Research Article

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Occurrence of thraustochytrids: the fungoid protists *vis-a-vis* marine macroalgae (seaweeds) along the coast of Goa, India

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Abstract: Thraustochytrids are fungoid protists ubiquitous in the marine environment and found to be associated with decaying macroalgae. Not much is known about their association with living macroalgae. Hence in the present study, different macroalgal samples were collected from various beaches of Goa to examine the presence of thraustochytrids during a four-year-long study. Brown, red and green algae were found to be substrata of thraustochytrids. Thraustochytrids were isolated on pine pollen baiting from 17 to 58% of the specimens. Thraustochytrids isolated from various macroalgae belonged to the genera *Oblongichytrium*, *Schizochytrium*, *Ulkenia*, and *Thraustochytrium*. *Labyrinthula* sp. was also found once on the green alga *Bryopsis hypnoides*. These were generally found during dry seasons rather than in monsoons. The seasonal occurrence of thraustochytrids was found to be associated with temporal variation in macroalgal diversity. The statistical analysis supported individual or interactive effects of both factors viz, seasons and macroalgal diversity, on the occurrence of thraustochytrids. Thraustochytrids were also isolated from seawater adjoining macroalgae and from estuarine water at all times of the year. *Oblongichytrium* sp. was isolated from the green alga *Ulva compressa* and Anjuna seawater samples at the same time, thus indicating that thraustochytrids from seawater could inhabit the macroalgae.

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Keywords: intertidal region; Labyrinthulomycetes protists; seaweeds; Stramenopiles; temporal variation.

1 Introduction

Seaweeds play a vital role in constructing intertidal communities and are known as ecosystem engineers (Jones et al. 1994). The microbial communities associated with seaweeds are crucial in determining seaweed morphology and their growth stages (Ghaderirdakani et al. 2020). These microbial communities include diverse groups of microorganisms such as fungi, protozoa, bacteria, larvae of marine invertebrates and diatoms (Singh and Reddy 2014). In some instances, bacterial communities associated with seaweeds are so specific that they are observed at all times of the year irrespective of the season as well as geologic habitats (Lachnit et al. 2011). In contrast, an individual seaweed species may show completely different bacterial communities in different seasons or different habitats (Singh and Reddy 2014).

Thraustochytrids are eukaryotic microorganisms that are scavengers in the marine environment (Raghukumar and Damare 2011). These are economically important organisms in the biotechnology industry owing to their production of polyunsaturated fatty acids (PUFAs), especially ω-3 docosahexaenoic acid and eicosapentanoic acid (Raghukumar 2008; Raghukumar and Damare 2011). These organisms are ubiquitous in the marine environment (Raghukumar 2002) and can be associated with marine animals such as tunicates, mollusks, etc. (Azevedo and Corral 1997; Rabinowitz et al. 2006). Thraustochytrids are found abundantly in mangrove environments and form an essential component of mangrove habitats (Raghukumar 2002). These protists are saprophytic in nature, bearing an osmoheterotrophic mode of nutrition similar to bacteria and fungi. They obtain nutrition by producing extracellular enzymes via the extensions of their plasma membrane called ectoplasmic net (EN) elements (Porter 1990). By

virtue of these EN elements, they can attach to any substratum and absorb nutrition (Raghukumar 2002). Thraustochytrids reproduce by means of motile biflagellate zoospores, which settle on any surface and round up to form young cells that multiply and colonize the surface, a characteristic similar to chytrid fungi (Gleason et al. 2018). These were earlier classified under oomycetan fungi (Sparrow 1973) and are now known as fungal-like organisms (Leaño and Damare 2012).

Despite the widespread occurrence of thraustochytrids in mangroves (Raghukumar 1988; Raghukumar et al. 1995), and their importance in the marine environment as decomposers, pathogens (Raghukumar 2002; Schärer et al. 2007) and as a source of nutrition to zooplankton (Damare and Raghukumar 2015; Damare et al. 2013), their associations with macroalgae are not well-studied. The few studies on the existence of thraustochytrids in the detritus of macroalgae (Sathe-Pathak et al. 1993; Sharma et al. 1994), and parasitic association of *Labyrinthula* (sister clade of thraustochytrids) with the green algae *Chaetomorpha media*, *Rhizoclonium* and *Cladophora* (Raghukumar 1987a, b) did not establish their contribution as a holobiont of macroalgae. The seaweed holobiont comprises a macroalgal host and microorganisms associated with it which help in maintenance of health, performance and resilience of the macroalga (Egan et al. 2013). There is a plethora of information on the role of epiphytic bacteria on macroalgae (Ghaderiardakani et al. 2020; Selvarajan et al. 2019). Fungal associations with macroalgae were also studied (Singh et al. 2018; Vallet et al. 2018). However, there is dearth of knowledge about thraustochytrids associated with living macroalgae. One-time sampling revealed a few thraustochytrids from *Centroceras clavulatum*, *Gelidium pusillum*, *Sargassum cinereum* and *Padina tetrastomatica* but further investigation showed growth inhibition in the presence of living algae (Raghukumar et al. 1992). Another one-time study revealed few thraustochytrids from drifting *Sargassum cinereum* (Damare 2015). The factors that drive the occurrence of these protists on macroalgae will remain ambiguous till a detailed study at a temporal scale is carried out. It is unclear whether thraustochytrids are a regular member of the seaweed holobiont or are sporadically found on macroalgae.

This study, therefore, investigated the diversity of thraustochytrids on brown, red and green macroalgae in different months in Goa to find a possible association between thraustochytrids and the macroalgae. Goa is in the tropical zone located on the southwest coast of India with the Arabian Sea at its west. Its area encompasses coastal plains, plateaus and hills, and is separated from the Deccan highlands by the Western Ghats, which is a global biodiversity hotspot (Myers et al. 2000). The Western Ghats block the southwest monsoon winds causing heavy rainfall in Goa from June to September, which is called the summer monsoon. Runoff during monsoons flows through several streams, rivers, and estuaries carrying it to the Arabian Sea. The estuaries are monsoonal in nature as their circulation is controlled by runoff during monsoons and by tides during the dry season. The dry season spreads from hot October and November (day time temperatures 28–32 °C), to winter with low humidity from December to February (day time temperatures 25–31 °C) and extremely hot and humid summer from March to May (day time temperatures of over 35 °C). The present study had two objectives: the first was to isolate thraustochytrids from macroalgae found on various beaches of Goa to check for spatial variation if any, and further study their temporal variation in association with macroalgae on one of the beaches. The second objective was to investigate the occurrence of thraustochytrids in the seawater surrounding the macroalgae and estuarine waters to study the factors responsible for thraustochytrid occurrence with the macroalgae.

