The fungal endobiome of seaweeds of the Andaman Islands

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Seventeen seaweed species (2 green algae, 9 brown algae and 6 red algae) of 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 including *Aspergillus niger, Aspergillus* sp.1, *Nodulisporium* sp., *Pestalotiopsis* sp., *Trichoderma yunnanense* and *Xylaria* sp. 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 indicated that fungi belonging to Eurotiomycetes which occur in low frequency as endophytes in terrestrial plants represent a significant percentage of endophytes of seaweeds and that environment

might have a significant role than host specificity in determining the endophyte community of seaweed mycobiome.

Keywords: *Trichoderma*, algal endophytes, marine algae, Eurotiomycetes, Sordariomycetes

Seaweeds (marine macroalgae) include the green, brown and red algae. They regulate ecosystem in coastal seas owing to their function as major primary biomass producers, and play a scritical role in founding and stabilizing the ecosystem, and 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 microbiome aids seaweeds in their growth, nutrition and development, and enhances their defense against pathogenic bacteria[6,7]. Most studies on seaweed microbiomes pertain to bacteria[7]. Investigations 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 the endophytes, are non-pathogenic and are associated with the internal tissues of seaweeds[10,11]. In this study, we screened seaweeds of Andaman Islands for their fungal endophytes. Although the 'brown algae' are Chromists and do not belong to the plant kingdom, we term them 'brown algae' in this communication.

Fresh and healthy algae belonging to 17 seaweed species (2 green algae, 9 brown algae and 6 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 size and surface sterilized as follows. One hundred segments of each seaweed species were immersed for 5 seconds in 70% ethanol and for 10 seconds in sterile water[11]. The tissue segments (10/Petri dish) were inoculated in potato dextrose agar medium (PDA) 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 with distilled water and not with seawater since we had found that there is 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 4 weeks at 26 ± 1ºC[11]. Emerging endophytes from the tissues were cultured in PDA 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, a PCR amplification was performed with ITS1 and ITS4 and 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 minutes, 30 cycles of 95°C for 1 minute, 55°C for 1 minute, 72°C for 90 seconds, and finally 72°C for 10 minutes. The PCR conditions for primer pair ITS1F and ITS4 were: 94°C for 5 minutes, 40 cycles of 94°C for 45 seconds, 53°C for 30 seconds, 72°C for 50 seconds, and finally 72°C for 10 minutes. The PCR products were purified by gel-elution and sequenced (in School of Life Sciences, Manipal using ABI 3130 Genetic Analyzer) using the ITS1 or ITS1F primer using ABI 3130 Genetic Analyzer 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 this study were aligned with ITS sequences from nearest matches (coverage >97% and identity >96%) and other species of *Trichoderma* available in the NCBI GenBank database. Multiple sequence alignment was performed with sequences which showed significant matches with default settings (limited to sequences from type material) employing ClustalW using MEGA 6. A phylogenetic tree was

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

The colonization frequency (CF%) of each endophyte is the % of tissue segments of an alga it colonized[17]. Chao 1, a nonparametric estimator, was used to know the extent of completeness of the sampling effort[18]. The data were randomized 100 times for plotting this and a 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 of 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 requirement for salt for growth. The number of endophytic species isolated increased rapidly initially and dropped gradually with increasing sample size as revealed by a species accumulation curve (Figure 1). The density of endophyte colonization varied across different host species. The CF% of endophytes ranged from 19% in *Halimeda opuntia* to 101% in *Boergesenia forbesii* (Table 2). The CF% was more than 100% in *B. forbesii* since more than one endophyte species grew out from a single tissue segment. A total of 6 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 these endophytes such as *Aspergillus niger*, *Aspergillus* sp. 1, *Aspergillus* sp. 2, *Nodulisporium* sp., *Pestalotiopsis* sp., *Trichoderma atrobrunneum*, *T. crassum*, *T. 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., *A. terreus*, *Penicillium oxalicum*, *Talaromyces* sp. 2, and *Trichoderma yunnanense* occurred in 7 or more species of the seaweeds screened (Table 2). Apart from yielding the highest number of endophyte strains, the genus *Trichoderma* was dominant in eleven and co-dominant in three 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, genomic DNA was isolated and their ITS region was amplified by polymerase chain reaction using primers specific to fungal ITS region. The purified amplicons were then sequenced and compared with the sequences of *Trichoderma* species type specimens available in 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 above 99% (Figure 2, 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 values on nodes being branch support values obtained from 1000 bootstrap replications (Figure 2). *Trichoderma rodmanii* was used as the outgroup for this analysis. The results showed that the *Trichoderma* isolates of our study clustered broadly into four different clusters. The identified *Trichoderma* species were deposited in Microbial Type Culture Collection (MTCC), Chandigarh and accession numbers obtained (Table 3).

