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Molecular characterization and antibiogram analysis of bacterial community from River Narmada, India

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ABSTRACT

Objectives: To investigate the microbial diversity of unculturable microbes in River Narmada, India, using molecular methods and undertake antibiogram analysis.

Methodology and results: Metagenomic DNA was extracted and cloned into high copy number plasmid vector and transferred to *E. coli* DH5α, the resulting gene bank was screened for positive clones by growth selection method. The library was then screened for the presence of aminoglycoside resistance genes among the recombinants. Genomic DNA of 10 Kb was obtained and upon preliminary screening, 24 clones expressing resistance to aminoglycoside antibiotics were detected. 1-6 Kb of plasmid DNA was obtained on plasmid profiling. Upon restriction digestion, molecular diversity of 16S *rDNA* genes showed similarity at 1.5 coefficient levels among the recombinants.

Conclusions and application of findings: Direct cloning method illustrates another way to access and exploit the immense pool of genes from microorganisms. No published reports exist on exploitation of the metagenomic approach to study the microbial diversity of River Narmada. The generation and analysis of (meta) genomic libraries provides a powerful approach to harvest and archive environmental genetic resources. The findings of the present work suggest that the occurrence of antimicrobial resistant bacteria in the aquatic ecosystem of river Narmada may contribute to the environmental dissemination of antibiotic resistance. This fact re-inforces the hypothesis that cultivation independent approach may estimate more accurately the prevalence of antibiotic resistance genes in the environmental samples though it may introduce bias in the recovery of their molecular variants.

Key words: Molecular characterization, antibiogram analysis, bacterial community.

INTRODUCTION

The multifarious uses of water for drinking, bathing, washing and cooking are well known. Water meant for human consumption should be free from pollution, safe and acceptable. Indeed, the microbial quality of water sources should not exceed the maximum limits specified in water quality guidelines. Several bacterial enteropathogens namely *Salmonella, Shigella, Aeromonas, Vibrio cholerae* and *Escherichia coli* have been isolated from the water of river Narmada (Sharma & Rajput, 1996; Sharma & Khokhale, 2005; Sharma *et al.,* 2009). The region of India where the river Narmada flows is predominantly rural and residents rely on untreated water sources for daily water needs. Fresh water is very important for human health and sound

ecosystem, especially aquatic ecosystem. However, freshwater resources have reduced and water quality has also deteriorated throughout the world. Due to contamination of fresh water, along with population explosion, one-fifth of the world's population has no access to safe water (WAO, 2009).

Fresh water and the related microbial population in the distribution system constitute one of the most extensively studied oligotrophic systems (Lechevaller *et al.,* 1987; Pedersen, 1990). River Narmada is proving to be no exception, and being an important source of fresh water in the states of MP and Gujarat, it is exploited in a variety of ways by the general public. It is the largest river flowing west in the Indian Peninsula originating from Amarkantak at 1151m altitude (latitude 21^{o} 40' and longitude 81^{o} 46' E) in Shahdol district from Maikal ranges in Madhya Pradesh.

Molecular phylogenetic analysis plays a very important role in the study of microbial diversity in riverine ecosystem. For both cultivated and uncultivated microbes, the comparative sequence analysis of 16S *rRNA* enables the investigation of phylogenetic relationships among microorganisms in a manner that was not feasible through traditional microbiology methods. It provides a method for biodiversity assessment and a guideline for biodiversity conservation. Also, it supports the goals of conservation, which are partly to obtain as much phylogenetic information as possible (Tian *et al.,* 2003). With molecular approaches it is possible to generate libraries of cloned DNA such as prokaryotic small subunit ribosomal RNA genes (SSU *rDNA*) which have illuminated the extensive diversity of known and novel taxa in aquatic systems (Crump *et al.,* 2003). The value of these molecular technologies in comprehensive environmental assessment also relies on the well known linkage between environmental parameters and the presence of specific microbial taxa (Wetzel, 1983).

