# Molecular characterization of fungi isolated from sea urchin S*tomopneustes variolaris* (Lamarck, 1816) – St. Mary's Island, west coast of India

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## ABSTRACT

**Introduction and Aim:** Sea urchins are commonly found along the rocky ocean floor in both shallow and deeper water and found inhabiting coral reefs. Sea urchins are omnivorous animals and eat both plant and animal matter. They mainly feed on coral and rocks along with decomposing matter. *Stomopneustes variolaris* species shows a remarkably patchy distribution and it can be pretty abundant where they are found. They can be encountered along the east African coast to the Philippines on rock and damaged reefs, most often in shallow waters but never too close to wave action. Aim of the study was to isolate and identify the fungal pathogens inhabiting in sea urchins and their molecular characterization using ITS sequencing method..

**Materials and Methods:** In the present study, sea urchin (*Stomopneustes variolaris*) samples were collected from St. Mary's Island, Malpe beach, west coast of India and tried to isolate some fungi as they are edible worldwide. Gut, gonad and coelomic fluids were separated from the sea urchin and subjected to isolation of different fungal forms.

**Results:** Four morphologically different species of fungi were isolated and identified as *Cladosporium cladosporiodes* SCS10 (coelomic fluid), *Corynespora cassiicola* ABS48 and *Cladosporium oxysporum* CBR23 (gonad) *Cladosporium colombiae* DFFSCS017 (gut). Fungal species were confirmed based on the morphological feature and through molecular characterization approach using ITS sequencing. The genomic DNA of fungi was extracted and a large-scale reaction of 100µl was set up using specific primers. This product was then purified and sequenced. The results obtained on sequencing were uploaded on the NCBI website and BLAST program was run to identify the fungi.

**Conclusion:** To our knowledge, this is the first report to investigate the diversity of fungi from sea urchin in the St. Mary's Island, Malpe beach, west coast of India.

**Keywords:** Coelomic; fungi; gonad; gut; S*tomopneustes variolaris*; St. Mary's Island, west coast.

## INTRODUCTION

Fungi are fundamental organisms in our ecological systems and will be found anywhere where the environment will support their growth, both on land and in water (1). Coastline habitats such as mangrove, sand, beach, river, and estuarine habitats have been contributed a diversity of fungi, suggesting that environmental influences such as floods and winds carry terrestrial fungi toward marine environments. Thus, morphological characteristics of marine-derived fungi are similar to their terrestrial counterparts (2, 3). Distribution and abundance of fungi in the marine environment can influence by many factors and different substrates such as sponges, algae, wood, tunicates, sediments, mollusks, corals, plants and fish have been contributed variety of marine fungi (4, 5). Marine habitats, such as sea water, sediment, marine animals and plants harbor marine fungi and play an important ecological role in recycling nutrients, decomposition of dead plant and animal tissues. Numerous studies have showed that sponge-associated fungi are one of the major marine sources of bioactive compounds (6, 7). Certain species of marine fungi are pathogenic to marine plants and animals and also form symbiotic relationships with other organisms (8). Marine fungi have been classified as obligate or facultative: Fungi are those that grow and sporulate exclusively in a marine or estuarine habitat are obligate fungi, whereas the fungi are those from freshwater or terrestrial origin that are able to grow and possibly sporulate in marine environments are facultative fungi (9, 10). Marine fungi form symbiotic relationships with other organisms (sponges, algae, corals, and calcareous tubes of mollusks), but the function of these associations is rarely known (11, 12).