It appears that 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 species accumulation curve with increasing sample size (Figure 1) suggested that the sample size used was adequate in reflecting the species richness. As in our earlier study on endophytes of seaweeds of 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 more 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 all the 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 various marine organisms such as sponges[25], seagrasses[26,27] and corals[28]. Furthermore, our molecular sequencing of 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 *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 elaborate anti-fungal compounds[11]. Thus, the secondary metabolites of a seaweed host and its native fungal endophyte assemblage together 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 evolved tolerance to or the ability to detoxify the host anti-fungal metabolites and to successfully interact with the existing microbiome of the host.

In our earlier study, we isolated endophytes from eleven brown and eight 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 current 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], environment plays a more critical role than host factors in structuring the endophyte community of the seaweed microbiome.

The fungal endophyte constituent of seaweed microbiome has hardly been explored for their diversity and ecology. Considering the important status of seaweeds in global market, it would be worthwhile knowing if endophyte association confers fitness benefit to seaweeds as they do 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 seaweed more diligently.

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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

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

Fungi	Green algae		Brown algae									Red algae					
	BF	**HO**	**CS**	**PG**	**PP**	**SI**	**SP**	**SW**	**SS**	**TC**	**TD**	**AS**	**DO**	**GE**	**GL**	**GS**	**HV**
Alternaria sp.		1						2									
Arthrinium sp.						1											
Aspergillus clavatus										1						2	
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							

(Contd)

Table 2. *(Contd)*

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

(Contd)

Table 2. *(Contd)*

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				
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**

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

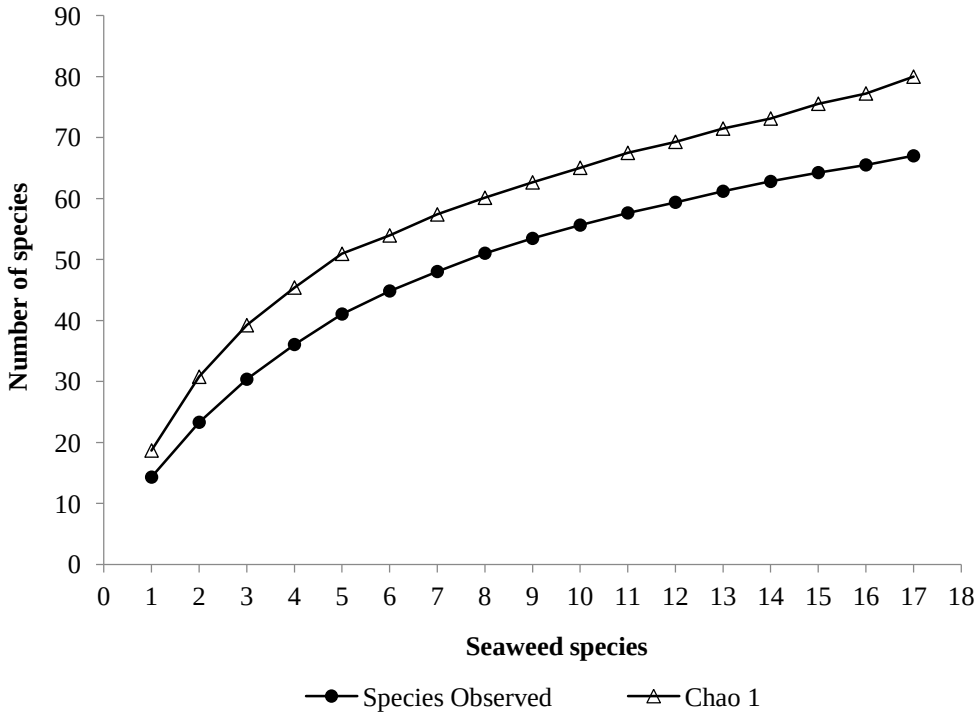

Figure 1. Species accumulation and species richness estimator (Chao1) plots for the fungal endophyte assemblages of seaweeds of Andaman Islands. The data were randomized 100 times for plotting the curves.

