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Phylogenetic diversity of cultivable bacteria associated with filamentous non-heterocystous marine cyanobacteria

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Abstract

Marine Cyanobacteria are known to be colonized by various heterotrophic bacteria. With a view to understand the diversity of associated bacteria, 12 different marine cyanobacterial species of *Oscillatoria*, *Lyngbya* and *Phormidium* were selected. Conventional and molecular techniques were employed to document the associated bacterial diversity. A total of 46 bacterial strains were isolated and their biochemical and cultural characteristics were studied. Based on these results, the bacterial isolates were grouped into 15 types and a representative from each group was subjected to RAPD fingerprinting using PCR to understand their phylogeny. The PCR amplification resulted in distinct banding profiles for all 15 representative strains. According to their banding profiles, they were clustered into two major clades, where the clade I being the smaller comprising only 2 strains (NTEB 27 and NTEB30) and the rest were grouped into clade II which clearly revealed high degree of variability of bacterial diversity associated with marine cyanobacteria.

Key words : Associative bacteria, Marine cyanobacteria, RAPD-PCR, Phylogeny

Introduction

Cyanobacteria are oxygen evolving photosynthetic prokaryotes that are known to grow in extreme environments (Thajuddin and Subramanian, 2005). In spite of being phototrophic metabolism, they are often

found to be associated with other group of organisms. Especially the marine cyanobacteria are often associated with bacterial groups (Fisher, *et.al* 1998; Hube, *et al.* 2009). The bacteria are found to be

associated with the extra cellular mucus zone of the phototrophic cells and attached more tightly with their cell surface (Bruno, *et al.* 2006; Salomen, *et al.* 2003; Tison, *et al.* 1980). In general, the epiphytes are heterotrophic (Hempel, *et al.*, 2008; Hube, *et al.* 2009; Meusnier, *et al.* 2001,) and in case of non-heterocystous marine cyanobacteria they are found to interact positively and improve their growth rate (Herbst and Overbeck 1978). They derive energy in form of sugars and peptides (Paerl, 1984) and in turn provide their host with remineralized nutrients (Hempel *et al.* 2008), antifouling compounds (Armstorng, *et al.* 2001; Roa, *et al.* 2006; Spragg, *et al.* 1998), supports the increased production of vitamin B12 by supplementing the hosts with cobalamin (Croft, *et al.* 2005) and improves the iron utilization (Liebson, *et al.* 1995). Thus the role of these epiphytes leads in maintaining the health of their host has received the attention of the researchers. Although the interaction between the marine cyanobacteria and bacteria has been documented in earlier studies, the ecological significance of such association remains unexplained (Bruno, *et al.* 2006; Kolmonen, *et al.* 2004; Margulis, 1981). Moreover, a clear picture on the actual

diversity of these epiphytes still remains to be recorded.

In order to understand the diversity of associated microbes, both conventional and molecular techniques were applied to 12 different filamentous non heterocystous marine cyanobacteria belonging to the genus *Oscillatoria*, *Lyngbya*, and *Phormidium*. The conventional culturing methods were used for preliminary characterization and their phylogenetic diversity was studied through genome PCR fingerprinting. RAPD-PCR (Randomly Amplified Polymorphic DNA - Polymerase Chain Reaction) technique is one of the best known fingerprinting techniques that are widely employed in molecular ecology especially to determine taxonomic identity (Hadrys, *et al.* 1992), was employed to study the phylogenetic diversity among the epiphytic bacterial isolates (Sakallah, *et al.* 1995).

Materials and Methods

Selection of cyanobacterial strains

Twelve marine cyanobacterial strains belonging to the genera *Oscillatoria*, *Lyngbya* and *Phormidium* were obtained from the germplasm of National Facility of Marine Cyanobacteria (Sponsored DBT, Govt. of India), Bharathidasan University,

Tiruchirappalli, Tamil Nadu, India for the present study.

