Curr Microbiol (2011) 62:7–15
DOI 10.1007/s00284-010-9663-9

Analysis of Bacterial Community Composition of a Spring Water from the Western Ghats, India Using Culture Dependent and Molecular Approaches

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Received: 2 September 2009 / Accepted: 27 April 2010 / Published online: 12 May 2010

Abstract Cultivation based and culture independent molecular approaches were used to characterize the composition and structure of bacterial community from a natural warm spring in the Western Ghats, a biodiversity 'hotspot'. Dilution plating was done on three types of media with varying nutrient levels. Relatively nutritionally poor medium supported growth of highest number of bacteria (4.98×10^3 ml^{-1}) compared to nutritionally rich media. On the basis of different morphological features on the plate, 62 aerobic and heterotrophic bacterial strains were isolated and their 16S rRNA genes were sequenced and analyzed. On the basis of sequence similarity these isolates were found to be distributed in 21 different genera belonging to *Proteobacteria* (58%) followed by *Firmicutes* (26%), *Actinobacteria* (13%) and *Bacteroidetes* (3%). Amplification of 16S rRNA gene of the community DNA using eubacterial primers, followed by cloning and sequencing revealed that predominant members of the habitat belong to the phylum *Cyanobacteria* (60%) followed by *Proteobacteria* (19.5%), *Bacteroidetes* (6.67%), *Actinobacteria* (4.4%) and *Firmicutes* (2.2%) and small ribosomal subunit of a plastid (of *Chlorophyta*, 2.2%).

Electronic supplementary material The online version of this article (doi:10.1007/s00284-010-9663-9) contains supplementary material, which is available to authorized users.

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Introduction

India has two of the "mega diversity hotspots" of the world. The Western Ghats is one among them. The Western Ghats is a forested tract of relatively smooth and very old mountain range starting from Central Maharashtra to the Sourthern tip of Kerala in India. There is high level of endemicity in a number of taxa with nearly 2000 species of higher plants, 87 species of amphibians, 89 species of reptiles, 15 species of birds and 12 species of mammals [4]. Diversity of fauna and flora of the Western Ghats has been reasonably well documented (http://wgbis.ces.iisc.ernet.in/biodiversity/). A few studies on fungal diversity of this region have also been reported [12, 16, 22]. But there is no idea about the diversity of bacteria in this region. We, therefore, initiated some work to find out the bacterial diversity in certain part of the Western Ghats, with the hope of understanding the complex interplay of microbes, plants and animals in this unique ecological niche. In any ecosystem, bacteria play a crucial role in the breakdown of organic matter and demineralization of nutrients. Evidences show that the bacterial community structure depends on dominant substrate source present in the environment [15]. The diversity of microorganisms and the role they play 'in situ' remain largely unknown as normally only a small proportion of the bacteria present in these environments are readily cultivable [1, 24] and could be studied in detail. Thus a combination of both molecular and cultivation based technique is expected to provide better insights into the components of microbial communities. To our knowledge, this is the first attempt to explore the composition of the bacterial community of a selective niche of the Western Ghats. In this paper we describe the bacterial diversity of water of one warm spring located in a village

in Puttur near Mangalore, India by using both culture dependent and culture independent approaches.

Materials and Methods

Site Description and Sampling

The present day Western Ghats forms a continuous chain of small to medium sized mountain ranges running along the Western coast of Southern India from central Maharashtra to Sourthern tip of Kerala between 8 and 210°N latitude. The mid Western Ghats region encompasses the states of Goa and Karnataka. A total of 22 soil and water samples were collected from four reserve forests of mid Western Ghats in and around Goa and two warm springs around Mangalore. Part of the soil and water samples were plated within 4–24 h after collection and the rest were stored at 4–6°C as well as preserved in 10% glycerol at −20°C. In case of water samples along with plating, water was first filtered through sterile Whatman filter paper no. 1 (pore size 11 μm) and then the filtrate was again filtered through 0.22 μm membrane filter (47 mm). Membrane filters were stored at 4°C and later used for DNA extraction.

The warm spring from which water sample collected is located next to a temple in a village, Irde in Puttur Taluka (12.77°N and 75.22°E) Mangalore district, Karnataka state, India and the water sample was collected (in two sterile plastic containers each of 10 l capacity) in the month of September, 2005.

Physicochemical Analysis

The temperature and pH of the sample were recorded on site and the total organic carbon, ammonia, phosphate and silica were analyzed at National Institute of Oceanography, Goa. The warm spring water, appeared little muddy and slightly greenish in colour presumably due to the presence of planktonic *Cyanobacteria* (see later). Temperature at the time of collection was 36°C and pH ~7.5. Chemical analysis of the water sample revealed the presence of phosphate (5.6 μM), ammonia (13.1 μM) and SiO_3 (381.8 μM). The total organic carbon was found to be 556.95 mg l^{-1}.