More importantly, *rDNA* also provides an environmental signal since microbial populations constantly fluctuate in response to changing conditions, and *rDNA* is generally a reflection of cell abundance. It is also easier to extract and purify DNA than RNA from natural samples without degradation. Thus, a profile of microbial community rDNAs ought to reflect the character of the surrounding environment (Marshall *et al.,* 2007).

Cultured microorganisms have been the source of almost all characterized antibiotic resistance; therefore, most previous studies have ignored the potential reservoir of antibiotic resistance genes in uncultured bacteria. The intense research efforts to elucidate mechanisms of resistance have focused on genes derived from a narrow range of environments. Most of the known resistance determinants have been discovered in bacterial isolates, whereas other environmental reservoirs of antibiotic resistance are not well characterized (Nwosu, 2001). Exhaustive characterization of species richness may ultimately demonstrate that "everything microbial is everywhere", but this approach often overlooks important quantitative data (Marshall *et al.,* 2007). Metagenomic profiles, however, encompass both qualitative and quantitative aspects of diversity, which are essential for a substantial environmental characterization. To do so, we compared metagenomic profiles of river Narmada by evaluating a relatively small number of clones taken from *rDNA* clone library and have been used to identify clones expressing antibiotic resistance. By using this strategy, we are able to determine whether nonculturable species or potentially culturable species predominate in fresh water systems.

MATERIALS AND METHODS

Sampling sites, bacterial strains and plasmids: For the construction of environmental DNA libraries, 16 water samples from surface and subsurface level were collected from river Narmada. *E.coli* DH5α (F^{-}Ø 80d lacz, A_l hsd R_{17} (rk^{-}, mk^{-}) pho A sup E 44 λ^{-}thi-l gyr A96, re^{l} A_l) (Life Technologies, Germany) was used as host strain and the plasmid pUC 4K, pUC 18 and pUC 19 were employed as vector for the cloning

experiments (Schaefer & Handelsman, 1997; Riesenfeld *et al.,* 2004).

DNA preparation and library construction: *rDNA* library was constructed by cloning DNA fragments obtained from river water into broad-host-range expression vectors pUC 4K (encoding kanamycin resistance), pUC 18 and pUC 19 (encoding ampicillin resistance). Strains were grown in Luria Bertani (LB) medium containing appropriate concentration of antibiotic kanamycin (30 µg/ml) for pUC 4K and ampicillin (50 µg/ml) for pUC 18 and 19) for plasmid maintenance. DNA was extracted from each water sample, using a cetyltrimethylammonium bromide (CTAB) buffer DNA isolation technique (Schaefer & Handelsman, 1997). Both the DNA and expression vectors were digested with restriction enzyme Eco R I. The 10 Kb fragment of the digested DNA was gel purified and ligated into the expression vectors using T4 DNA ligase. The reaction mixture consisted of T4 DNA ligase 20 U, 10X buffer 2 µl, DNA 2 µl, and vector 6 µl and incubated for 4 h at 16 ^{o}C. The vector containing the insert was transformed into competent cells of *E.coli* DH5α by $CaCl_2$ method for library construction (Ausubel *et al.,* 1995). Enzymes used for the study were purchased from Fermentas, Life Sciences (USA) and used according to manufacturer's instructions. *E.coli* transformants were selected on Luria Bertani agar plates containing ampicillin (50 µg/ml) for pUC 18 and 19, kanamycin (30 µg/ml) for pUC 4K, 5 bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) (20 mg/ml) and Isopropyl β-D-1 thiogalactopyranoside (IPTG) (24 mg/ml) by blue white selection. To determine the average insert size for each library, the plasmids from 24 colonies were extracted and digested with EcoR I and the fragments were analyzed through agarose gel electrophoresis (Sambrook *et al.,* 1989). Pools of library clones were stored at -10^{o}C.