Isolation and preliminary characterization of associated bacteria

The cyanobacterial cultures were rinsed with sterile distilled water for two to three times, crushed and were spread on marine nutrient agar plates containing Peptone – 0.1%, Yeast extract – 0.1%, agar – 1.5% and filtered sea water – 25% (Anderson and Heffernan 1965) and incubated in dark for 2-4 days. The distilled water used for final rinsing was also spread on agar plate and maintained as a control. Then the colonies developed were scrapped and plated repeatedly on marine nutrient agar plates to isolate the bacterial strains in pure form. Then the preliminary characterization of the isolates was carried out by carefully recording cultural characteristics. They were also tested for their Gram staining behavior and preserved in stocks containing 17.4% glycerol.

DNA extraction from the associated bacteria

Extractions of the genomic DNA from 15 representative associated bacterial isolates were carried out following the protocol of Smoker and Barnum(1998).

According to the method 1ml of overnight grown cultures were centrifuged at 10000 rpm for 5 minutes and the pellet was washed and suspended in 500µl of STE buffer (50mM Tris, 5mM EDTA and 50mM NaCl) and vortexed. To this, 20µl of lysozyme (10mg/ml) was added and incubated in water bath at 55°C for 30 minutes. After incubation added 10µl of proteinase K (10mg/ml) and 20µl of 10% SDS and again incubated in water bath at 55°C for 30 minutes. Following this, the mixture was cooled in ice and extracted with equal volume of phenol:chloroform:Isoamyl alcohol mixture (25:24:1) and centrifuged at 12,000 rpm for 10 minutes. The supernatant was separated with care and equal volume of 4M ammonium acetate and two volumes of Isopropanol were added. This was again centrifuged at 14,000 rpm for 15 minutes. Supernatant was decanted and the pellet was washed with 70% ethanol and dried. Finally the dried pellet of genomic DNA was dissolved in 100µl of TE buffer and stored at -20°C until next use.

RAPD-PCR fingerprinting:

RAPD-PCR fingerprinting analysis was done by using RAPD P7 primer 5' CCAAGCTGCC 3' (2) using a Unieqiup Thermal Cycler (Germany) following the

instruction of the manufacturers of PCR Kit (Finzymes, Finland). A 50µl reaction was carried out with 5µl of 10x reaction buffer, 1µl of dNTPs, 1µl of RAPD primer, 1 unit (0.5µl) of Taq polymerase and 1µl (50ng) of DNA template in 41.5µl of sterile double distilled water. The PCR reaction was started as 300 seconds of initial denaturation at 94°C followed by the amplification with 30 cycles of cyclic denaturation for 45 seconds at 94°C, 60 seconds at 47°C for primer annealing and 90 seconds at 72°C for primer extension, followed by a final extension of 7 minutes at 72°C and hold at 4°C.

The amplified product was then electrophoresed at 70V on 1.2% agarose gel, with ethidium bromide (0.5µg/ml), prepared with 1x TAE buffer (pH 8.0). The banding profiles were documented under UVP GelDoc-it TS imaging system.

Phylogeny construction:

Phylogeny construction was performed using Vision Works Image Analysis Software (UVP imaging systems). Analysis was made using the inbuilt software depending on the dataset obtained.

Results and Discussions

Cyanobacterial cultures

A total of 12 filamentous marine cyanobacteria namely *Oscillatoria amphibia* BDU 110121, *O. subtilissima* BDU 92183, *O. pseudogeminata* BDU 142171, *O. cortiana* BDU 142551, *Phormidium valderianum* BDU 141851, *P. lucidum* BDU 10141, *P. tenue* BDU 92361, *P. foreolarvum* BDU 916681, *Phormidium* sp., BDU 141601, *Lyngbya confervoides* BDU 140301, *Lyngbya* sp., BDU 91711, *Lyngbya* sp., BDU 141561 were taken for the study. These cultures were maintained in germplasm for almost 15 years. In general the cyanobacterial species under the order *Oscillatoriales* were selected based on the previous reports which suggests that the filamentous cyanobacteria may harbour large number of associated bacteria (Fisher *et al.*, 1998) and in particular the species of *Oscillatoria*, *Lyngbya*, and *Phormidium* were selected based on their sheath properties, where the *Lyngbya* sp., were known to possess very massive thick and firm sheath, *Phormidium* sp., produces sheath that may be more or less firm in nature while in *Oscillatoria* sp., sheath are generally absent and rarely if present that too are very delicate (Desikachary, 1959).