2 Materials and methods

2.1 Isolation of thraustochytrid protists from seaweeds and seawater

Seaweed or macroalgal samples were collected at low tide from intertidal zones during four time periods (August–December 2015; August 2016–April 2017; August 2017–March 2018; and September 2018–February 2019) from the coastal belts of north and south Goa (Figure 1). Details of the beaches and sampling period are given in Table 1. Different macroalgal samples were collected in clean zip-lock plastic bags. The samples were transported to the laboratory on ice. Macroalgae were identified on the basis of morphology (Dhargalkar and Kavlekar 2004; Kamboj et al. 2019; www.algaebase.org), and Simpson's Diversity Index (D) and Margalef Species Richness (S) were calculated for the samples collected using the following formulae (De Jong 1975; Gamito 2010; Okpiliya 2012).

$$D = 1 - \frac{\Sigma n(n-1)}{N(N-1)} \quad \text{and} \quad S = \frac{s-1}{\ln n}$$

The macroalgal samples (Table 2) were washed with sterile seawater 4–5 times in a laminar flow hood and processed further for isolation of thraustochytrids. While collecting macroalgae from Anjuna beach, surface seawater was also collected in sterile 50-mL Falcon tubes for isolation of thraustochytrids. Surface seawater was collected also from Dona Paula, Ribandar jetty and Divar island along the Mandovi estuary, and Madkai and Durbhat jetty along the Zuari estuary in Goa, India, from September to November 2018 during high tide. Dona Paula lies at the mouth of both the Mandovi and Zuari estuaries, the monsoonal estuaries in Goa (Figure 1).

Macroalgal samples were cut into small pieces of 25 mm^2 and put in small Petri dishes or multiwell plates containing sterile seawater.

Figure 1: Map showing locations in Goa, India, where sampling was carried out. Closed circles: Beaches where macroalgae were obtained. Open stars: The estuarine locations (Divar, Ribandar, Durbhat and Madkai) and Dona Paula.

The seawater samples from various locations were also placed in separate multiwell plates. Sterile pine pollen was used as a bait and added to each Petri dish and/or well. These were incubated for 3–4 days at room temperature and monitored daily by microscopy for thraustochytrid growth. The pine pollen baited macroalgal samples that showed thraustochytrid cells on microscopy were streaked on MV (modified Vishniac) agar plates (0.001% liver infusion powder, 0.01% yeast extract, 0.15% peptone, 0.4% dextrose, 0.8% agar in natural seawater), supplemented with AMPILOX-*c* containing 500 mg Ampicillin and 500 mg Cloxacillin per 100 mL. The plates were incubated at room temperature for 1–2 days. The colonies obtained were purified by sub-culturing at an interval of 4–5 days on MV agar plates without the antibiotics.

All the seawater samples were tested for total viable count (TVC) of bacteria, and various physicochemical parameters such as pH, salinity, nitrates and phosphates. TVC was carried out by spread plating 0.1 mL of seawater dilutions on Zobell Marine Agar. pH and salinity of water samples were checked soon after collection using pH-indicator strips (HiMedia) and a portable refractometer (RHS-10ATC), respectively. Nitrates were determined by the spectrophotometric method of Howse (1997). Ten ml of each water sample were added to volumetric flasks. A pinch of zinc dust was added with 80 mL of distilled water. Later 1 mL of sulfanilamide solution (1%) was added and mixed, and after 3–4 min, 1 mL of 0.1% N-(1-naphthyl)-ethylene-diamine dichloride (NED) was added. The pink-coloured complex produced was measured at 543 nm. Phosphate analysis was done by the Murphy and Riley (1962) method. Eight ml of reagent mix (containing sulphuric acid, ascorbic acid, ammonium molybdate and potassium antimonyl tartarate) were added to 40 mL of water sample and topped up to 50 ml with distilled water.

Table 1: Details of time period during which seaweed samples were collected from various beaches of Goa.

Sampling period	Beach	Geographical location	District	Seaweeds found
Aug–Dec 2015	Anjuna	15.573418°N, 73.740785°E	North Goa	+
	Vainguinim Valley	15.455658°N, 73.813388°E	North Goa	+
	Siridao	15.445130°N, 73.855871°E	North Goa	+
	Cabo de Rama	15.193606°N, 73.925520°E	South Goa	+
Aug 2016–Apr 2017	Anjuna	15.573418°N, 73.740785°E	North Goa	+
Aug 2017–Mar 2018	Anjuna	15.573418°N, 73.740785°E	North Goa	+
	Miramar	15.483093°N, 73.807399°E	North Goa	–
	Odxel	15.453517°N, 73.830257°E	North Goa	–
	Cacra	15.450988°N, 73.837641°E	North Goa	–
	Siridao	15.445130°N, 73.855871°E	North Goa	–
	Betalbatim	15.293586°N, 73.907732°E	South Goa	–
	Colva	15.281209°N, 73.911641°E	South Goa	–
	Benaulim	15.257193°N, 73.918688°E	South Goa	–
	Mobor	15.156811°N, 73.945616°E	South Goa	–
	Betul	15.146801°N, 73.948175°E	South Goa	+
	Rajbagh	14.985055°N, 74.038474°E	South Goa	–
Sep 2018–Feb 2019	Anjuna	15.573418°N, 73.740785°E	North Goa	+

'+' represents occurrence of seaweeds in that location; '–' represents no seaweeds.

Blue-coloured phosphomolybdic acid was formed and the optical density was measured at 882 nm within 30 min. The standard curves for nitrates and phosphates were constructed using sodium nitrate (0.5–5 μM) and potassium dihydrogen orthophosphate (0.5–5 μM), respectively.

2.2 Identification of thraustochytrid isolates

Identification of the isolates was carried out by examining their life cycle morphology (Bennett et al. 2017; Leaño and Damare 2012). Each culture was grown in MV broth (without the addition of agar), and a loopful was placed on a glass slide of a continuous flow chamber (Raghukumar 1986). Life cycle was monitored under the microscope for two days (Damare and Raghukumar 2006). A continuous flow of MV broth was maintained to provide nutrients to the growing cultures.

Table 2: Macroalgae collected at different sampling sites and during different seasons positive for thraustochytrid cells on pine pollen and on modified Vishniac (MV) agar.