Isolation, Enumeration and Characterization of Bacteria

The serial dilution and plating of the sample were done within 24 h of collection. The water sample was plated on three different types of media of varying nutrient conditions, tryptic soy broth agar (TSBA), plate count agar (PCA) and 100 times diluted TSB and solidified with 1.5% agar (TD) (nutritionally rich, moderate and poor, respectively). The plates were incubated at 37°C for up to 96 h. Colony counts were made every 24 h and colonies of different morphotypes were picked, purified and preserved in 10% glycerol initially at −20°C and subsequently at −70°C. Genomic DNA from 62 isolates was extracted according to Pitcher et al. [21]. PCR amplification of 16S rRNA gene was done using universal eubacterial primers (27F and 1492R) as reported earlier [19].

Amplified DNA was sequenced using Big Dye terminator cycle sequencing ready reaction kit (Applied Biosystems). Sequencing PCR was done using primers, 357fp-(5′-CTCCTACGGGAGGCAGCAG-3′), 518rp-(5′-GTGCCAGCAGCCGCGG-3′), 907rp-(5′-CCGTCAATTCMTTTRAGTTT-3′), 1100rp-(5′-GGGTTGCGCTCGTTG-3′), 1492rp-(5′-TACGGYTACCTTGTTACGACTT-3′). PCR cycling parameters included initial denaturation at 96°C for 2 min, followed by 25 cycles of denaturation at 96°C for 10 s, annealing at 50°C for 5 s and extension at 60°C for 4 min. The amplified products were purified and sequencing was carried out using ABI 3130 Genetic Analyzer (Applied Biosystem).

Extraction of Community DNA, Amplification of 16S rRNA Gene, Construction of Library and Sequence Analysis

Water was first filtered through sterile Whatman filter paper no. 1 (pore size 11 μm) and then the filtrate was again filtered through 0.22 μm membrane filter (47 mm). The membrane filters were cut into small pieces and DNA was extracted by using Fast spin DNA kit (Qbiogene, USA) according to the manufacturer's instruction.

The community DNA was used for PCR amplification of 16S rRNA gene using universal bacterial primers (27F and 1492R) as described above except that annealing temperature was increased from 50 to 55°C and addition of 1.0 μg of BSA (Sigma, USA) in the PCR reaction. PCR amplified 16S rRNA genes from the environmental DNA sample were cloned directly into *E. coli* DH5α using plasmid vector pGEM®-T Easy (Promega). A total of 200 recombinant clones were randomly picked and preserved at −70°C. Plasmid DNA was extracted from randomly selected clones using Qiaprep spin Mini Prep Kit (Qiagen). The extracted plasmids were directly used for sequencing PCR using both vector specific T7 promoter primer as well as 16S rRNA gene specific primers. The sequencing of the amplified community DNA was done as described above for the culturable isolates. Sequences were screened for the presence of chimaeric artifacts by using CHIMERA_CHECK program of Ribosomal Database Project (RDP version 8.1) and also by Mallard and Pintail

programs [3]. Vector contamination was checked by VecScreen program of NCBI.

A total of 45 clones from a single eubacterial library and 62 cultivable isolates were analyzed. The 16S rRNA gene sequences of the isolates and the clones were compared with the sequences available in GenBank nucleotide database using BLAST. Sequences were aligned using BioEdit sequence alignment editor [8] and identity matrix was created using sequence identity matrix feature of the software. Sequences with identity greater than or equal to 97% were grouped into OTUs. The clone sequences along with reference sequences from known taxa were used to construct maximum likelihood phylogenetic trees after aligning the sequences with Clustal X program [30]. All phylogenetic analyses were performed in PAUP v4.0b10 [26]. Likelihood model parameters were estimated with MrModeltest v2.2 [17]. A general-time-reversible likelihood model [28] was selected. Bayesian probabilities for the nodes were calculated with the MrBayes v3.1 [10]. Trees were figured in Treeview [18].

The extent of diversity covered by both culture dependent and independent approaches has been analyzed using the program RarefactWin [9].

Nucleotide Sequence Accession Numbers

The 16S rRNA gene sequences of isolates and clones obtained from this study have been deposited in Gen Bank and EMBL under the accession numbers, EF633683–EF 633687; EU000448–EF000455; EF208921; EF208922; EF 187228; AM992174–AM992203; FM164624–FM164637; FJ765236; FJ765237 for isolates and EU091538, EU09 1540–EU091553, EU091555–EU091562, EU000439–EU000447; FM165295–FM165308 and FJ765238 for clones.