The sheath property was considered as important criteria because the bacteria were usually known to be associated with the mucilaginous sheath (Fisher *et al.*, 1996).

Isolation and preliminary characterization of associated bacteria

The optimal growth was obtained at 28-30°C in the washed marine cyanobacterial cultures spread onto marine nutrient agar plates. The number of bacterial colonies per plate varied from 25 to more than 100. The control plates showed no bacterial growth which was actually inoculated with the water used for final rinsing of the cyanobacterial filaments. As many as 46 bacterial isolates were obtained upon repeated sub culturing, which includes both commonly occurring bacteria as well as the undescribed ones (Salomen *et al.* 2003).

The number of associated bacterial isolates varied from 1 to 6 from each cyanobacterial sample (Table 1) in which *Lyngbya* sp., BDU 141561 was found to harbor maximum number of cultivable bacteria (6 isolates). The cultural and microscopical characteristics of these isolates were also showing their diversity. The variable number of bacterial colonies per plate suggests that not all cyanobacterial cultures had same numbers of bacterial association.

This may be depended on various factors such as the readily available nutrients from the host, competence towards the association etc. Apart from these factors, the degree of variability in the association was also known to be influenced by the age of the cyanobacterial cultures (Salomen *et al.* 2003). Of the total of 46 bacterial isolates 12 were found to be pigmented and the remaining 34 were either white or colourless. The bacteria isolated from *Lyngbya* sp. BDU 141561 (NTEB26, NTEB41, NTEB42) showed yellow brown pigmentation, while 3 isolates (NTEB06, NTEB12, NTEB36) were lemon yellow pigmented and was isolated from *O. pseudogeminata* BDU 142117, whereas about 6 (NTEB05, NTEB09, NTEB13, NTEB18, NTEB32, and NTEB46) isolates were found to form pink coloured colonies were isolated from different sources i.e., *Phormidium lucidum* BDU 10141 and *Phormidium* sp. BDU 141601. Previous studies were also suggestive that the pigmentation of bacteria could be considered as a significant character in diversifying the associated bacteria (Hube *et al.* 2009). Apart from pigmentation, all their colonial parameters were recorded and the isolates were found to differ significantly in their colonial morphology. Most of them

formed smooth mucoid colonies showing their nature to produce exopolysaccharides. The colonial morphology of the isolates were illustrated in Fig. 1. Especially colonies of NTEB29 & NTEB19 strains originally isolated from *O. subtilissima* BDU 921183 and *Phormidium* sp. BDU 141601 respectively were highly mucous and non sticky while some strains (NTEB 30 & 35) showed very rough and irregular colonies (Fig. 1).

Microscopically, the isolates were cocci (single and clusters), bacilli (single and chains) and coccobacilli in shape among which bacilli type cells were found to dominate the population (68%). The Gram staining of these epiphytes revealed that about 82% of them were Gram positive and 12 were found to be Gram negative and that too are bacilli. This result was completely a reversal when compared to (Hube *et al.* 2009) where no Gram positive bacteria were reported. In general, two different cultures showing same cultural morphology need not to be the same always (Pinhassi and Berman 2002). For instance, even though the associated bacterial isolates NTEB20 and NTEB27 were forming smooth, white irregular colonies, through Gram staining they were differentiated significantly, while the former being Gram negative bacilli the

latter was found to be Gram positive bacilli. These results showed that the colonial characteristics alone can not serve a tool to discriminate the bacterial isolates. With this in mind considering both cultural and microscopic morphology, the isolates were carefully examined and grouped in to 15 types and selected for the further analysis. The results of the preliminary characterization of these selected isolates were summarized in Table 2. The classification was done with primary weightage to their colonial characteristics followed by their microscopic morphology and Gram's reaction (Worm and Sondergaard 1998).