Sea urchin (*S. variolaris*) which belongs to the phylum Echinodermata, widely distributed in all the seas from the Arctic to Antarctic regions (13). They are distributed from Kanyakumari to Vishakapatnam shores of India and most commonly found in the shallow sub tidal zone on rocky bottoms, commonly known as slate pencil sea urchin. Only 16 species of sea urchins are harvested for food worldwide among 1000 (14). *Stomopneustes variolaris* are dark-coloured, omnivores and warm-water echinoderm species. They are widely distributed in the tropical

and subtropical of Indo-Pacific Ocean in shady littoral areas with water depth up to 18 m (15). They are popular in Korean and Japanese cuisine and are also a traditional food in Chile (16). *S. variolaris* is one of the edible sea urchin species found in the Indian Ocean (17). The World annual consumption of sea urchins has been steadily increasing over the last decade and is one of the important fisheries in several areas of the world (18). Sea urchins have long been harvested for their gonads (roe), are in high demand and economically important food across the world as they have high nutritional contents. Shells and spines of sea urchins are generally discarded as food waste without further utilization after removal of the edible gonads (19). But the shells are containing various bioactive (20). Presence of *Cladosporium* sp. and other fungal species in sea urchin would lead to potential risk for human consumption (21). Hence, the present study was undertaken to isolate and identify some fungi in sea urchin as a first attempt.

## MATERIALS AND METHODS

### Sample collection and processing

Sea urchins were collected from St. Mary's Island using scuba diving at 15-meter depth. It is an island of white sand beaches, rock monoliths and great wildlife making it a mesmerizing place to be in. It is a group of four individual islands namely Coconut Island, North Island, South Island and Daryabahadurgarh Island lying a little to the north of the Malpe port of Udupi District, West coast of India, Karnataka (Latitude 13º 20′ 60″ N; Longitude 74º 40′ 60″ E). The sea urchin samples were transferred directly to a sterile plastic bag with seawater and brought to the laboratory for the isolation of fungi. The samples were preserved at 4ºC for the further process

### Isolation and identification of fungi

Sea urchin samples were thoroughly washed with sterile seawater to remove any loosen associated microbes on the surface of sea urchin. Then they were dissected to separate the coelomic fluid, gut and gonad for isolation of fungi. The gut, gonad and coelomic fluid samples were transferred to sterile 50 mL falcon tubes separately. These were then macerated and teased gently with the help of sterile glass rod and sterile forceps in order to release any organisms adhered to the tissue into the surrounding saline. The samples were centrifuged at 8000 rpm for 10 minutes and then the supernatant was collected in separate sterile falcon tubes. 5 mL of each aliquot was diluted to 1:1 with sterile 2.5% saline. This was done in order to enrich the organisms present in the sample since a higher salt concentration would mimic their natural habitat (sea water). 0.1 mL of enriched sample of the gut, gonad and coelomic fluid was spread on sterile Potato dextrose agar (PDA) plates to obtain marine fungi. 100µg/mL of Ampicillin was added to the PDA plates in order to avoid bacterial growth. The enriched samples were spread onto the Petri plates in triplicates. Petri plates were incubated for 10-15 days at room temperature until the morphology of the fungi could be distinguished. Isolated fungi were sub-cultured on PDA Petri plates and they were subjected to the lacto-phenol cotton blue staining. All the procedures were performed aseptically in order to avoid contamination. Morphological characteristics were determined according to the methodology (22).

### Fungal DNA extraction

Fungal isolates were transferred onto a new corresponding agar plates based on their morphological differences, growth characteristics, mycelia, and diffusible pigments. Then the plates were incubated at 10 °C to obtain the pure cultures. Fungal isolates were identified by comparing the morphological observations with internal transcribed spacer sequences (ITS). Fungal genomic DNA extraction was done according to the method described (23). From the genomic DNA, nearly full-length ITS sequences were amplified by polymerase chain reaction (PCR) using the following primers: ITS1 (50 TCC GTA GGT GAA CCT GCG G -30) and ITS4 (50 -TCC GCT TAT TGA TAT GC -30) (24). PCR amplification conditions using ITS1 and ITS4 primers were as follows: the initial denaturation was 7 min at 94 °C followed by 30 cycles of denaturation for 30s at 94 °C, annealing for 30s at 55 °C, extension for 30s at 72 °C and the final 7 min extension at 72 °C (25). 1 % agarose gel was used for the diffusion of PCR products and visualized by ethidium bromide. Each band on a gel electrophoresis represented the single species of fungi. The results obtained on sequencing were uploaded on the NCBI website and BLAST program was run to identify the fungi. Fungal ITS gene sequencing and identification was done according to the following method (26). By sequenced data a phylogenetic tree was constructed using the Clustal Omega tool software, in Molecular Evolutionary Genetics Analysis Tool (MEGA X) (27).

