Fungal diversity studies using cultured-based and isolation-independent methods have shown that fungi occupy an important ecological niche in the marine environment. Although it has been suggested that the culture-based approach overlooks a large diversity of fungi, this method is still favored by research programs aimed at studying the biotechnological potential of fungi. Interactions between fungi and marine sponges are far less studied when compared with interactions between sponges and bacteria in terms of host specificity and microbial diversity. Living, apparently healthy, marine sponges were collected by scuba from 12 sites along the Caribbean and Pacific coasts of the Isthmus of Panama, at depths between 3 and 30 m. Sponges were collected in an area of ca. 3 m$^2$ at each site. Measures were taken to prevent cross contamination among samples during collection and isolation of the fungi. Total genomic DNA was extracted from 570 representative fungal strains and the ITS and LSU regions of the nuclear
ribosomal DNA were amplified and sequenced bidirectionally. Sequences were compared against the NCBI GenBank database using BLAST to estimate taxonomic placement. A total of 198 Operational Taxonomic Units (OTU) was found with 58% of the OTUs represented by singletons. Marine sponges were highly dominated by fungi within the Ascomycota (80.9%), with a minority within Basidiomycota (2.8%). Ascomycota fungi were represented by several major lineages, including Sordariomycetes, Dothideomycetes and Eurotiomycetes. Species accumulation curves for both geographical region and sponge orders indicated no obvious saturation. Several unique clades were found during phylogenetic analysis. Our results indicated that marine sponges from Panama could be a “hot spot” of fungal diversity as well as a rich resource for capturing, cataloguing, and assessing the pharmacological potential of previously undiscovered fungi associated with marine sponges.

Introduction

Fungi occupy an important ecological niche in the marine environment. This is revealed by phylogenetic analysis of increasingly available molecular data which promise to unravel the yet to be described marine fungal diversity (Manohar and Raghukumar, 2013). Sponges can harbor microorganisms that are either acquired by the surrounding water or by parental sponges through reproductive stages (Taylor, 2007). Interactions between sponges and bacteria are well studied and characterized in terms of host specificity and bacterial diversity (Taylor, 2007; Simister et al., 2012; Taylor et al., 2012); studies regarding archaea and fungi interacting with sponges are far less numerous. Although, it has been suggested that culture-based methodology overlooks a large diversity of fungi, this method is still favored by research programs aimed at studying the biotechnological potential of fungi (Blunt et al., 2009; Caballero-George et al., 2010; Kjer et al., 2010; Liu et al., 2010).

The Pacific shelf of Panama is wide and is divided by the southward projecting Azuero Peninsula, into two large areas: the Gulf of Panama (east-side) subject to strong seasonal wind-driven upwelling, and the Gulf of Chiriqui (west) where high mountains block the wind and prevent wind-induced upwelling (D’Croz and O’Dea, 2007). In contrast, the Caribbean coast of Panama is relatively straight with a narrow continental shelf exposed to ocean water except for the Bocas del Toro Archipelago on the west side, where rainfall and river discharge are higher (D’Croz et al., 2005; Collin et al., 2009). Thus, Panama’s “four oceanic zones” provide unique opportunities for understanding how and why low-latitude marine ecosystems vary and function as they do (Robertson et al., 2009). We used these widely differentiated oceanographic zones to compare fungal communities associated with 39 species of sponges collected by scuba in areas of high biodiversity.

Methods

Living, apparently healthy, marine sponges were collected by scuba at depths between 3 and 30 m from 12 primary sites locations along the Caribbean and Pacific coasts of the Isthmus of Panama (Figure 1). Three individuals of each sponge species were collected in an area of ca. 3 m² at each site. To prevent cross contamination among samples, each sponge was cut and sealed in situ (Figure 2) in an individual plastic bag after removing excessive water; they were processed for fungal isolation within 2 h. Sponges were identified following Zea (1987); Hooper and Van Soest (2002); and Collin et al. (2005). Collected sponges represented the following orders: Chondrosida, Dendroceratida, Dictyoceratida, Hadromerida, Halichondrida, Haplosclerida, Homosclerophorida, Lithistida, Poecilosclerida, Spirophorida, and Verongida.