Results and Discussion

Diversity of Culturable Bacteria

It is well known that the type of bacteria that are recovered by plating is influenced by the type of media and conditions of growth used in the study of bacterial diversity from natural environments [27, 31]. While it may not be practical to use all possible media compositions and growth conditions for the study of bacterial diversity, culturing in more than one media will support more diverse type of bacteria than the use of a single medium. One may also choose to use enrichment media for the isolation of specific group of bacteria. In this study, diversity of culturable bacteria was investigated using three different types of media of varying nutrient conditions, rich (TSBA), moderate (PCA) and poor (TD) and the number of colonies (CFU) per millilitre in each of these media were as follows: TD (4.98×10^3 ml^{-1}); PCA (1.82×10^3 ml^{-1}) and TSBA (7.8×10^2 ml^{-1}). Among these three types of media it appears that the nutritionally poor media (TD) supported the growth of highest number of bacteria even though the number of morphologically distinct colonies was less. A total of 62 strains from all the three types of media were picked and preserved. Their 16S rRNA gene sequences were determined and analyzed.

Diversity of culturable bacteria as determined by the 16S rRNA gene sequence analysis revealed the presence of members of four phyla, the predominant being *Proteobacteria* (58%) followed by *Firmicutes* (26%), *Actinobacteria* (13%) and *Bacteroidetes* (CFB group) (3%). It appears that these four phyla dominate the freshwater ecosystem since similar observations were reported from fresh water sediment of Lake Kasumigaura [27] and from fresh water of Lake Taihu in China [34]. In a similar study of a warm spring water from Borpung reserve forest in Assam (North East India), from our laboratory [23] it was found that the predominance of members belonging to phyla *Proteobacteria* (49.1%) and *Firmicutes* (45.5%) with minor representations of *Bacteroidetes* (CFB group) and *Deinococcus-Thermus* existed. Out of the 62 isolates studied 24 were Gram-positive and the rest were Gram-negative organisms. Among the low G + C *Firmicutes*, the genus *Bacillus* was present in majority (12 isolates) and was distributed in eight species (sequence similarity 98–100%; Table 1), all of them recovered from PCA and TSBA plates and none from nutritionally poor TD medium. Two genera, *Staphylococcus* and *Paenibacillus* (sequence similarity 100 and 98%, respectively) were the other representatives of this phylum. A detailed look into the diversity of culturable bacteria in this warm spring water showed that in the phylum *Proteobacteria* members belonging to *Gammaproteobacteria* were predominant and were represented by the genera *Pseudomonas, Aeromonas, Enterobacter, Kluyvera, Klebsiella, Acinetobacter* and *Halomonas* (sequence similarity 97–100%). Nine isolates belonged to *Aeromonas* distributed in three species and 12 belonged to *Acinetobacter* distributed in five species (with >97–100% sequence similarity; Table 1). In this sample *Betaproteobacteria* was represented by a single genus *Chromobacterium* and *Alphaproteobacteria* by two genera *Roseomonas* and *Sphingomonas* (both with 97% sequence similarity with existing species). Species belonging to seven genera of the phylum *Actinobacteria* were recovered from this sample and these are *Dermococcus, Microbacterium, Arthrobacter, Janibacter, Micrococcus*, *Nocardioides* and *Cellulosimicrobium. Bacteroidetes* (CFB group) was represented by two isolates belonging to same taxon with 95.7% sequence similarity. The tentative affiliations

Table 1 Diversity of IW isolates at the species level based on 16S rRNA gene sequence analysis