## RESULTS

### Identification of fungi and molecular characterization

Our result represents the first report of isolation and identification of fungi from sea urchin (Stomopneustes variolaris). In the previous study we have isolated Vibrio sp. from coelomic fluid of sea urchin using rDNA sequencing as a first report from sea urchin (Stomopneustes variolaris). In the present study, four morphologically different fungal isolates were identified after incubation of 10-15 days. Based on the morphological characteristic fungi were identified and sub-cultured. The isolated fungal strains were identified on the basis of colour, colony morphology, shape, texture, and microscopic

characteristics like the presence of specific reproductive structures, shape of conidia and mycelium (Table1).

**Table 1:** Cultural and morphological characteristics of fungi and their identification.

| Cultural characteristics | Morphological characteristics | Identification of fungi |
|---|---|---|
| Colonies olive to olive-green, velvety surface. | Conidiophores are either straight or slightly bent, conidia oval to lemon-shaped, unbranched or branched chains arising from cylindrical base cells. | *Cladosporium oxysporum* |
| Colonies are olive-grey to dull green, Colony edges olive-green to white. | Conidiophores are unbranched, rarely branched, darkly pigmented hyphae not constricted at the base. Many long and branched chains of conidia. | *Cladosporium cladosporoides* |
| Colonies are olivaceous-brown to blackish-brown sometimes grey, or brown. | Conidiophores macro and micronematous, slightly narrower, walls slightly thickened, conidiogenous cells non-nodulose, usually with a single apical scar, conidia formed in chains. | *Cladosporium colombiae* |
| Colonies are green to light. olivaceous green to brown or dark blackish brown. | Conidia are solitary or in chains, variable in shape, cylindrical or curved, pale olivaceous brown or brown with pseudosepta. | *Corynespora cassiicola* |

Fungal strains such as *Cladosporium cladosporiodes* SCS10 isolated from coelomic fluid (F/GC4), *Cladosporium oxysporium* CBR23 (F/Go2) and *Corynespora cassiicola* ABS48 (F/Go3), *Cladosporium colombiae* (F/G5) from gonad and gut respectively (fig. 1). The isolated fungal species were identified by ITS sequencing method.