RAPD-PCR fingerprinting and phylogeny

In order to evaluate the diversity among the associated bacterial isolates, RAPD – PCR fingerprinting was carried out using genomic DNA (50ng) of 15 representative isolates. With this, distinct banding profiles were obtained and revealed a high degree of variability among the epiphytic isolates (Fig. 2). The number of bands ranged from 2 to 11 each where NTEB06 (lane 2) and NTEB21 (lane 9) with maximum number of bands while NTEB10 (lane 3) and NTEB27 (lane 13) was recorded with minimum bands. On a whole

except for a 1000 bp band, which was commonly found in most of the strains, there were relatively fewer bands in common. In the present study, the RAPD-P7 primer is found suitable for differentiating the associated bacterial populations in the cyanobacterial cultures. Based on the RAPD-PCR fingerprints associated bacterial isolates, a dendrogram was constructed to understand their phylogeny. The phylogenetic tree of the associated bacterial isolates has clearly revealed their genetic relatedness among the isolates (Fig. 3). According to their banding profiles, they were clustered into two major clades (I and II), where the clade I being the smaller included only two strains (NTEB 27 and NTEB30) which was found to have 80% genetic similarity and while comparing their microscopic morphology both were Gram positive bacilli. This reveals the possibility of these two strains to be in same genus. While considering the major clade it was broadly clustered into two groups the first group was dominated by cocci cells and the later was grouped with rod shaped cells,. Thus the molecular phylogeny was found to be clear and comparable with that of the morphological data (Webster, *et al.*2001; Jasti *et al.* 2005; Andreote *et al.* 2009).

In the present study, the culturable associated bacterial communities from 12 filamentous non-heterocystous marine cyanobacteria were isolated in using the marine nutrient agar medium has clearly revealed the range of diversity and phylogenetic relatedness of the associated bacteria. Still for a better understanding of the intimate association between the bacteria and cyanobacteria, further studies are required for the formulation and optimization of new media and conditions, a combination of different biochemical, physiological techniques and culture independent metagenomic approaches have to be employed.

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References

Anderson, J.I.W. & W.P., Heffernan 1965
Isolation and Characterization of Filterable

Marine Bacteria, *Journal of Bacteriology*,
90(6): 1713 – 1718

Andreote, F.D., V.C.Pietrobon, F.D.

Andreote, A.S. Romao, M.B. Sposito &
W.L. Araujo 2009 Genetic variability of
Brazilian isolates of *Alternaria alternata*
detected by AFLP and RAPD techniques,
Brazilian Journal of Microbiology, 40: 670
– 677

Armstorng, E., L.Yan, K.G. Boyd,
P.C.Wright & J.G. Burges 2001 The
symbiotic role of marine microbes on living
surfaces, *Hydrobiologia*, 461:37-40

Bruno, L., D.Billi, & P. Albertano 2006
Genetic characterization of epilithic
cyanobacteria and their associated bacteria,
Geomicrobiology Journal, 23: 293-299

Croft, M.T., A.D.Lawrence, E.R. Deery,
M.J. Warren & A.G.Smith 2005 Algae
acquire vitamin B₁₂ through a symbiotic
relationship with bacteria, *Nature*, 483:90-
93

Desikachary, T.V. 1959 Cyanophyta, *Indian
Council Agricultural Research, New Delhi*,
PP. 686.

Fisher, M.M., & E. Margoliash 1996
Desmid-Bacterial association in *Sphagman-*

Bacteria associated with filamentous marine cyanobacteria

dominated Wiscosin peatlands, *Journal of
Phycology*, 32: 543-549

Fisher, M.M., L.W. Wilcox & L.E. Graham
1998 Molecular characterization of
epiphytic bacterial communities on
Charophycean green algae, *Applied and
Environmental Microbiology*, 64(11): 4384-
4389

Hadrys, H., M. Balick & B. Schierwater
1992 Applications of random amplified
polymorphic DNA (RAPD) in molecular
ecology, *Molecular Ecology*, 1:55-63