Phylum		Closest genus	Closest species (% similarity)	Isolate identity (nt)
Actinobacteria (13%)		*Dermacoccus*	*nishinomyaensis* (99.9%)	PCIW 01
		Dermacoccus	*barathri* (96.6%)	**PCIW 02**
		Arthrobacter	*pascens* (99.4%)	PCIW 08
		Nocardioides	*fonticola* (99.6%)	TDIW 07
		Janibacter	*limosus* (99%)	TSIW 22
		Micrococcus	*luteus* (99.6%)	TSIW 23
		Microbacterium	*testaceum* (99.8%)	PC IW 05
		Cellulosimicrobium	*funkei* (99%)	TSIW 35
Proteobacteria (58%)	*Alpha*	*Roseomonas*	*roseus* (97%)	**PCIW 03**
		Novosphingobium	*mathurense* (97.6%)	**TDIW 02, TDIW 05**
	Beta	*Chromobacterium*	*aquaticum* (99.4%)	PCIW 06, PCIW 24
	Gamma	*Klebsiella*	*variicola* (100%)	TDIW 16, TDIW 18
		Aeromonas	*aquariorum* (99.8–99.9%)	PCIW 33, PCIW 14, PCIW 38, PCIW 39, TSIW 09, TSIW 32
			punctata (99.9%)	PCIW 40, PCIW 41
			veronii (99.9%)	PCIW 37
		Enterobacter	*aerogens* (99.8%)	PCIW 35,TS IW 13
			ludwigii (100%)	PCIW 26
			hormaechei (99.2%)	TD IW 03
		Kluyvera	*cryocrescnes* (99.9%)	PC IW 29
		Halomonas	*aquamarina* (99%)	TSIW 20
		Pseudomonas	*pseudoalcaligenes* (99.2%)	TSIW 01
			aeruginosa (95.63%)	PCIW 25
		Acinetobacter	*junii* (100%)	TSIW 07, TDIW 15
			schindleri (99%)	TSIW 21
			beijerinckii (97.7–98.4%)	TDIW 13, TSIW11, TSIW29, TSIW 30, TSIW 37, TDIW 10, TDIW 17
			tandoii (98%)	TD IW 08
			gyllenbergii (99.3%)	PCIW 04
Firmicutes (26%)		*Staphylococcus*	*arlettae* (100%)	PCIW 10, PCIW 11
		Bacillus	*anthracis* (99.6%)	TSIW 25
			subtilis (99.7%)	PCIW 31
			firmus (99.4%)	TSIW 24
			megaterium (99.5–99.8%)	PCIW 13, TSIW 17, TSIW 26, TSIW 36, TSIW 31
			luciferans (100%)	PCIW 15
			thioparans (99.4%)	PCIW 34
			infantis (97.8%)	TS IW 33
			flexus (99.8%)	TSIW 14
		Paenibacillus	*lautus* (98.2, 98.9%)	TSIW 08, TSIW 27
Bacteroidetes (CFB group) (3%)		*Chryseobacterium*	*haifense* (95.7%)	**TSIW 15, PCIW 30**

Strains shown in bold are potentially novel taxa

of the 62 isolates to the closest taxa are shown in Table 1. Phylogenetic positions of these isolates along with some related species are presented in Supplementary Fig. 1.

The study using culture dependent approach not only reflects part of the diversity of bacteria in this habitat, it also revealed four potential novel taxa because of their low sequence similarity ($\sim$97% or less) and some differences in phenotypic characteristics with the closest validly described species. The currently accepted definition of *Archaeal* and *Bacterial* species at present is a collection of

strains showing 70% or greater DNA–DNA relatedness and with 5°C or less ΔT_m of the re-associated DNA molecules [33]. It is generally observed that the 16S rRNA gene sequences showing less than 97% similarity normally show less than 70% genomic relatedness [25]. These strains of potential novel taxa are being characterized by polyphasic taxonomic approach.

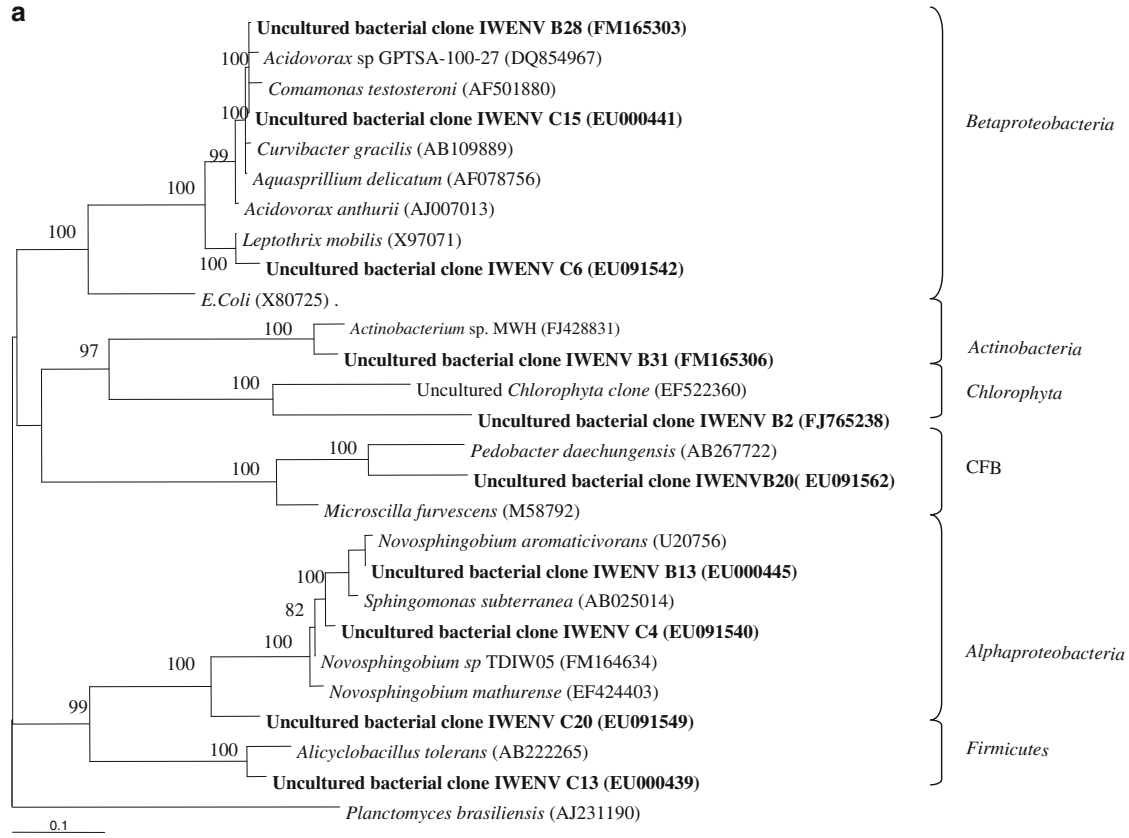
Culture Independent Analyses of Bacterial Diversity

