Chapter 2: Development of a Deinococcus specific hemi-nested PCR method for the environmental detection and study of culture-independent diversity of Deinococcus sp.

When you have eliminated the impossible, whatever remains, however improbable, must be the truth.
- Sir Arthur Conan Doyle

2.1 Introduction

The family *Deinococcaceae* is represented by more than 40 validly described species, all (except one) belonging to a single genus, *Deinococcus* (Taxonomy Browser NCBI, *http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi),* which has members that are non-spore forming, gram positive, aerobic, chemorganotrophs, that exhibit remarkable resistance to radiation, desiccation and DNA damaging agents such as mitomycin (Battista, 1997; Cox and Battista., 2005; Blasisus et al., 2008; Slade and Radman, 2011). Study of this group of organisms is central for understanding the mechanism of radiation resistance (Battista, 1997; Liu et al., 2003; Daly et al., 2004; Daly et al., 2007). Their potential application in bioremediation of radioactive waste sites is also an important aspect (Daly, 2000; Fredrickson et al., 2000).

Environments from which deinococci have been isolated include diverse locations such as desert sand (de Groot et al, 2005, Rainey et al., 2005, Chanal et al., 2006), canned meats, (Anderson et al., 1956) geothermal springs (Ferreira et al., 1997), plant rhizospheres (Lai et al., 2006, Yang et al., 2008), and Antarctic soil (Hirsch et al., 2004). However the properties of these environments do not show any correlation that could proffer an explanation, based on natural selection, of their ionizing radiation (IR) resistant phenotype. It has been postulated that the IR resistance of *D. radiodurans* R1 (DR1) and its desiccation tolerance may be attributed to common machinery that repairs double-strand breaks in DNA (Slade and Radman, 2011).

Interest in the isolation of deinococal cultures has led to the rapid expansion of the list of validly known species from only seven a decade ago (Battista, 1997) to about 47 in 2011 (Slade and Radman, 2011). Most often, for the isolation of deinococci, exposure of the total population to ionizing radiation is utilized as a means of enrichment (Ferreira et al, 1997; Rainey et al., 2005; Shukla et al., 2007), since the slow growth and fastidious nutrient requirements of deinococci makes them difficult to be isolated without eliminating the co-existing faster growing radiation–sensitive population. The utilization of strong selective conditions for their isolation, however, obliterates information about their relative abundance in different environments. As a result, despite their wide spread distribution in several niches, comprehensive study on deinococcal ecology is lacking.

In addition to deinococcal isolates, a number of environmental 16S rRNA gene sequences having deinococcal affinities are reported form different habitats (Ribosomal database Project *http://rdp.cme.msu.edu/index.jsp)*. These nucleotide sequences are mostly obtained by culture-independent approaches involving construction of rRNA gene libraries of the bacterial community present in the environment, followed by sequencing and identification of deinococcal clones from these libraries on the basis of their sequence similarity with the 16S rRNA gene sequences of identified isolates (Carpenter et al, 2000; Chanal et al, 2006). Based on the abundance of deinococcal clones in 16S rRNA gene libraries, it could be surmised that deinococci comprise a small fraction of the total bacterial community.

An alternative to total community rRNA gene libraries for culture independent study of bacterial populations is to selectively target the 16S rRNA gene sequences of a specific subpopulation, to study the composition and diversity of only that population from the environment. Such a study necessitates the development of unique DNA probes and/or group specific PCR primers for the selective study of that subpopulation. Such primers/probes may be species specific (Pulawska et al., 2006; Wong-Villarreal et al., 2010), genus specific (Jurado et al., 2006; Klocke et al.,2006; Kim et al., 2011), phylum specific (Blackwood et al., 2006; Muhling et al., 2008) or they could be specific towards a gene involved in a common functional attribute of that subpopulation (Táncsics et al., 2010; Dandie et al., 2007; Gomes et al., 2007; Antony et al., 2010). Group specific primers give better insight into the distribution, abundance and diversity of a specific sub-population. Of the several radiation resistant genera described *Rubrobacter* has been investigated using group specific primers (Holmes et al., 2000).

Wise et al., (1996) developed DNA probes based on the four deinococcal species described then. The designed probes were claimed to species specific. Peltola et al., (2008) have described 16S rRNA gene based PCR primers for quantification of *D. geothermalis*, so that they serve as species specific primers. Thus, PCR primers/DNA probes specifically and exclusively targeting majority if not entire deinococcal community are lacking and this precludes culture-independent studies addressing specifically the diversity and species composition deinococci.

Radiation resistant bacteria can severe as sensitive indicators of radiation pollution or excessive exposure to radiation. Development of group-specific primers for the detection of the members belonging to the genus *Deinococcus* will aid in easy and rapid detection and quantification of their distribution in several different environment that can aid in understanding of the evolution of the radiation resistance mechanism of the deinococci. This chapter deals with the development of 16S rRNA gene based PCR method for the rapid detection of deinococci from the environmental samples. The method is successfully applied to comprehend the diversity of deinococci by PCR-denaturing gradient gel electrophoresis (DGGE) and to establish the deinococcal identity of isolates obtained.

2.2 Materials and Methods

2.2.1 Bacterial strains and growth conditions

The bacterial strains used in this study are listed in Table 2.1. The deinoocccal cultures were grown in tryptone-glucose-yeast extract (TGY) medium (0.5 % tryptone, 0.1 % glucose and 0.3 % yeast extract) at 30 °C while non-deinoccal cultures were grown in Luria-Bertani (LB) medium at 30 °C except *E. coli* which was grown at 37 °C. *Bradyrhizobium japonicum* 61A152 was grown on Congo red-yeast extract-mannitol agar at 30 °C.

2.2.2 Bioinformatics tools used for the designing of *Deinococcus* specific primer

The 16S rRNA gene sequences of genus *Deinococcus* as listed in the Ribosomal Database Project (RDP) (*http://rdp.cme.msu.edu/*) were aligned using Clustal W (*http://www.ebi.ac.uk/clustalw*). The specificities of the conserved sequences were examined using the RDP PROBE MATCH tool by using sequences of approximately 20 nucleotide length as the query.

2.2.3 Soil sample collection

Soil samples from several arid and non-arid regions in India were collected. Top 5 cm layer of soil was collected and stored at 4 °C for culturable studies. An aliquot of the sample was preserved at -20 °C for community DNA isolation. The physico-chemical characteristics of the soil samples were determined at the Soil testing laboratory, Gujarat State Fertilizer Company (GSFC), Vadodara. The total Cr^{6+} concentration of chromium contaminated soil sample was estimated by heat digestion of soil sample by

HNO_3 + $HClO_4$ and analyzed by Atomic absorption spectrophotometer (AAS) (GBC, Australia) at GSFC, Vadodara.

2.2.4 Isolation of radiation resistant bacteria

One gram of soil sample was irradiated for 10 kGy using Co_{60} source at 4 kGy/h. The irradiated soil sample was suspended in 4.5 ml of saline (0.85 % NaCl), vortexed thoroughly and appropriate dilutions were plated on TGY agar plates and incubated for 4-5 days at 30 °C. Morphologically distinct colonies were purified and maintained on TGY plates.

Table 2.1 Bacterial strains used in this study

Bacterial Strain	Description	Source or Reference
Deinococcal strains		
D. radiodurans R1 ATCC 13939 (DR1)	Type strain	Prof. Mary Lidstrom, University of Washington, USA
D. radiophilus ATCC27603	Type strain	Dr. Y. N. Lee, Chungbuk National University, Korea
D. indicus MTCC 4913	Arsenic resistant	Microbial Type Culture Collection (MTCC), Chandigarh, India
Deinococcus sp. X2	Lab. isolate	Shukla et al., 2007
Deinococcus sp. G8	Lab. isolate	Shukla et al., 2007
Non- Deinococcal strains		
Rhodococcus rhodochorous MTCC 2569	High G+C gram positive, Family *Nocardiacae*	MTCC, Chandigarh
Micrococcus flavus, NCIM 2763	High GC gram positive, Family *Micrococcacae*	National centre for industrial microorganisms, National Chemical Laboratory, Pune, India
Bacillus subtilis	Low GC gram positive, Phylum *Firmicutes*	Department culture collection

Bradyrhizobium japonicum 61A152	Phylum *Alpha-Proteobacteria*	Joshi et al., 2008
Escherichia coli DH5α	Phylum *Gamma-Proteobacteria*	Department culture collection, Sambrook and Russell, 2001

2.2.5 Determination of UV tolerance of the bacterial isolates

The bacterial cultures were grown in TGY broth to late exponential phase, pelleted, washed with saline and OD_{600} adjusted to 0.4 and 10 ml of it was exposed to UV in a sterile petri plate using UV chamber (Wilber Lourmat, France) with 254 nm UV source. Aliquots of 1 ml were withdrawn at an interval of 200 J/m^2, appropriately diluted, and plated on TGY agar plates, incubated at 30 °C. The colonies were enumerated after 72 h.