Hempel, M., M. Blume, I. Blindow &
E.M.Gross 2008 Epiphytic bacterial
community composition on two common
submerged macrophytes in brackish water
and freshwater, *BMC Microbiology*, 8(58):1-
10

Herbst, V. & J. Overbeck 1978 Metabolic
coupling between the alga *Oscillatoria
redekei* and accompanying bacteria,
Naturwissenschaften, 65: 598 – 599

Hube, A.E., B.H. Soller & U. Fischer 2009
Phylogenetic classification of heterotrophic
bacteria associated with filamentous marine
cyanobacteria inculture, *Systematic and
Applied Microbiology*, 32: 256-265

Jasti, S., M.E. Sieracki, N.J. Poulton, M.W. Giewat & J.N.R. Varga 2005 Phylogenetic diversity and specificity of bacteria closely associated with *Alexandrium* spp. and other phytoplankton, *Applied and Environmental Microbiology*, 71(2): 3483-3494

Kolmonen, E., K. Sivonen, J. Rapala & K. Haukka 2004 Diversity of cyanobacteria and heterotrophic bacteria in cyanobacterial blooms in Lake Joutikas, Finland, *Aquatic Microbial Ecology*, 36: 201 -211

Liebson, E.K., Y. Hadar & Y. Chen 1995 Oligotrophic bacteria enhance algal growth under iron-deficient conditions, *Applied and Environmental Microbiology*, 61(6):2439-2441

Margulis, I. 1981 Symbiosis in cell evolution, *W.H. Freeman and Co., San Francisco, Calif* : 164

Meusnier, I., J.L. Olsen, W.T. Stam, C. Destombe & M. Valero 2001 Phylogenetic analyses of *Caulerpa taxifolia* (Chlorophyta) and of its associated bacterial microflora provide clues to the origin of the Mediterranean introduction, *Molecular Ecology*, 10:931-946

Paerl, H.W. 1984 Transfer of N₂ and CO₂ fixation products from *Anabaena*

oscillarioides to associated bacteria during inorganic carbon sufficiency and deficiency, *Journal of Phycology*, 20:600–608

Pinhassi, J., & T. Berman 2002 Differential Growth Response of Colony-Forming α and γ Proteobacteria in Dilution Culture and Nutrient Addition Experiments from Lake Kinneret (Israel), the Eastern Mediterranean Sea, and the Gulf of Eilat, *Applied and Environmental Microbiology*, 69(1): 199–211.

Roa, D., J.S. Webb & S. Kjelleberg 2006 Microbial colonization and competition on the marine alga *Ulva australis*. *Applied and Environmental Microbiology*, 72(8): 5547 – 5555

Sakallah, S.A., R.W. Lanning & D.L. Cooper 1995 DNA fingerprinting of crude bacterial lysates using degenerate RAPD primers, *Genome Research*, 4:265-268

Salomen, P.S., S. Janoson & E. Graneli 2003 Molecular identification of bacteria associated with filaments of *Nodularia spumigena* and their effect on the cyanobacterial growth, *Harmful Algae*, 2:261-272

Smoker, J.A. & S.R. Barnum 1988 Rapid small-scale DNA isolation from filamentous

cyanobacteria, *FEMS microbiology letters*,
56(1): 119 – 122

Spragg, A.M., M. Bergu, K.G. Boyd & J.G. Burgess 1998 Cross-species induction and enhancement of antimicrobial activity produced by epibiotic bacteria from marine algae and invertebrates, after exposure to terrestrial bacteria, *Letters in Applied Microbiology*, 27: 142-146

Thajuddin, N. & G. Subramanian 2005 Cyanobacterial biodiversity and potential applications in biotechnology, *Current Science*, 89(1): 47 – 57

Tison, D.L., D.H. Pope, W.B. Cheryy & C.B. Fliermans 1980 Growth of *Legionella pneumophila* in association with blue-green algae (Cyanobacteria), *Applied and*

Environmental Microbiology, 39(2): 456-459

Webster, N.S., K.J. Wilson, L.I. Blackall & R.T. Hill 2001 Phylogenetic diversity of bacteria associated with the marine sponge *Rhopaloeides odorabile*, *Applied and Environmental Microbiology*, 67(1):434-444