Season	Location	Sample	Thraustochytrid cells on pine pollen	Thraustochytrid cells on MV agar
Oct 2015	Siridao	*Sargassum* sp.	+	–
	Vainguinim	*Sargassum tenerrimum* J. Agardh	–	–
		Sargassum cinereum J. Agardh	–	–
		Sargassum polycystum C. Agardh	–	–
		Sargassum ilicifolium (Turner) C. Agardh	+	+
	Anjuna	*Lomentaria hakodatensis* Yendo	+	+
		Sargassum tenerrimum	–	–
		Sargassum sp.	+	–
		Stoechospermum marginatum (C. Agardh) Kützing	+	+
		Spatoglossum asperum J. Agardh	–	–
		Cheilosporum spectabile Harvey ex Grunow	+	–
		Laminaria sp.	–	–
		Padina tetrastomatica Hauck	+	–
Dec 2015	Cabo de Rama	*Cutleria multifida* (Turner) Greville	–	–
		Ulva lactuca Linnaeus	+	–
		Caulerpa peltata J.V.Lamouroux	–	–
		Caulerpa racemosa (Forsskål) J. Agardh	+	–
		Sargassum sp.	+	–
		Padina tenuis Bory	+	–
Aug 2016	Anjuna	*Chaetomorpha* sp.	–	–
		Ulva intestinalis Linnaeus	–	–
		Ulva compressa Linnaeus	–	–
		Ulva flexuosa Wulfen	–	–
Sep 2016	Anjuna	*Ulva intestinalis*	–	–
		Ulva compressa	–	–
		Ulva flexuosa	–	–
		Ulva fasciata Delile	–	–
Oct 2016	Anjuna	*Padina gymnospora* (Kützing) Sonder	+	–
		Hypnea valentiae (Turner) Montagne	+	–
		Gracilaria sp.	+	–
		Hypnea sp.	–	–
		Sargassum tenerrimum	+	–
		Sargassum cinereum	+	–
		Ulva intestinalis	+	–
		Ulva clathrata (Roth) C. Agardh	–	–
Nov 2016	Anjuna	*Padina gymnospora*	–	–
		Gracilaria sp.	–	–
		Caulerpa sertularioides (S.G. Gmelin) M. Howe	–	–
		Chaetomorpha media (C. Agardh) Kützing	–	–
		Sargassum cinereum	+	–
Dec 2016	Anjuna	*Chaetomorpha media*	–	–
		Caulerpa sertularioides	–	–
		Stoechospermum marginatum	–	–
		Sargassum cinereum	+	+
		Padina gymnospora	+	–
Jan 2017	Anjuna	*Caulerpa peltata*	+	–
		Sargassum tenerrimum	+	–
		Padina gymnospora	+	–
		Ulva faciata	–	–
Feb 2017	Anjuna	*Caulerpa peltata*	–	–
		Sargassum tenerrimum	+	–

Table 2: (continued)

Season	Location	Sample	Thraustochytrid cells on pine pollen	Thraustochytrid cells on MV agar
		Sargassum cinereum	+	–
		Padina gymnospora	+	–
		Caulerpa sertularioides	+	–
		Gracilaria corticata (J. Agardh) J. Agardh	+	+
March 2017	Anjuna	*Dictyota dichotoma* (Hudson) J.V. Lamouroux	+	–
		Asparagopsis taxiformis (Delile) Trevisan	+	+
		Sargassum cinereum	–	–
		Caulerpa peltata	+	–
		Hypnea cervicornis J. Agardh	+	–
		Bryopsis hypnoides J.V. Lamouroux	+	+
		Padina gymnospora	+	–
		Caulerpa sertularioides	–	–
		Gracilaria corticata	+	–
Apr 2017	Anjuna	*Ulva flexuosa*	–	–
		Sargassum cinereum	+	–
		Hypnea cervicornis	+	–
		Asparagopsis taxiformis	+	–
		Caulerpa sertularioides	+	–
		Gracilaria corticata	+	–
Oct 2017	Betul	*Sargassum cinereum*	–	–
		Ulva fasciata	–	–
		Padina gymnospora	–	–
		Stoechospermum marginatum	–	–
Jan 2018	Anjuna	*Sargassum* sp.	–	–
		Stoechospermum marginatum	–	–
		Ulva fasciata	+	+
		Stoechospermum sp.	–	–
		Sargassum cinereum	+	–
		Ulva compressa	+	+
		Padina gymnospora	–	–
Feb 2018	Anjuna	*Ulva fasciata*	–	–
		Padina gymnospora	–	–
		Sargassum cinereum	–	–
		Stoechospermum sp.	–	–
Feb 2018	Betul	*Ulva fasciata*	–	–
		Sargassum cinereum	+	–
		Stoechospermum sp.	–	–
March 2018	Anjuna	*Padina gymnospora*	–	–
		Gracilaria spp.	–	–
		Sargassum cinereum	+	–
		Ulva fasciata	+	–
Sep 2018	Anjuna	*Chaetomorpha* sp.	–	–
		Ulva fasciata	+	+
		Ulva intestinalis	–	–
Oct 2018	Anjuna	*Asparagopsis taxiformis*	–	–
		Ulva fasciata	–	–
		Gracilaria corticata	–	–
		Padina gymnospora	–	–
		Dictyota dichotoma	–	–
		Chaetomorpha media	–	–
		Caulerpa peltata	–	–
		Lomentaria sp.	+	+
Nov 2018	Anjuna	*Caulerpa sertularioides*	–	–
		Caulerpa peltata	–	–
		Hypnea valentiae	–	–

Table 2: (continued)

Season	Location	Sample	Thraustochytrid cells on pine pollen	Thraustochytrid cells on MV agar
		Padina gymnospora	–	–
		Gracilaria corticata	–	–
		Sargassum tenerrimum	–	–
Jan 2019	Anjuna	*Asparagopsis taxiformis*	–	–
		Lomentaria hakodatensis	+	+
		Ulva fasciata	+	+
		Chaetomorpha media	–	–
Feb 2019	Anjuna	*Sargassum tenerrimum*	+	–
		Ulva fasciata	–	–
		Caulerpa peltata	–	–
		Caulerpa sertularioides	–	–
		Dictyota dichotoma	–	–

'+' indicates positive sample; '–' indicates no thraustochytrids obtained.

