# The fungal endobiome of seaweeds of the Andaman Islands, India

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**Seventeen seaweed species (two green algae, nine brown algae and six red algae) of the Andaman Islands, India, were studied for their culturable fungal endophyte assemblage. A total of 796 endophytic isolates (67 species of fungi belonging to 22 genera and 10 sterile forms) were recovered from the 17 seaweeds. All the fungi were marine-derived forms and many belonged to Eurotiomycetes and Sordariomycetes of the Ascomycota group. More species of *Aspergillus*, *Fusarium*, *Penicillium* and *Trichoderma* were present as endophytes. While most endophytic species recovered were present in low frequency, some fungi like *Aspergillus niger*, *Aspergillus* sp. 1, *Nodulisporium* sp., *Pestalotiopsis* sp., *Trichoderma yunnanense* and *Xylaria* sp. 1 exhibited more than 40% frequency of colonization. Apart from yielding the maximum number of endophytic isolates, different *Trichoderma* species showed the highest colonization frequency in 11 of the 17 seaweeds. The results of this study indicate that fungi belonging to Eurotiomycetes which occur in low frequency as endophytes in terrestrial plants represent a significant percentage in the seaweeds and that the environment might have a more critical role than host specificity in determining the endophyte community of seaweed mycobiome.**

**Keywords:** Algal endophytes, eurotiomycetes, marine algae, Sordariomycetes, *Trichoderma*.

SEAWEEDS (marine macroalgae) include green, brown and red algae. They regulate the ecosystem in coastal seas because they function as primary biomass producers, play a critical role in founding and stabilizing the ecosystem, and do nutrient recycling[1–3]. Considering their importance in ecosystem functions, market value as food[4] and as feedstock for third-generation biofuels[5], it is essential to study the microbiome of seaweeds. This is imperative since the microbiome aids seaweeds in their growth, nutrition and development and enhances their defence against pathogenic bacteria[6,7]. Most studies on seaweed microbiomes pertain to bacteria[7]. Studies on their fungal component are mainly related to parasitic and saprotrophic fungi[8,9], less information is available on the fungi of the endobiome of seaweeds. These fungi, called endophytes, are non-pathogenic and associated with the internal tissues of seaweeds[10,11]. In the present study, we screened seaweeds of the Andaman Islands, India, for their fungal endophytes. In this communication, we refer to the brown seaweeds as 'brown algae' though according current classification they are chromists and not algae.

Fresh and healthy algae belonging to 17 seaweed species (two green algae, nine brown algae and six red algae) were collected from tourist spots of Corbyn's Cove Beach, Andaman Islands (Table 1). The seaweeds were washed in running water, cut into small segments of approximately 0.5 cm in size and surface-sterilized as follows: 100 segments of each seaweed species were immersed for 5 sec in 70% ethanol and 10 sec in sterile water[11]. The tissue segments (10/petri dish) were inoculated in potato dextrose agar (PDA) medium containing chloramphenicol (150 mg/l) and screened for the presence of endophytes[11]. The effectiveness of surface sterilization was confirmed by imprint method[12]. The PDA medium was made up of distilled water and not seawater since we had observed no significant difference between these media with reference to the emergence of endophytes from the tissues[11]. The petri dishes were exposed to a 12 h light : 12 h dark cycle for four weeks at 26° ± 1°C (ref. 11). Emerging endophytes from the tissues were cultured in PDA medium as axenic cultures, and identified based on microscopic and molecular methods. Isolates which did not sporulate were treated as morphospecies and assigned codes depending on culture morphology.

