Marine and Freshwater Research **50**:839-866.

Hughes, T. P. 1994. Catastrophes, phase shifts, and large-scale degradation of a Caribbean coral reef. Science **265**:1547-1551.

Hughes, T. P., A. H. Baird, D. R. Bellwood, M. Card, S. R. Connolly, C. Folke, R. Grosberg, O. Hoegh-Guldberg, J. B. C. Jackson, J. Kleypas, J. M. Lough, P. Marshall, M. Nyström, S. R. Palumbi, J. M. Pandolfi, B. Rosen, and J. Roughgarden. 2003. Climate change, human impacts, and the resilience of coral reefs. Science **301**:929-933.

Jolles, A. E., P. Sullivan, A. P. Alker, and C. D. Harvell. 2002. Disease transmission of aspergillosis in sea fans: Inferring process from spatial pattern. Ecology **83**:2373-2378.

Keesing, F., R. D. Holt, and R. S. Ostfeld. 2006. Effects of species diversity on disease risk. Ecology Letters **9**:485-498.

Kim, K., A. P. Alker, K. Shuster, C. Quirolo, and C. D. Harvell. 2006. Longitudinal study of aspergillosis in sea fan corals. Diseases of Aquatic Organisms **69**:95-99.

Kim, K., and C. D. Harvell. 2004. The rise and fall of a six-year coral-fungal epizootic. The American Naturalist **164**:S52-S63.

Kinzie, R. A. 1970. The ecology of the gorgonians (Cnidaria, Octocorallia) of Discovery Bay, Jamaica. PhD. Yale University, New Haven, Connecticut.

Klich, M. A. 2002. Identification of common *Aspergillus* species. Centraalbureau voor Schimmelcultures, Utrecht, Netherlands.

Lasker, H. R., and M. A. Coffroth. 1988. Temporal and spatial variability among grazers: Variability in the distribution of the gastropod *Cyphoma gibbosum* on octocorals. Marine Ecology Progress Series **43**:285-295.

Lasker, H. R., M. A. Coffroth, and L. M. Fitzgerald. 1988. Foraging patterns of *Cyphoma gibbosum* on octocorals: the roles of host choice and feeding preference. Biological Bulletin **174**:254-266.

Lilleskov, E. A., and T. D. Bruns. 2005. Spore dispersal of a resupinate ectomycorrhizal fungus, *Tomentella sublilacina*, via soil food webs. Mycologia **97**:762-769.

Malaquias, M. A. E., S. Condinho, J. L. Cervera, and M. Sprung. 2004. Diet and feeding biology of *Haminoea orbygniana*. Journal of the Marine Biological Association of the United Kingdom **84**:767-772.

McCallum, H., D. Harvell, and A. Dobson. 2003. Rates of spread of marine pathogens. Ecology Letters **6**:1062-1067.

Mullen, K. M., E. C. Peters, and C. D. Harvell. 2004. Coral resistance to disease. Pages 377-400 *in* E. Rosenberg and Y. Loya, editors. Coral Health and Disease. Springer-Verlag New York, Incorporated, New York.

Nagelkerken, I., K. Buchan, G. W. Smith, K. Bonair, P. Bush, J. Garzon-Ferreira, L. Botero, P. Gayle, C. D. Harvell, C. Heberer, K. Kim, C. Petrovic, L. Pors, and P. Yoshioka. 1997a. Widespread disease in Caribbean sea fans: II. Patterns of infection and tissue loss. Marine Ecology Progress Series **160**:255-263.

Nagelkerken, I., K. Buchan, G. W. Smith, K. Bonair, P. Bush, J. Garzon-Ferreira, L. Botero, P. Gayle, C. Heberer, C. Petrovic, L. Pors, and P. Yoshioka. 1997b. Widespread disease in Caribbean sea fans: I. Spreading and general characteristics. Proceedings of the 8th International Coral Reef Symposium **1**:679-682.

Nowlis, J. P. 1993. Mate- and oviposition-influenced host preferences in the coral-feeding snail *Cyphoma gibbosum*. Ecology **74**:1959-1969.

Opresko, D. M. 1973. Abundance and distribution of shallow-water gorgonians in the area of Miami, Florida. Bulletin of Marine Science **23**:535-558.