Identification of some of the isolates was confirmed by molecular analysis (Damare et al. 2020). This involved 18S rRNA gene amplification using two sets of universal primers NS1–NS4 and NS3–NS8 (White et al. 1990). Amplification conditions were the same as mentioned in Damare and Raghukumar (2010). The PCR products were sequenced by the 3130XL Genetic Analyser (ABI Sequencer 2200) after purification with PCR Clean Up kit (Promega Cat. No. A9282).

2.3 Effect of seasons and type of macroalgae on occurrence of thraustochytrids

Macroalgae collected from the intertidal region of Anjuna beach during the second and fourth sampling period corresponded to the following seasons, summer monsoon (August–September), summer post-monsoon (October), autumn post-monsoon (November), winter post-monsoon (December–February), and summer pre-monsoon (March–April). Five pieces of 25 mm^2 each of different seaweeds (Table 2) were incubated in separate wells of 24-well plates along with sterile pine pollen. The wells were observed after 3–5 days under a microscope for the presence of thraustochytrid-like cells. Wet mounts of the pollen grains in the wells were prepared and observed under 10× magnification of bright field microscopy. Thirty pollen grains with cells were observed (Supplementary Figure S1), and the number of cells found attached to them was recorded with an average taken.

2.4 Statistical analysis

Effects of seasons and type of macroalga (substratum) on the occurrence of thraustochytrids were analysed statistically by a non-parametric test to check which of the two factors affected thraustochytrid isolation. At first, Friedman's test was carried out with all the factors together, i.e., seasonality and macroalgal diversity, to examine multiple dependent factors. Single-factor influence was analysed by Kruskal–Wallis test. Friedman's test represents a non-parametric version of two-way ANOVA, and the Kruskal–Wallis test represents a non-parametric version of one-way ANOVA.

The effect of different physicochemical parameters of the seawater samples on the presence of thraustochytrids was also analysed by non-parametric Friedman's test because the data did not follow a normal distribution. All statistical analysis was carried out using Statistica 8 (Weiß 2007). The data were also correlated by multidimensional scaling (MDS) using Primer 6 software (Clarke and Gorley 2006). The data was log-transformed prior to MDS analysis. The original distance between the samples was measured as Euclidean distance.

3 Results

3.1 Diversity of macroalgae and their associated thraustochytrids

Aug–Dec 2015: different beaches sampled during this period showed different macroalgal diversity (Table 2). The most common macroalgae found in the intertidal zone of all the beaches were *Sargassum* spp. In addition, *Lomentaria hakodatensis*, *Stoechospermum marginatum*, *Spatoglossum aspermum*, *Cheilosporum spectabile*, *Laminaria* sp., *Padina tetrastomatica* were present at Anjuna beach. *Cutleria multifida*, *Ulva* sp., *Caulerpa* spp. and *Padina tenuis* were found on Cabo de Rama beach. No thraustochytrid cultures were isolated from the macroalgae of Siridao and Cabo de Rama beaches. One culture was obtained from *Sargassum ilicifolium* from Vainguinim beach. Two cultures were recovered from Anjuna beach, one from *Lomentaria hakodatensis* and the other from *Stoechospermum marginatum*. However, only the culture from *Lomentaria hakodatensis* survived after subsequent subculturing and this isolate A1 was identified as *Oblongichytrium* sp. based on its life cycle (Figure 2a).

Aug 2016–Apr 2017: thraustochytrid cells were observed on pine pollen baiting of macroalgae from October 2016 onwards till April 2017, i.e., post-monsoon to pre-monsoon, excluding the monsoon period. Three

