

The ITS-based phylogeny of fungi associated with tarballs

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Abstract

Tarballs, the remnants of crude oil which change into semi-solid phase due to various weathering processes in the sea, are rich in hydrocarbons, including toxic and almost non-degradable hydrocarbons. Certain microorganisms such as fungi are known to utilize hydrocarbons present in tarballs as sole source of carbon for nutrition. Previous studies have reported 53 fungal taxa associated with tarballs. There is apparently no gene sequence-data available for the published taxa so as to verify the fungal identification using modern taxonomic tools. The objective of the present study is to isolate fungi from tarballs collected from Candolim beach in Goa, India and investigate their phylogenetic diversity based on 5.8S rRNA gene and the flanking internal transcribed spacer regions (ITS) sequence analysis. In the ITS-based NJ tree, eight tarball-associated fungal isolates clustered with 3 clades of *Dothideomycetes* and 2 clades of *Saccharomycetes*. To the best of our knowledge, this is the first study that has employed ITS-based phylogeny to characterize the fungal diversity associated with tarballs. Further studies are warranted to investigate the role of the tarball-associated fungi in degradation of recalcitrant hydrocarbons present in tarballs and the role of tarballs as carriers of human pathogenic fungi.

Keywords: Bioremediation, DNA barcoding, Hydrocarbons, Identification, Marine pollution, Tarballs

Introduction

Tarballs are the remnants of crude oil which change into semi-solid phase due to various weathering processes in the sea (Suneel et al. 2013). There have been tarball deposits on the beaches of Goa, especially during the southwest monsoon (Dhargalkar et al. 1977, Suneel et al. 2013). Tarballs are rich in hydrocarbons, including toxic and almost non-degradable hydrocarbons (Suneel et al. 2013). Certain microbes such as fungi are capable of sustaining and increasing their population on hydrocarbon-contaminated surfaces. These fungi can utilize hydrocarbons as sole source of carbon for nutrition (e.g. Snellman et al. 1988, Das and Chandran 2011, Al-Nasrawai 2012, Al-Jawhari 2014). Previous studies from India and other parts of the world have so far reported 53 fungal taxa that are associated with tarballs (Table 1). It is to be highlighted here that the reported tarball-associated fungal taxa are apparently not represented by gene sequence-data. It is essential to improve our understanding of diversity and genetic capabilities of tarball-associated microbes before these microbes are tested for their suitability in bioremediation of hydrocarbon contaminated beaches. This study is therefore initiated as part of our ongoing investigations into microbial diversity associated with tarballs along the coastline of Goa, India. The objective of the present study is to collect tarball samples from one of the popular tourist beaches of Goa: Candolim, and isolate fungi from the collected tarballs, followed by characterization of their phylogenetic diversity based on ITS sequence analysis.

Materials and methods

Sampling: Tarball samples were collected from Candolim beach located on the northern part of Goa during the monsoon period on 23rd July, 2015. They were handpicked wearing gloves and put into sterile zip-lock bags. The samples were brought to the lab and kept in refrigerator at 4°C until further processing.

Isolation of fungi: The tarball samples were processed aseptically in a laminar airflow in the laboratory. They were kept in a petri-dish and washed twice with filtered sea-water using a sterile cotton swab to remove the excess sand adhering to their surface. The almost sand-free tarballs were surface-sterilized using 1% Sodium hypochlorite and then immersed in sea-water, taken off and kept aside. A sterilized toothpick was used to prick-out a small portion of the tarball onto potato dextrose agar (PDA) (prepared with filtered sea-water) amended with Chloramphenicol (100 mg/l), followed by streaking and incubation at $28 \pm 2^\circ\text{C}$ for 48-72 hours. The exuberant fungal

mycelia were sub-cultured on the PDA medium and maintained at $28 \pm 2^\circ\text{C}$ to allow optimal growth of fungi.