Phylogenetic diversity estimation: A single colony was inoculated into 50 µl of sterile water and lysed by boiling for 10 minutes. Suspension was then centrifuged at 13,000 rpm for 1 minute at 4^{o}C and 1µl of supernatant was used directly as template DNA (Holmes *et al.,* 2003). Bacterial 16S *rDNA* sequences were amplified using the primer B_{27}F (5'AGAGTTTGATCCTGGCTCAG3') & -1541-1522R (5'AAGGAGGTGATCCAGCCG CA3') (Edwards *et al*., 1989; Bruce e*t al*., 1992). Total reaction (25 µl) mixtures for PCR amplification contained 1.94 µl of 10X buffer, 1.6 µl of 25 mM $MgCl_2$, 1.6 µl of 2.5 mM dNTP mixture, 0.2 µl of each 200 µm (each) forward and reverse primer, 13.3 µl of milli Q water, 0.5 U of Taq DNA polymerase and 1 µl of template DNA (Waters & Davies, 1997). The reaction mixtures were initially heated at 95^{o}C for 5 minutes, followed by 35 cycles at 95^{o}C for 1 minute, 55.5^{o}C for 30 seconds, 72^{o}C for 1 minute with a final extension period of 5 minutes at 72^{o}C. Amplified product was digested with Rsa I and banding patterns were analysed by gel electrophoresis (Sambrook *et al.,* 1989; Ausubel *et al.,* 1995). The phylogenetic analysis of ARDRA profile was carried out using the software version NTsys 2.02i.

Isolation of clones expressing antibiotic resistance: Selection for the aminoglycoside resistant clones was conducted by plating pools of clones from library on to LB medium containing inhibitory concentrations of the aminoglycoside antibiotics, i.e. , amikacin (2.5 µg/ml), streptomycin (5 µg/ml), kanamycin (2.5µg/ml) and tobramycin (2.5 µg/ml). Plates were incubated overnight at 37^{o}C. The plasmid of 24 antibiotic resistant recombinants were isolated and digested with the same enzyme used to clone isolated DNA. Plasmids with unique restriction fragment length polymorphism (RFLP) pattern were retransformed into *E.coli* DH5α, and the phenotypes were verified by patching cells on to the appropriate media to ensure that the cloned DNA was responsible for the phenotype (Risenfeld *et al.,* 2004). Antibiotics were purchased from Himedia (Mumbai) and Life Technologies (Germany).

Antiobiotic susceptibility testing: Minimum inhibitory concentrations (MICs) were determined using the macro dilution assay in LB broth with about 1x 10^5 cells per ml using amikacin, tobramycin, streptomycin and kanamycin antibiotics (Andrews, 2001). All the experiments were performed in duplicate.

RESULTS

For each sample, libraries of SSU *rDNA* clones were produced. Library comparisons were made to determine if similar estimates of species diversity indicated similar community composition. For each pairwise composition, a similarity coefficient was calculated. This analysis showed some overlap between every pair of samples but the extent of commonality varied to coefficient level 1.50 (Fig. 1).

Amplified Ribosomal DNA Restriction Analysis (ARDRA): Twenty-four clones of different genomospecies were subjected to ARDRA of 16S *rDNA* genes using restriction enzyme Rsa I. Similarity coefficient was analysed by UPGMA cluster analysis. Among the recombinants studied 12 showed the same pattern while 11 isolates showed different pattern from previous one (Fig. 2).

Table 1: Antibiotic resistance profile of clones (%) isolated from river Narmada, India.

S.No.	Antibiotics	pUC 18 Clones(%)	pUC 19 Clones(%)	pUC 4K Clones(%)
1	Tobramycin	62.5	75	75
2	Sterptomycin	87.5	75	62.5
3	Kenamycin	62.5	87.5	75
4	Gentamycin	50	62.5	62.5

Effect of Antibiotic and Minimum Inhibitory Concentration (MIC): The effect of aminoglycoside antibiotic in metagenomic library against 24 clones is presented in table 1 and the minimum inhibitory concentration of four different aminoglycosides against 24 clones is presented in table 2

Plasmid profiling: The analysis of plasmid profile of 24 clones showed that each clone harbored multiple plasmids. The plasmids detected among these clones were diverse, showed different size ranging from 1 – 6 kb. Among the 24 different clones screened, 17 of them showed almost same types of banding pattern. The plasmids of 1 kb to 6 kb were most frequently found in 17 isolates (60%). Majority of clones from the DNA library showed multi drug resistance. The plasmid profiling revealed that most of the clones showed similarity with each other at 1.55 coefficient level (Fig. 3).