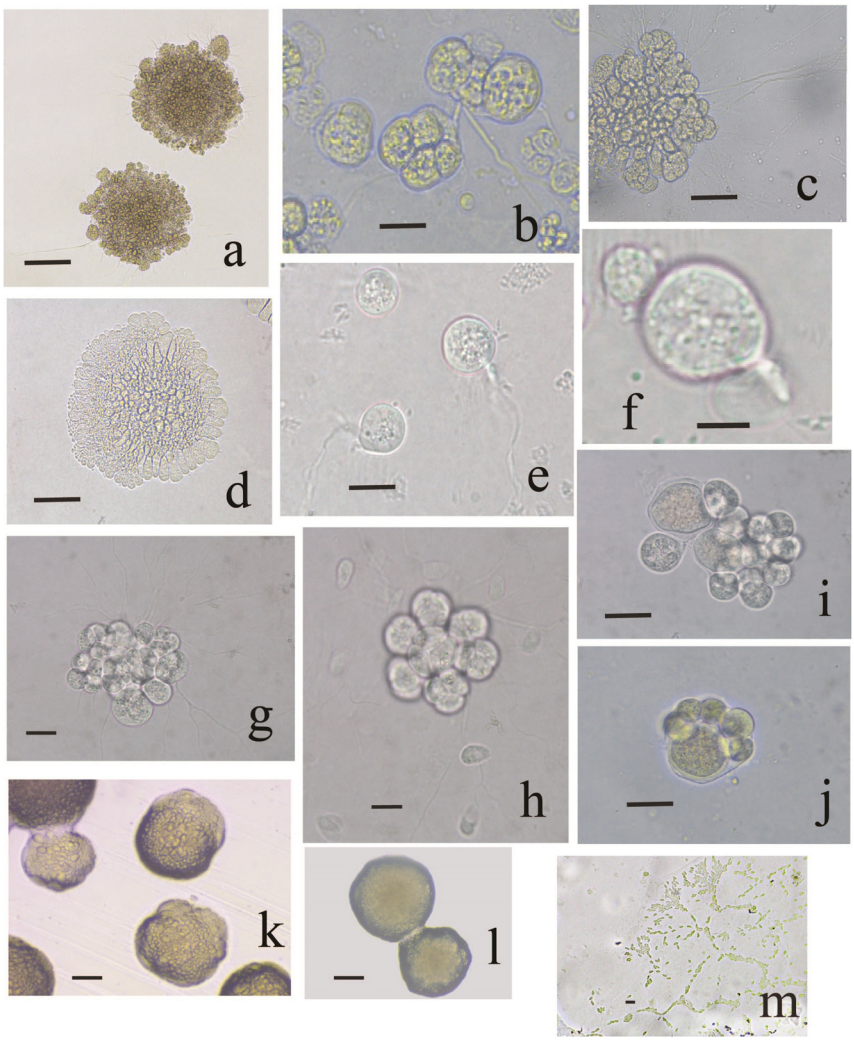

Figure 2: Micrographs of thraustochytrid isolates: (a) colonies of *Oblongichytrium* sp. A1; (b) mature cell and zoosporangia of *Oblongichytrium* sp. B6; (c) a colony of *Oblongichytrium* sp. B6; (d) colony of *Ulkenia* sp. C1; (e–f) cells of *Thraustochytrium* sp. SAC1 showing emergence of ectoplasmic net element from one point on the cell wall and apophysis beneath; (g) cells of *Oblongichytrium* sp. #VD4; (h) cells and oblong zoospores of *Oblongichytrium* sp. #VD4; (i) cells of *Parietichytrium* sp. #VD12 on maturity become amoeboid; (j) mature sporangium of *Parietichytrium* sp. #VD12 showing radiated cell division; (k) colonies of *Ulkenia* sp. AUS obtained from *Ulva*; (l) colonies of *Schizochytrium* sp. ALN obtained from *Lomentaria*; (m) *Labyrinthula* cells obtained after baiting green alga *Bryopsis hypnoides* in sterile seawater and pine pollen collected in March 2017. Scale bars: (a) 100 µm; (b), (c), (i), (j) 20 µm; (d) 50 µm; (e), (g), (h), (m) 10 µm; (f) 5 µm; (k), (l) 200 µm.

thraustochytrid isolates were obtained in culture, one during December (isolate SAC1) and two during February (isolates B6 and C1). Isolates B6 and C1 were obtained from the red alga *Gracilaria corticata* and were identified as *Oblongichytrium* sp. (Figure 2b and c) and *Ulkenia* sp. (Figure 2d, Supplementary Figure S2), respectively. Isolate SAC1 was obtained from the brown alga *Sargassum cinereum* and identified as *Thraustochytrium* sp. based on its life cycle (Figure 2e and f).

Aug 2017–Mar 2018: no seaweeds were found on the beaches of Cacra, Siridao, Colva, Benaulim, RajBagh and Mobor during August–September (monsoon) and October (post-monsoon) 2017. *Sargassum*, *Ulva*, *Padina* and *Stoechospermum marginatum* were recorded during October 2017 on Betul beach, but none of them revealed thraustochytrid cells on incubation with pine pollen. However, only *Sargassum cinereum* collected from the same beach in February 2018 showed thraustochytrids on incubation. *Ulva fasciata* and *Stoechospermum* sp. from the Betul beach in February 2018 did not produce any thraustochytrids on pine pollen baiting. Macroalgae found on Anjuna beach during this study period were *Sargassum cinereum*, *Padina gymnospora* and *Ulva fasciata* (during January–March 2018), *Stoechospermum* sp. (during January–February 2018) and *Gracilaria* sp. only (in March 2018). Amongst the macroalgae from Anjuna beach, only *Sargassum cinereum* and *U. fasciata* showed the presence of thraustochytrids in January and March but not in February. Thraustochytrid cells were also seen in pollen baiting samples of the stem and air bladders of *Sargassum cinereum* and other seaweeds such as *Stoechospermum marginatum* and *Padina gymnospora*. However, these lost their viability during subculturing. The isolates obtained from Anjuna beach during January 2018 were #VD4 obtained from *U. compressa* identified as *Oblongichytrium* sp. (Figure 2g and h) and isolate #VD12 obtained from *U. fasciata* identified as *Parietichytrium* sp. (Figure 2i and j) based on their life cycle and 18S rDNA sequencing (Damare et al. 2020).

Sep 2018–Feb 2019: only the green algae *Ulva* and *Chaetomorpha* were found in September 2018. A higher diversity of algae as compared to September was seen from October 2018 onwards up to February 2019 (Table 2). Two thraustochytrid isolates were obtained during this period, *Ulkenia* sp. (isolate AUS, Figure 2k) from *Ulva* and *Schizochytrium* sp. (isolate ALN, Figure 2l) from *Lomentaria* during September and November 2018, respectively, as identified by their life cycle studies.

3.2 Occurrence of thraustochytrids on macroalgae and seawater

Of the 19 macroalgal samples collected during the first study period (August–December 2015), 58% of the samples were positive for thraustochytrids: 2 of 3 green algae, 2 of the two red algae and 7 of 14 brown algae (Figure 3). Out of 50 macroalgal samples collected during the second study period (August 2016–April 2017), 48% of the samples were positive for thraustochytrids. Almost 82% of the brown algae were positive for thraustochytrids. During the third study period (August 2017–March 2018), only 24% of samples showed the presence of thraustochytrids: 3 of 17 brown algae, 4 of 10 green algae and none for the red algae. During the fourth study period (September 2018–Februrary 2019), only 17% of seaweed samples gave rise to thraustochytrid cells after incubation: 2 of 15 green algae, 2 of 8 red algae, and 1 of 6 brown algae. The number of seaweed specimens positive for thraustochytrids correlated with high Simpson's Diversity Index (D) and Species Richness (S) values of the seaweed samples (Table 3).

Pine pollen baiting of seawater from all the locations in Mandovi and Zuari estuaries, and at Dona Paula showed the presence of thraustochytrids. The seawater collected from Anjuna beach along with seaweeds also revealed the presence of thraustochytrids. Isolate #VD6 obtained from the seawater at Anjuna during January 2018 was identified molecularly and based on its life cycle as *Oblongichytrium* sp., which was the same as that obtained from *Ulva compressa* from Anjuna during the same period (Damare et al. 2020). The other isolates were #RBSS (*Botryochytrium* sp.) obtained from Ribandar (Mandovi estuary) and #MSS (*Thraustochytrium* sp.) obtained from Madkai (Zuari estuary) in September. Isolate #DPSO (*Thraustochytrium* sp.) was isolated from Dona Paula and #DBSO (*Thraustochytrium* sp.) from Durbhat (Zuari estuary) in October. The isolates from estuarine waters were identified based on their life cycle and morphology (Supplementary Figure S3).

Table 3: Diversity indices and isolation frequency of thraustochytrids on macroalgal samples collected during the four time periods.