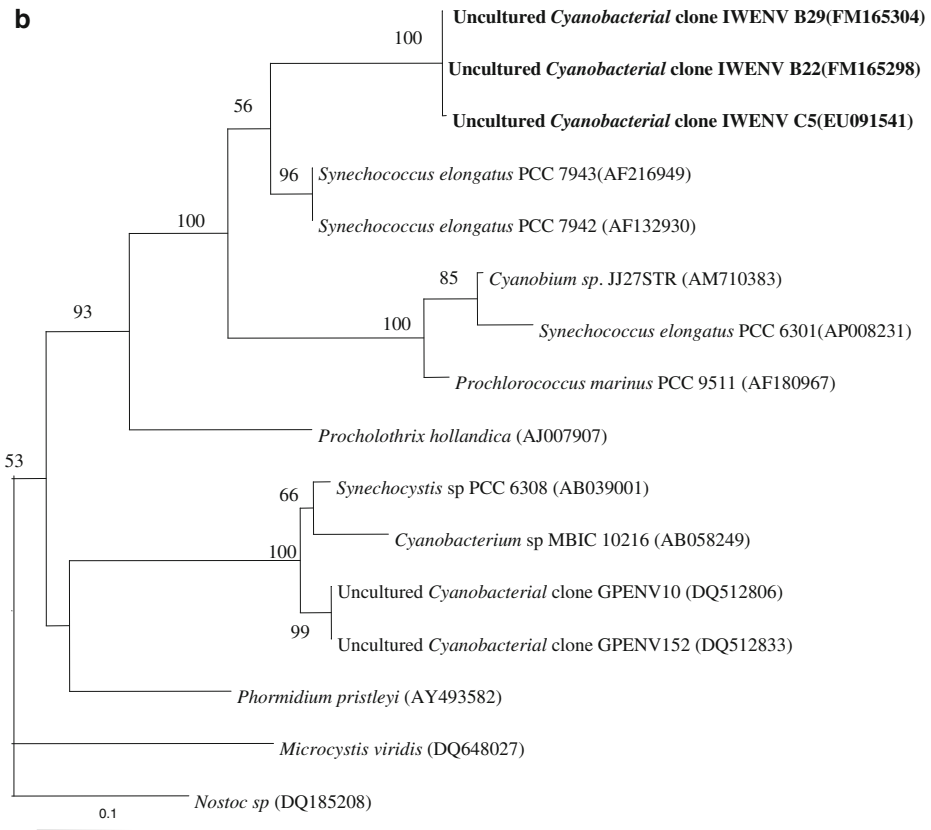
When the 16S rRNA gene sequences of 47 randomly picked clones were analyzed, two among them were found to be chimaeras and removed from further consideration. A maximum likelihood phylogenetic tree with Bayesian support was constructed using 45 clone sequences (Fig. 1). Summary of the sequence analysis of the clones is given in Table 2. The clone library can be grouped into 11 OTUs based on a cut off value of 97% or greater sequence similarity. The 16S rRNA clones examined in this study showed affiliations with five major phyla, overwhelming majority belonging to *Cyanobacteria,* followed by *Proteobacteria, Actinobacteria, Firmicutes* and CFB group. Incidentally, one of the clones showed 86% sequence similarity with the small subunit ribosomal RNA of plastid of the photosynthetic green algae *Pedimonas minor* belonging to *Chlorophyta* (Fig. 1a). Out of these 45 clones analyzed 27 turned out to be of *Cyanobacterial* origin, showing varying degree of sequence similarity (91–94%) with the species *Synechococcus elongatus* PCC 7942 (Fig. 1b). With such low similarity of the cloned sequences with cultured *Cyanobacteria,* they may represent new taxa but require further investigation to establish their correct status. From sequence analysis it is apparent that genotypic variants do exist among them. The existence of *Synechococcus* related organisms in this spring water in abundance is not surprising as they are the major photosynthetic bacteria in some aquatic habitats [14, 29]. It was shown that in *Synechococcus* community phylogenetic clusters could be demarcated and correlated with the variation in the environment [32]. Similarly genetic diversity was found in the *Synechococcus* population in a water column in the Red sea although there was a predominance of a single clade [6]. In a similar culture independent analysis of water of a warm spring from Borpung reserve forest of Assam, it was found that the cloned sequences belong to two phyla, *Cyanobacteria* (~56%) and *Proteobacteria* (~44%) [23]. Most of these *Cyanobacterial* clones showed 87.2–96.7% sequence similarity with *Synechocystis* sp. Strain PCC6308 and four with *Cyanobacterium* sp. MBIC10216; S*ynechococcus* related sequences were not present in this Borpung sample.