2.2.6 Biochemical characterisation of the radiation resistant isolates

2.2.6 a Biochemical characters

Biochemical characterisation of the radiation resistant isolates were done in accordance to the Bergey's Manual

2.2.6 b Single carbon utilisation

Carbohydrate utilisation test was done using the Hi-Carbo test kit (Hi-media, Bombay, India). The bacterial cultures were grown to late exponential phase and washed with saline. A 50 µl of this was inoculated in each of the carbohydrate well provided in the kit and incubated at 30 °C for 48 h.

2.2.6 c Antibiotic –senstivity test

A 100 µl of overnight grown culture was spreaded on TGY agar plates. The desired antibiotic octadisc was placed on the plate and incubated at 30 °C for 48 h.

2.2.6 d pH and temperature optima for growth of radiation-resistant bacterial isolates

To determine the temperature optima the cultures were grown in TGY and incubated at different temperatures. OD_{600} was monitored after 48 h. pH optima for the bacterial isolates was determined in TGY broth prepared in 0.1 M buffer of varying pH range. Acetate buffer for pH range 3, 4 and 5; phosphate buffer for pH 6 and 7; and Tris buffer for pH 8, 9, 10 were employed for determining the pH optima.

2.2.7 Soil community DNA isolation

Community DNA was isolated by the method described by Porteous et al., (1997). Briefly 0.5 g of soil was added to 925 µl of SDS lysis buffer (0.25 M NaCl, 0.1 M Na_2EDTA, 4 % SDS) and 75 µl of guanidine isothiocyanate, homogenized for 1-2 min by vortexing and then incubated for 1 h at 68 °C. The samples were then centrifuged at 13,000 g for 15 min at 4 °C and to the supernatant 300 µl of 30 % PEG 8000 in 1.5 M NaCl was added. The DNA was precipitated at -20 °C for 1 h followed by centrifugation at 13,000 g for 15 min at 4°C. Pellet was dissolved in 900 µl 2x CTAB solution (2 % hexadecyl trimethyl ammonium bromide, 1.4 M NaCl and 0.1 M Na_2EDTA) and incubated for 15 min at 68 °C. To the above solution 925 µl chloroform was added and gently mixed and centrifuged at 13,000 g for 10 min at room temperature. The aqueous DNA solution was precipitated with 1 ml isopropanol for at least 15 min at -20 °C, centrifuged at 13000 g for 15 min at 4 °C to collect the pellet. Pellet was dissolved in 450 µl 2.5M ammonium acetate (NH_4OAc) and 1ml of 95 % ethanol was added, kept for incubation at – 20 °C for 15 minutes and centrifuged at 13000 g for 15 min at 4 °C. The pellet obtained was then washed with 70 % ethanol and dissolved in minimum amount of double distilled water.

2.2.8 PCR amplification of 16S rRNA gene using universal primers

Universal eubacterial 16S rRNA PCR primers, Eub27F and Eub1107R (Table 2.2), obtained from MWG Biotech, Germany, were used for amplification of 16S rRNA gene from either pure cultures or from soil community DNA. PCR was carried out in 30 µl reaction mixture consisting of 1 ng of template DNA, 30 pmole of each of the primers, 1 µl of mixture of dNTPs 2.5 mM each, 1.5 U of Taq polymerase, combined with appropriate amount of 10X Taq polymerase buffer. Amplification was carried out in a thermal cycler (Applied Biosystems, USA) with an initial denaturation at 94 °C for 3 min, followed by 30 cycles of denaturation at 94 °C for 45 s, annealing at 58 °C for 30 s, elongation at 72 °C for 1 min and 30 s, with a final elongation at 72 °C for 10 min. Amplicons were detected by electrophoresis on 1.0 % (w/v) agarose ethidium bromide gels.

2.2.9 PCR Amplification of 16S rRNA gene using *Deinococcus* specific primer

16Sr RNA gene amplicon obtained as above (Section 2.2.8) was used as template for hemi-nested PCR using forward *Deinococcus* specific primer Deino202F (Table

2.2), (numbering corresponding to the 202-222 *D. indicus* 16S rDNA gene sequence) and 1107R as the reverse primer. Following additives were added to the PCR mixture individually or in combinations mentioned at a final concentration as given: betaine, 1M; DMSO, 10 %; glycerol, 10 % and formamide, 10 %. About 10- 50 ng of the template DNA was combined with 30 pmole of each of the primers, 1 µl of 2.5 mM of dNTPs, 1.5 U of *Taq* DNA polymerase in 10X *Taq* polymerase buffer. The PCR was carried out at an initial denaturation at 94 °C for 3 min followed by 30 cycles each consisting of denaturation at 94 °C for 45 s, annealing at 58 °C for 30 s, elongation at 72 °C for 1 min and 30 s repeated for 30 cycles with a final elongation at 72 °C for 10 min.

For amplification in presence of competing DNA, mixture of 20 ng each of 16S rRNA gene fragments amplified from different organisms was combined with varying concentrations of 16S rDNA of DR1 at final concentration and used as template for *Deinococcus* specific nested PCR. .

Table 2.2 PCR primers used in this study

Primer	Sequence (5′-3′)	Reference
Eub27F	AGAGTTTGATCCTGGCTCCAG	Shukla et al., 2007
Eub1107R	GCTCG TTGCGGGACTTAACC	Shukla et al., 2007
Deino202F	GGGTTGCGTTCCATCAGC	This study
GC-P341F	CGCCCGCCGCGCGCGGCGGGCGGGGCGGG GGCACGGGGGGCCTACGGGAGGCAGCAG	Muyzer et al., 1993
Eub534R	ATTACCGCG GCTGCTGG	Muyzer et al., 1993

2.2.10 Construction and analysis of deinococcal 16S rRNA gene fragment library

The community rDNA gene fragments obtained by PCR amplification of soil community DNA using *Deinococcus* specific PCR was purified and cloned in the

pTZ57R/T (InsTAclone, MBI Fermentas, USA) following the manufacturer's instructions and transformed in to *E.coli* DH5α. The presence of the recombinant plasmids in different transformants was confirmed by PCR with Deino202f and Eub1107R. The clones were analyzed by restriction fragment length polymorphism (RFLP) using restriction enzymes *Hha*I and *Msp*I. The digested products were electrophoresed using 8 % polyacrylamide gel and visualized by silver staining.

2.2.11 PCR-DGGE of 16S rRNA gene

An appropriate dilution of PCR product obtained upon *Deinococcus* specific hemi-nested PCR, using either environmental DNA or genomic DNA of a pure culture, was reamplified using GC- P341F containing the GC clamp and Eub534R by the protocol described by Muyzer et al., (1993). The products were analysed on 8 % polyacrylamide gel with denaturing gradient of 50 to 70 %, where 100 % denaturant contained 7 M urea and 40 % formamide. Electrophoresis was carried out using the D-code system (Bio-Rad, USA) for 16 h at 60 °C in 1X TAE buffer at 75 V, following which the DNA bands were visualized by silver staining. The DGGE gels were photographed and analysed by AlphaEase 4.0 software (Alpha Innotech , USA). The Shannon–Weaver index was calculated as

$H= -\Sigma(n_i/N) \log (n_i/N)$

Where n_i is the area of the peak and N is the sum of all the area covered under individual peaks of the densitometric curve. The DGGE bands were excised and gel pieces crushed in 50 µl distilled water, followed by centrifugation at 10,000 rpm for 3min. A 5µl aliquot of the supernatant was added to PCR reaction mixture for reamplification using the GCP341F and Eub534R primers. The reamplified bands were checked for their purity on DGGE gel as mentioned above and sequenced.