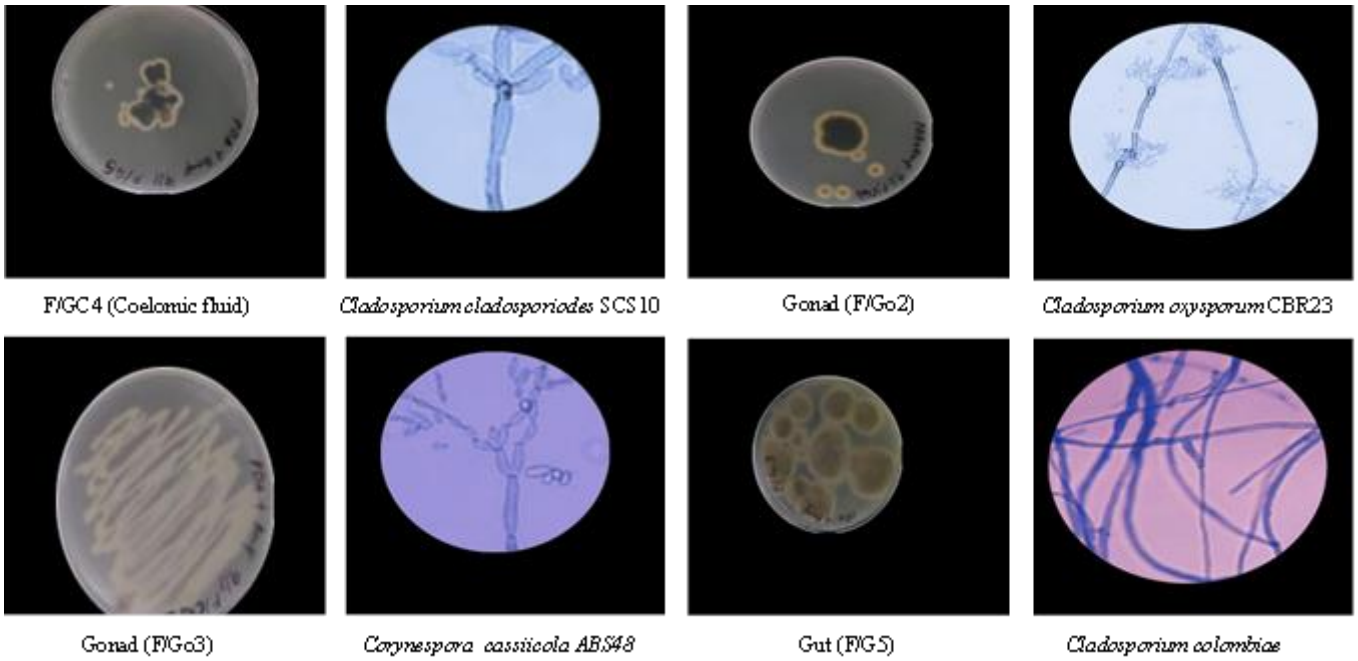



**Fig. 1:** Morphological characteristics of fungi (sub-cultured and isolated fungi)

**F/Go2:**

5'GGGTGCGTCGCCAGTATACGCGGGAGTTCA
TACCCTTTGTTGTCCGACTCTGTTGCCTCCGG
GGCGACCCTGACTACGGGCGGGGGCTCCGGG
TGGACACTTCAAACTCTTGCGTAACTTTGCA
GTCTGAGTAAACTTAATTAATAAATTAAAAC
TTTTAACAACGGATCTCTTGGTTCTGGCATCG
ATGAAGAACGCAGCGAAATGCGATAAGTAA
TGTGAATTGCAGAATTCAGTGAATCATCGAA
TCTTTGAACGCACATTGCGCCCCCTGGTATTC
CGGGGGGCATGCCTGTTCGAGCGTCATTTCA
CCACTCAAGCCTCGCTTGGTATTGGGCAACG
CGGTCCGCCGCGTGCCTCAAATCAACCGGCA
GGGTCTTCTGTCCCCTAAGAGTTGTGG3'

**F/Go3:**

5’GGCGCCGGGGCGGGAGTCGCCCTTCGAGA
AGCACCCTTTGTTTATGAGCACCTCTCGTTTC
CTCGGCAGGCTCGCCTGCTAACGGGGACCCA
CCACAAACCCATTGTAGTACAAGAAGTACAC
GTCTGAACAAAACAAAACAAACTATTTACAA
CTTTCAACAACGGATCTCTTGGTTCTGGCATC
GATGAAGAACGCAGCGAAATGCGATAAGTA
GTGTGAATTGCAGAATTCAGTGAATCATCGA
ATCTTTGAACGCACATTGCGCCCTTTGGTATT
CCTTAGGGCATGCCTGTTCGAGCGTCATTTCA
ACCCTCAAGCCTAGCTTGGTGTTGGGCGTCT
GTCCCGCCTCCGCGCGCCTGGACTCGCCTCA
AAAGCATTGGCGGCCGGTTCCCAGCAGGCCA
CGAGCGCAGCAGAGCAAGCGCTGAAGTGGC

TGGGGTCGGCGCACCATGAGCCCCCCCACAC
CAGAATTTTGACCTCGGATCAGGTAGGGATA
CCCGCTGAACTTAAGCATATCAGTAAGCGGA
AGAAA3’