Standard phenol–chloroform extraction protocol to isolate genomic DNA from fungal endophyte cultures was followed. From the genomic DNA, PCR amplification was performed with ITS1 and ITS4 as well as ITS1F and ITS4 primers that target the internal transcribed spacers (ITS) of ribosomal DNA[13]. The PCR conditions for primer pair ITS1 and ITS4 were: 95°C for 10 min, 30 cycles of 95°C for 1 min, 55°C for 1 min, 72°C for 90 sec, and finally 72°C for 10 min. The PCR conditions for primer pair ITS1F and ITS4 were: 94°C for 5 min, 40 cycles of 94°C for 45 sec, 53°C for 30 sec, 72°C for 50 sec and finally 72°C for 10 min. The PCR products were purified by gel-elution and sequenced (in the School of Life Sciences, Manipal employing the ABI 3130 Genetic Analyzer) using the ITS1 or ITS1F primer and following standard protocol. The sequences were manually edited and the closest match to type sequences in the NCBI database was identified using the BLASTN tool. A total of 21 fungal ITS sequences from fungal endophytes were deposited in GenBank (MN158327–MN158347). The sequences of *Trichoderma* isolates from the present study were aligned with ITS sequences from the nearest matches (coverage >97% and identity >96%) and other species of *Trichoderma* available in GenBank. Multiple sequence alignment was performed with sequences that showed significant matches with the default settings (limited to sequences from type material) employing ClustalW and using MEGA 6. A phylogenetic tree was generated after applying the maximum

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**Table 1.** Seaweeds from Andaman Islands screened for their fungal endophyte assemblage

| Seaweed species | Family | Code |
|---|---|---|
| Green algae | | |
| *Boergesenia forbesii* (Harvey) Feldmann | Siphonocladaceae | BF |
| *Halimeda opuntia* (L.) J.V. Lamouroux | Halimedaceae | HO |
| Brown algae | | |
| *Colpomenia sinuosa* (Mertens ex Roth) Derbès & Solier | Scytosiphonaceae | CS |
| *Padina gymnospora* (Kützing) Sonder | Dictyotaceae | PG |
| *Padina pavonica* (L.) Thivy | Dictyotaceae | PP |
| *Sargassum ilicifolium* (Turner) C. Agardh | Sargassaceae | SI |
| *Sargassum polycystum* C. Agardh | Sargassaceae | SP |
| *Sargassum wightii* Greville | Sargassaceae | SW |
| *Sargassum* sp. | Sargassaceae | SS |
| *Turbinaria conoides* (J. Agardh) Kützing | Sargassaceae | TC |
| *Turbinaria decurrens* Bory de Saint-Vincent | Sargassaceae | TD |
| Red algae | | |
| *Acanthophora spicifera* (M. Vahl) Børgesen | Rhodomelaceae | AS |
| *Dichotomaria obtusata* (J. Ellis & Solander) Lamarck | Galaxauraceae | DO |
| *Gracilaria edulis* (S.G. Gmelin) P.C. Silva | Gracilariaceae | GE |
| *Gracilaria lantaensis* Muangmai *et al*. | Gracilariaceae | GL |
| *Gracilaria salicornia* (C. Agardh) E.Y. Dawson | Gracilariaceae | GS |
| *Hypnea valentiae* (Turner) Montagne | Cystocloniaceae | HV |

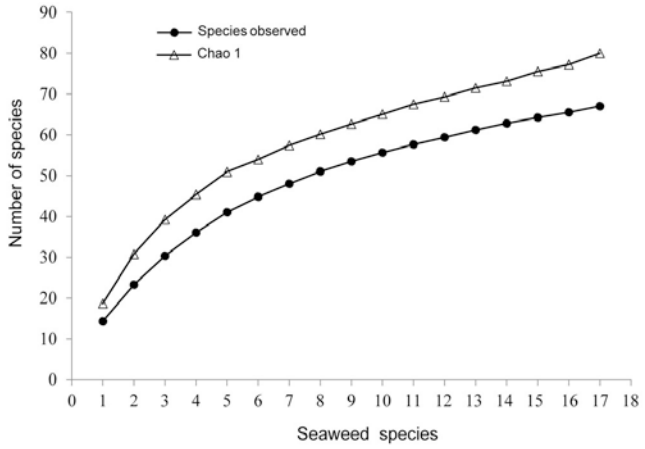


**Figure 1.** Species accumulation and species richness estimator (Chao 1) plots for the fungal endophyte assemblages of seaweeds of the Andaman Islands India. The data were randomized 100 times for plotting the curves.

likelihood method based on Kimura 2-parameter model[14–16]. Maximum likelihood tree with the highest log likelihood was constructed after calculating bootstrap support based on 1000 replications.