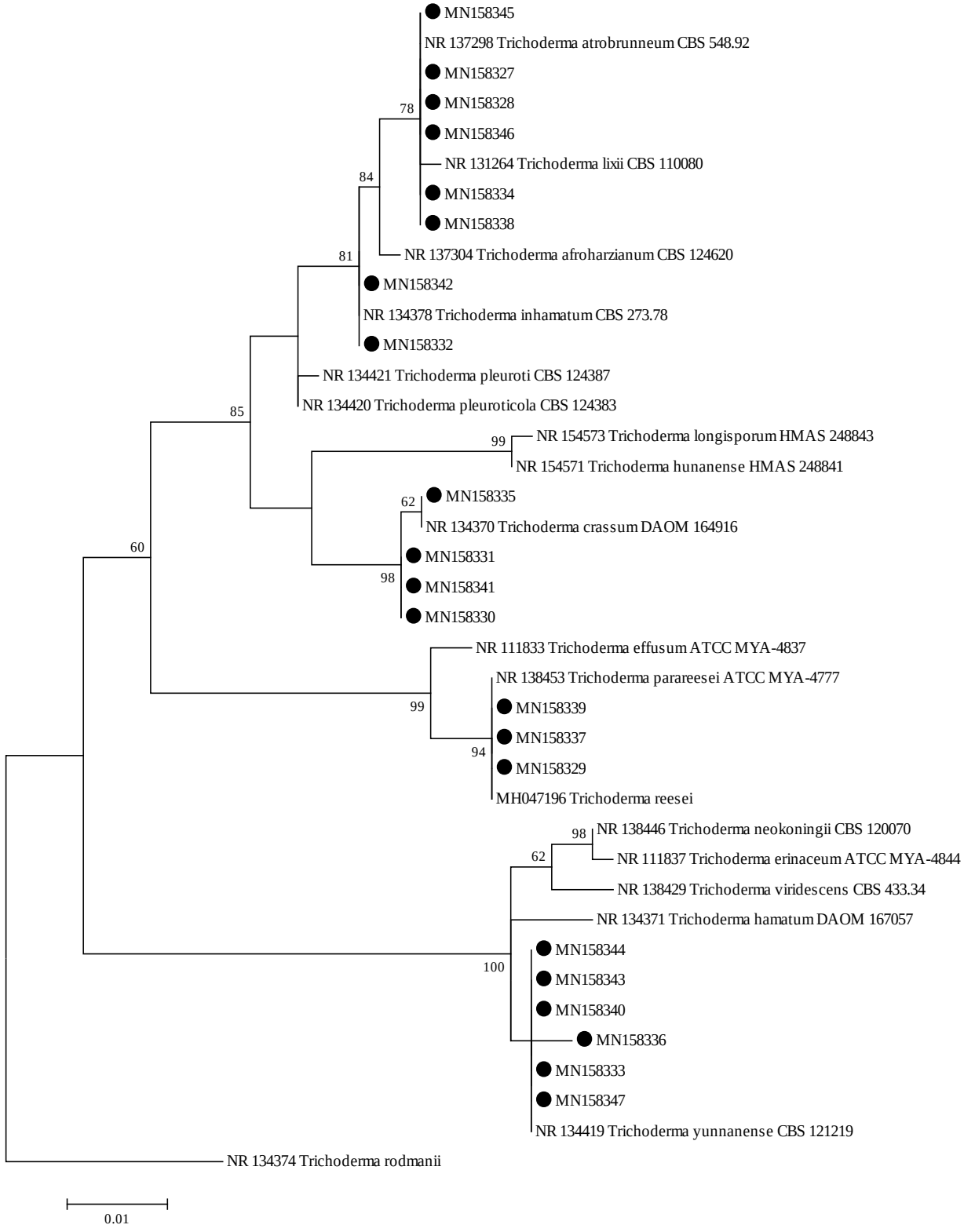

Figure 2. Phylogenetic analysis of *Trichoderma* isolates obtained in the present study. A maximum-likelihood method was used to infer evolutionary history after 1000 bootstrap replications. Tree with highest log likelihood is shown and branches with more than 50% support are only shown. Isolates from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