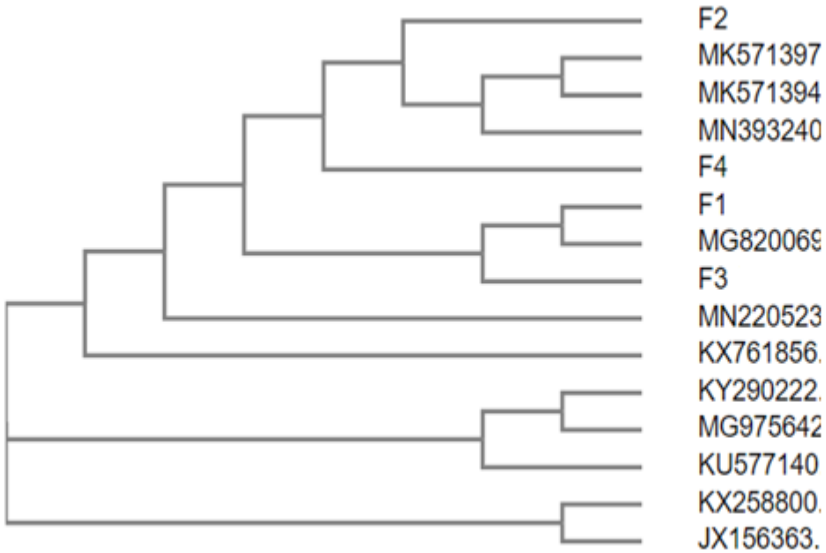
**F/GC4:**

5’GGTGGTGTGACCCGATTACCCCGGGAGTTC
ATACCCTTTGTTGTCCGACTCTGTTGCCGGGG
CGACCCTGCCTTCGGGCGGGGGCTCCGGGTG
GACACTTCAAACTCTTGCGTAATCCCTTGCA
GGTCTGAGTAAACTTAATTAATAAATTAAAA
CTTTTAACAACGGATCTCTTGGTTCTGGCATC
GATGAAGAACGCAGCGAAATGCGATAAGTA
ATGTGAATTGCAGAATTCAGTGAATCATCGA
ATCTTTGAACGCACATTGCGCCCCCTGGTATT
CCGGGGGGCATGCCTGTTCGAGCGTCATTTC
ACCACTCAAGCCTCGCTTGGTATTGGGCAAC
GCGGTCCGCCGCGTGCCTCAAATCGACCGGC
TGGGTCTTCTGTCCCCTAAGCGTTGTGGAAA
CTATTCGCTAAAGGGTGCTCGGGAGGCTACG
CCGTAAAACAACCCCATTTCTAAGGTTGACC
TCGGATCAGGTAGGGATACCCGCTGAACTTA
AGCATATCAATAAGCGGAGGAA3’

**F/G5:**

5’GGGGGGTCTCCGCCAGTATCTACACCGGGA
GTTCTATCCCTTTGTTGTCCGAACTCTGTTGA
CTCAAGGGCGACCCTGTTAAAAGGCGGGGGC
TCCGGGTGGACACTTCAAACTCTTGCGTAAC
TTTGCAGTCTGAGTAAACTTAATTAATAAATT
AAAACTTTTAACAACGGATCTCTTGTTCTGGC
ATCGATGAAGAACGCAGCGAAATGCGATAA
GTAATGTGAATTGCAGAATTCAGTGAATCAT
CGAATCTTTGAACGCACATTGCGCCCCCTGG
TATTCCGGGGGGCATGCCTGTTCGAGCGTCA
TTTCACCACTCAAGCCTCGCTTGGTATTGGGC
AACGCGGTCCGCCGCGTGCCTCAAATCGACC
GGCTGGGTCTTCTGTCCCCTAAGCGTTGTGG
AAACTATTCGCTAAAGGGTGCTCGGGAGGCT
ACGCCGTAAAACAACCCCATTTCTAAGGTTG
ACCTCGGATCAGGTAGGGATACCCGCTGAAC
TTAAGCATATCAATAAGCGGGAGGAA3’.

Based on the above sequences, phylogenetic tree was constructed using Custal Omega tool (fig. 2).