DNA extraction, PCR amplification and sequencing of the ITS region: Fungal DNA was extracted from mycelia using ZR Fungal/ Bacterial DNA MiniPrep kit (Zymo Research, catalogue number D6005) following the manufacturer's protocol. The ITS region was amplified by polymerase chain reaction (PCR). The reactions were carried out in a 50 μl of volume, containing 5 μl of (10X) PCR buffer (supplemented with 15 mM MgCl_2) (Merck Biosciences), 38 μl of nuclease-free water, 0.5 μl of 10 mM dNTPs, 1 μl of (20 pM) forward primer ITS1 (5'-TCCGTAGGTGAACCTGCGG-3'), 1 μl of (20 pM) reverse primer ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White et al. 1990), 1 μl of (3 units/ μl) Taq polymerase (Chromous) and 1.5 μl (~50ng) of DNA template. The following PCR cycling conditions were followed: initial denaturation at 95°C for 2 minutes, denaturation at 95°C for 1 minute, annealing at 52°C for 30 seconds, extension at 72°C for 1 minute and final extension at 72°C for 10 minutes. The denaturation, annealing and extensions steps were repeated 35 times (White et al. 1990). The PCR products were purified using QIAquick PCR purification kit (QIAGEN, Catalogue number 28106) following manufacturer's protocol. The sequencing of ITS gene region was done using Genetic Analyzer 3130xl (ABI) based on the Big Dye terminator v 3.1 (Chain terminator) chemistry.

Sequence alignment and phylogenetic tree construction: The raw sequences obtained from the forward primer were checked for quality in MEGA version 7 (Kumar et al. 2015) and trimmed to obtain good quality sequences. A multiple sequence alignment was prepared in MEGA using the newly-generated ITS sequences (Fig. 1, in bold) and the reference sequences retrieved from NCBI-GenBank (Fig. 1). The evolutionary tree was inferred in MEGA using Neighbour-Joining method (Saitou and Nei 1987). The sequences generated in this study have been deposited in NCBI-GenBank (Table 2).

Results

The culture morphology of eight tarball-associated fungal isolates from Candolim beach is presented in Fig. 2 and described in Table 2. The evolutionary relationships of the fungal isolates are presented in the NJ tree (Fig. 1). The tree details as retrieved from MEGA software are reproduced here, with minor modifications: "The phylogenetic analysis involved 55 nucleotide sequences, including 8 ITS sequences from this study. All ambiguous positions were removed for each sequence pair. There were a total of 773 positions in the final dataset. The optimal tree with

the sum of branch length = 2.67369481 is shown (Fig. 1). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al. 2004) and are in the units of the number of base substitutions per site. The rate variation among sites was modeled with a gamma distribution (shape parameter = 1). The differences in the composition bias among sequences were considered in evolutionary comparisons". *Colletotrichum gloeosporioides* is the designated outgroup (Fig. 1).

In the NJ tree (Fig. 1), the eight fungal isolates associated with tarballs collected from Candolim clustered with 3 clades each of *Dothideomycetes* (Clades 1, 2 and 5) and *Saccharomycetes* (Clades 3 and 4). In the class *Dothideomycetes*, Clade 1 consisted of isolate Tar06, isolate Tar08 and *Curvularia/ Biploaris* sequences, while isolate Tar10 clustered with *Alternaria* sequences in the Clade 2. Isolate Tar01 clustered with *Cladosporium* sequences in Clade 5. In the class *Saccharomycetes*, isolate Tar09 clustered with the *Candida* sequences (Clade 3), while the isolates Tar03, Tar04 and Tar05 clustered with *Issatchenkia orientalis* (AY939808) in Clade 4.

Discussion

Though there have been a few studies on tarball-associated fungi (Table 1), our understanding of fungal diversity associated with tarballs needs improvement. *Rhodotorula* sp., a yeast species, was apparently the first report on fungus from tarballs (Nair et al. 1972). Later, Nair and Lokhabharati (1977) isolated *Fusarium* sp. from tarballs collected from Goa and studied the effects of glucose, kerosene and petrol on its growth. They observed that the addition of up to 4% of kerosene and petroleum in the growth medium could enhance the fungal growth, above which it actually declined. They concluded that kerosene was a better substrate for growth of the *Fusarium* sp., in comparison with petrol.