	Aug–Dec 2015	Aug 2016–March 2017	Sep 2017– March 2018	Sep 2018–Feb 2019
Simpson's diversity index (D)	0.977	0.952	0.837	0.941
Margalef species richness (S)	5.094	5.087	2.079	3.86
Percentage of seaweed samples positive for thraustochytrids	58	48	24.14	17.24

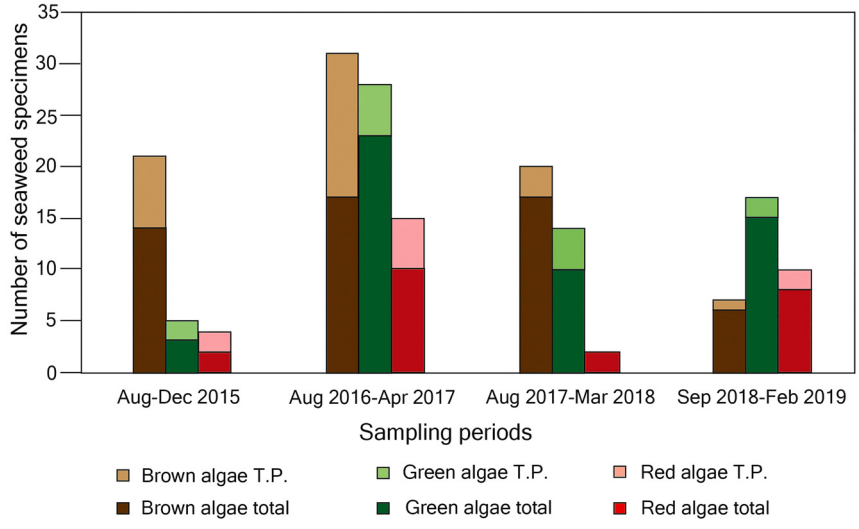

Figure 3: Presence of thraustochytrids on the three different macroalgal types in different sampling periods. T.P., thraustochytrid-positive specimens.

Physicochemical properties and TVC counts of bacteria in seawater (Table S1) clearly differentiated between estuarine and beach waters as seen by the clustering pattern in multidimensional scaling (Supplementary Figure S4). Except for the October seawater sample of Dona Paula and December's sample of Anjuna, all the samples from these two sites formed one cluster. Salinity at Anjuna and Dona Paula ranged from 35 to 44 which was higher than the estuarine waters (14–28 in Mandovi and 20–34 in Zuari estuary). The pH of all these locations was between 6.5 and 8. Similarly, phosphates ranged from 0.36 to 5.04 μM, and nitrates ranged from 0.11 to 2.09 μM in all the samples. The TVC of bacteria ranged between 40 and 242.5×10^5 cfu (colony forming unit) mL^{-1}.

3.3 Effect of seasons and type of macroalgae on occurrence of thraustochytrids

Temporal variation in the diversity of macroalgae was observed in the intertidal region of Anjuna beach. Species richness was lowest during the monsoon of both study periods, i.e., the monsoons of 2016 and 2018 (Table 4). Monsoon samples did not yield thraustochytrids except for one instance, from *Ulva fasciata* during Sep 2018 (Figure 4). The highest diversity was observed in summer pre-monsoon (March and April) and summer post-monsoon (October) (Table 4), followed by post-monsoon November and winter months (December–February). MDS reflected the clustering of monsoon samples together (Figure 5). The post-monsoon samples of October and pre-monsoon samples of March and April dispersed far away from the rest of the samples, standing clearly from the rest, as reflected in their high seaweed diversity. The number of seaweed types observed was 21 during 2016–2017 and 14 during 2018–2019; out of these, only a few produced thraustochytrid cells on pine pollen, i.e., 10 during the former and only four during the latter period (Figure 6). *Padina*, *Sargassum*, and *Caulerpa* were obtained consistently from October onwards till April, and all these showed the presence of thraustochytrids in 2016 (Figure 6) but no thraustochytrids in 2018. In 2016–2017, *Asparagopsis taxiformis* was observed in March–April, while in 2018–2019, it was observed in January. *Dictyota dichotoma* was observed only during March in 2017 and in February 2019. Both *A. taxiformis* and *Dictyota dichotoma* showed the presence of thraustochytrids on pine

Table 4: Margalef species richness (S) of macroalgal specimens collected every month during two sampling periods August 2016–April 2017 and September 2018–February 2019.

Sampling period	Species richness (S)
Aug 2016	2.16
Sep 2016	2.16
Oct 2016	3.37*
Nov 2016	2.49
Dec 2016	2.49
Jan 2017	2.16
Feb 2017	2.79
Mar 2017	3.64*
Apr 2017	3.35*
Sep 2018	1.82
Oct 2018	2.92
Nov 2018	2.79
Jan 2019	2.16
Feb 2019	2.49

Higher richness indices were observed during post-monsoon (October) and pre-monsoon (March and April) (marked by an asterisk) than in other months.

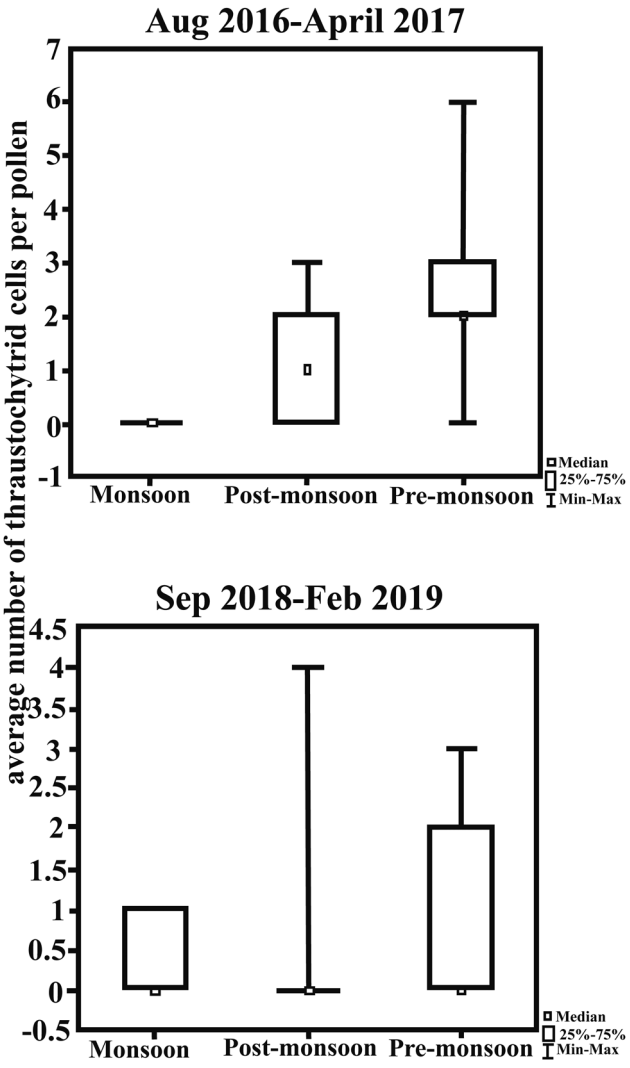

Figure 4: Box plots showing the effect of seasons on the presence of thraustochytrids in association with macroalgae.