2.2.12 DNA sequencing and phylogenetic analysis

Cloned DNA samples were sequenced at Bangalore Genei Pvt. Ltd. (India). The phylogenetic analysis of the sequences was performed using the Tree Building tool at RDP (http://rdp.cme.msu.edu/). The BLASTn search tool (Altschul et al., 1990) was used to determine sequence homology and the most similar sequences in the GenBank database. All 16S rRNA gene sequences obtained in this study have been deposited in the GenBank and accession numbers are provided in the results section.

2.3 Results and Discussion

2. 3.1 Designing and in-silico validation of *Deinococcus* specific primer

All the type strains representing genus *Deinocoocus* listed at RDP (*http://rdp.cme.msu.edu/*) were used for the alignment using Clustal W and several patches of sequences conserved in all deinococci were obtained. The conserved sequences comprising of 18-mers were analysed using for their presence in other taxonomically distinct groups of bacteria. Table 2.3 lists the data for all the conserved sequences and the hits in other phyla such as *Proteobateria, Firmicutes* etc. The sequence corresponding to nucleotide positions 202-222 (numbered according to *D. indicus* sequence) had maximum coverage in the genus *Deinococcus.* This oligonucleotide sequence identified had 99.4 % hits in *Deinococcus*, demonstrated excellent match to this group specifically (Table 2.3). Whether all deinococci show conservation of this sequence was ascertained and it was found that this sequence was present with 100 % match in 27 of the 40 species listed at the RDP database. The type strains *D. alpitudinis, D. claudioa and D. radiomolis,* were recognized with a single mismatch at 5′ end of the primer, indicating that these may be amplified by primers complementary to this sequence. Seven species viz. *D. misasensis, D. roseus, D. papagonenesis, D. periaridlitoris, D. sonorensis, D. maricopensis* and *D. pimensis,* form a coherent group in the *Deinococcus* clade not efficiently recognized by this primer. Earlier probes reported by Wise et al., (1996) as *Deinococcus* specific, when subjected to a similar analysis, were found to be present in maximum of 4 type strains of genus *Deinococcus*. Thus, an oligonucleotide primer corresponding to the 202-222 region, Deino202F (Table 2.2), was identified as a potential *Deinococcus* specific primer, targeting majority of species.

2.3.2 Development of *Deinococcus* specific hemi-nested PCR protocol

A two step hemi-nested PCR approach was developed, in which the first amplification was done with universal eubacterial primers Eub27F and Eub1107R, followed by a second PCR, that utilizes the 16S rDNA form the first round as template, with *Deinococcus* specific primer, Deino 202F and universal eubacterial primer 1107R (Fig. 2.1).

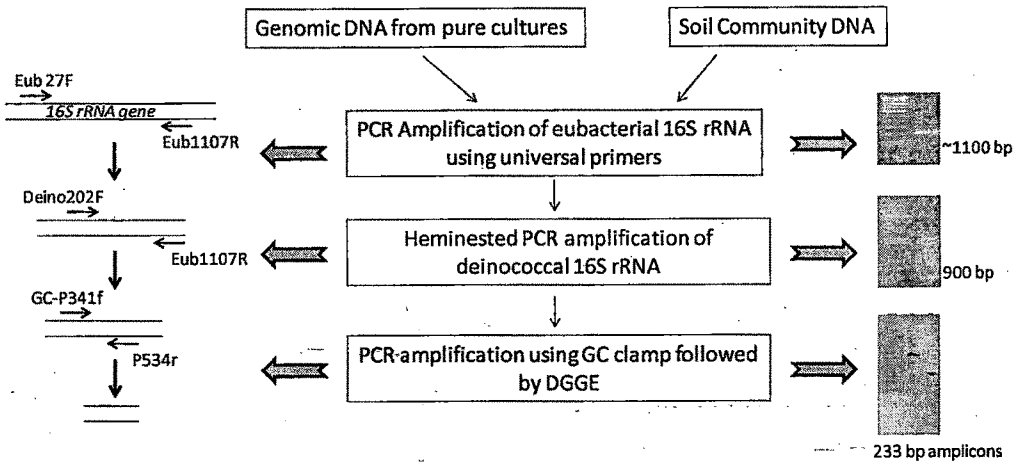

Fig. 2.1: Schematic representation of the *Deinococcus* specific heminested PCR protocol and culture-independent DGGE analysis of deinococcal diversity

The initial standardization of the protocol was done using genomic DNA as template, of DR1 as a positive control and of *E. coli* as a negative control. PCR reaction mixture containing 1M betaine was found to improve specificity at 58 °C, as opposed to other additives, to yield a band of expected band size, 900 bp, only with the deinococcal template DNA (Fig. 2.2). The GC content of the deinococci varies between 60-70 %; betaine, an isostabilizing agent, equalizes the contribution of GC- and AT-base pairing to the stability of the DNA duplex (Henke et al., 1997), possibly thereby improving the amplification. Therefore, subsequent PCR amplifications were carried out at annealing temperature of 58 °C in presence of 1 M betaine. Positive amplification was observed with *D. radiophilus* ATCC 27603, *D. indicus* MTCC 4913 and our laboratory isolates *Deinococcus* sp. X2 and *Deinococcus* sp. G8 (Shukla et al., 2007), but not with other phylogenetically diverse bacteria tested (Fig. 2.3).

For the suitability of the *Deinococcus* specific PCR to explore the diversity of this group from natural environments, the protocol should be robust in terms of its sensitivity so as to detect deinococci from mixed communities, in which deinococci are usually far out-numbered by other taxonomically distinct bacteria. An amplicon of 900 bp was obtained using 16S rDNA of *D. radiodurans* R1 ATCC 13939 with a sensitivity of 1 pg of first PCR product (Fig. 2.4a).

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Table 2.3: Specificity of the conserved regions of the aligned 16S rRNA genes of *Deinococcus* sp.

Region	Nucleotide position	Sequence	% Hits			
			Deinococcus	*Proteobacteria*	*Firmicutes*	Others
1	73-90	AGTGGCGCACGGGTGAGTA	1.166	66.66	0.84	31.260
2	202-222	GGGTTGCGTTCCATCAGC	99.4	0.5	0	0
3	265-285	CCGGCCTGAGAGGGTGGCCGG	33.8	6.08	12.5	47.52
4	288-308	ACAGGGGCACTGAGACACGGG	89.6	0.30	0	10.03
5fl	310-327	CCCACTCCTACGGGAGGC	61.062	0.871	0.435	37.63
5fr	328-345	AGCAGTTAGGAATCTTC	95.97	0.53	0.13	3.35
6	367-388	GAGCGACGCCGCGTGAGGGA	41.16	1.405	21.108	35.54
7fl	477-492	CGTGCCAGCAGCCGCG	0.209	35.008	28.36	36.41
7r	493-508	GTAATACGGAGGGTGC	0.86	81.53	0.04	90.09
8fl	510-529	AAGCGTTACCCGGAATCACTG	73.55	1.23	0.41	24.79
8fr	530-546	GGCGTAAAGGGCGTGTA	14.3	28.1	30.56	27.22
9fr	642-657	CTGGTGTAGCGGTGG	2.155	0.407	1.117	96.33
9fl	658-673	AATGCGTAGATACCAG	83.5	1.877	1.877	12.67
10	725-743	GCGCGAAAGTGTGGGGAGC	17.908	6.805	68.33	8.38
11fr	743-764	AACCGGATTAGATACCCGGG	19.46	0.38	3.8	76.34
11fl	765-785	TAGTCCACACCCTAAACGATG	65.14	15.22	0.07	18.37
12fr	839-857	CCGCCTGGGAAGTACGGCC	10.604	58.3	0.46	30.62
12fl	858-876	GCAAGGTTGAAACTCAAAG	0.521	17.96	65.86	15.65
13fr	878-899	AATTGACGGGGGCCCGCACAAG	0.345	37.37	21.651	59.37
13fl	900-922	CGGTGGAGCATGTGGTTTAATT	0.512	49.731	34.197	15.558