Of the remaining 18 clones, seven were members of *Alphaproteobacteria* belonging to three genera, the predominant one (five clones) showing 97% sequence similarity with *Novosphigobium mathurense* of *Sphingomonadaceae* family [7]. Incidentally these five clones show 99% sequence similarity with two strains (TD IW 02 and TD IW 05) recovered in the culture dependent approach of the present study. One of the *Alphaproteobacteria* showed 97% sequence similarity with *Novospingobium aromaticivorans.* The other clone belonged to *Mesorhizobium*. *Betaproteobacteria* was represented by three different genera (Table 2). Three 16S rRNA clones showed low (86%) sequence similarity with a culturable bacteria belonging to the CFB group. Such a low sequence similarity indicates that these may represent new taxa possibly at a higher taxonomic hierarchy. Two of the clones from this sample showed 95.7% sequence similarity with Candidatus *Planktophila limnetica*, an *Actinobacterium* representing one of the most numerically important taxa in freshwater bacterioplankton. Further down the BLAST analysis reveals these clones to have 91% sequence similarity with *Sporichthya polymorpha*. Phylum *Firmicutes* was represented by one clone showing 95% sequence similarity with *Alicylocbacillus*. It is to be noted here that the type of bacteria represented in the clone library are the ones which are mostly retrieved from aquatic habitat, like drinking water source, fresh water springs or marine water or sediments of pond or sludge bioreactors [2, 5, 11]. The rarefaction curve for the diversity of isolates and clones did not plateau when OTUs were defined at 97% sequence identity (Fig. 2a, b) and it appears that inclusion of some more clones was desirable to get a better picture of the diversity of bacteria in this warm spring.

Comparison of Diversity by Culture Dependent and Culture Independent Approaches

The diversity pattern obtained by culture dependent approach does not necessarily overlap, especially at generic level, with the one generated by culture independent approach. In this study no attempt was made to isolate and cultivate *Cyanobacteria*. Even from the limited sample size it is quite apparent that bacterial community of this spring water is dominated by *Cyanobacteria* (Table 2). It is to be noted here that members of *Gammaproteobacteria* were not represented in the limited number of sequences analyzed although they were a major component in culture dependent approach (Fig. 3a, b). We could only conclude that these heterotrophic bacteria may be a minority or less abundant in the environment but get selectively enriched and appear as a dominant population in the culture media. Since the media used did not support the phototrophic bacteria which were actually found to be dominant by the 16S rRNA cloning, preferential growth of these heterotrophic were observed in the culture media. A comparative phylogenetic tree including sequences of both cultivable


a
Uncultured bacterial clone IWENV B28 (FM165303)
Acidovorax sp GPTSA-100-27 (DQ854967)
Comamonas testosteroni (AF501880)
Uncultured bacterial clone IWENV C15 (EU000441)
Curvibacter gracilis (AB109889)
Aquasprillium delicatum (AF078756)
Acidovorax anthurii (AJ007013)
Leptothrix mobilis (X97071)
Uncultured bacterial clone IWENV C6 (EU091542)
E.Coli (X80725) .
Actinobacterium sp. MWH (FJ428831)
Uncultured bacterial clone IWENV B31 (FM165306)
Uncultured Chlorophyta clone (EF522360)
Uncultured bacterial clone IWENV B2 (FJ765238)
Pedobacter daechungensis (AB267722)
Uncultured bacterial clone IWENVB20(EU091562)
Microscilla furvescens (M58792)
Novosphingobium aromaticivorans (U20756)
Uncultured bacterial clone IWENV B13 (EU000445)
Sphingomonas subterranea (AB025014)
Uncultured bacterial clone IWENV C4 (EU091540)
Novosphingobium sp TDIW05 (FM164634)
Novosphingobium mathurense (EF424403)
Uncultured bacterial clone IWENV C20 (EU091549)
Alicyclobacillus tolerans (AB222265)
Uncultured bacterial clone IWENV C13 (EU000439)
Planctomyces brasiliensis (AJ231190)
0.1
Betaproteobacteria
Actinobacteria
Chlorophyta
CFB
Alphaproteobacteria
Firmicutes


