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Diversity of culturable yeasts associated with zoanthids from Brazilian reef and its relation with anthropogenic disturbance[☆]

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ABSTRACT

Some of the main threats to coral reefs come from human actions on marine environment, such as tourism, overfishing and pollution from urban development. While several studies have demonstrated an association between bacteria and corals, demonstrating how these communities react to different anthropogenic stressors, yeast communities associated with corals have received far less attention from researchers. The aim of this work was therefore to describe cultivable yeasts associated with three coral species and to evaluate the influence of sewage discharge on yeasts community. We obtained 130 isolates, mostly belonging to phylum Ascomycota and many of them had previously been isolated from human samples or are considered pathogens. The mycobiota was more similar among corals collected from the same reef, indicating that the composition of reef yeast community is more influenced by environmental conditions than host species. We suggest further studies to elucidate which factors are most influential on the composition of the coral-associated yeast community.

1. Introduction

Zoanthids (Cnidaria: Anthozoa: Hexacorallia: Zoantharia) are sessile and suspensivorous organisms (Santana et al., 2013), that are symbiotic with photosynthetic dinoflagellates of genus *Symbiodinium* (Reimer et al., 2012). As the group lacks a calcium carbonate skeleton, the taxon is often considered an intermediary between hard corals (Scleractinia) and sea anemones (Actiniaria) (Reimer et al., 2008) and are commonly dominate sites where stress conditions exclude scleractinian corals (Silva et al., 2015). Zoanthids also generate a wide range of microniches for microorganisms such as bacteria, viruses and fungi (Bourne and Munn, 2005), including yeasts.

Yeasts are fungi whose asexual growth predominantly results from budding or fission and do not form their sexual states within or upon a fruiting body (Kutzman and Fell, 2015). They are distinguished from filamentous fungi by their unique unicellular growth and are considered a polyphyletic group of basidiomycetous and ascomycetous fungi (Kutty and Philip, 2008). Marine yeasts are defined as yeasts isolated from marine environments that grow better in seawater than in fresh water (Chi, 2012). Marine yeasts can be obligate (confined to a marine environment) or facultative if they are also found in terrestrial

habitats (Jones et al., 2013). Marine yeasts have been implicated in several ecologically significant marine processes, including decomposition of plant substrates, nutrient-recycling, and parasitism of marine animals (Jones and Pang, 2012). Nevertheless, fungi in the marine environment are poorly studied compared to those in terrestrial and freshwater systems. Marine yeasts may be saprophytic, mutualistic, or parasitic, and can occur on a wide range of substrates or be associated with various marine invertebrates including oysters, clams, mussels, prawns, and crabs (de Araujo et al., 1995; Kosawa da Costa et al., 1991; Pagnocca et al., 1989). The most frequently isolated yeasts from these organisms that are also generally the ones prevalent in the surrounding water (de Araujo et al., 1995). This symbiotic association between microorganisms and marine invertebrates can benefit the hosts by protecting them from diseases (for example, by producing killer toxins by yeasts) and by providing nutrients (Bourne et al., 2016; Ritchie, 2006). However, some fungi species are considered opportunistic and may cause various mycoses, allergies, and fungemia (Hagler, 1998).

One of the main threats to coral reefs is the impact of anthropic actions. These include tourism, overfishing and pollution, mainly from sewage, resulting from urban development in coastal areas (Wear and

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Thurber, 2015). Such sewage contains a diverse array of polluting agents, such as ammonium, nitrite, nitrate and phosphate, in addition to diverse pathogens (Islam and Tanaka, 2004). Studies have shown that allochthonous bacteria, including many human pathogens, may persist from one to several weeks after introduction into the marine environment, replacing native microbiota and disrupting ecosystem processes (Nagvenkar and Ramaiah, 2009).

Despite the ecological and cultural importance of reef ecosystems, there have been few studies on the effects of anthropogenic pollution on filamentous fungi and even less on coral-associated yeasts. However, the relationship between bacteria and coral has been well studied (Ainsworth et al., 2007; Bourne and Munn, 2005; Frias-Lopez et al., 2002; Rohwer et al., 2002), including some species of zoanthids. For example, as the zoanthid *Palythoa caribaeorum* was shown to possess a high bacterial diversity associated (Carlos et al., 2013; Paulino et al., 2017) with several groups with nitrogen metabolisms (Shieh and Lin, 1992) and production of bioactive compounds with antimicrobial activity that would contribute to defense of the coral host (Sun et al., 2014). Moreover, sewage exposure generally results in a disruption of the bacterial microbiota, which become more heterogeneous with a greater abundance of groups considered pathogenic for both humans and coral host (Thurber et al., 2009; Ziegler et al., 2016).

Along the Brazilian coast, zoanthids are widely distributed, forming dense aggregates in intertidal environments and shallow waters (Rabelo et al., 2015) and playing key roles in community structuring (Pérez et al., 2005). Unfortunately, many of these coral reefs suffer from as the effects of climate change and impact of human actions, especially sedimentation and pollution (Ferreira and Maida, 2006). Nevertheless, the diversity and tolerance to pollution of coral-associated yeasts, especially zoanthids, has not been investigated. Here, we isolate and identify the yeasts associated with three species of cnidarians belonging to the family Zoanthidae (*Palythoa caribaeorum*, *Palythoa variabilis* and *Zoanthus sociatus*), collected in two Brazilian coastal reefs (one reef exposed to high levels of sewage and one reef with low levels of contamination).