14	934-947	AAGAACCTTACCAG	1.13	15.71	65.64	17.50
15	983-997	GGTGCCCTTCGGGGA	3.81	29.42	64.70	2.050
16fr1	1012-1029	GCTGCATGGCTGTCGTCA	0.229	75.25	0.2921	24.22
16fr2	1030-1048	CTCGTGTCGTGAGATGTTG	0.339	37.59	37.85	24.21
16fl	1049-1076	GGTTAAGTCCCGCAACGAGCGCAAC CC	0.338	36.988	35.085	27.58
17fl	1133-1154	GGAGGAAGGCGGGGATGACGTC	2.377	11.438	71.85	14.328
17fr	1155-1174	TAGTCAGCATGGTCCTTACG	98.63	0	0	1.36
18	1185-1201	ACACACGTGCTACAATG	0.44	46.667	35.56	17.31
19	1271-1286	AGTCTGCAACTCGACT	0.307	59.47	21.07	19.14
20	1296-1313	TGGAATCGCTAGTAATCG	0.34	29.64	44.05	25.95
21fl	1328-1349	GCGGTGAATACGTTCCCGGGCCT	0.461	30.214	25.480	43.843
21fr	1350-1373	TTGTACACACCGCCCGTCACACCA	0.151	57.56	32.244	10.041

fl and fr refer to the left and right half of the longer alignment blocks.

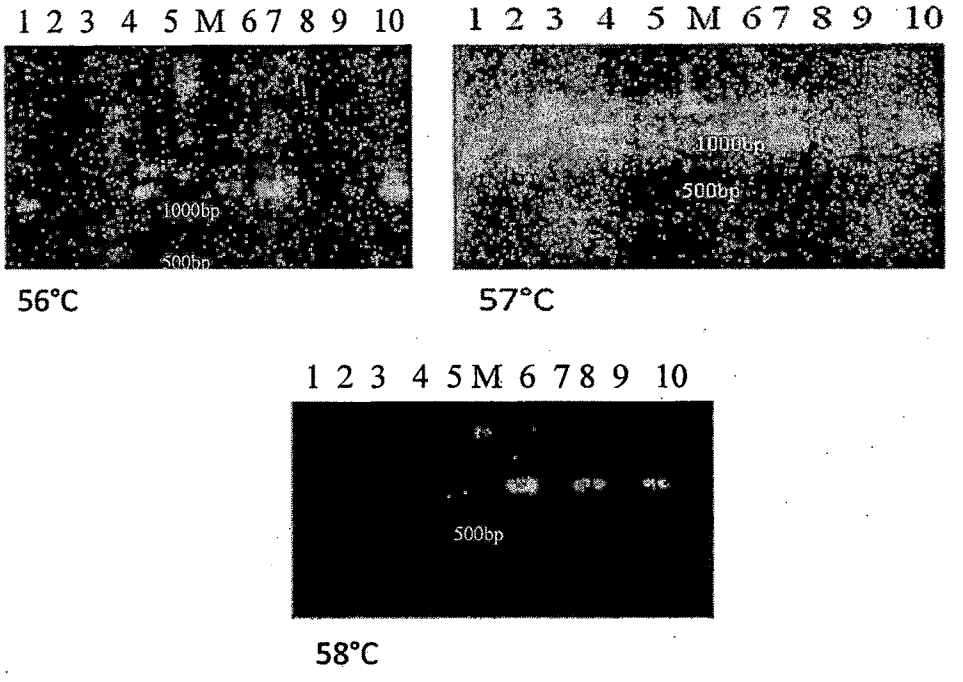


Fig. 2.2: Optimization of the *Deinococcus* specific hemi-nested PCR. 16S rDNA was used as the template for the second round of nested PCR. Lanes 1-5 in each gel represent PCR products obtained using *E. coli* genomic DNA as template; lanes 6-10 with *D. radiodurans* R1 genomic DNA as template. Additives used for PCR are as follows: Lanes 1 and 6, 1M Betaine; Lanes 2 and 7, 2.5 M Betaine; Lanes 3 and 8, 10 % DMSO and 10 % Glycerol; Lanes 4 and 9, 10 % Formamide and 10 % Glycerol; Lanes 5 and 10, without any additive. M, (marker) 500 bp ladder. Annealing temperatures are given alongside the gel picture.

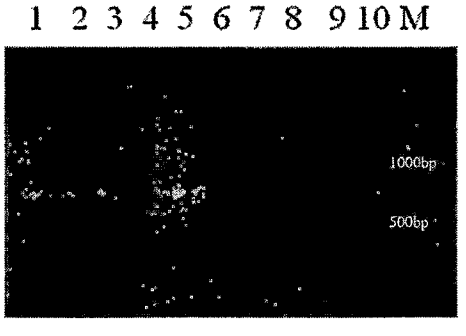


Fig. 2.3: Specificity of the *Deinococcus* specific hemi-nested PCR. Lanes : 1, *D. radiodurans* R1 ATTC13939; 2, *Deinococcus* sp.G8; 3, *D. indicus*; 4, *Deinococcus* sp. X2; 5, *D. radiophilus*; 6, *R. rhodochorous* MTCC 2569; 7, *M. flavus*; 8, *B. japonicum* 61A152; 9, *E. coli*; 10, *B. subtilis*; Lane M, 500 bp ladder.

In order to study whether these primers are suitable for specific amplification of deinococcal 16S rRNA gene from a heterogenous mixture containing template DNA from non-target organisms, a concoction of 20ng of each of 16S rDNA of the non-deinococcal representatives, *R. rhodochorous* MTCC 2569, *M. flavus, B. japonicum* 61A152, *E.coli* and *B. subtilis* was made and to this 16S rDNA of DR1 was added at different concentrations. This was used as a template for the second step of the *Deinococcus* specific nested PCR with Deino202F and 1107R as primers. An expected amplification of 900 bp was obtained in presence of competing non-target template DNA at all the concentrations (Fig. 2.4b). The sequence of the amplified product showed 100 % sequence similarity with DR1 16S rRNA. This signifies the specificity of designed primers in the detection of deinococci from a mixed population such as in soils.

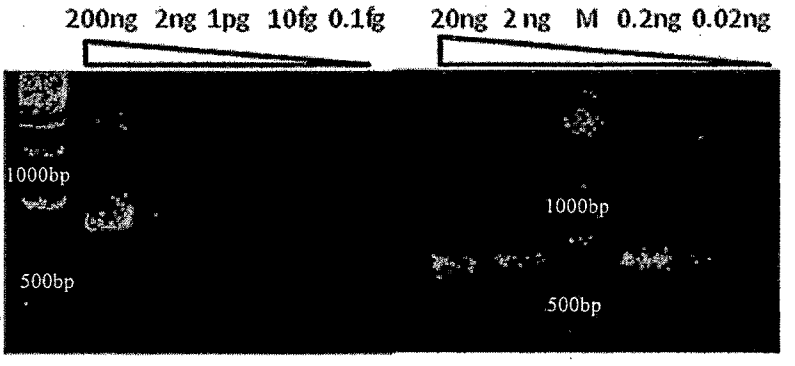


Fig. 2.4: Sensitivity of the hemi-nested PCR amplification for the amplification of deinococcal 16S rRNA gene fragments. a) Amplification using different amounts (as indicated) of DR1 16S rDNA as template. b) Amplification using mixture of 20 ng each of 16S rDNA from different pure cultures and spiked with varying concentrations of DR1 16S rDNA as indicated.