The colonization frequency (CF%) of each endophyte is the percentage of tissue segments of an alga it colonizes[17]. Chao 1, a nonparametric estimator, was used to determine the extent of completeness of the sampling effort[18]. The data were randomized 100 times for plotting Chao 1 and the species accumulation curves. Fisher's alpha was used to determine the species diversity and correspondence analysis for discerning any difference in the endophyte assemblage of seaweeds from Tamil Nadu (results from our earlier study) and the Andaman Islands.

A total of 796 endophytic isolates comprising 67 species of fungi belonging to 22 genera and 10 sterile forms (non-sporulating) were recovered from the 17 seaweeds. All the fungi were marine-derived forms showing no obligate salt requirement for growth. The number of endophytic species isolated increased rapidly initially and then dropped gradually with increasing sample size, as revealed by the species accumulation curve (Figure 1). The density of endophyte colonization varied across different host species. CF% of endophytes ranged from 19 in *Halimeda opuntia* to 101 in *Boergesenia forbesii* (Table 2). CF% was more than 100 in *B. forbesii*, since more than one endophyte species grew from a single tissue segment. A total of six endophyte species occurred in *Gracilaria edulis*, while 21 different species colonized *Colpomenia sinuosa* (Table 2). The species diversity of endophytes was the lowest for *G. edulis* and highest for *C. sinuosa* (Table 2).

More species of some fungal genera were encountered as endophytes. These included *Aspergillus* (14 species/167 isolates), *Fusarium* (5 species/21 isolates), *Penicillium* (6 species/48 isolates) and *Trichoderma* (8 species/184 isolates). Some fungi showed a wide host range as endophytes. *Aspergillus niger* and *Xylaria* sp. 1 were isolated from 14 and 13 seaweeds respectively (Table 2). Apart from exhibiting a wide host range, some of the endophytes such as *A. niger*, *Aspergillus* sp. 1, *Aspergillus* sp. 2, *Nodulisporium* sp., *Pestalotiopsis* sp., *Trichoderma atrobrunneum*, *Trichoderma crassum*, *Trichoderma yunnanense* and *Xylaria* sp. 1, showed higher frequencies of colonization in different seaweeds (Table 2). The rest of the 58 species of endophytes recovered were present in low frequencies. *Aspergillus* sp. 2, *Nodulisporium* sp., *Penicillium digitatum*, *Pestalotiopsis* sp., *Aspergillus terreus*, *Penicillium oxalicum*, *Talaromyces* sp. 2 and *T. yunnanense* occurred in seven or more species

**Table 2.** Colonization frequency (CF%) of endophytic fungi isolated from 17 seaweed species (refer Table 1 for name of seaweeds)