DISCUSSION

Microbes affect the physicochemical and biological parameters of the environment. People of India and other developing countries use river water directly not only for drinking but also for various recreational purposes. Narmada is the fifth largest river of India. Majority of river water sources harbor enteropathogens and have been reported to be of poor microbiological quality and unsafe for consumption. The objective of this study was to determine if partial profiles of microbial communities could be compared to make meaningful ecological inferences and to provide guidance in the development of environmental assays based on such profiles. To do so, we randomly drew a total of 24 clones from libraries of prokaryotic SSU *rDNA* to identify the more common *rDNA* sequence from each sample.

The underlying principal was that the community profile could act as a comprehensive bio-indicator of water quality because microorganisms respond rapidly to environmental cues. To the best of our knowledge, no other report of such an approach on river Narmada has been published. The sequences of the primers used were derived from conserved regions of known genes or protein families. Thus, the identification of entirely new genes or gene products by PCR-based methods is very limited (Waters & Davies, 1997). Direct cloning approach illustrates another way to access and to exploit the immense pool of genes from microorganisms, which have not been cultivated so far. The aim of present study was to construct a stable large insert DNA library representing bacterial genomic DNA, in order to gain information about the genetic and physiological potential of representative group in the bacterial community especially of river Narmada.

The existence of sequence information prior to cloning is not required. In addition, the existing environmental libraries can be employed for screening of various targets. This has significant implications for microbial biotechnology in the future (Henne *et al.,* 1999). Amplified Ribosomal DNA Restriction Analysis (ARDRA) with a probe complementary to 16S *rRNA* was

performed. Repeated ARDRA analysis established the similarities between the isolates and thus suggests ARDRA as an authentic and precise detection protocol. Variation in the species composition of the fresh water communities obtained at the same sampling site but at different level is due to seasonal fluctuations of organic substrate in fresh water. The presence of organic matter would promote bacterial regrowth that in turn would lead to deterioration of water quality in distribution system (Lee, 2008). However, the question remains whether the population changes in the distribution system are primarily due to regrowth phenomenon, as described by LeChevaller in 1990 or whether these changes reflect population shifts within the microbial community of the raw water sources. The use of antibiotics is generally considered to be the major factor determining the prevalence of antibiotic resistance in a population (Bruin's *et al.,* 2003). Another important factor contributing to the development of antibiotic resistance is the dissemination of drug resistance genes from the donor to a susceptible microbial host. Subsequent colonization by drug resistant bacteria in the host could result in the transfer of the resistant genes to normal intestinal flora (Lester *et al.,* 1987).

Table 2: Minimum Inhibitory Concentration of antibiotics against clones isolated from river Narmada. Range tested is 0.25 – 128 mg/L.