Worm, J. & M. Sondergaard 1998 Dynamics of heterotrophic bacteria attached to *Microcystis* spp. (Cyanobacteria), *Aquatic Microbiology and Ecology*, 14: 19 – 28

Table 1: The list of cyanobacterial cultures and their associated bacterial isolates

S. No.	Cyanobacterial strains	Bacterial isolates
1	<i>Lyngbya confervoides</i> BDU 140301	NTEB07
2	<i>Lyngbya sp.</i> , BDU 141561	NTEB26, 27, 41, 42, 14, 15
3	<i>Lyngbya sp.</i> , BDU 91711	NTEB01, 03, 11, 23
4	<i>Oscillatoria amphibia</i> BDU 110121	NTEB02, 08, 16
5	<i>O. cortiana</i> BDU 142551	NTEB31, 33, 38, 43, 45
6	<i>O. Pseudogeminata</i> BDU 1421171	NTEB06, 30, 12, 36, 35
7	<i>O. subtilissima</i> BDU 921183	NTEB04, 29, 25
8	<i>Phormidium lucidum</i> BDU 10141	NTEB13, 05, 09, 18
9	<i>P. tenue</i> BDU 92361	NTEB17, 24, 28, 44
10	<i>P. foreolarvum</i> BDU 91681	NTEB34, 37, 39, 40
11	<i>P. valderianum</i> BDU 141851	NTEB10, 22
12	<i>Phormidium sp.</i> , BDU 141601	NTEB46, 19, 20, 21, 32

Table 2: Cultural and morphological characteristics of the associated bacteria isolated from non-heterocystous filamentous marine cyanobacteria

Cyanobacterial cultures	Bacterial Groups	Representative Bacterial isolates	Cultural characteristics	Gram reaction and microscopic morphology
<i>Lyngbya confervoides</i> BDU 140301	1	NTEB22	Pure white, round, small opaque smooth colonies	Gram negative bacilli
<i>Lyngbya sp.</i> , BDU 141561	2	NTEB26	Pin pointed yellow brown smooth colonies	Gram positive cocci in cluster
	3	NTEB27	Small, white irregular smooth colonies	Gram positive bacilli
<i>Oscillatoria amphibia</i> BDU 110121	4	NTEB02	Transparent dirty white colonies	Gram positive cocci in cluster
<i>O. cortiana</i> BDU 142551	5	NTEB31	White dirty translucent colonies	Gram positive cocci
<i>O. Pseudogeminata</i> BDU 1421171	6	NTEB06	Pin pointed lemon yellow colonies	Gram positive cocci in cluster
	7	NTEB30	Large white rough irregular colonies	Gram positive bacilli
<i>O. subtilissima</i> BDU 921183	8	NTEB04	Pin pointed colonies	Gram negative cocco bacilli
	9	NTEB29	White mucoid raised irregular translucent colonies	Gram positive bacilli
<i>Phormidium lucidum</i>	10	NTEB13	Translucent pink colour	Gram positive cocci

BDU 10141			colonies	in cluster
<i>P. valderianum</i> BDU 141851	11	NTEB10	Pin pointed dirty white colonies	Gram positive bacilli
<i>Phormidium sp.</i> , BDU 141601	12	NTEB46	Pin pointed pink colour colonies	Gram positive bacilli
	13	NTEB19	Smooth round white muciod colonies	Gram positive bacilli
	14	NTEB20	White irregular colonies	Gram negative bacilli
	15	NTEB21	White opaque smoth colonies	Gram positive streptobacilli