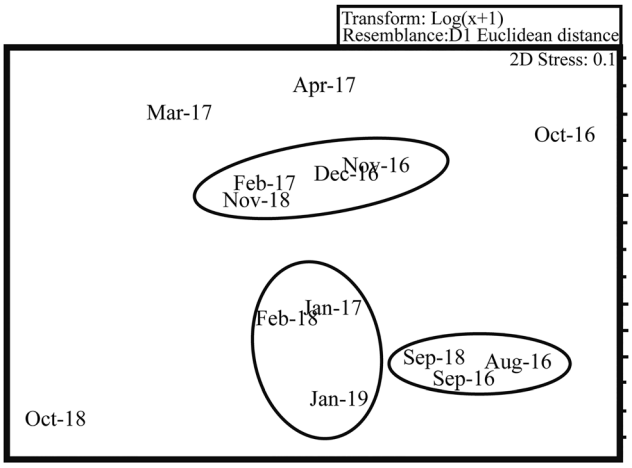

Figure 5: Two-dimensional MDS (multi-dimensional scaling) plot of the macroalgal samples based on similarities and groups.

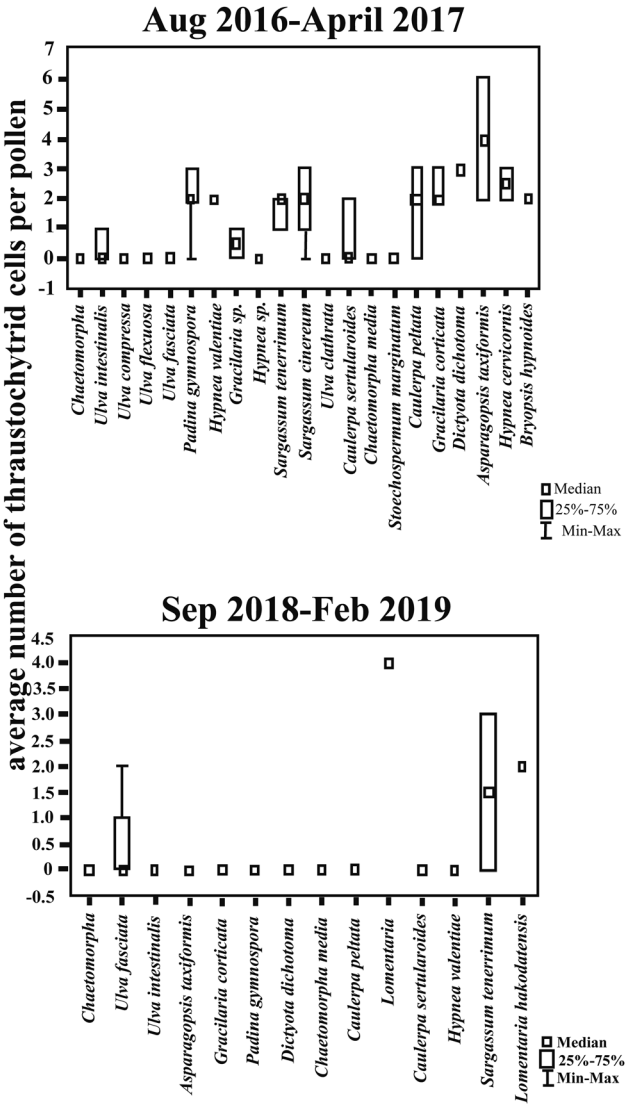

Figure 6: Box plots showing the presence of thraustochytrids in association with macroalgae during two sampling periods.

pollen in the first temporal study (2016–2017) but not in the second (2018–2019). *Bryopsis hypnoides* and its associated thraustochytrids were observed only during March 2017 and not during 2018–2019. This green alga also revealed *Labyrinthula*-like cells on baiting during March 2017 (Figure 2m).

3.4 Factors governing thraustochytrid occurrence

Temporal variation in the macroalgae-thraustochytrid association was confirmed by non-parametric statistical analysis (Table 5). The ANOVA Chi-Square value by Friedman test and Kendall's coefficient of concordance were significant during both sampling periods indicating that seasons and macroalgal type together influenced the occurrence of thraustochytrids. When these two parameters were analysed separately by Kruskal–Wallis test, both were found to significantly affect thraustochytrid occurrence separately as seen by their p-values <0.05. However, this significance was found only during the 2016–2017 study period. During 2018–2019, the Kruskal–Wallis test showed no significant effect of any of the two parameters, seasons or macroalgae type.

In the estuarine water samples, it was difficult to determine if any physicochemical factors or bacterial counts influenced thraustochytrid occurrence as thraustochytrids were present in all samples despite varied physicochemical properties and TVC of bacteria. Significant ANOVA Chi Square value by Friedman test and Kendall's coefficient of concordance indicated the cumulative effect of all these parameters on thraustochytrid occurrence (Table 6).

4 Discussion

Thraustochytrids were found to be associated with seaweeds (macroalgae) in the present study. Temporal variation in the association between thraustochytrids and macroalgae was studied at the rocky beach of Anjuna, as macroalgae were consistently found there. Monsoons were generally marked by the presence of green algae only. Monsoon samples never produced thraustochytrids after pine pollen baiting except in September 2018 from *Ulva fasciata*. An *Ulkenia* isolate was cultured successfully from *Ulva fasciata*. Many other specimens of *Ulva* gave rise to thraustochytrids on pine pollen though not in culture. Green algae were found during other seasons also, but only 25% of them recovered thraustochytrids on baiting. *Caulerpa* spp. and *Ulva* spp., though frequently found

Table 5: Results of nonparametric statistical analyses (i.e. Friedman's test and Kruskal–Wallis tests) to analyse two different parameters (seasons and macroalgal diversity) influencing thraustochytrid numbers (average number of thraustochytrid cells per pollen).