Patterson, K. L., J. W. Porter, K. B. Ritchie, S. W. Polson, E. Mueller, E. C. Peters, D. L. Santavy, and G. W. Smith. 2002. The etiology of white pox, a lethal disease of the Caribbean elkhorn coral, *Acropora palmata*. Proceedings of the National Academy of Sciences **99**:8725-8730.

Prom, L. K., and J. D. Lopez Jr. 2004. Viability of *Claviceps africana* spores ingested by adult corn earworm moths, *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae). Journal of Economic Entomology **97**:764-767.

Slattery, M. 1999. Fungal pathogenesis of the sea fan *Gorgonia ventalina*: Direct and indirect consequences. Chemeoecology **9**:97-104.

Smith, G. W., L. D. Ives, I. A. Nagelkerken, and K. B. Ritchie. 1996. Caribbean sea-fan mortalities. Nature **383**:487.

Smith, G. W., and E. Weil. 2004. Aspergillosis of gorgonians. Pages 279-288 *in* E. Rosenberg and Y. Loya, editors. Coral Health and Disease. Springer-Verlag New York, Incorporated, New York.

Sussman, M., Y. Loya, M. Fine, and E. Rosenberg. 2003. The marine fireworm *Hermodice carunculata* is a winter reservoir and spring-summer vector for the coral-bleaching pathogen *Vibrio shiloi*. Environmental Microbiology **5**:250-255.

Ward, J. R., K. Kim, and C. D. Harvell. 2007. Temperature affects coral disease resistance and pathogen growth. Marine Ecology Progress Series **329**:115-121.

Ward, J. R., and K. D. Lafferty. 2004. The elusive baseline of marine disease: Are diseases in ocean ecosystems increasing? PLoS Biology **2**:542-547.

Ward, J. R., K. L. Rypien, J. F. Bruno, C. D. Harvell, E. Jordán-Dahlgren, K. M. Mullen, R. E. Rodríguez-Martínez, J. Sánchez, and G. Smith. 2006. Coral diversity and disease in Mexico. Diseases of Aquatic Organisms **69**:23-31.

Wilkinson, C. 2002. Status of coral reefs of the world: 2002. Australian Institute of Marine Science, Townsville, Australia.

Williams, D. E., and M. W. Miller. 2005. Coral disease outbreak: pattern, prevalence and transmission in *Acropora cervicornis*. Marine Ecology Progress Series **301**:119-128.

Yoshioka, P., and B. Yoshioka. 1989. A multispecies, multiscale analysis of spatial patterns and its application to a shallow-water gorgonian community. Marine Ecology Progress Series **54**:257-264.

APPENDIX

ISOLATION AND CHARACTERIZATION OF MICROSATELLITE LOCI IN *ASPERGILLUS SYDOWII*, A PATHOGEN OF CARIBBEAN SEA FAN CORALS

ABSTRACT

Here we report on nine microsatellite loci designed for *Aspergillus sydowii*, a widely distributed soil saprobe that is also the pathogenic agent of aspergillosis in Caribbean sea fan corals. Primers were tested on 20 *A. sydowii* isolates from the Caribbean, 17 from diseased sea fans and 3 from environmental sources. All loci were polymorphic and exhibited varying degrees of allelic diversity (three to nine alleles). Gene diversity (expected heterozygosity) ranged from 0.353 to 0.821. These primers will enable future research into the epidemiology of *A. sydowii* as an emergent infectious disease.

INTRODUCTION

The haploid filamentous fungus *Aspergillus sydowii* is a globally distributed saprophyte, most commonly found in soil (Raper and Fennell 1965, Klich 2002). This fungus has also recently been identified as the pathogenic agent of an infectious disease of Caribbean sea fan corals (Smith et al. 1996, Geiser et al. 1998b). The aspergillosis epizootic originated in the mid 1990s, and has caused major declines in sea fan populations throughout the Caribbean (Nagelkerken et al. 1997, Kim and Harvell 2004). The source of this normally terrestrial fungus in marine ecosystems is not known, but current hypotheses include local terrestrial runoff (Smith et al. 1996, Geiser et al. 1998b), Saharan dust (Shinn et al. 2000), and marine sources (Roth Jr. et al. 1964). To effectively manage marine disease, an understanding of pathogen origins and mechanisms of transmission is critical. We developed nine novel

microsatellite loci for *A. sydowii* to assess population genetics and phylogeography in this species.