In late 1980s, Snellman et al. (1988) isolated fungi from tarballs collected from certain areas in the north Atlantic and north Pacific Oceans and investigated their ability to degrade tarballs. They reported 42 filamentous fungi associated with the tarballs samples (Table 1). Majority of the isolates from their study belonged to fungal genera *Aspergillus* and *Penicillium*. Later, Itah and Essien (2005) isolated fungi and bacteria from tarballs collected from Ibeno beach in Nigeria and tested their ability to degrade tarballs. They reported mycological counts in the range of $1 (\pm 0.3) \times$

10^2 to $2 (\pm 0.4) \times 10^4$ c.f.u. g^{-1} of tarball. In their study, *Cladosporium resinae*, *Candida marina* and “*Saccharomyces estuari*” were found to be the most efficient utilizers and biodegraders of tarballs. Elshafie et al. (2007) isolated fungi from tarballs collected from the beaches of Oman and tested these fungi for their abilities to grow and degrade n-alkanes and crude oil. In their study, *Aspergillus* and *Penicillium* were the most dominant taxa, while *A. niger*, *A. terreus* and *P. chrysogenum* were found to be active bio-degraders of selected hydrocarbons.

Recently, Lotfinasabasl et al. (2012) isolated fungi from soil and tarball samples collected from mangrove forest of Alibaug and Akshi coastal area, Maharashtra, India and tested their ability to degrade kerosene. They reported *Aspergillus terreus* and *Rhizopus* sp. from the tarball samples and observed that *Rhizopus* sp. exhibited the highest growth diameter in 5% kerosene. Though, so far 53 fungal taxa have been reported to be associated with tarballs collected from various parts of the world, we believe that the present report is the first study on generation of sequence-data for tarball-associated fungi, followed by ITS-based phylogenetic analysis to place them in closest fungal clades. There are already two such reports on tar-ball associated bacteria (Bacosa et al. 2016, Nkem et al. 2016).

In our study, four fungal isolates (Tar03, Tar04, Tar05 and Tar09) were found to belong to the *Saccharomycetes*. While the placement of isolate Tar09 within the *Candida* clade (Fig. 1, Clade 3) is strongly supported, the placement of isolates Tar03, Tar04 and Tar05 within *Issatchenkia orientalis* subclade (Fig. 1, Clade 4) is tentative for the reason that limited ITS sequences from the type materials could be retrieved for the genus *Issatchenkia* from the NCBI BLAST search analysis. Though the placements of isolates Tar06 and Tar08 in the *Bipolaris/ Curvularia* clade (Fig. 1, Clade 1), isolate Tar10 in the *Alternaria* clade (Fig. 1, Clade 2), and isolate Tar01 in the *Cladosporium* clade (Fig. 1, Clade 5) are statistically well-supported, we refrain from giving species names to the fungi just based on monophasic data (i.e. ITS sequence-data).

Further studies employing a polyphasic approach are required to accurately identify these fungi to species level. Additional studies are required from India to obtain better insights into genetic diversity of fungi and other microbes involved in different stages of degradation of tarballs. Detailed assessment of ability of tarball-associated fungi and other microorganisms to degrade toxic hydrocarbons present in tarballs is expected to assist in bioremediation of tarballs (Nadapdap et al. 1996).

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Table 1: List of fungi reported from tarballs

[§]Legend: 1 = Snellman et al. (1988); 2 = Elshafie et al. (2007); 3 = Itah and Essien (2004); 4 = Lotfinasabasl et al. (2012); 5 = Nair and Lokabharathi (1977); 6 = Nair et al. (1972)