2. Materials and methods

2.1. Yeast isolation and collection

Yeasts strains were isolated from three marine zoanthids species (*Palythoa caribaeorum*, *Palythoa variabilis* and *Zoanthus sociatus*). Collections were obtained in 2014 and 2015 at two points located in Maceió, northeast Brazil: seven collections on *Ponta Verde* reef (9° 66' 32" S 35° 69' 82" W), located in an urban coastal setting and subject to direct discharge of untreated sewage from illegal connections to storm sewers. Another seven collections on *Sereia* reef (9° 56' 52" S 35° 64' 49" W), a sandstone reef less affected by anthropic actions where the main stressor is tourism (Fig. 1). The collections from the *Ponta Verde* reef began in April 2014 and ended in March 2015, while the collections from *Sereia* reef began in September 2014 and ended in September 2015. Physicochemical parameters of the water such as the pH, temperature, and salinity were collected using a Hanna HI 9828 multi-parameter probe, and data about bathing (thermotolerant coliforms) were obtained from the Institute of the Environment of Alagoas (IMA). Permission for this study was obtained from the regulatory institution *Instituto Brasileiro do Meio Ambiente* (license no. L. 32723-1).

The marine soft corals samples were washed with sterile seawater to remove the debris, cut into small pieces, and pounded with a mortar and pestle. We inoculated 100 µL of decimal dilutions (10^{-1} , 10^{-2} , 10^{-3} , 10^{-4}) in Wickerham's YM medium (0.3% yeast extract, 0.3% malt extract, 0.5%, 2% agar, 1% glucose) with seawater, supplemented with 0.04% chloramphenicol, and incubated samples at 22 °C for up to 26 days. One representative morphotype of each different colonial per sample were purified twice in YEPD medium (2% dextrose, 1% peptone, 0.5% yeast extract and 2% agar). For conservation, the isolates

were kept in vials with YEPD media covered with sterile mineral oil at 4 °C.

2.2. Molecular identification

The yeast strains were cultured aerobically in Sabouraud broth at 22 °C. Samples were centrifuged at $8000 \times g$ for five minutes to recover cells. The pellets were then washed with sterile distilled water and centrifuged again (as above). Samples were diluted with 500 µL of buffer lysis (0,15 M NaCl, 50 mM Tris-HCl, 10 mM EDTA, 2% SDS pH 8,0) and incubated at 65 °C for one hour, after which 200 µL potassium acetate (5 M, pH 4,8) was immediately added and the tubes were placed in an ice bath for 30 min. The samples were then centrifuged at $14,100 \times g$ for 5 min after which the supernatant was transferred to another tube and centrifuged. The supernatant was transferred to another tube; 600 µL of absolute isopropanol was added, and the sample was incubated at -20 °C for 15 min. After a centrifugation at $14,100 \times g$ for 20 min, the DNA was collected by centrifugation at $13,000 \times g$ for 15 min and washed with 70% ethanol. The supernatant was discarded and, after drying, the DNA was resuspended in a TE buffer (Tris-EDTA pH 7,4) and stored at -20 °C. To amplify the divergent D1/D2 domain of the LSU rDNA gene and the ITS region, universal primers NL1, NL4 and ITS1, ITS4 were used (Kurtzman and Robnett, 1998). Amplification conditions were: 94 °C for 5 min, 35 cycles at 94 °C for 15 s, 56 °C for 45 s, and 72 °C for 90 s, and a final extension at 72 °C for 6 min. Sequencing was performed as described by Péter et al. (2009) in the ABI-PRISM 3100 Genetic Analyzer (Applied Biosystems) according to the manufacturer's instructions. Sequences were compared with those deposited at the GenBank database (<http://www.ncbi.nlm.nih.gov/>) using the BLAST tool.

2.3. Statistical analyses

Yeast diversity was quantified using the Shannon-Weaver index for all collection points and for each species of zoanthid (Shannon, 1948). A linear regression was used to verify the influence of sewage on yeast diversity. In order to compare the similarity between the collection sites, using the data from the same sampling period, beta diversities was calculated using the Whittaker coefficient. Data was analyzed using the PAST v.3 statistical program (Hammer et al., 2001) and PCAs were generated with ClustVis (Metsalu and Vilo, 2015).

3. Results

We isolated 130 yeasts, mainly at concentrations of 10^0 and 10^{-1} , 50 of which were obtained from samples at the *Ponta Verde* reef, and 80 were from the *Sereia* reef (Table 1 and Supplementary Materials 2 and 3). The molecular identification based on the D1/D2 domain of the LSU rDNA of the isolates resulted in a total richness of 38 species, of which 25 belonged to the phylum Ascomycota and 13 to the phylum Basidiomycota. The Ascomycota prevalence in coastal marine environments has been reported in several studies (Jones and Pang, 2012; Vogel et al., 2007; Zhang et al., 2015). This fact is possibly associated with the greater availability of organic matter and the chemical composition of coral compartments. For example, coral mucus, is composed mainly of monosaccharides such as glucose, galactose and lipids (Ducklow and Mitchell, 1979; Glasl et al., 2016). These compounds facilitate the growth of this phylum's members, which are specialists in degrading simple compounds in environments rich in organic carbon and sugars (Suh et al., 2006).

The physicochemical parameters measured during the collections (pH, temperature and salinity) were homogeneous (Supplementary Material 1) and were not used to responsible for influencing the yeasts distributions. However, the average values related to thermotolerant coliform counts showed a high variation between the two reefs being, in *Ponta Verde*, the Most Probable Number (MPN) = 3502 supporting the

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