METHODS

Whole genomic DNA was extracted using four different methods: DNeasy Plant Kit (Qiagen), DNeasy Tissue Kit (Qiagen), lysis by vortexing with 425 - 600 μm glass beads (Sigma) for 30 seconds, cooling on ice for 30 seconds (repeated three times), followed by organic clean up with phenol-chloroform (Sambrook and Russell 2001), and digestion in lysis buffer with proteinase K, followed by standard phenol-chloroform purification and ethanol precipitation (Sambrook and Russell 2001). For all extraction methods, isolates of *A. sydowii* were grown on potato dextrose agar at 25°C seven days (minimum) in the dark. Fungal spores and hyphae were scraped from the plate using a sterile wooden dowel. The quality and quantity of whole genomic DNA was compared by running samples on a 1.5% agarose gel, analyzing concentration using a spectrophotometer, and amplifying a 485 base-pair portion of the 5′ non-translated region of the *trpC* gene using methods from Geiser et al. (1998a).

For construction of the microsatellite library, we used isolates of *A. sydowii* collected from the Caribbean, including individuals cultured from diseased sea fans in the Bahamas, Florida, Mexico, and the Netherland Antilles (Geiser et al. 1998b, Alker et al. 2001), and from environmental sources (soil collected from Jamaica, Florida and Venezuela). Whole genomic DNA was extracted from five isolates of *A. sydowii* grown on potato dextrose agar at 25°C for seven days in the dark using the method which yielded the highest concentration and quality of DNA (see descriptions above).

The genomic DNA was enriched for microsatellite repeats using a protocol adapted from Hamilton et al. (1999). Following digestion with *BsaA I* and *Hinc II* (New England Biolabs), fragments were ligated to a double-stranded SNX linker using

T4 DNA ligase. The pool of genomic fragments was enriched by hybridization with synthetic single-stranded biotinylated di- (GT, TC, TA), tri- (GAT, GTT, GTA, TTC, GCT, GTG, GTC, TCC, TTA), and tetranucleotide (GAAT, GATA, GATT, GTAT, GTTA, GTTT, TTAC, TTTC, GATG, GGTT, GCTT, GTAG, GTCA, GTCT, GTTC, TCAC, TTCC, GGGT, GCCT, GCTG, GCTC, GTGC, GTCG, GTCC, TCCC) repeats. Fragments were captured magnetically using Strepavidin-coated magnetic beads (New England Biolabs), and were made double-stranded using a polymerase chain reaction (PCR) with the SNX primer. Following digestion with *Nhe I* and *Xba I* (New England Biolabs), fragments were ligated into a pUC 19 cloning vector and transformed into ElectroMAX DH5α-E Cells (Invitrogen). Transformed cells were selected using Luria-Bertani agar amended with ampicillin, colonies were transferred to nylon membranes and screened using a ^{33}P-labelled probe of pooled oligonucleotides, the identity of which was the same as those used to enrich the library. Approximately 200 positively hybridized clones were sequenced using M13 forward primers and the BigDye Terminator Cycle Sequencing Kit v. 3.1 (PerkinElmer) on an ABI Prism 377 DNA sequencer (Applied Biosystems). After trimming the pUC19 vector and SNX linker, all clone sequences containing microsatellite regions were aligned to identify duplicates. Primers were designed for 34 unique loci using PrimerSelect software (DNASTAR, Inc.), 9 of which produced reliable amplification products, and were polymorphic (Table 1).

The resulting primers were optimized to yield clean products. We found that PCR reactions were more successful if *Taq* polymerase (Roche) was used, rather than an enzyme mixture, such as Platinum *Taq* DNA polymerase high fidelity (Invitrogen). All primers were first tested on two individuals along a temperature gradient (40°C to 55°C, and 55°C to 70°C). If non-specific binding occurred (i.e. multiple bands were observed on an agarose gel), reactions were run across a $MgCl_2$ gradient (Innis and

Gelfand 1990). Once temperature and $MgCl_2$ concentrations were optimized each primer pair was tested on ten isolates spanning the geographic range of the fungus, to ensure that the locus amplified in all individuals.