2.3.3 PCR amplification of deinococcal 16S rRNA gene fragments from environment using *Deinococcus* specific primers

Samples were collected from western (GRK, LRK, CS, PP, CRC, BK), northern Himalayan samples (LD , LH, BR) and southern parts of India (CH) (Fig. 2.5). Sampling sites included diversified environments like salt deserts (LRK and GRK), cold arid environments (LD and LH) and contaminated soils (PP and CRC).The physico-chemical properties of the soil samples, as given Table 2.4, show that

samples varied in their physico-chemical properties, pH ranging from 7 to 8.5, while organic carbon ranged from 0.11-1.52 %. Soils with electrical conductivity (EC) > 4.0, such as samples LRK, CS, CH, are considered of high salinity (Yuan et al., 2007). The 16S rRNA gene was amplified from the total community DNA extracted from environmental samples and the resulting amplicon was subjected to hemi-nested PCR. Interestingly all the samples tested gave positive reaction however CS sample consistently gave a significantly smaller sized PCR product than expected (Fig. 2.6) indicating differences in this group. This infers the sensitivity of the designed *Deinococcus* specific PCR protocol to detect deinoocci from diverse environments.

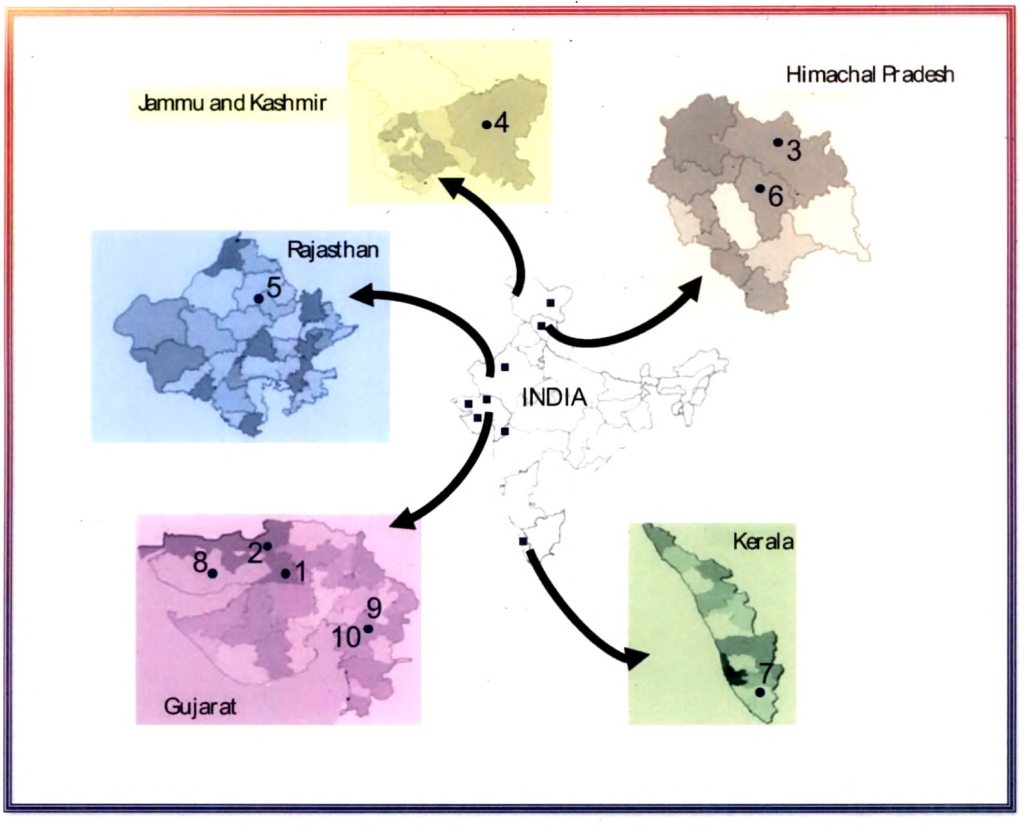


Fig. 2.5: Map showing the location of soil sampling sites. Central map shows locations of the sites along the country and the maps on the sides show more precisely the location within the states, with sample number given at the location in individual states. Samples locations are as follows with sample designations given in brackets

1: Little Rann of Kutch (LRK); 2: Greater Rann of Kutch (GRK) 3: Lahaul (LH); 4: Ladakh (LD); 5: Bikaner (BK); 6: Soil at the banks of river Baes, Kullu (BR); 7: Chavra (CH); 8: Coastal soil (CS); 9: Petrol Pump (PP), Vadodara; 10: Chromium contaminated soil (CRC), Vadodara;

Table 2.4: Physico-chemical analysis of the soil samples

Sample Number	Location of soil sample collection site (Sample designation)	Sample collection period	pH	Organic Carbon (%)	Organic Nitrogen (%)	Electrical conductivity (1:2) $mScm^{-1}$
1.	Little Rann of Kutch, Gujarat (LRK)	Dec, 2007	7.45	0.50	0.043	17.31
2.	Greater Rann of Kutch, Gujarat (GRK)	Jan, 2007	8.15	1.30	0.1118	2.98
3.	Lahaul, Himachal Pradesh (LH)	July, 2006	7.13	1.43	0.122	0.22
4.	Ladhak, Jammu & Kashmir (LD)	June, 2006	8.23	1.25	0.1075	0.29
5.	Bikaner, Rajasthan (BK)	May, 2007	7.75	0.25	0.0215	1.08
6.	Beas River bank soil, Himachal Pradesh (BR)	May, 2006	7.15	0.14	0.0129	0.14
7.	Chavara, Kerala (CH)	Jan, 2007	8.55	0.11	0.00946	4.84
8.	Coastal soil, Kandla (CS)	Jan, 2005	8.37	1.25	0.1075	17.70
9.	Soil near petrol pump, Vadodara (PP)	Nov, 2006	6.97	1.52	0.1307	0.30
10.	Chromium contaminated soil, Vadodara (CRC)	May, 2007	8.30	0.82	0.070	2.32

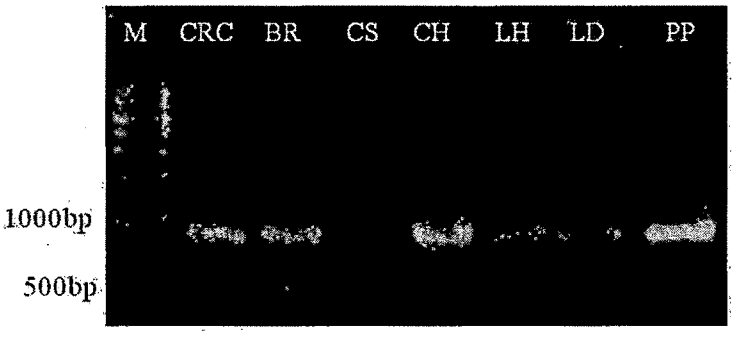


Fig. 2.6: *Deinococcus* specific nested PCR amplification from representative soil samples used in the study. M: 500 bp Marker. Sample names as given in Table 2.4.

The ability to withstand high doses of radiation in bacteria is considered as an outcome of repeated cycles of hydration and dessication despite the apparent absence of natural radioactivity on Earth (Cox and Battista, 2005; Shukla et al., 2007; Slade

and Radman, 2011). Our results of positive amplification from xeric environments further affirm that such environments naturally select upon organisms that may be radiation resistant. Positive results from desert samples BK, LD, LH, GRK, is in coherence with the fact that deinococci have been routinely isolated from xeric environments such as the deserts of Sonoran (Rainey et al., 2005), Sahara (de Groot et al., 2005), Tataouine (Chanal et al., 2006), Kutch (Shukla et al., 2007), as well as cold deserts of Antarctica (Hirsh et al., 2004) and constitute about 37 % of all the type strains (Slade and Radman, 2011).

Recently several deinococci have been reported from a radioactive spring samples (Asker et al., 2008; Wang et al., 2010; Peng et al., 2009; Asker et al., 2011). Chhavra (CH) lies in the south Indian state of Kerala, the coast of which contains the world's highest levels of natural radioactivity due to the local abundance of monazite, a mineral containing 10 % thorium phosphate (Forster et al., 2002). Detection of deinococci in CH sample emphasizes the enrichment of the deinococci due to high natural radioactivity. However deinococcal clones were not detected amongst 16S rRNA gene library from uranium mining impacted soils in South Dakota, USA (Rastogi et al., 2010). Detection of deinococci from CRC sample, with chromium levels of 13.5 g kg^{-1} as determined by atomic absorption spectroscopy, as well as from petrol contaminated soil sample is interesting, since it is indicative of presence of strains possessing metal resistance or hydrocarbon tolerance, which are of significance in bioremediation of radioactive waste sites.