b
Uncultured Cyanobacterial clone IWENV B29(FM165304)
Uncultured Cyanobacterial clone IWENV B22(FM165298)
Uncultured Cyanobacterial clone IWENV C5(EU091541)
Synechococcus elongatus PCC 7943(AF216949)
Synechococcus elongatus PCC 7942 (AF132930)
Cyanobium sp. JJ27STR (AM710383)
Synechococcus elongatus PCC 6301(AP008231)
Prochlorococcus marinus PCC 9511 (AF180967)
Procholothrix hollandica (AJ007907)
Synechocystis sp PCC 6308 (AB039001)
Cyanobacterium sp MBIC 10216 (AB058249)
Uncultured Cyanobacterial clone GPENV10 (DQ512806)
Uncultured Cyanobacterial clone GPENV152 (DQ512833)
Phormidium pristleyi (AY493582)
Microcystis viridis (DQ648027)
Nostoc sp (DQ185208)
0.1

◀ **Fig. 1** Phylogenetic relationships between 16S rRNA clone sequences from IW sample (labelled IWENVB1-33; C1-22) along with sequences of related strains. (Only one representative of each OTU is taken.) Both phylogenetic trees represent maximum likelihood trees inferred by Bayesian analysis. The Bayesian support values are given at the nodes. *Bolded* ones are sequences obtained from this study. **a** Tree depicting clones other than *Cyanobacterial* clones. The *Planctomyces barasiliensis* is taken as an outgroup. **b** Tree depicting only *Cyanobacterial* clones. Sequence of *Nostoc* is taken as outgroup

isolates and clones from this study has been depicted in Supplementary Fig. 2. From the results presented here it could be argued that in the absence of any enrichment treatment the bacteria that appear on general purpose media like PCA, TSBA, etc., are a minority in the community and therefore, their representation gets severely diluted in the 16S rRNA sequence library which are dominated by bacteria which are not amenable to cultivation on such media.

For isolation of the culturable bacteria we have used only three types of media of different nutritional conditions such as PCA, TSBA and TD. Although they may support growth of a diverse type of organisms, many may still remain unrepresented. Thus the choice of the media plays a crucial role in culture dependent study. For example the photosynthetic bacteria, *Cyanobacteria* require special basal media with specific photoperiod for its culturing. Moreover it is time consuming and difficult to obtain

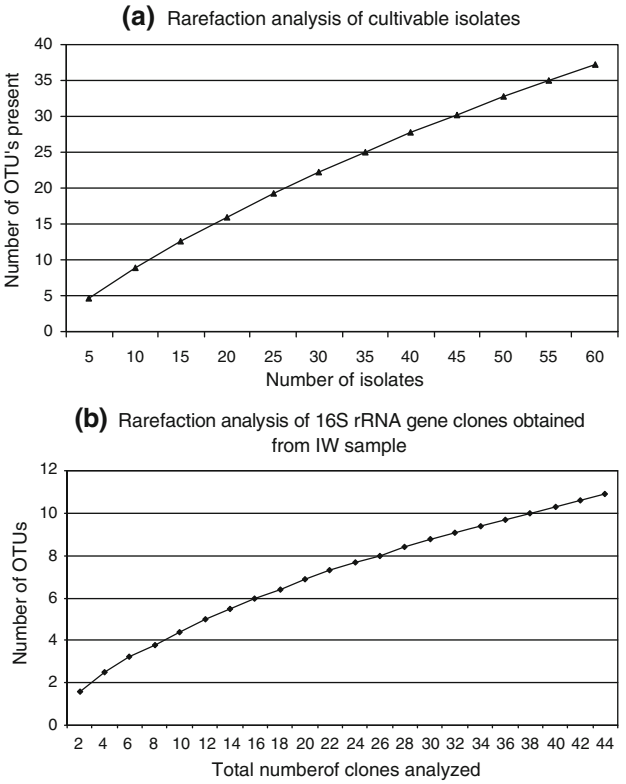


Fig. 2 Rarefaction analyses of bacterial diversity of the IW water sample. **a** Represent cultivable isolates. **b** Represent 16S rRNA gene clones