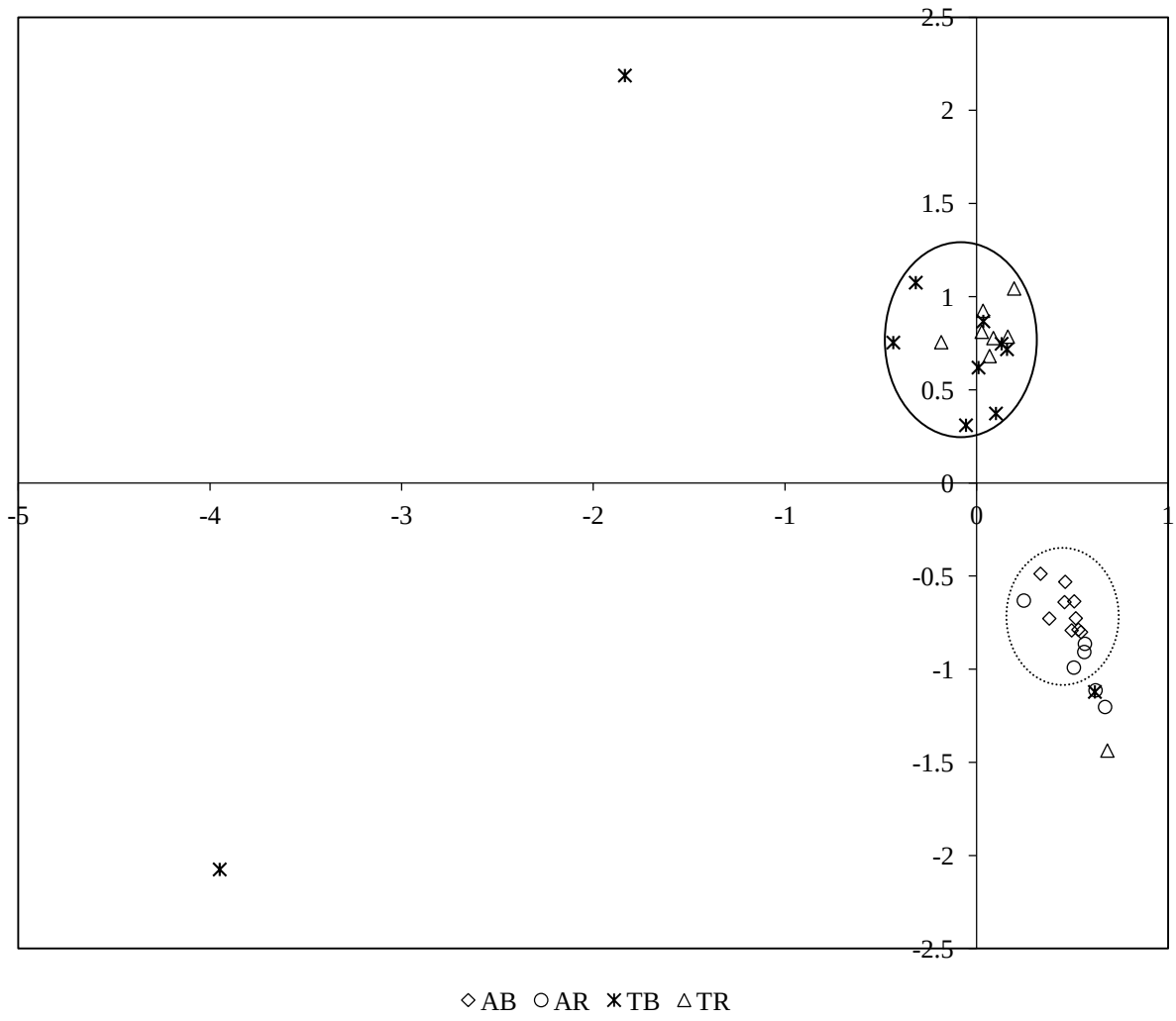

Figure 3. Correspondence Analysis for fungal endophyte assemblages of red and brown algae between Andaman Islands and Tamil Nadu. AB - Andaman brown algae, AR - Andaman red algae, TB - Tamil Nadu brown algae, TR - Tamil Nadu red algae.