Sl. no.	Taxon name	Sampling location	Ref. [§]
1	<i>Acremonium</i> sp.	North Atlantic and north Pacific oceans	1
2	<i>Alternaria alternata</i>	Muscat coastline, Gulf of Oman	2
3	<i>Alternaria</i> sp.	North Atlantic and north Pacific oceans	1
4	<i>Amorphotheca</i> (= <i>Cladosporium</i>) <i>resinae</i>	North Atlantic and north Pacific oceans; Ibeno beach, near Akwa Ibom State, Nigeria	1, 3
5	<i>Aspergillus ficuum</i>	North Atlantic and north Pacific oceans	1
6	<i>Aspergillus fumigatus</i>	North Atlantic and north Pacific oceans	1
7	<i>Aspergillus heteromorphus</i>	North Atlantic and north Pacific oceans	1
8	<i>Aspergillus niger</i>	North Atlantic and north Pacific oceans; Muscat coastline, Gulf of Oman	1, 2
9	<i>Aspergillus ochraceus</i>	Muscat coastline, Gulf of Oman	2
10	<i>Aspergillus pseudoglaucus</i> (= <i>A. repens</i>)	North Atlantic and north Pacific oceans	1
11	<i>Aspergillus</i> sp.	North Atlantic and north Pacific oceans	1
12	<i>Aspergillus terreus</i>	North Atlantic and north Pacific oceans; Muscat coastline, Gulf of Oman; Akshi coastal area, Maharashtra, India	1, 2, 4
13	<i>Aspergillus ustus</i>	North Atlantic and north Pacific oceans	1
14	<i>Aspergillus versicolor</i>	North Atlantic and north Pacific oceans	1
15	<i>Beauvaria</i> sp.	North Atlantic and north Pacific oceans	1
16	<i>Chaetomium globosum</i>	North Atlantic and north Pacific oceans	1
17	<i>Chaetomium murorum</i>	North Atlantic and north Pacific oceans	1
18	<i>Chaetomium</i> sp.	North Atlantic and north Pacific oceans	1
19	<i>Chaetomium spirale</i>	North Atlantic and north Pacific oceans	1
20	<i>Cladosporium cladosporoides</i>	North Atlantic and north Pacific oceans; Muscat coastline, Gulf of Oman	1, 2
21	<i>Cryptococcus albidus</i>	Ibeno beach, near Akwa Ibom State, Nigeria	3
22	<i>Cryptococcus marinus</i> (= <i>Candida marina</i>)	Ibeno beach, near Akwa Ibom State, Nigeria	3
23	<i>Cunninghamella elegans</i>	North Atlantic and north Pacific oceans	1
24	<i>Emericella</i> (<i>Aspergillus</i>) <i>nidulans</i>	Muscat coastline, Gulf of Oman	2
25	<i>Epicoccum purpurascens</i>	North Atlantic and north Pacific oceans	1

26	<i>Eurotium chevalieri</i> (<i>Aspergillus</i>)	North Atlantic and north Pacific oceans	1
27	<i>Eurotium rubrum</i> (<i>Aspergillus ruber</i>)	North Atlantic and north Pacific oceans	1
28	<i>Fusarium</i> sp.	Undesignated beach in Goa, India	5
29	<i>Graphium</i> sp.	North Atlantic and north Pacific oceans	1
30	<i>Humicola fuscoatra</i>	North Atlantic and north Pacific oceans	1
31	<i>Microascus cinereus</i>	North Atlantic and north Pacific oceans	1
32	<i>Mucor</i> sp.	North Atlantic and north Pacific oceans	1
33	<i>Paecilomyces</i> sp.	Muscat coastline, Gulf of Oman	2
34	<i>Passalora</i> (= <i>Cercosporidium</i>) sp.	North Atlantic and north Pacific oceans	1
35	<i>Penicillium chrysogenum</i>	North Atlantic and north Pacific oceans; Muscat coastline, Gulf of Oman	1, 2
36	<i>Penicillium citrinum</i>	North Atlantic and north Pacific oceans; Ibeno beach, near Akwa Ibom State, Nigeria	1, 3
37	<i>Penicillium herquei</i>	North Atlantic and north Pacific oceans	1
38	<i>Penicillium javanicum</i>	North Atlantic and north Pacific oceans	1
39	<i>Penicillium oxalicum</i>	North Atlantic and north Pacific oceans	1
40	<i>Penicillium</i> sp. 1	Muscat coastline, Gulf of Oman	2
41	<i>Penicillium</i> sp. 2	North Atlantic and north Pacific oceans	1
42	<i>Pseudallescheria</i> (= <i>Lophotrichus</i>) <i>ampullus</i>	North Atlantic and north Pacific oceans	1
43	<i>Rhizopus</i> sp.	Akshi coastal area, Maharashtra, India	4
44	<i>Rhizopus stolonifer</i>	Muscat coastline, Gulf of Oman	2
45	<i>Rhodotorula</i> sp.	Goa, India	6
46	" <i>Saccharomyces estuary</i> "	Ibeno beach, near Akwa Ibom State, Nigeria	3
47	<i>Sarocladium (Acremonium) kiliense</i>	North Atlantic and north Pacific oceans	1
48	<i>Sarocladium (Acremonium) strictum</i>	North Atlantic and north Pacific oceans	1
49	<i>Scopulariopsis brevicaulis</i>	North Atlantic and north Pacific oceans	1
50	<i>Sordaria fimicola</i>	North Atlantic and north Pacific oceans	1
51	<i>Sordaria</i> sp.	North Atlantic and north Pacific oceans	1
52	<i>Syncephalastrum racemosum</i>	North Atlantic and north Pacific oceans	1
53	<i>Tetracoccusporium</i> sp.	North Atlantic and north Pacific oceans	1