| | Green algae | | Brown algae | | | | | | | | | Red algae | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Fungus | BF | HO | CS | PG | PP | SI | SP | SW | SS | TC | TD | AS | DO | GE | GL | GS | HV |
| *Alternaria* sp. | | 1 | | | | | | 2 | | | | | | | | 2 | |
| *Arthrinium* sp. | | | | | | 1 | | | | | | | | | | | |
| *Aspergillus clavatus* | | | | | | | | | | 1 | | | | | | | |
| *Aspergillus flavus* | 1 | | | | | | | 2 | | | | | | | | | |
| *Aspergillus fumigatus* | | | | | | | 1 | | 1 | | | | | | | 1 | |
| *Aspergillus giganteus* | | | | 3 | | 2 | | | | | 1 | 1 | | | | | |
| *Aspergillus nidulans* | | | | 1 | | | | | | 1 | | | | | 1 | | |
| *Aspergillus niger* | 3 | 3 | 2 | | 3 | 3 | 1 | 2 | 3 | 5 | 3 | 4 | | | 4 | 2 | 3 |
| *Aspergillus terreus* | 1 | | 1 | | | | | | | 1 | 2 | 1 | 1 | | | 1 | |
| *Aspergillus* sp. 1 | 52 | | | | | | | | | | | | | | | | |
| *Aspergillus* sp. 2 | 1 | | 3 | 5 | 2 | 1 | | 2 | 3 | 2 | 5 | | | 2 | 5 | | |
| *Aspergillus* sp. 3 | 1 | | | | 2 | | 2 | | | 2 | 1 | | | | | 2 | |
| *Aspergillus* sp. 4 | | | 1 | | | | | | | | | | | | | | |
| *Aspergillus* sp. 5 | | | | | | | | | | | | | | | | 1 | |
| *Aspergillus* sp. 6 | | | 1 | | | | | 1 | | | 1 | | | | | | |
| *Aspergillus* sp. 7 | | | | | | | 1 | | | | | | | | | | |
| *Aureobasidium pullulans* | 1 | | | | 1 | | | | | | | | | | | | |
| *Cladosporium* sp. | | | 3 | | | 2 | | | | | | 1 | | | | | |
| *Colletotrichum* sp. | | | | 1 | | | | | | | | | | | | | |
| *Curvularia* sp. | | 1 | | | | | | | | | | | | | | | |
| *Eupenicillium* sp. | | | | | 1 | | | | | 2 | | | | | | | |
| *Fusarium* sp. 1 | | | 1 | | | | | 1 | | | | | 1 | | 1 | | 2 |
| *Fusarium* sp. 2 | 1 | | | 3 | | | 1 | | | | | | | | | 1 | |
| *Fusarium* sp. 3 | | | | | 4 | | | | | | | 2 | | | | | |
| *Fusarium* sp. 4 | | | | | | | | | | | | | | | 1 | | |
| *Fusarium* sp. 5 | 2 | | | | | | | | | | | | | | | | |
| *Lasiodiplodia theobromae* | | 1 | 1 | | | 1 | | | | | | 2 | | | 2 | | |
| *Mucor* sp. | | | 2 | | 1 | | | | | 1 | | | 4 | | | | |
| *Nigrospora oryzae* | 1 | | | | | | | | | | | | | | | | |
| *Nodulisporium* sp. | 13 | 1 | 1 | 2 | | | 10 | | | | 5 | 10 | 1 | 3 | | 3 | 10 |
| *Paecilomyces* sp. 1 | | | | | | 3 | | | | | | | | | | | |
| *Paecilomyces* sp. 2 | | | | | 1 | | | | | | | | | | | | |
| *Paecilomyces* sp. 3 | | | | | | | | | | | | | 1 | | | | |
| *Penicillium digitatum* | 1 | | | | 3 | 4 | | 5 | 3 | | 1 | 1 | 1 | | 1 | 1 | 1 |
| *Penicillium oxalicum* | 2 | | 2 | 1 | | | | | | | 1 | 3 | | 1 | | | 1 |
| *Penicillium purpurogenum* | | | | | | | 1 | | | | | | | | | | |
| *Penicillium* sp. 1 | | | 3 | | 3 | | 2 | | 1 | 1 | | | | | | | |
| *Penicillium* sp. 2 | | | 2 | | | | | | | | | | | | | | 1 |
| *Penicillium* sp. 3 | | | | | | | 1 | | | | | | | | | | |
| *Pestalotiopsis* sp. | | 2 | 11 | | 5 | | | | | 14 | 2 | 17 | 1 | | 8 | 7 | 3 |
| *Phoma* sp. | | 1 | | | | | | | | | | | 1 | | | | |
| *Phomopsis* sp. | | | 2 | | | | 1 | | | | | | | | | | |
| Sterile form 1 | | | | 2 | | | | 1 | | 2 | | | | 8 | | 2 | 1 |
| Sterile form 2 | 1 | | | 4 | 4 | | | | 1 | 1 | | | 2 | | | | |
| Sterile form 3 | 1 | | 1 | | | | 1 | | | | | | 1 | | | 1 | |
| Sterile form 4 | | | | | 1 | 1 | | 1 | | | | 2 | | | 1 | | 1 |
| Sterile form 5 | | | | | | | | 1 | | | | | | | | | |
| Sterile form 6 | | | | | | | | | | | | | | 2 | | | |
| Sterile form 7 | | | | | | | | | | | | 2 | | | | | |
| Sterile form 8 | | | | 2 | | | | | | | | | | | | | |
| Sterile form 9 | | | | | | | | | | | 1 | | | | | | |
| Sterile form 10 | | | 2 | | | | 1 | | | | | | 3 | | | | |
| *Talaromyces* sp. 1 | | | 1 | | | 2 | | | 1 | | 2 | | | | | | |
| *Talaromyces* sp. 2 | 4 | 1 | | | | 2 | 2 | | 2 | | | | 2 | | | | 3 |
| *Talaromyces* sp. 3 | | | | | | | 2 | 5 | | 3 | | | | | | | |
| *Torulomyces* sp. | | | 1 | | | | | 1 | 1 | | 1 | | | | | | |
| *Trichoderma atrobrunneum* | | | 8 | | | | | | | 12 | | 18 | | | | | |
| *Trichoderma crassum* | | | | | 8 | | | | | | | | | 4 | 11 | 9 | |
| *Trichoderma inhamatum* | | | | | | | | | | | | | | | 6 | | |
| *Trichoderma lixii* | | | | 6 | | | | 4 | | | | | 4 | | | | |