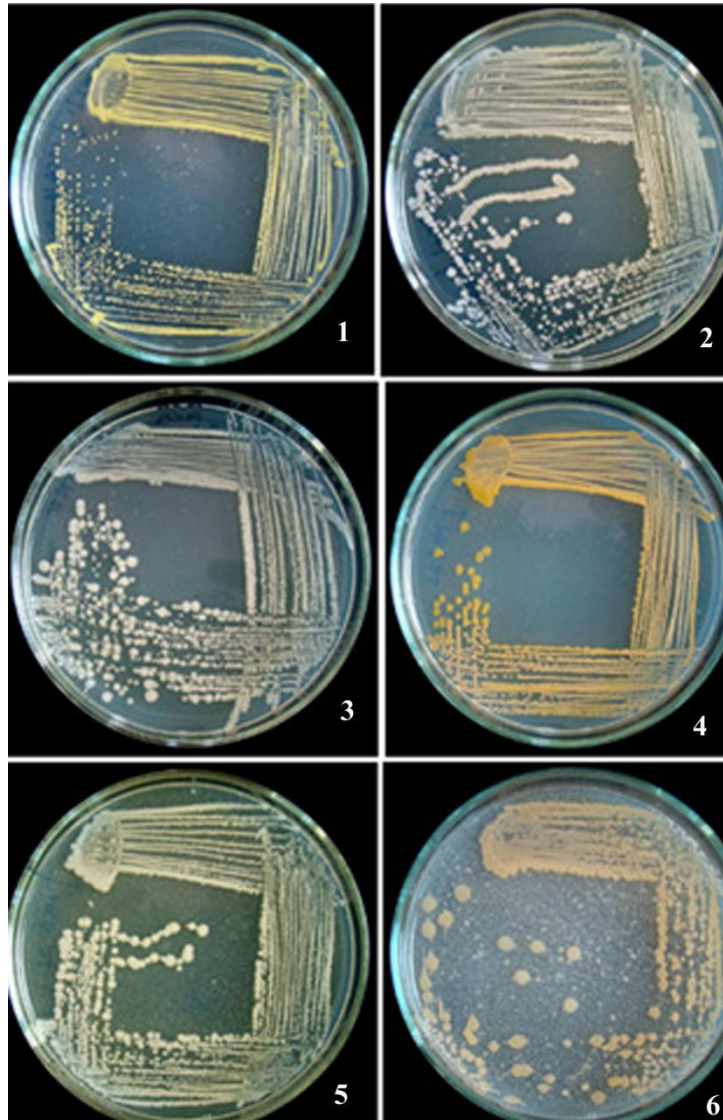


Fig 1: Cultural Morphology of the Epiphytic bacterial isolates of non-heterocystous filamentous marine cyanobacteria. 1 – NTEB06, 2 – NTEB19, 3 – NTEB21, 4 – NTEB26, 5 – NTEB29, 6 – NTEB41

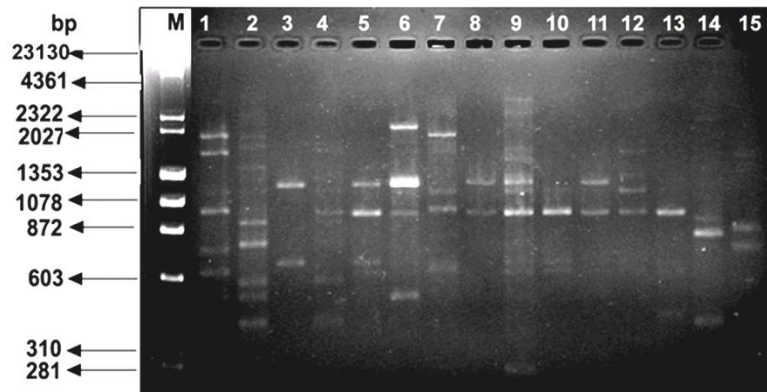


Fig 2: RAPD – PCR fingerprints of Associated bacteria isolated from non-heterocystous filamentous marine cyanobacteria. M – marker lane, lane 1 – NTEB26, lane 2 – NTEB06, lane 3 – NTEB10, lane 4 – NTEB02, lane 5 – NTEB13, lane 6 – NTEB46, lane 7 – NTEB19, lane 8 – NTEB20, lane 9 – NTEB21, lane 10 – NTEB22, lane 11 – NTEB04, lane 12 – NTEB29, lane 13 – NTEB27, lane 14 – NTEB30, lane 15 – NTEB31.