Isolation of sponge-associated fungi was carried out under sterile conditions in a laminar flow cabinet (Portable Clean Air Unit, Liberty Industries). Whole fresh sponges were placed on a sterilized strainer
and washed thoroughly with sterile artificial seawater (ASW, adjusted to 36 g/l for Caribbean sites and 32 g/l for Pacific sites). Samples were pressed on sterile, absorbent paper and cut with a sterile scalpel into ca. 1 x 0.5 cm pieces, making a cross section from the osculum to the holdfast. Pieces were washed three times with sterile ASW and dried as above. From the cleaned mesophyll of each sample, 50 cubes of 2-3 mm$^3$ were cut. Ten cubes were placed onto each of five solid isolation media (P15, P30, EM, M1D, SNA) prepared prior to sterilization in 1 l volumes of distilled water (Caballero-George et al., 2010).

Figure 2. Diver cutting and sealing sponge individually in situ.
Total genomic DNA was extracted from 570 representative fungal strains; the Internal Transcribed Spacers (ITS) and Large Subunit (LSU) of the nuclear ribosomal DNA were amplified in a single reaction using ITS5 (or ITS1) and LR3 (or ITS4) primers (White et al., 1990) and sequenced bidirectionally. Sequences were grouped at 97% similarity, 40% overlap in sequencher as a proxy for species definition. OTUs were confirmed comparing the sequences against the NCBI GenBank database using BLAST analysis. The following criteria were used to interpret the sequences of the GenBank database: for sequence identities ≥97%, the genus and species were accepted; for sequence identities between 91% and 96%, only the genus was accepted; and for sequence identities ≥90%, isolates were labeled as the order or family name. All three criteria were defined on the basis of sequence similarity over shared sequence lengths with at least 80% overlap. We used the Shannon-Weaver diversity index in order to estimate the diversity of isolated fungal species from different regions (Caribbean, Pacific) and from different sponge orders. We used analysis of variance (ANOVA) to compare relative abundance of fungal species as a function of regions and sponge orders. Finally, we generated accumulation curves to test for the cumulative number of fungal species in relation to our sampling effort. These analyses were performed using the R (R Development Core Team, 2008).

Results

A total of 198 different species were found as defined by the Operational Taxonomic Units (OTU) with 58% of the OTUs found only once (singletons). The majority of the isolated fungi were Ascomycota (80.9%), with a minority within Basidiomycota (2.8%). Ascomycota fungi are represented by 15 orders dominated by Hypocreales, Xylareales and Pleosporales (Figure 3a) while Basidiomycota was represented by Agaricales, Polyporales and Russulales (Figure 3b).

![Figure 3](http://archive.rubicon-foundation.org)
Shannon-Weaver diversity index revealed a high diversity of cultivable fungi, and this diversity was comparable between the two geographical regions (3.51 for Caribbean, 3.52 for the Pacific). The following diversity indices were found for fungi isolated from different taxonomic orders of sponges: Dictyoceratida (1.20), Chondrosida (1.68), Hadromerida (1.68), Haplosclerida (2.74), Haplosclerida Verongida (2.55), non-identified species from the Caribbean (N.I.C) (3.40), Spirophorida (1.09), Homosclerophorida (2.45), Halichondrida (2.10), Poecilosclerida (3.02), non-identified species from the Pacific (N.I.P) (1.53). The analyses of variance indicated significant differences in the relative abundance of fungal species between geographical regions ($F_{2}= 31.2, \ P<0.001$) and among sponge orders ($F_{10}= 7.55, \ P<0.001$). Finally, our species accumulation curves for both geographical region and sponge orders indicate an increasing trend of species accumulation with no obvious saturation (Figure 4).

Figure 4. a) Species-accumulation curves per site of collection; b) Species-accumulation curves per taxonomic order of host sponge.