(*Contd*)

**Table 2.** (*Contd*)

| Fungus | Green algae | | Brown algae | | | | | | | | | Red algae | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | BF | HO | CS | PG | PP | SI | SP | SW | SS | TC | TD | AS | DO | GE | GL | GS | HV |
| *Trichoderma parareesei* | | | | | | | | | | | | | | | | 5 | |
| *Trichoderma pleuroticola* | | | | | | 9 | 1 | | | | | | | | | | |
| *Trichoderma reesei* | | | 2 | 10 | | | | | | | 5 | | | | | | 5 |
| *Trichoderma yunnanense* | | 7 | | | 10 | 6 | | 8 | 8 | | 11 | | 7 | | | | |
| *Xylaria* sp. 1 | 7 | 1 | | 5 | 3 | 8 | 6 | 2 | 1 | | | 14 | 2 | | 2 | 8 | 37 |
| *Xylaria* sp. 2 | | | | | | | 3 | | | | | 4 | 2 | | | | |
| *Xylaria* sp. 3 | 8 | | | | | | | | | | | | | | | | |
| Total CF% | 101 | 19 | 51 | 45 | 52 | 45 | 37 | 38 | 25 | 48 | 42 | 82 | 34 | 20 | 43 | 46 | 68 |
| Total no. of species | 18 | 10 | 21 | 13 | 16 | 14 | 17 | 15 | 11 | 14 | 15 | 15 | 16 | 6 | 12 | 15 | 12 |
| Fisher's alpha | 6.4 | 8.5 | 13.4 | 6.1 | 7.9 | 7.0 | 12.2 | 9.1 | 7.5 | 6.6 | 8.3 | 5.4 | 11.8 | 2.9 | 5.5 | 7.7 | 4.2 |