S.No.	Clones	Antibiotics	MIC Value mg/l	S.No.	Clones	Antibiotics	MIC Value mg/l
1	BGCC#C1	Amikacin	≤ 2	28	BGCC#C16	Tobramycin	0.5
2	BGCC#C1	Tobramycin	1	29	BGCC#C18	Amikacin	2
3	BGCC#C2	Amikacin	1	30	BGCC#C18	Tobramycin	0.5
4	BGCC#C2	Tobramycin	2	31	BGCC#C18	Streptomycin	1
5	BGCC#C2	Kanamycin	1	32	BGCC#C18	Kanamycin	0.5
6	BGCC#C4	Kanamycin	1	33	BGCC#C19	Tobramycin	1
7	BGCC#C4	Streptomycin	1	34	BGCC#C19	Streptomycin	0.5
8	BGCC#C6	Amikacin	1	35	BGCC#C19	Kanamycin	1
9	BGCC#C7	Kanamycin	1	36	BGCC#C20	Amikacin	1
10	BGCC#C8	Tobramycin	≤ 2	37	BGCC#C20	Tobramycin	1
11	BGCC#C8	Streptomycin	0.5	38	BGCC#C20	Kanamycin	≥ 2
12	BGCC#C9	Streptomycin	≤ 0.5	39	BGCC#C20	Streptomycin	≤ 0.5
13	BGCC#C9	Tobramycin	≥2	40	BGCC#C21	Streptomycin	4
14	BGCC#C9	Kanamycin	≥ 4	41	BGCC#C21	Kanamycin	≥ 4
15	BGCC#C10	Amikacin	2	42	BGCC#C22	Amikacin	2
16	BGCC#C10	Streptomycin	1	43	BGCC#C22	Tobramycin	≤ 2
17	BGCC#C10	Tobramycin	≤ 2	44	BGCC#C22	Streptomycin	1
18	BGCC#C12	Tobramycin	2	45	BGCC#C22	Kanamycin	≥ 2
19	BGCC#C12	Streptomycin	1	46	BGCC#C23	Amikacin	≤ 1
20	BGCC#C14	Tobramycin	2	47	BGCC#C23	Tobramycin	1
21	BGCC#C14	Streptomycin	1	48	BGCC#C24	Kanamycin	4
22	BGCC#C14	Kanamycin	≥ 0.25	49	BGCC#C24	Amikacin	2
23	BGCC#C15	Streptomycin	0.5	50	*E.coli*	Amikacin	-
24	BGCC#C15	Kanamycin	1	51	*E.coli*	Streptomycin	-
25	BGCC#C15	Tobramycin	0.25	52	*E.coli*	Tobramycin	-
26	BGCC#C16	Amikacin	≤ 1	53	*E.coli*	Kanamycin	-
27	BGCC#C16	Streptomycin	0.5				

BGCC: Bacterial Germplasm Culture collection.
Concentration range (0.25-128 mg/l): 0.25, 0.5, 1.0, 2, 4, 8, 16, 32, 64, 128 mg/l

Table 3: Plasmid profiling of DNA library isolated from river Narmada, India.

S. No	Clone Code	No. of fragment	Fragment (bp)
1	BGCC#C1	2	1000, 6000
2	BGCC#C2	1	2500
3	BGCC#C3	2	1000, 6000
4	BGCC#C4	1	2500
5	BGCC#C5	2	1000, 6000
6	BGCC#C6	1	2500
7	BGCC#C7	2	1000, 6000
8	BGCC#C8	1	2500
9	BGCC#C9	2	1000, 6000
10	BGCC#C10	1	2500
11	BGCC#C11	2	1000, 6000
12	BGCC#C12	1	2500
13	BGCC#C13	1	3000
14	BGCC#C14	1	3000
15	BGCC#C15	1	3000
16	BGCC#C16	-	-
17	BGCC#C17	1	1000
18	BGCC#C18	-	-
19	BGCC#C19	-	-
20	BGCC#C20	-	-
21	BGCC#C21	-	-
22	BGCC#C22	-	-
23	BGCC#C23	-	-
24	BGCC#C24	-	-

BGCC: Bacterial Germplasm Collection Centre;

The findings of this study suggested that the plasmids harbor the genes for broad spectrum aminoglycosides resistance. During the present investigation, 70.2 % recombinants were resistant to four commonly used antibiotics of aminoglycoside class, i.e. tobramycin, streptomycin, kanamycin and gentamycin, which might be due to indiscriminate use of these drugs. The present study demonstrated that clones expressing antibiotic resistance obtained from fresh water metagenomic libraries harbor antibiotic resistance genes with considerably more genetic diversity than reported previously for the culturable microorganism in our laboratory from the same reverine ecosystem. The strategy used in this study provides the possibility to correlate information about the phylogenetic identity with physiological parameters of the environment.