The microsatellite repeats were amplified using polymerase chain reaction (PCR) on a DNA Engine® (PTC-200™) Peltier Thermal Cycler (MJ Research) with the following protocol: an initial denaturation at 95°C for 5 minutes, followed by 35 cycles of 95°C for 1 minute, a specified annealing temperature for 1 minute (Table 1), 72°C for 1 minute, and a final extension at 72°C for 30 minutes. PCR amplification reactions were 10μL total volume, and included approximately 10ng genomic DNA, 10mM Tris-HCl (pH 8.3), 50mM KCl, 1.5mM $MgCl_2$, 0.2μM dNTPs, 0.2μM fluorescently-labeled primer (Applied Biosystems), 0.2μM unlabelled primer, and 0.25U *Taq* polymerase (Roche). Locus AS93 amplified best at 2.0mM $MgCl_2$. Fragments were analyzed on an ABI 3100 Automated Capillary DNA Sequencer using the GeneScan-500 LIZ size standard (Applied Biosystems). Allele sizes were estimated using GeneMapper *ID* v3.5 (Applied Biosystems) and were verified by eye.

RESULTS & DISCUSSION

A comparison of four DNA extraction methods found that traditional phenol-chloroform methods resulted in the highest concentration and quality of DNA. Both Qiagen kits had extremely low yields, with amplification of the *trpC* gene often failing. Of the two phenol-chloroform protocols, there was no obvious difference in the concentration or quality of genomic DNA. Therefore, we used lysis with glass beads followed by phenol-chloroform purification for all future DNA extractions, as it was the least time-intensive.

Twenty isolates of *A. sydowii* from the Caribbean representing a single population were tested to quantify variation at the nine microsatellite loci. Seventeen

isolates were from diseased sea fans sampled throughout the Caribbean, and three were from environmental sources (specific collection locations discussed earlier). The number of detected alleles at the nine loci ranged from three to nine, and expected heterozygosity (Nei 1987) ranged from 0.353 to 0.821 (Table A.1). The program Genepop on the web version 3.4 (Raymond and Rousset 1995) was used to test for departures from linkage disequilibrium. A Markov chain method with Bonferroni corrections for multiple comparisons was used to determine significance. One pair of loci showed significant linkage disequilibrium in global tests (AS251 and AS260). Null alleles were rare, with total amplification for all individuals at seven of the loci, and low frequencies of null alleles in the remaining two loci (AS251 5%, AS262 10%).

Preliminary data suggests that the microsatellite loci described here will be informative for population genetic studies of *A. sydowii*, an emergent fungal pathogen in coral reef communities. These markers were also found to amplify in isolates of *A. sydowii* from a wide range of substrates and geographic locations (data not shown). Four other microsatellite loci designed for *A. fumigatus* (Bart-Delabesse et al. 1998) also amplify successfully and are polymorphic in this species. The combination of these markers will provide a powerful tool to analyze molecular variation and patterns of population genetics in one of the few identified and culturable coral pathogens.

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Table A.1. Primer sequences, amplification conditions and diversity for 9 microsatellite loci in *Aspergillus sydowii*. F, forward primer; R, reverse primer; T_a, annealing temperature; N, number of individuals scored; N_a, number of alleles. Gene diversity (ie. expected heterozygosity) computed according to Nei (1987).

Locus (GenBank)	Primer Sequence (5′-3′)	T_a (°C)	Repeat	N/N_a	Size (bp)	Gene Diversity
AS93	F: PET-	40.4	$(TTC)_{21}$	20/3	244-	
(EF688266)	CTATGATCAAGAGAATACTTAAAGAAAGAAAAGACAA				288	0.353
	R: ATTCCAAATAGATGAACAATAACAATCAAAGTG					
AS202	F: 6-FAM-TACTTAGTCTCATGGCTGCCGCTGAAA	70	$(CA)_{29}$	20/5	303-	0.505
(EF688270)	R: ATACTCGCTTCGCAAGGTCATTGAGGTA				352	
AS203	F: VIC-CTTCGTGCTCGATTCCAATGAGTGC	69.7	$(GAA)_{21}$	20/9	215-	0.821
(EF688271)	R: CCCGTTTGCCAATTTCCCTATGGT				278	
AS206	F: 6-FAM-GACTCCTCCCCCGCTCCTCAAACAG	70	$(GTT)_{10}$	20/5	205-	0.774
(EF688269)	R: CTCGGTCGCCAAAGGTCAAAGTCGTCGTAT				243	
AS210	F: NED-GGGCTTCCGTAGGTGTGCTCA	70	$(ATT)_3(GTT)_7$	20/5	138-	0.753
(EF688268)	R: AGGTTGTTTCATCGGGCTTCTCATTC				154	
AS214	F: VIC-GATCGTCCTCTTCTTGCCTGCCTCCATTTAT	70	$(CT)_{28}$	20/6	409-	0.716
(EF688267)	R: GCGCTGCGGTGTTTTTGAAGAGGTGCTGTGA				427	
AS251	F: 6-FAM-CCTCTGGGCAATCTGGTTCCTGTAA	70	$(CAA)_{13}$	19/5	344-	0.717
(EF688273)	R: TCTTCCGGGCTCTCCTGACTCCT				379	