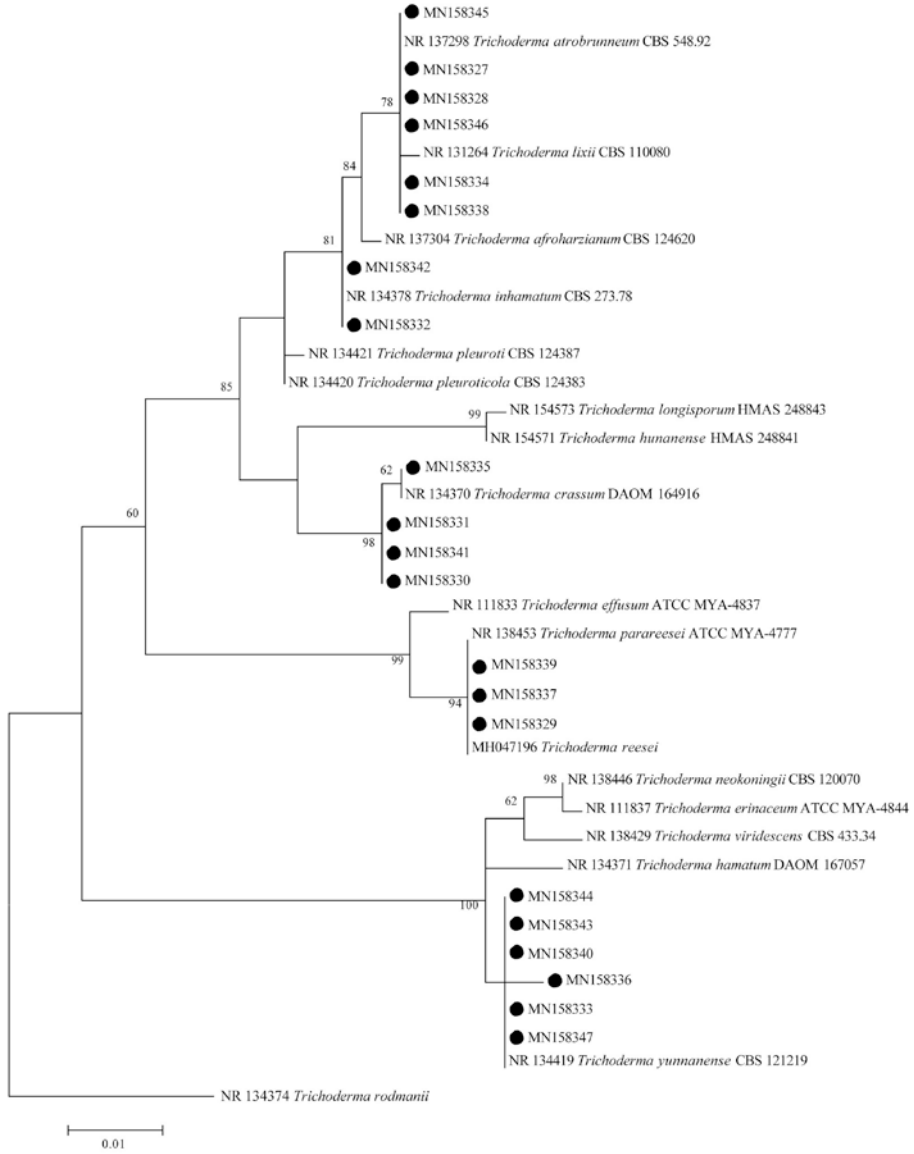


**Figure 2.** Phylogenetic analysis of *Trichoderma* isolates obtained in the present study. Maximum likelihood method was used to infer evolutionary history after 1000 bootstrap replications. The tree with the highest log likelihood and branches with more than 50% support are only shown. Isolates from the present study are marked with a black circle.

of the seaweeds screened (Table 2). Apart from yielding the highest number of endophyte strains, the genus *Trichoderma* was dominant in 11 and co-dominant in 3 of the 17 seaweeds. Hence, the isolates belonging to *Trichoderma* were chosen for further molecular characterization.

A total of 21 *Trichoderma* isolates from different seaweeds were cultured axenically, the genomic DNA was isolated and their ITS region was amplified by polymerase chain reaction using primers specific to the fungal ITS region. The purified amplicons were then sequenced and compared with the sequences of *Trichoderma* species-type specimens in the NCBI database to arrive at species names. In all the cases, species names were assigned only when the query coverage was 100% and the similarity was above 99% (Figure 2 and Table 3). Furthermore, a maximum likelihood tree was generated after aligning the ITS sequences to arrive at the phylogenetic relationship among various isolates. The highest log likelihood tree was plotted, with the values on nodes being branch support values obtained from 1000 bootstrap replications (Figure 2). *Trichoderma rodmanii* was used as the outgroup for the analysis. The results showed that the *Trichoderma* isolates could be grouped broadly into four different clusters. The identified *Trichoderma* species were deposited in the Microbial Type Culture Collection (MTCC), Chandigarh and the accession numbers were obtained (Table 3).

More sampling effort would report more endophytic species since the species accumulation curve and Chao 1 estimator did not reach an asymptote[18] (Figure 1). However, the deceleration of the species accumulation curve with increasing sample size (Figure 1) suggested that the sample size used adequately reflected the species richness. As in our earlier study on endophytes of seaweeds on the Tamil Nadu coast, only marine-derived fungi and not obligate marine fungi were present as endophytes in all the seaweeds screened. The use of selective growth media and molecular tools may reveal a complete picture of the endophyte facet of the seaweed microbiome. The endophytes isolated belonged to the classes Eurotiomycetes (33% of the isolates recovered) and Sordariomycetes (57% of the isolates recovered) (Table 2). These lineages along with the Dothideomycetes represent the major fungal groups associated with various marine life[19,20]. We isolated several marine-derived fungi, including *Aspergillus*, *Penicillium* and *Trichoderma*, as endophytes from different seaweed hosts. These genera occur in seaweeds of Brazil[21], southern India[11], North Atlantic[22] and the Antarctica[23,24], endorsing their broad host range and high ecological amplitude. These genera are also the common fungal associates of marine organisms such as sponges[25], seagrasses[26,27] and corals[28]. Furthermore, our molecular sequencing of the ITS region and phylogenetic analysis of different *Trichoderma* isolates showed that different species of *Trichoderma* (such as *T. atrobrunneum*, *T. crassum*, *T. inhamatum*, *T. lixii*, *T. parareesei*, *T. pleuroticola*, *T. reesei* and *T. yunnanense*) are endophytic in different seaweeds. In addition, isolates belonging to *T. lixii* and *T. atrobruunneum*, which were earlier separated from the *T. harzianum* species complex, formed a tight clade with a bootstrap support of 78%. Seaweeds produce many anti-fungal metabolites[11,29]; furthermore, endophytes associated with seaweeds also produce anti-fungal compounds[11]. Thus, the secondary metabolites of a seaweed host and its native fungal endophyte assemblage could play a role in determining the composition of its endophyte assemblages[4,29,30]. It is likely that the multi-host endophytes of seaweeds have