ACKNOWLEDGEMENTS: [redacted]

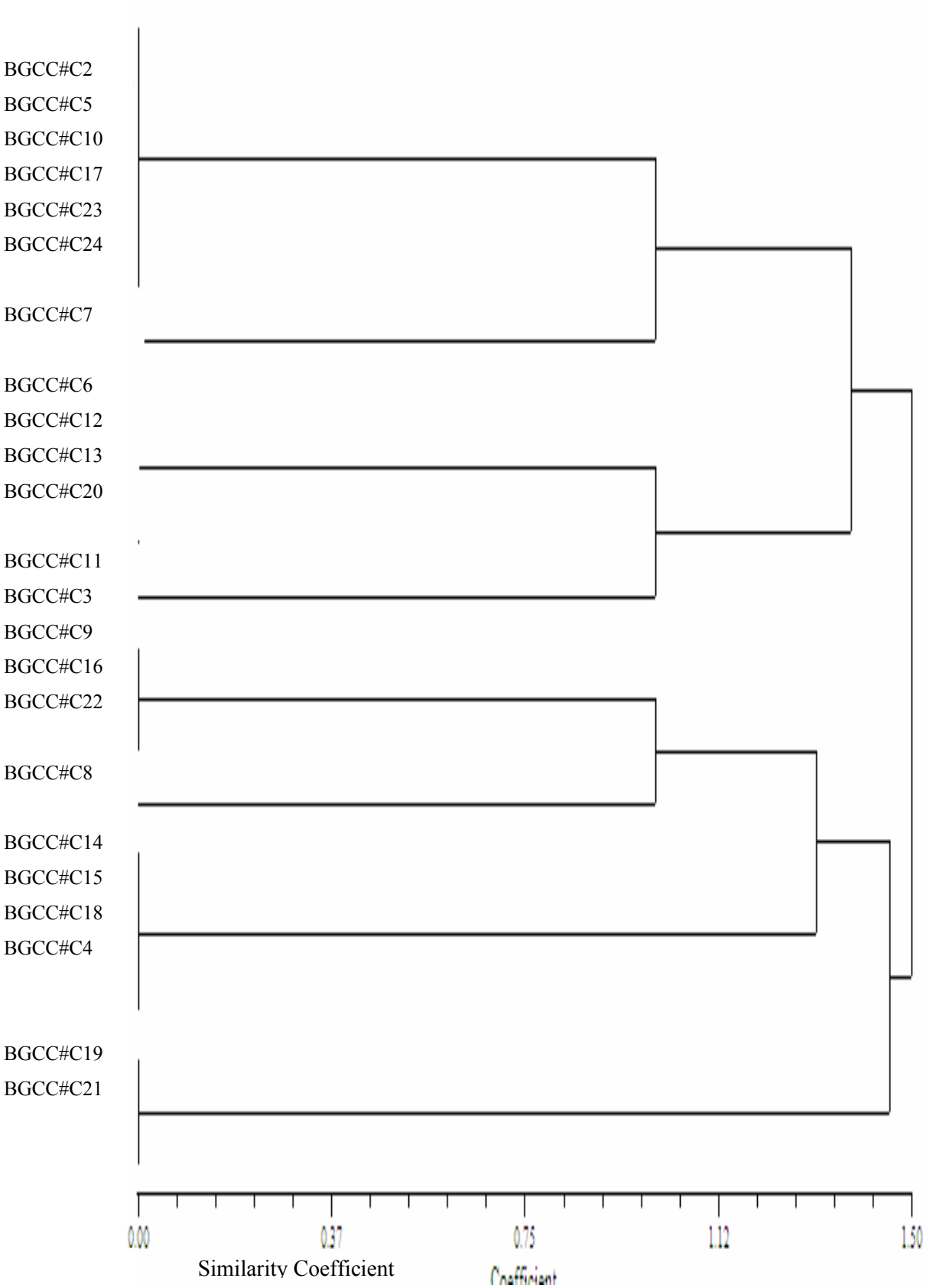

Figure 1: UPGMA cluster analysis of recombinants isolated from river Narmada, India.