Two clone libraries using *Deinococcus* specific 16S rRNA gene were constructed from CRC and GRK samples. A total of 75 clones were obtained and Amplified Ribosomal DNA Restriction Analysis (ARDRA) revealed the presence of a single dominant clonal population in CRC sample, whereas GRK sample showed three different phylotypes. The sequences of four random clones, designated 6crc, 19crc, 22crc, and 25crc showed all of them to be affiliated to *D. radiopugnans* (Fig. 2.7) (Appendix II), suggesting the abundance of this species. *D. radiodurans* has been reported from vadose zone at the Hanford site having high Cr concentration as well as high background radioactivity. The Cr concentration at the Hanford site ranged from 0.02- 829.76 μg g^{-1} soil which is considerably lower than that found at CRC site (Fredrickson et al., 2004). The presence of deinococcal strains from polluted soils indicates the robust physiology of this group of organism.

GRK represents a salt desert habitat. Shukla et al., (2007) have earlier reported the isolation of deinococcal strain from such habitat however no attempt to study the un cultivable deinoccocal diversity from such environments was made. This is the first attempt to assay the deinococcal diversity from such environment. Three different phylotypes were obtained from the clone library showing maximum homology to *D. hopeinsis*, and *D. murrayi* (Appendix II). Clone library from Calamita, a black ferromagnetic sand from a marine iron ore on Elba Island (Italy) showed predominance of the clones that had affiliation to radiation resistant bacteria *Trupera* from the *Deinococcus –Thermus* decent and *Rubrobacter*. However no representation of deinococci was reported (Perfumo et al., 2011). *D. hopeinsis* has been earlier reported from Sonoran desert while there are no reports of isolation of *D. murrayi,* a moderate thermopile, from such environments.

2.3.4 *Deinococcus* diversity analysis using PCR-DGGE

To ascertain the suitability of the deinococcal nested PCR protocol for studying deinococcal diversity by a sensitive molecular fingerprinting technique, viz. PCR-DGGE a three step PCR-DGGE was adopted wherein the nested PCR product was used as the template for amplification using GC- P341F and Eub 534 R (Fig. 2.1). The protocol was adopted to enhance the representation of the *Deinococcus* specific sequences in the 16S rRNA gene pool of the community.

Various deinococcal pure cultures, when subjected to the three-step DGGE protocol, showed single bands at different positions, indicating 16S rRNA gene fragments showed sufficient sequence diversity to differentiate *Deinococcus* species. Environmental samples when subjected to three step deinococcal specific PCR-DGGE, showed several bands of varying intensities, with majority of samples having a single predominant band, suggesting dominance of a specific species as indicated by the clone library for CRC sample. Taking into consideration all the samples, about ten differently migrating major bands and several lighter bands could be discerned, demonstrating the high resolution of the method. It is known that culturable members of *Deinococcus* species isolated from similar environments are not necessarily phylogenetically closely related (Fig. 2.7) and our results are in coherence with the earlier observation.

Dar et al., (2005) have demonstrated that three step method increased the detection sensitivity of the sulfate reducing bacteria, otherwise a poorly represented population.

The novelty of our approach is that the hitherto unknown diversity of major and minor representatives of deinococci was revealed as distinct ribotypes in the DGGE profile (Fig. 2.8 and Fig. 2.9). Fig. 2.9 demonstrates the relationship between the several sampling sites, the desiccated samples such as Ladhak and Little Rann of Kutch were related while those of Lahaul and Bikaner were related. Shannon–Weaver diversity index showed values ranging from 0.64 to 2.7 (Table 2.5), indicating variation in the species abundance. The higher indices of samples from salt desert of Gujarat (GRK and LRK) are suggestive of high species richness. The identity of the DGGE bands marked in Fig. 2.8, as revealed by their nucleotide sequences, is provided in Table 2.6. The sequence of the major band in CRC sample is in congruence with the observation from CRC group specific clone library. 16S rRNA gene sequences similar to *D. radiopugnans* were represented from diverse environments such as coastal region of Chhavra and banks of Baes river. Several *D. radiopugnans* have been demonstrated as a predominant member in river soil samples from Nottingham, UK (Masters et al., 1991). Ladakh is a region belonging to the northern Greater Himalayas characterized by extreme environments and low precipitation. 16S rRNA based studies embarked to elucidate the diversity of regions of Himalaya do not show representation of deinococci (Pradhan et al., 2010). Our protocol was able to detect deinococci from this sample and sequences showing maximum similarity to *D. deserti*, a strain earlier isolated from Sahara desert (de Groot et al., 2005). Sequences from CS (8B3) and PP (9B1) showed maximum identity to *D. proteolyticus*, and *D. radiodurans*, respectively. The 16S rRNA gene library from cyanobacterial mats at the petroleum contaminated at the Arabian Gulf coast was shown to have clones of deionococcal affiliation (Abed et al., 2006). *D. murrayi*, represented diversity of GRK. Nevertheless none of these isolates, showing best match with the DGGE bands, have been described from any other region expect from their initial source of isolation.