Sampling period	Interaction effect of macroalgal type and seasons on thraustochytrid cell numbers per pollen					Individual effect on thraustochytrid cell numbers per pollen			
	N	ANOVA Chi-square	p value	*df*	Kendall's coefficient of concordance (W)	Effect of	Kruskal–Wallis (H)	p value	*df*
2016–17	51	101.0297*	0.000001	2	0.99049	Macroalgal type	31.81663*	0.0453	20
						Seasons	16.86812*	0.0002	2
2018–19	29	53.22124*	0.00001	2	0.91761	Macroalgal type	17.43275	0.1803	13
						Seasons	3.091239	0.2132	2

Significant H statistic values are marked by an asterisk; *df*, degrees of freedom.

Table 6: Results of Friedman's test to check the effect of physicochemical parameters of seawater and estuarine water samples and their bacterial counts on the presence of thraustochytrids.

N	ANOVA Chi-square	p value	*df*	Kendall's coefficient of concordance (W)
15	69.42748	0.00001	5	0.9257

The p value shows the significant Chi-square value; *df*, degrees of freedom.

across beaches and seasons, produced thraustochytrids on baiting only at a few instances. In the study by Raghukumar et al. (1992), no thraustochytrids were isolated from *Ulva fasciata*.

A higher percentage of red algae than of green algae was positive for thraustochytrids (41%). Raghukumar (1987a) also observed the association of thraustochytrids with red algae. Red algae deposit fatty acid derivatives on cell wall surfaces as an antifouling strategy (Paradas et al. 2016). Thraustochytrids could use the osmolytes produced by red algae to build up their metabolites similar to algae-associated bacteria (Ramanan et al. 2016) and, in turn, help the alga by controlling fouling, probably due to their fatty acids. Epibiotic thraustochytrids may protect the algae from biofouling by serving as prey to zooplankton that prefer the algal habitat and feed on all biofouling organisms (Damare and Raghukumar 2015; Damare et al. 2013; Eereveld et al. 2013).

As compared to green and red algae, a greater percentage of brown algae showed the presence of thraustochytrid cells on pine pollen baiting during the post-monsoon and pre-monsoon period (46%). Brown algae were not found during monsoons. Live brown algae have a lower percentage of phenolics than red and green algae (Raghukumar et al. 1992). Hence brown algae would serve to be a good niche for survival and multiplication of thraustochytrids. In this study, *Sargassum* spp. showed greater incidence of thraustochytrid cells on pine pollen baiting followed by *Stoechospermum*, *Padina* spp., and *Dictyota dichotoma*. In culture, only one *Thraustochytrium* sp. was recovered from *Sargassum* during December 2016. Previous studies showed isolation of different thraustochytrid species such as *Ulkenia visurgensis, Schizochytrium* sp., *Thraustochytrium motivum* and one species of aplanochytrid (*Aplanochytrium minuta*) from *Sargassum cinereum* and *Padina tetrastomatica* (Raghukumar et al. 1992).

Labyrinthula sp., belonging to sister clade of thraustochytrids, was recovered during pine pollen baiting of the green alga *Bryopsis hypnoides* during March 2017. *Labyrinthula* is the causative agent of wasting disease of eelgrass *Zostera marina* (Miller and Jones 1983; Sullivan et al. 2013). The nature of association between *Labyrinthula* sp. and *B. hypnoides* could not be deduced as the former could not be cultured for further studies. Raghukumar (1987b) also observed *Labyrithula* sp. growing inside *Rhizoclonium* sp. and *Cladophora* sp.

In January 2018, *Oblongichytrium* sp. was isolated from the green alga *Ulva compressa* and the seawater of Anjuna. The presence of the same culture in seawater and in association with the macroalga indicated that thraustochytrids present in the seawater inhabit the algae and could therefore be cultured from them. Thus, the seasonal occurrence of thraustochytrids with macroalgae could be related to the seasonal incidence of macroalgae in nature. The statistical analysis gave further evidence that both the seasons and the type of macroalgae affected the occurrence of thraustochytrids. Pine pollen baiting of macroalgal specimens giving rise to thraustochytrids in post-monsoon and pre-monsoon seasons but not in monsoons during the first three study periods pointed towards the seasonal occurrence of thraustochytrids in association with seaweeds. In order to determine if the seasonal occurrence is not restricted to the macroalgal association but is prevalent

in other habitats as well, seawater from various locations was sampled during different seasons in 2018 to study the presence of thraustochytrids. Seawater from Mandovi and Zuari estuaries, Dona Paula and Anjuna beach revealed thraustochytrid cells in pine pollen baiting at all the times sampled showing no seasonal occurrence.

Thus seasonal variation was observed only in macroalgae-associated thraustochytrids. This agrees with the observations on other macroalgae-associated organisms, such as mollusks, limpets, mussels (Antit et al. 2013; Benedetti-Cecchi et al. 1996; Cox et al. 2017). The seasonality of thraustochytrids observed in macroalgae follows that of their macroalgal hosts. Moreover, the sporadic occurrence of thraustochytrids with macroalgae during one monsoon contrasts with their higher frequency of occurrence during the dry season than the rainy season (Jaritkhuan and Suanjit 2018).

Interestingly, about 88% of thraustochytrid isolates cultured from the macroalgae were pigmented, whereas only 40% of those cultured from the seawater were pigmented. Pigments are usually secondary metabolites produced by microorganisms in response to stress, as protection against UV radiation and as virulence factors (Liu and Nizet 2009; Moss 2002). Macroalgae found in the intertidal region are subjected to intense solar radiation during low tide, thus pigment formation in macroalgae-associated thraustochytrids may be a defense strategy against UV exposure since pigments are known to be UV-protectant (Boulay et al. 2008; Borić et al. 2011).

5 Ecological implications of thraustochytrid-seaweed associations

The present study showed that thraustochytrids were associated with living algae. The greater the species richness of the macroalgae, the higher was the percentage occurrence of thraustochytrids on them. The presence of thraustochytrids in seawater determines the health of that environment since they are a rich source of PUFAs to the plankton and fish (Atienza et al. 2012; Castillo et al. 2009; Damare and Raghukumar 2015; Kumar et al. 2018; Song et al. 2007). In addition to that, their occurrence indicates organic matter in the environment, since thraustochytrids are known to produce various degradative enzymes to obtain nutrition by decomposing organic matter (Raghukumar 2002). Macroalgae harbour a variety of fauna in their habitat (Bhaduri and Wolf 2017; Hinz et al. 2019). Thraustochytrids are one of the groups of organisms found in this habitat. These will therefore serve to be an important component of the seaweed ecosystem. More environmental studies are necessary to probe into the presence of thraustochytrids in and among seaweeds, as well as laboratory studies to elaborate on their association.

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