Table 2 Grouping of the 16S rDNA clones based on sequence similarity

Similarity groups (OTU)	Clone ID	Closest culturable match	Percentage similarity (%)	Taxonomic affiliation
1	IWENV B22, C18, C21, C22	*Synechococcus elongatus* PCC 7942	94	Cyanobacteria (27 clones)
	IWENV B4, B7, B9, B12, B14, B17, B18, B19, B21, B23, B24, B25, B26, B30, B32, B33, C1, C5, C10, C12, C14, C19		93	
	IWENV B29		91	
2	IWENV C20	*Mesorhizobium amorphae*	97	*Alpha proteobacteria* (seven clones)
3	IWENV B13	*Novosphingobium aromaticivorans*	97	
4	IWENV B15, B16, C16, C4, C17	*Novosphingobium mathurense*	97	
5	IWENV B1, B11, B20	*Pedobacter daechungensis*	86	CFB group (three clones)
6	IWENV B3, C6	*Leptothrix mobilis*	97	Beta-proteobacteria (four clones)
7	IWENV C15	*Curvibacter gracilis*	97	
8	IWENV B28	*Comamonas testosteroni*	97	
9	IWENV C13	*Alicyclobacillus tolerans*	95	Firmicutes (one clone)
10	IWENV B5, B31	Candidatus *Planktophila limnetica* strain MWH-EgelM2-3.acI	95.7	Actinobacteria (two clones)
		Sporichthya polymorpha	91	
11	IWENV B2	Chloroplast DNA of *Pedimomonas minor*	86	(one clone)

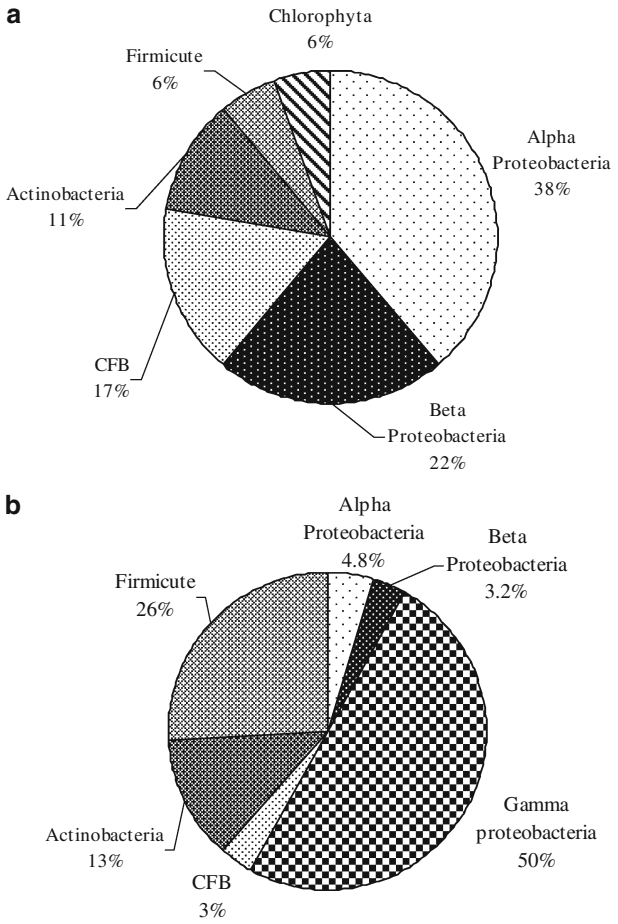


Fig. 3 Comparison of bacterial diversity from the warm spring water as revealed by **a** culture independent (except *Cyanobacteria*) and **b** culture dependent approaches

axenic culture. Thus even though *Cyanobacteria* was found to be dominating in the culture independent approach, it is not surprising that this group could not be retrieved in the media we have used. For practical purposes only limited types of media and growth conditions are normally used for isolation of cultivable bacteria. Culture independent approach on the other hand can indicate the nature of media and condition of growth one need to use for isolation of desired organisms. The use of gellan gum instead of agar on the media was found to support growth of "a diverse array of novel microbes, and reducing the gaps between molecular and cultivation based analysis" [27]. Even though culture independent molecular approaches based on 16S rRNA gene sequence have been used for many of the fresh water niches they have mainly focused on particular group of organisms and moreover most of the studies on bacterial communities in natural ecosystem are based solely on molecular analyses [13, 20, 34].

Obtaining bacteria in pure culture is still important to understand the ecological and physiological roles they play in the environment. Therefore, a combination of both the approaches, in our opinion, is the ideal way to study the bacterial diversity of any natural habitat.

Acknowledgements The authors thank Prof. D. J. Bhat, Department of Botany, Goa University, India for his help during sample collection and generosity to use his laboratory for preliminary processing of the samples. Help of Dr. S. Roy Choudhury, Mr. A. Bhattacharya and Mr. Malkit Singh of this institute and Ms. Puja Gawas of Goa University in sample collection and of Dr. S. Achuthankutty of NIO, Goa in chemical analysis of the spring water is thankfully acknowledged. The authors acknowledge Dr. Belle D. Shenoy of this institute for his help in phylogenetic analyses. Financial assistance from DBT, Government of India and CSIR is duly acknowledged. AR is a recipient of DBT post doctoral fellowship. This is IMTECH Communication No. 5/2009.

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