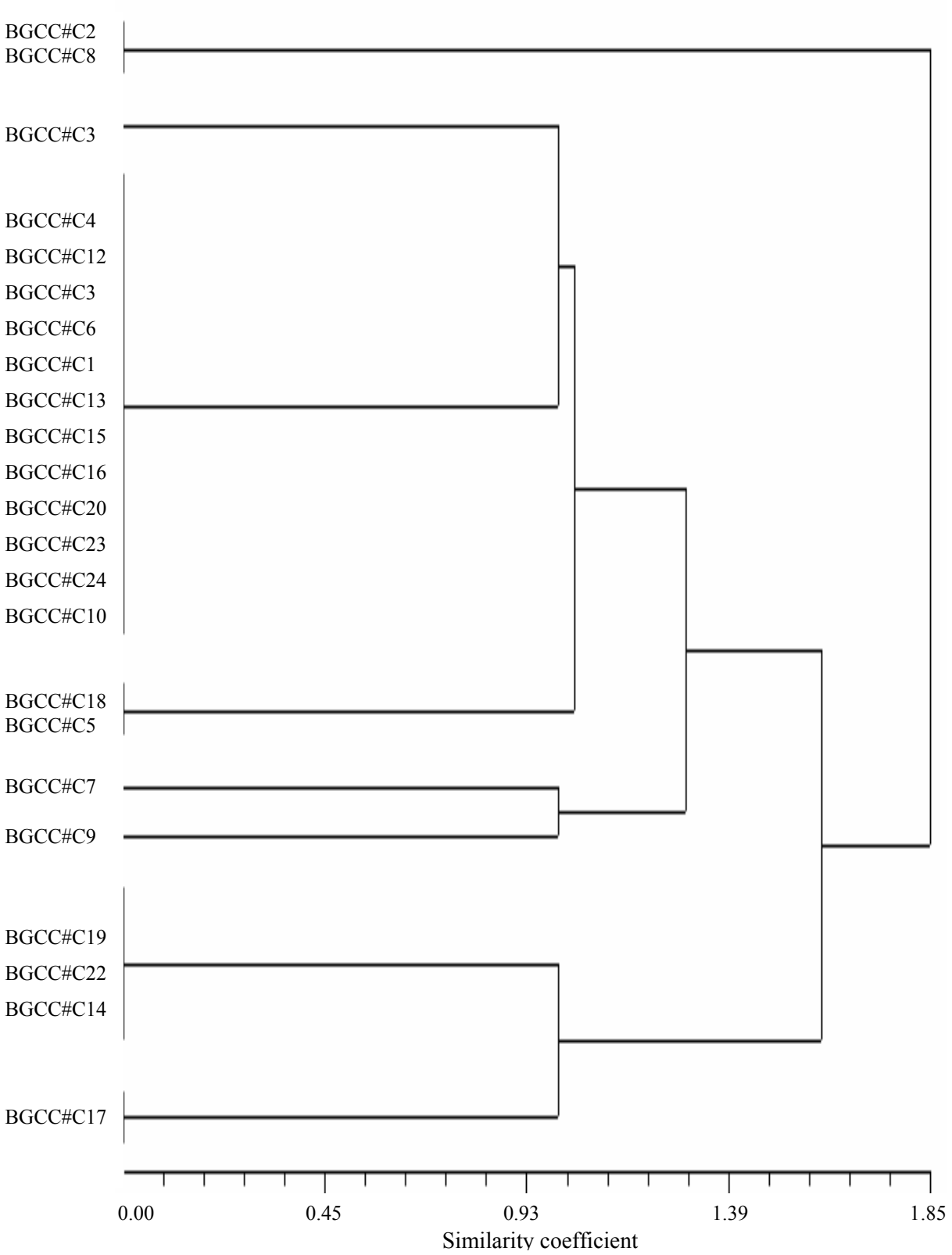

Figure 2: UPGMA cluster analysis on the basis of ARDRA with Rsa I of recombinants isolated from river Narmada, India.