Effect of isolation media on isolation of sponge associated-fungi

In general, isolation media based on malt extract (Baker et al., 2009; Caballero-George et al., 2010) like EM appeared as a good culture media to obtain a high number of fungal isolates. Nevertheless, the question remains whether these high numbers also involve a high diversity. Figure 5 shows that EM media allowed isolation of the highest number of different fungal species (25%) followed by P15 and P30 (20%), which are based on glucose, peptone and yeast extract (Caballero-George et al., 2010). However, fungal species like Armillaria tabescens and Microdiplodia hawaiensis were only found in M1D culture media, and Colletotrichum gloeosporioides, Microdiplodia miyakei, Corynespora cassicola and Eutypella kochiana were found in PD culture media. In the present study, fungi from the order Hypocreales, particularly the genera Acremonium and Hypocrea would grow more readily on EM than the other five media. Interestingly, the genus Fusarium showed equal preference for media P15, SNA and EM, while Lecanisillium kalimantanense showed no preferences. The order Dothideales and unidentified strains of Ascomycota had equal preference for media EM as well as P30. Fungi from the order Xylariales had equal preference to grow in EM and P15. This was also the case for Eutypa consobrina. However, Eutypella sp. was isolated mainly from P15. Interestingly, Annulohypoxylon stygium, Anthostomella conorum, Arthrinium phaeospermum and Cryptosphaeria eunomia var. eunomia were isolated from medium EM and Daldinia cf. loculatoides was isolated only from PD and SNA. Fungi from the order Pleosporales were isolated mainly from media P30. The order Diaporthales was isolated in media EM, P30 and PD and the order Eurotiales was only isolated with PD and EM. The order Agaricales showed no preference for any media.
Figure 5. Number of different species of fungi isolated with each type of culture media.

**Discussion**

Very little is still known about diversity, nature of association and ecological significance of sponge-associated fungi. It is crucial to improve this knowledge in order to estimate a more precise fungal diversity (Richards et al., 2012) and to maximize their biotechnological application (Suryanarayanan, 2012).

The majority of the fungi isolated from this study belong to the Phylum Ascomycota and many branched close to clades of known terrestrial fungi. Marine ascomycetes are described as primary or secondary inhabitants of marine environments (Kohlmeyer, 1986). Primary marine species are hypothesized to be derived from ancestral lineages that originated in marine environments, while secondary marine species represent the reintroduction of fungi into the marine environment and are suggested to share a more recent common ancestry with terrestrial lineages (Spatafora et al., 1998).

Most of the current knowledge about the diversity of sponge-associated fungi comes from culture-based studies that aim to discover pharmacologically active metabolites within individual strains. Although these studies are conservative and might not be truly representative of natural diversity (Richards et al., 2012) they remain the goal of research programs aimed at isolating bioactive compounds from fungi. This is why special efforts have been made to optimize culture media in order to isolate the largest possible diversity. In the present study, media based on malt extract was suitable to isolate a large number of fungi with the highest number of different species. Nevertheless, isolates like M2034-2-M1D (*Microdiplodia hawaiiensis* DQ885897 ID 90%) and M188I-1-M1D (*Armillaria tabescens* AY213589 ID 83%) that were found only once came from isolating media that yielded a low number of isolates such as M1D. This suggests that the use of diverse cultivation media increases the efforts to capture a large diversity of fungi. Our results suggest that fungi from different taxonomical orders may show preferences regarding culture media. This knowledge might facilitate the isolation of specific groups of fungi.

Together, these results suggest that there is a high diversity of sponge-associated fungal species in neotropical waters and that this diversity varies both between geographical regions and among host-sponge species. Interestingly, our results also indicate that our sampling design was rather limited and that
a more systematic effort could help in a better understanding of the species richness and diversity of sponge-associated fungi.

Acknowledgment

Catherina Caballero-George thanks financial support by the Panamanian Secretariat of Science and Technology (SENACYT) through the incentive program of the National System of Innovation (SNI). The authors acknowledge financial support by grants of international collaboration by SENACYT numbers COL08-014 and COL10-070, and by a partnership program between the Organization for the Prohibition of Chemical Weapons (The Hague, Netherlands) and the International Foundation for Science (Stockholm, Sweden). The College of Agriculture and Life Sciences at The University of Arizona is gratefully acknowledged for technical and logistical support for molecular analyses, Juan B. Del Rosario for research assistance, and the Smithsonian Tropical Research Institute for laboratory facilities, boats and technical support. The authors also want to thank the Panamanian Authority of the Environment (ANAM) for their collaboration.

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