**Table 3.** *Trichoderma* species identified based on ITS-sequences, their GenBank accession and culture deposition accession numbers

| Seaweed | Identified as | GenBank accession no. | MTCC accession no. |
|---|---|---|---|
| AS | *Trichoderma atrobrunneum* | MN158327 | 13204 |
| CS | *Trichoderma atrobrunneum* | MN158328 | 13205 |
| GS | *Trichoderma parareesei* | MN158329 | 13206 |
| GE | *Trichoderma crassum* | MN158330 | 13207 |
| GL | *Trichoderma crassum* | MN158331 | 13208 |
| GL | *Trichoderma inhamatum* | MN158332 | 13209 |
| DO | *Trichoderma yunnanense* | MN158333 | 13210 |
| DO | *Trichoderma lixii* | MN158334 | 13211 |
| GS | *Trichoderma crassum* | MN158335 | 13212 |
| HO | *Trichoderma yunnanense* | MN158336 | 13213 |
| HV | *Trichoderma reesei* | MN158337 | 13258 |
| PG | *Trichoderma lixii* | MN158338 | 13234 |
| PG | *Trichoderma reesei* | MN158339 | 13235 |
| PP | *Trichoderma yunnanense* | MN158340 | 13254 |
| PP | *Trichoderma crassum* | MN158341 | 13270 |
| SI | *Trichoderma pleuroticola* | MN158342 | 13261 |
| SS | *Trichoderma yunnanense* | MN158343 | 13267 |
| SW | *Trichoderma yunnanense* | MN158344 | 13255 |
| SW | *Trichoderma lixii* | MN158345 | 13256 |
| TC | *Trichoderma atrobrunneum* | MN158346 | 13268 |
| TD | *Trichoderma yunnanense* | MN158347 | 13269 |

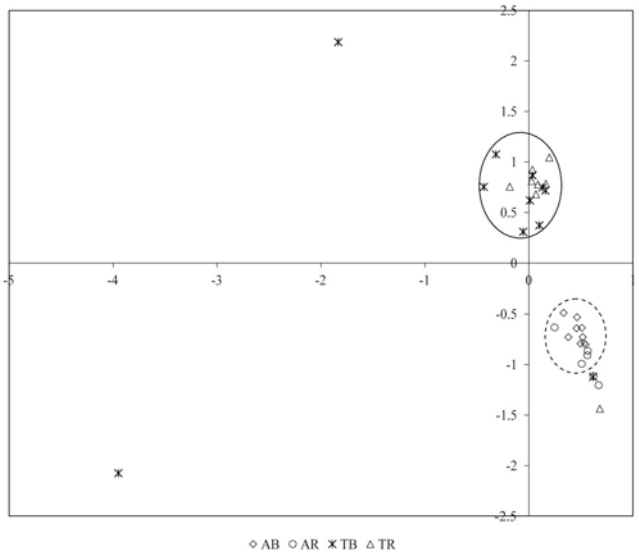


**Figure 3.** Correspondence analysis for fungal endophyte assemblages of red and brown algae between Andaman Islands and Tamil Nadu. AB, Andaman brown algae, AR, Andaman red algae; TB, Tamil Nadu brown algae and TR, Tamil Nadu red algae.

evolved tolerance to or the ability to detoxify the host antifungal metabolites, and interact with the host's existing microbiome successfully.