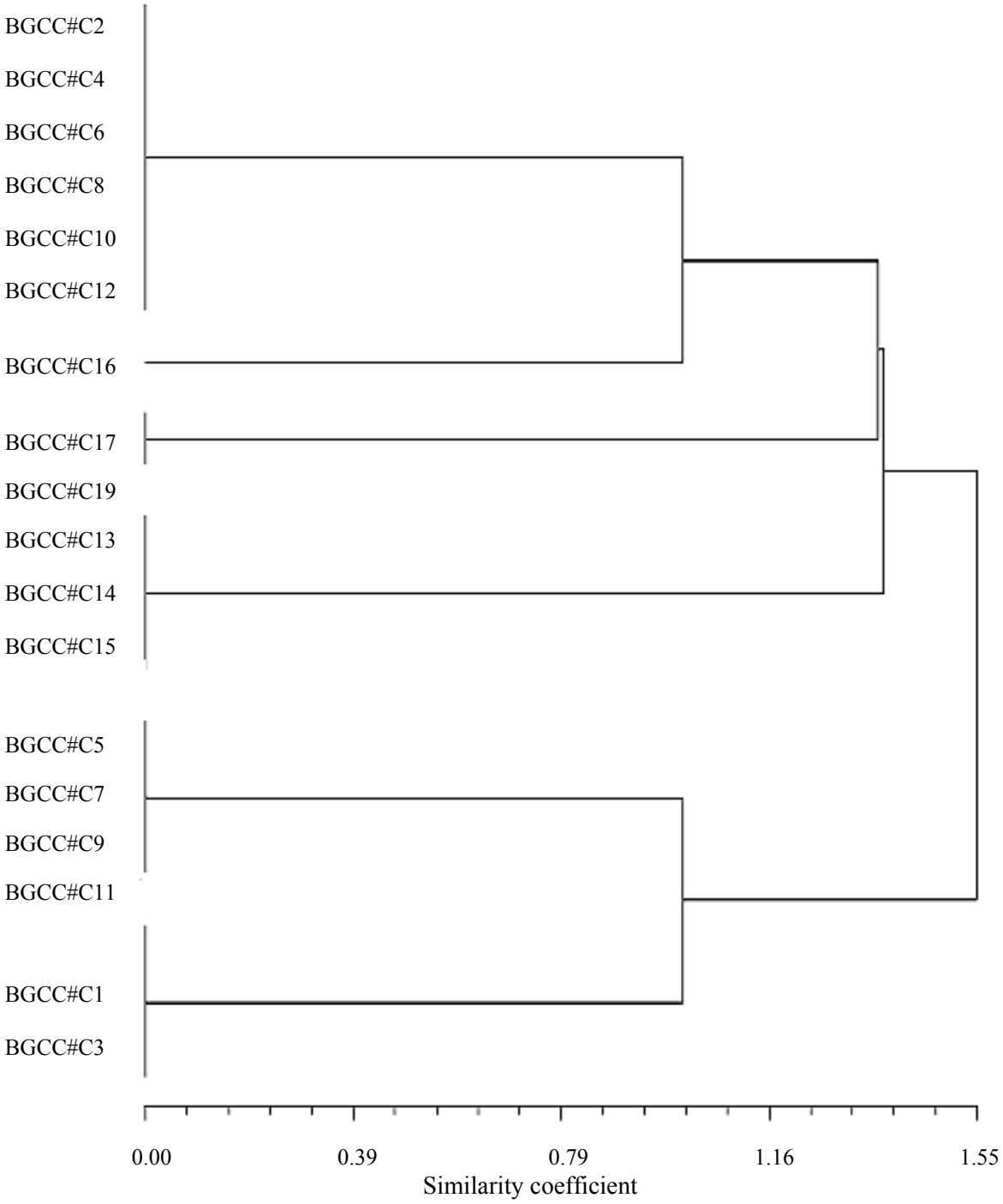

Figure 3: UPGMA cluster analysis on the basis of plasmid profiling from river Narmada, India.

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