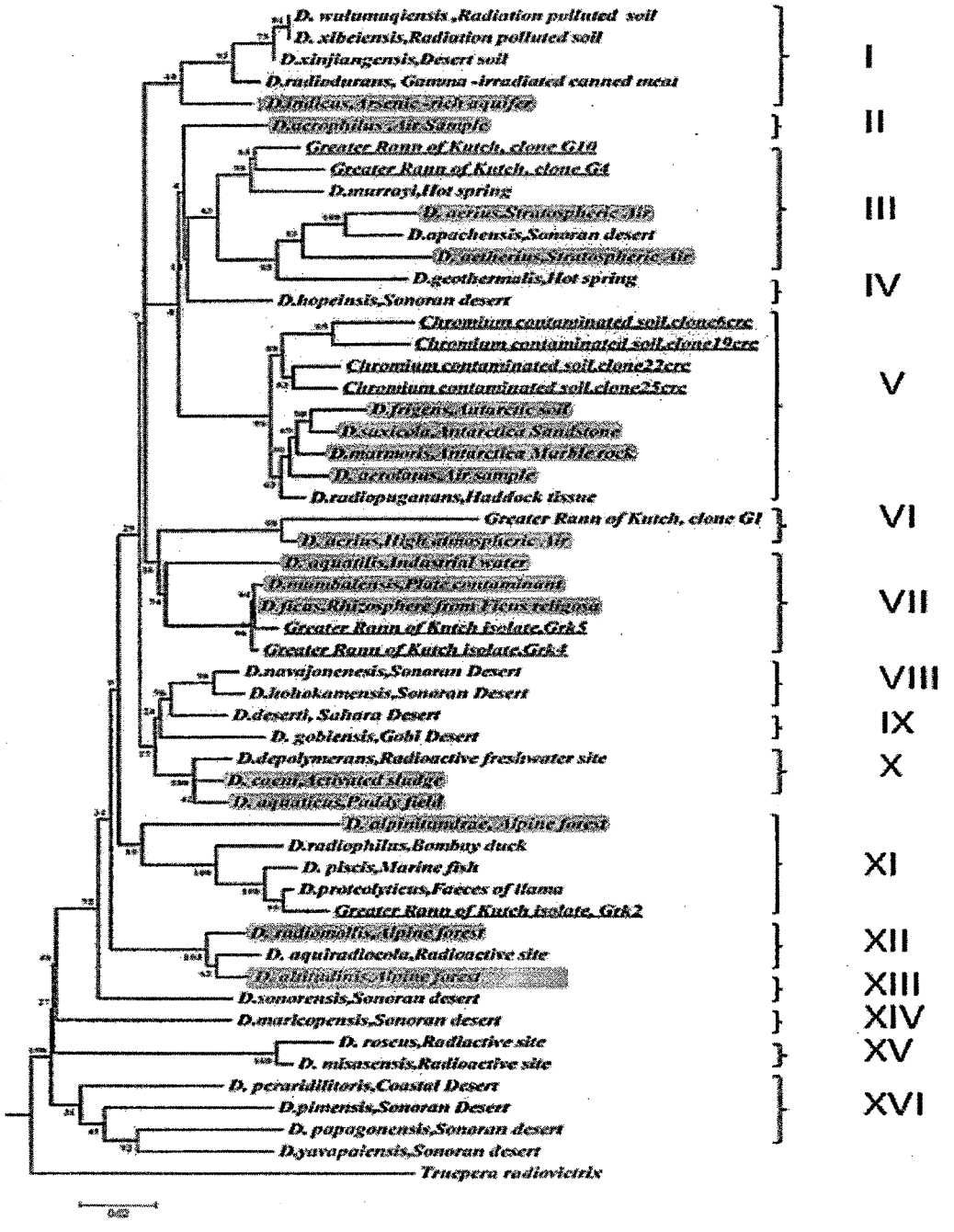


Fig. 2.7: Neighbor-joining tree based on 16S rRNA gene sequences showing the phylogenetic affiliation with type strains of *Deinococcus* species as listed in RDP. *Trupera radiovitrix* is used as outgroup; The cloned sequences from the CRC, GRK sample and the isolates from Grk2 are underlined. Bootstrap values based on 100 replicates are shown near the branch points. The bar indicates the Jukes–Cantor distance of 0.02. Information regarding the environmental niche from where the strains were isolated has been added against the strain name and highlighted isolates

are the strains that were isolated from samples that had no exposure to radiation treatment prior to isolation.

Table 2.5: Diversity indices for deinococcal community from different soil samples

Sample	LRK	GRK	LD	LH	BK	BR	CH	MS	PP	CRC
Shannon Weaver index (H')[a]	1.861	2.233	3.019	2.494	2.702	0.951	2.156	2.451	2.104	0.604
Eveness (E)	0.895	0.970	0.976	0.920	0.935	0.866	0.936	0.986	0.957	0.871
Simpson's Dominance (D)	0.152	0.112	0.059	0.102	0.077	0.432	0.132	0.101	0.132	0.585

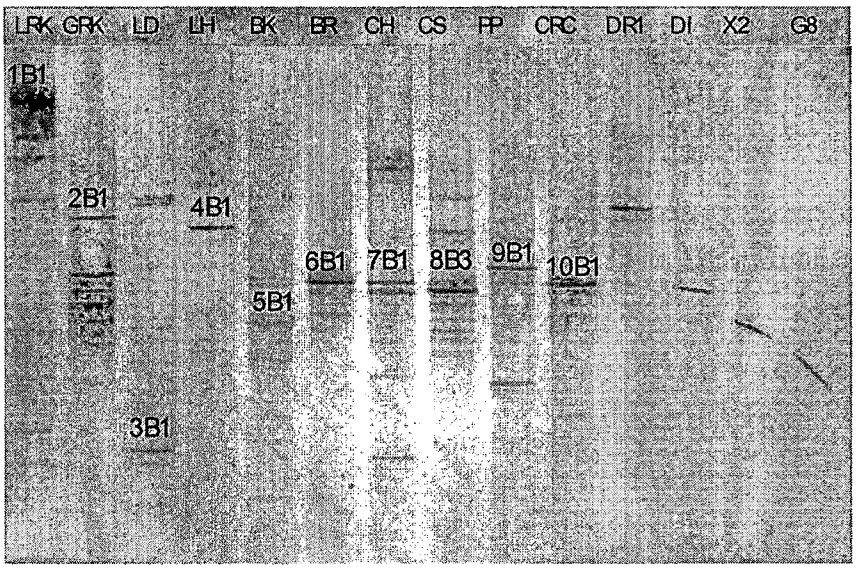


Fig. 2.8: PCR-DGGE analysis of deinococcal diversity from environmental samples. R1, DI, X2, G8 are control samples using pure cultures of *D. radiodurans* R1, *D. indicus, Deinococcus* sp. X2 and *Deinococcus* sp. G8. Lanes with environmental samples are named according to the soil sample designations are as given in Table 2.6.

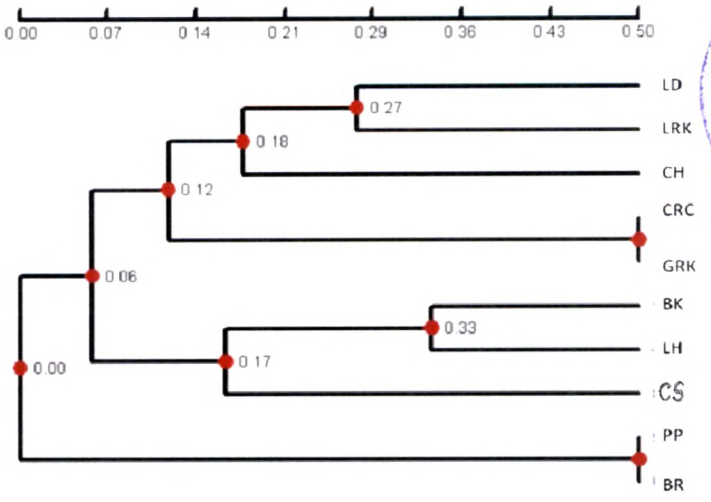


Fig. 2.9: Dendrogram for the DGGE profile from soil bacterial communities. UPGMA dendrogram was constructed using Dice's similarity index. The scale indicates similarity amongst the soil samples.

Table 2.6: Sequence similarity of representative bands excised from the DGGE gel shown in Fig. 2.8 (Appendix I).

Sample	DGGE band	Genbank accession No.	Best Match (Genbank accession No.)	% similarity
GRK	2B1	FJ548964	*D.murrayi* DSM11303 (NR026416)	96
LD	4B1	FJ548965	*D.deserti* VCD115 (NR043243.1)	98
BR	6B1	FJ548966	*D.radiopugnans* ATCC19172 (NR026403.1)	97
CH	7B1	FJ548967	*D.radiopugnans* ATCC19172 (NR026403.1)	97
CS	8B3	FJ548968	*D.proteolyticus* MRP (NR026400.1)	95
PP	9B2	FJ548969	*D.radiodurans* DSM20539 (NR026401.1)	95
CRC	10B1	FJ548970	*D. radiopugnans* ATCC19172 (NR026403.1)	97

2.3.5 Culturable diversity of the radiation resistant bacteria

In an effort to isolate ionising radiation bacteria from the soils described in the study, the samples from different habitats were subjected to γ radiation and the surviving organisms were plated. *Deinococcus* sp. are known to tolerant to both ionizing as well as non-ionizing radiation, therefore, UV radiation resistance of the isolates was studied (Table 2.7). Of the 25 isolates obtained from 5 samples, isolates Grk4, Grk5, Lrk4, Crc4, PP2, PP3 could tolerate up to 1000J/m^2 while Grk2, Crc1, PP1 showed an appreciable resistance of up to 800 J/m^2 (Table 2.7). The hemi-nested approach was employed to ascertain whether the isolates belonged to the genus *Deinococcus*. Interestingly, only four of the UV resistant isolates i.e. three isolates of GRK sample, Grk2, Grk4 and Grk5, while one isolate of LRK sample, isolate Lrk4 could be assigned to the *Deinococcus* as they gave positive amplification in hemi-nested PCR approach. The other isolates may not be deinococci but may belong to other eubacteria genera that have have been reported to be radiation resistant such as the *Rubrobacter*, *Kinecococcus*, or members of genus *Methylobacteria*, *Lactobacillus* (Cox and Battista, 2005). Alternatively they may belong to the deinococcal clade which is not recognized by the specific primer Deino202f (Fig. 2.7). The UV resistance of the GRK isolates (Fig. 2.10) is comparable to the type strain DR1. Grk4, Grk5 are more resistant to UV while Grk2 is sensitive to radiation. Partial sequence of Grk2, Grk4, Grk5 confirmed them to be *D. proteolyticus* and *D. ficus* respectively Table 2.8 and Fig. 2.7.