Table A.1 (Continued)

AS260 (EF688272)	F: NED-CGCGGTGAGCAATGGCGTAGAT R: TACCGCACCGTCTTCCTTGTCCTCT	70	$(GTT)_{10}(GGT)_4$	20/4	142-151	0.621
AS262 (EF688274)	F: NED-CCGGGCTTCCGTAGGTGTGCT R: GTTGTTTCATCGGGCTTCTCATTCATTT	68.9	$(GTT)_{10}$	18/3	150-155	0.578

for assistance in the development of the microsatellite library. Fungal isolates were transported and maintained according to USDA permit P526P-06-00465. All molecular work was conducted in the Evolutionary Genetics Core Facility at Cornell University.

REFERENCES

Alker, A. P., G. W. Smith, and K. Kim. 2001. Characterization of *Aspergillus sydowii* (Thom et Church), a fungal pathogen of Caribbean sea fan corals. Hydrobiologia **460**:105-111.

Bart-Delabesse, E., J. F. Humbert, E. Delabesse, and S. Bretagne. 1998. Microsatellite markers for typing *Aspergillus fumigatus* isolates. Journal of Clinical Microbiology **36**:2413-2418.

Geiser, D. M., J. I. Pitt, and J. W. Taylor. 1998a. Cryptic speciation and recombination in the aflatoxin-producing fungus *Aspergillus flavus*. Proceedings of the National Academy of Sciences of the United States of America **95**:388-393.

Geiser, D. M., J. W. Taylor, K. B. Ritchie, and G. W. Smith. 1998b. Cause of sea fan death in the West Indies. Nature **394**:137-138.

Hamilton, M. B., E. L. Pincus, A. Di Fiore, and R. C. Fleischer. 1999. Universal linker and ligation procedures for construction of genomic DNA libraries enriched for microsatellites. Biotechniques **27**:500-507.

Innis, M. A., and D. H. Gelfand. 1990. Optimization of PCRs. Pages 3-13 *in* M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White, editors. PCR Protocols. A Guide to Methods and Applications. Academic Press, San Diego.

Kim, K., and C. D. Harvell. 2004. The rise and fall of a six-year coral-fungal epizootic. The American Naturalist **164**:S52-S63.

Klich, M. A. 2002. Identification of common *Aspergillus* species. Centraalbureau voor Schimmelcultures, Utrecht, Netherlands.

Nagelkerken, I., K. Buchan, G. W. Smith, K. Bonair, P. Bush, J. Garzon-Ferreira, L. Botero, P. Gayle, C. Heberer, C. Petrovic, L. Pors, and P. Yoshioka. 1997. Widespread disease in Caribbean sea fans: I. Spreading and general

characteristics. Proceedings of the 8th International Coral Reef Symposium **1**:679-682.

Nei, M. 1987. Molecular Evolutionary Genetics. Columbia University Press, New York.

Raper, K. B., and D. I. Fennell. 1965. The genus *Aspergillus*. The Williams & Wilkins Company, Baltimore.

Raymond, M., and F. Rousset. 1995. GENEPOP (version 1.2): Population genetics software for exact tests and ecumenicism. Journal of Heredity **86**:248-249.

Roth Jr., F. J., P. A. Orpurt, and D. G. Ahearn. 1964. Occurrence and distribution of fungi in a subtropical marine environment. Canadian Journal of Botany **42**:375-383.

Sambrook, J., and D. W. Russell. 2001. Molecular Cloning: A Laboratory Manual. 3rd edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Shinn, E. A., G. W. Smith, J. M. Prospero, P. Betzer, M. L. Hayes, V. Garrison, and R. T. Barber. 2000. African dust and the demise of Caribbean coral reefs. Geophysical Research Letters **27**:3029-3032.

Smith, G. W., L. D. Ives, I. A. Nagelkerken, and K. B. Ritchie. 1996. Caribbean sea-fan mortalities. Nature **383**:487.