In our earlier study, we isolated endophytes from 11 brown and 8 red algal species occurring along the Tamil Nadu coast of southern India, which lies about 1500 km west of the Andaman Islands[11]. Since all the methods used were essentially the same for this and the present study, we used correspondence analysis to visualize the distribution of endophytes as influenced by seaweed species and their geographic locations (Figure 3). The endophyte communities differed more between location than between the type of seaweed, suggesting that, as has been observed in terrestrial plants[31], the environment plays a more critical role than host factors in structuring the endophyte community of the seaweed microbiome.

The fungal endophytes of the seaweed microbiome have hardly been explored for their diversity and ecology. Considering the important status of seaweeds in the global market, it would be worthwhile knowing if endophyte association confers fitness benefits to seaweeds as it does for their terrestrial plant hosts[32]. Furthermore, seaweed fungal endophytes, especially *Trichoderma* species, produce several technologically exploitable metabolites. These include anti-malarial, anti-bacterial, anti-algal and anti-fungal metabolites, novel salt and ionic liquid-tolerant xylan-degrading enzymes[33], as well as novel chitin-modifying enzymes[34]. These observations underscore the need to explore fungal endophytes of seaweeds more diligently.


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# Conducting clinical trials only in India's large cities is unlikely to sample the country's ethnicity sufficiently well

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**Around the world, there have been calls to include participants of diverse ethnicities in every clinical trial. In India, some years ago, a Parliamentary Committee was informed that trials are run in cosmopolitan cities of the country, and that this ensured suitable ethnic representation. The Indian Council of Medical Research, New Delhi, has defined six zones of the country from where sampling needs to be done to ensure good ethnic coverage. We found that no city has adequate representation from all the zones. However, possibly, a suitable sampling strategy in some cities could replace running trials from a few zones.**

**Keywords:** Clinical trials, cosmopolitan cities, diversity, ethnicity, sampling strategy.

PATIENTS of any age, gender, ethnicity, etc. may require a particular drug. However, clinical trials that are conducted to confirm whether a candidate drug is efficacious and not toxic usually do not adequately include representatives of the various sub-populations that will need it[1–3]. As such, much clinical research is sub-optimal and can result in unexpected adverse or serious adverse events when a drug is rolled out to the general population[4].

Increasing the diversity of trial participants is a non-trivial task. Nevertheless, as a result of a range of efforts[5–8], the diversity in trials in the USA, for instance, has increased in the last couple of decades[9].

In India, too, the need to include various ethnic groups in trials has received attention. In 2012, the 59th Parliamentary Report, by the Parliamentary Standing Committee on Health and Family Welfare, Government of India[10], commented that 'In response to a question as to how various ethnic groups are being enrolled in Phase III clinical trials, the Committee was informed that "most trials were taking place in cosmopolitan towns. It is understood that cosmopolitan cities have a heterogeneous population comprising various ethnic groups. Otherwise, there is no proactive, specific procedure to test new drugs on different ethnic groups" '[10]. However, the Standing Committee considered it inadequate that a drug was only trialled in Mumbai, for instance. Furthermore, in response to the Standing Committee highlighting various inadequacies with the drug testing and approval processes, the Ranjit Roy Chaudhury Committee was established in order to, inter alia, provide guidance on clinical trials. In 2013, this Committee, too recommended that diverse ethnicities be included in trials run in India[11].

Let us return to the issue of diversity in metropolitan India. Assuming for the moment, that sufficient ethnic diversity does exist in some of India's largest cosmopolitan cities, this raises the question, 'How should one define a city as cosmopolitan?'. We examine this below.

In terms of defining the variety of ethnicities in India at the broadest level, the Indian Council of Medical Research (ICMR), New Delhi, has divided the country into six zones, viz. East, West, North, South, Central and North East[12]. Although there has been migration within the country, each zone is characterized by a dominant ethnicity[13]. Therefore, we define each zone as being characterized by ethnicity different from that in another zone. We list the constituent states and Union Territories (UTs) of each zone, largely based on Government categorizations (Table 1).

Separately, we use the Census 2011 data at the town level, which records the total number of speakers of each of the

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