Table 2: Colony morphology of fungal cultures and the GenBank accession numbers of the ITS region

Sl. no.	Strain no.	Fungal colony morphology after 21 days of inoculation on the PDA medium	GB# of the ITS
1	Tar01	Dark green; Powdery texture; Mycelial growth; Colonies raised and uplifted from the surface; Colony diam. 5.6 mm	KX757230
2	Tar03	Off white; Non-mycelial growth; Smooth yeast like texture; Colonies were not raised from the surface; Colony diam. 46 mm	KX757231
3	Tar04	White; Non-mycelial growth; Rough texture; Colonies not raised above the surface; Colony diam. 32.1 mm	KX757232
4	Tar05	White; Rough textured; Non mycelial growth; Colonies raised a bit from the surface; Colony diam. 40 mm	KX757233
5	Tar06	Light grey; Mycelial growth; Cottony texture; Colonies not raised from the surface; Colony diam. 68.7 mm	KX757234
6	Tar08	Whitish grey; Mycelial growth; Shrinkable cottony texture; Colonies not raised from the surface; Colony diam. 52.2 mm	KX757235
7	Tar09	White; Non-mycelial growth; Rough texture; Colonies not raised from surface; Colony diam. 34.5 mm	KX757236
8	Tar10	Whitish on outer surface and dark grey on inner surface; Mycelial growth; Shrinkable feathery texture; Colonies raised from the surface; Colony diam. 63 mm	KX757237