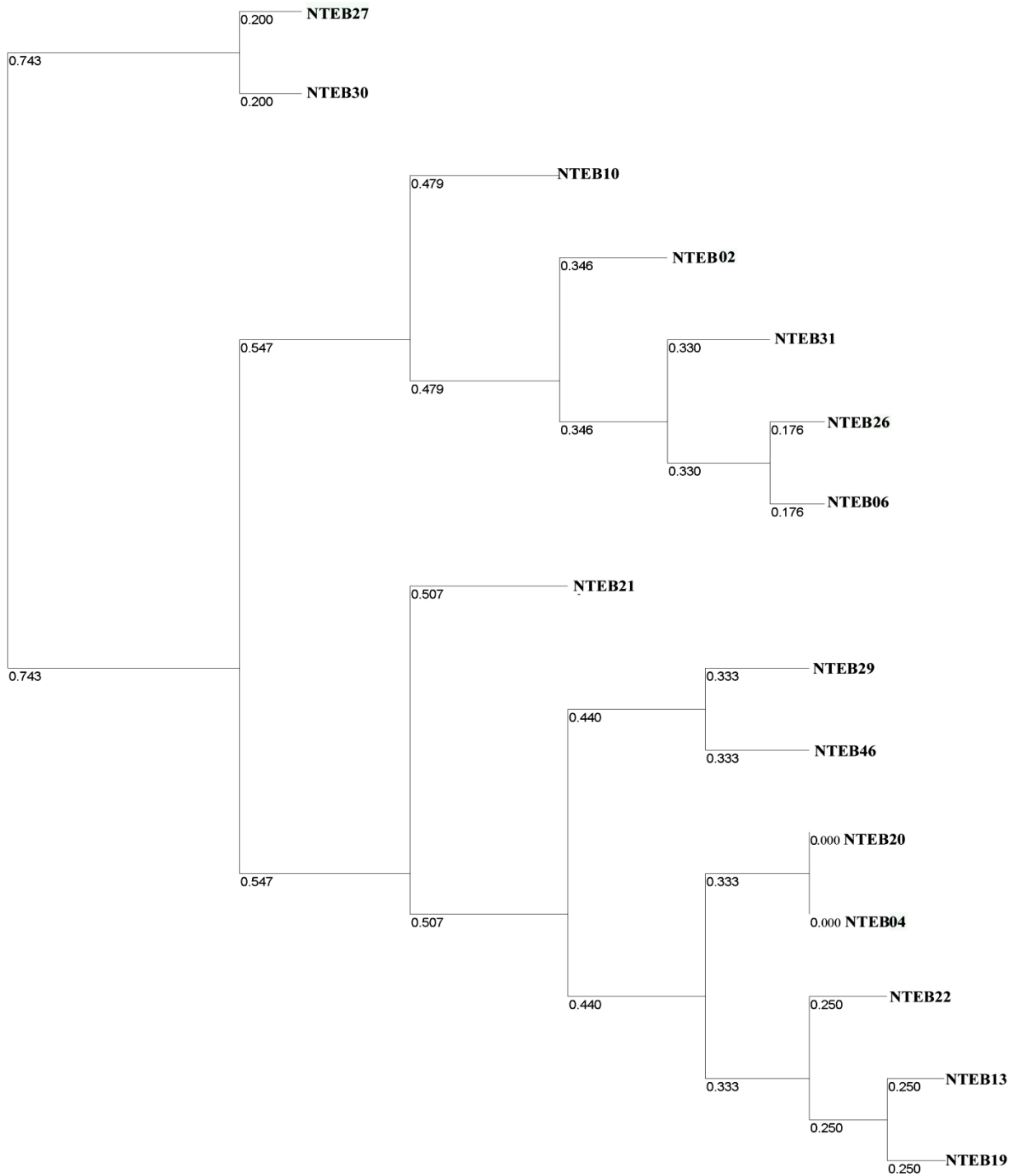


Fig 3: Phylogenetic tree of associated bacterial isolates on the basis of their RAPD fingerprints.