1. *Corynespora cassiicola* ABS48
2. *Corynespora cassiicola* clone PaCcHNSY1705: MK571397.1
3. *Corynespora cassiicola* clone LsCcHNSY1102: MK571394.1
4. *Corynespora cassiicola* isolate JD001: MN393240.1
5. *Cladosporium colombiae* DFFSCS017
6. *Cladosporium oxysporum* CBR23
7. *Cladosporium cladosporioides* strain BYCDW7: MG820069.1
8. *Cladosporium cladosporiodes* SCS10
9. *Cladosporium asperulatum* isolate LH4: MN220523.1
10. *Cladosporium sp. strain* jyzA2: KX761856.1
11. *Cladosporium cladosporiodies* culture FCBP: 1493: KY290222.1
12. *Cladosporium sp*. isolates RT42: MG975642.1
13. *Cladosporium oxysporum* isolate CBR23: KU577140.1
14. *Cladosporium cladosporioides* strain SCSIO z015: KX258800.1
15. *Cladosporium colombiae* isolate DFFSCS017: JX156363.1

The above phylogenetic tree depicts the evolutionary relation of the selected sequences. The sequences are classified into 3 different family groups as shown above. The first group has a total of 10 sequences which are indicated by their accession number. All the query sequences (F1, F2, F3 and F4) are sharing the first group in the phylogeny. F2 and F4 and F1and F3 are closer to each other in the course of

evolution. Among the sequences compared the closest organism of F1, F2, F3 and F4 were found to be *Cladosporium cladosporioides* strain BYCDW7, *Corynespora cassiicola* isolate JD001, *Cladosporum cladosporioides* strain BYCDW7 and *Corynespora cassiicola* ABS48.

## DISCUSSION

*Cladosporium* sp. is the genus of the fungi found most frequently in outdoor air in temperate climates. It is a major colonizer of plant litter and has been isolated from many different types of soil. Unless there is an indoor source of contamination, it is found indoors as well, but usually in less numbers. In indoors, this fungus is often encountered in dirty refrigerators, especially in reservoirs where condensation is collected. The ability to sporulate heavily, ease of dispersal and buoyant spores, makes this fungus an important fungal allergen. Due to its ability to invade rapidly, (many different ecological niches including marine habitat), *Cladosporium* is ubiquitous and therefore sometimes problematic (28). *Cladosporium* is a member of the form class Deuteromycetes, family Cladosporiaceae under the order Hyphomycetes. It is one of the largest and most heterogeneous genera of hyphomycetes. *Cladosporoids* are common and distributed worldwide (29). Several endolithic *Aspergillus* sp. *Penicillium* sp. and *Cladosporum* sp. (among others) were isolated in 15 reef building corals and hydrozoans from the Caribbean and Australia (30). Endophytic fungal strain such as *Cladosporium tenuissimum, Cladosporium cladosporioides* and *Cladosporium* sp. were isolated from the Red Sea sponge *Niphates rowi* (31). *Cladosporium* sp. has been recorded from the South China Sea and from marine sponges of Antarctic (Xiao-Yong (32, 33). Marine fungi have been isolated from macroalgae from maritime, Antarctica (34). Fungal species including *Cladosporium cladosporioides* derived from sand and seawater of coastal regions of Red Sea and marine animals of Antarctica respectively (35, 36). *Corynespora cassiicola* is an Ascomycetus fungi found from a piece of fresh tissue from the inner part of an unidentified sponge collected from the Xisha Islands coral reef in the South China Sea (37). *Cladosorium* species including *Cladosporium oxysporum* was isolated from a natural mangrove environment in Zhanjiang Bay, Guangdong Province, China and they showed pathogenic effects on the mangrove plants (38).

## CONCLUSION

According to the literature surveyed *Cladosporium* species are rarely pathogenic to humans but have been reported to cause infections of the skin and toenails as well as sinuses and lungs. The airborne spores of *Cladosporium* species are significant allergens and do produce volatile organic compounds. Sea urchins are long been harvested for human consumption worldwide because they have various bioactive compounds. Hence, more research studies are required to look into the presence of such pathogens in marine environment and their potential risks need to be evaluated.

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## CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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