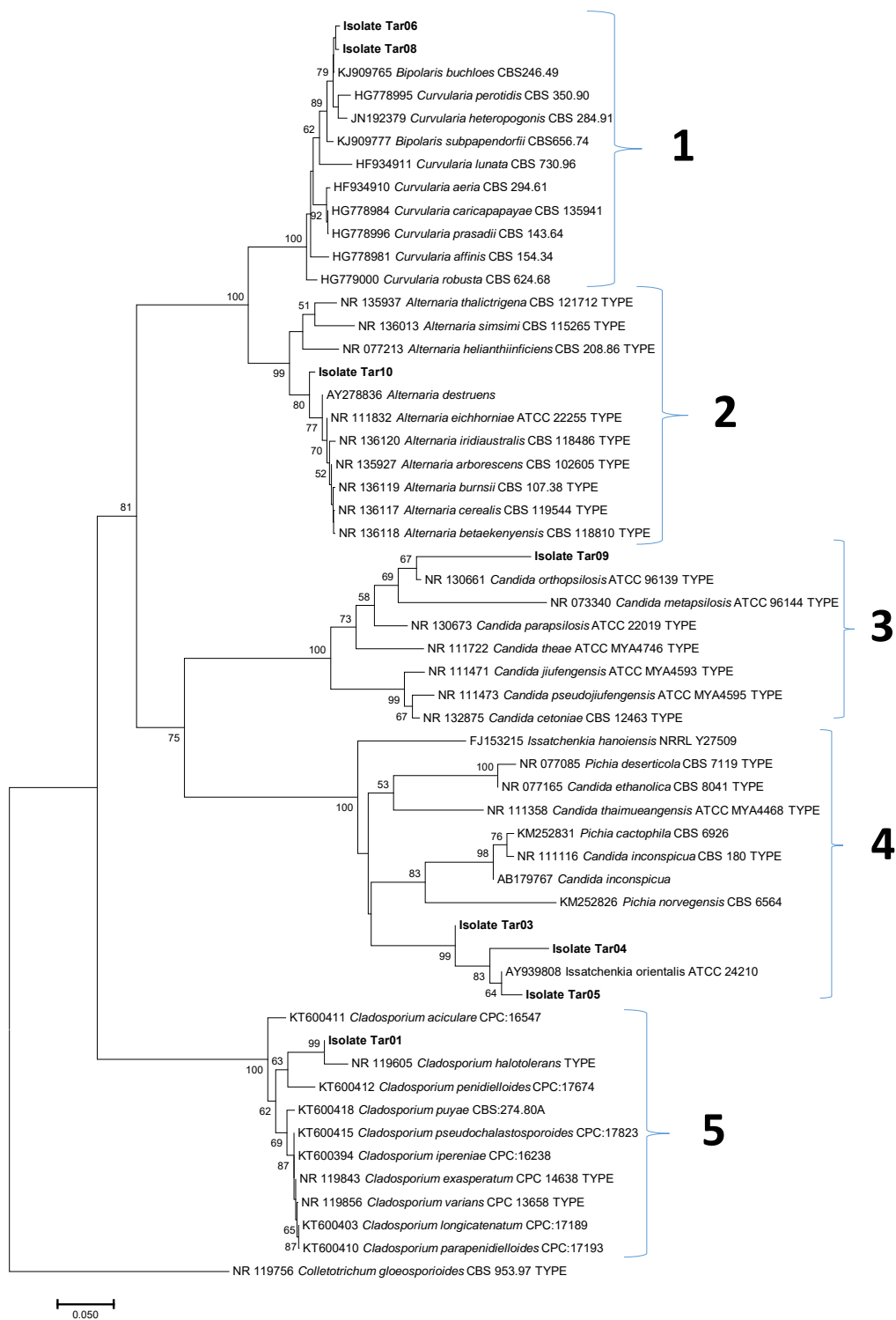


Fig. 1. Phylogenetic relationships of fungi associated with tarballs from Candolim beach, Goa, inferred from NJ analysis of the ITS sequences using MEGA. The tree is rooted with *Colletotrichum gloeosporioides* (NR_119756).

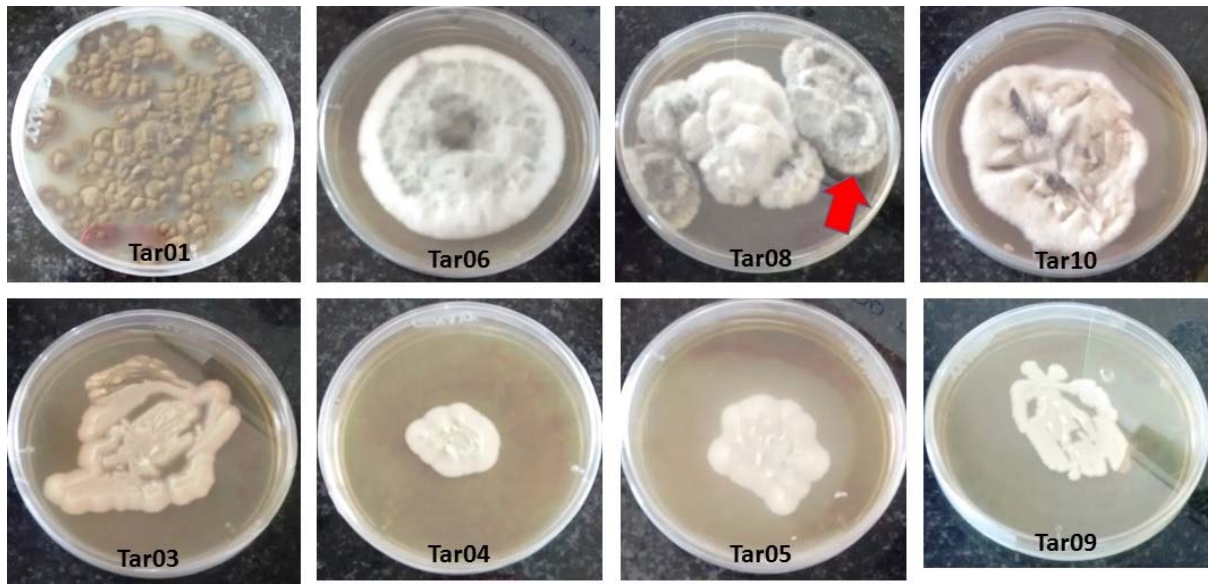


Figure 2: Culture morphology of tarball-associated fungi from Candolim, Goa, India (Tar01, Tar06, Tar08, Tar10 - Dothideomycetes; Tar03, Tar04, Tar05, Tar09 – Saccharomycetes; Red arrow indicates the colony of fungal isolate Tar08 in a mixed culture)