Table 2.7: Characterization of gamma radiation enriched colonies for UV resistance and PCR with *Deinococcus* specific hemi-nested protocol

Sample	Isolate	UV resistance	*Deinococcus* specific hemi-nested PCR
Greater Rann of Kutch (GRK)	Grk1	-	-
	Grk2	+	+
	Grk3	-	-
	Grk4	++	+
	Grk5	++	+
Little Ran of Kutch (LRK)	Lrk1	-	-
	Lrk2	-	-

	Lrk3	-	-
	Lrk4	++	+
Chromium Contaminated Soil (CRC)	Crc1	+	-
	Crc2	-	-
	Crc3	-	-
	Crc4	++	-
	Crc5	-	-
Petrol Pump soil (PP)	PP1	+	-
	PP2	++	-
	PP3	++	-
	PP4	-	-
	PP5	-	-
Bikaner Soil (BK)	BK1	+	-
	BK2	-	-
	BK3	-	-
	BK4	-	-
	BK5	-	-

+ Tolerates up to 800J/m^2; ++ Tolerates up to 1000J/m^2.

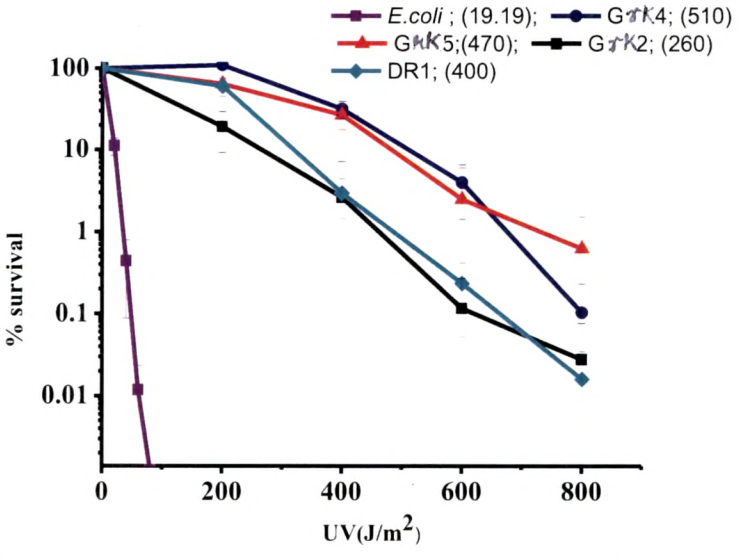


Fig. 2.10: UV tolerance of the radiation–resistant bacterial isolates.

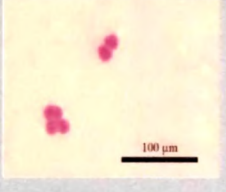
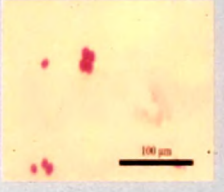
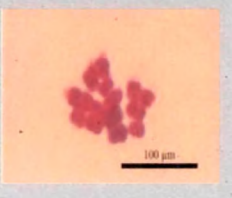
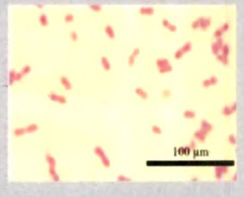
Values in parentheses against the strain names are D_{10} values, i.e. the UV dose required for 90 % killing, in J/m^2.

Table 2.8: 16S rRNA gene sequence match of the radiation resistant isolates (Appendix III)

Isolate	Genbank Accession No.	Best Match (Genbank Accession Number)	% similarity
Grk2	HQ738629	*D. proteolyticus* MRP (NR_026400.1)	98
Grk4	HQ876598	*D. ficus* CC-FR2-10 (NR_043282.1)	98
Grk5	HQ738630	*D. ficus* CC-FR2-10 (NR_043282.1)	93

The biochemical test that characterize genus *Deinococcus* show that all the three isolates could grow on 1 % NaCl, were catalase positive and could reduce nitrate. Grk2 and Grk5 could hydrolyse esculin while Grk2 like the standard strain DR1 could not hydrolyse esculin (Table 2.9).

Table 2.9 Morphological and biochemical characteristics of the radiation resistant bacterial isolates.

Characterstics	DR1[a]	Grk2	Grk4	Grk5
Arrangement of cells	Gram positive, Cocci[†] 	Gram positive, Cocci[†] 	Gram positive, Cocci[†] 	Gram positive, Cocci[†] 
Motility	-	-	-	-
Growth with 1% NaCl	+	+++	+++	+++
NO_3^-	+	+	+	+

reduction				
Esculin hydrolysis	-	++	-	++
ONPG[b] hydrolysis	-	-	-	-
Catalase	++	++	++	++

[a] DR1 refers to *D. radiodurans* R1

[b] ONPG ortho-nitrophenyl-β-D-galactoside

[†] Gram- stained cells and bar each of the micrograph denotes 100 μm.

The isolates Grk2, Grk4, Grk5 were compared for carbohydrate utilisation (Table 2.10) and antibiotic resistance (Table 2.11).

Table 2.10: Carbohydrate utilisation of the radiation resistant bacterial isolates

Carbohydrate	DR1	Grk2	Grk4	Grk5
Lactose	-	-	-	-
Xylose	-	-	+	-
Maltose	-	-	-	-
Fructose	++	++	++	++
Dextrose	+	++	++	++
Galactose	-	-	-	-
Raffinose	+	-	-	-
Trehalose	+	-	+	-
Melibiose	-	-	-	-
Sucrose	+	++	++	-
L-Arabinose	-	-	-	-
Mannose	+	++	+	++
Inulin	-	++	++	-
Sodium gluconate	+	-	-	-
Glycerol	-	-	-	-
Salicin	-	+	-	-
Glucosamine	-	-	-	-

Dulcitol	-	-	-	-
Inositol	-	-	-	-
Sorbitol	-	+	+	-
Mannitol	-	-	-	-
Adonitol	-	-	-	-
α-methyl-D-glucoside	±	+	-	-
Ribose	-	+	+	-
Rhamnose	-	+	++	++
Cellobiose	-	-	-	++
Melezitose	-	++	-	++
α-methyl-D-mannoside	-	++	-	++
Xylitol	-	++	-	++
ONPG	-	-	-	-
Esculin	-	++	-	++
D-Arabinose	-	-	-	-
Citrate	-	-	-	-
Malonate	-	-	-	-
Sorbose	-	+	-	-

Grk2 and Grk5 showed similar carbohydrate utilization. All the isolates could utilize fructose as the standard strain DR1. The isolates have broader carbohydrate utilization spectrum as opposed to the standard strain DR1 indicating biochemical versatilty.

The strain Grk4 was sensitive to all the antibiotics tested while Grk5 and Grk2 were resistant to colistin. Grk2 also showed resistance to oflaxcin. From the comparison of the biochemical, carbohydrate utilization and antibiotic resistance the isolates Grk2 and Grk5 were demonstrated to be similar.

Table 2.11: Antibiotic susceptibility of the radiation resistant bacterial isolates

Antibiotic	Grk2	Grk4	Grk5
Cephalothin (Ch) 30µg	S(--)	S(-)	S(--)
Clindamycin (Cd) 2 µg	S(--)	S(--)	S(--)
Co-Trimoxazole(Co) 25 µg	S(-)	S(--)	S(--)
Erythromycin (E) 15 µg	S(--)	S(--)	S(--)
Gentamycin (G) 10 µg	S(-)	S(-)	S(-)
Oflxacin (Of) 1 µg	R	S(-)	S(--)
Penicillin G (P) 10U	S(--)	S(--)	S(--)
Vancomycin (Va) 30 µg	S(--)	S(-)	S(--)
Ampicillin (A) 25 µg	S(-)	S(--)	S(--)
Tetracycline (T) 10 µg	S(-)	S(--)	S(-)
Cephradine (Cv) 30 µg	S(-)	S(--)	S(--)
Carbenicillin (Cb) 100 µg	S(--)	S(--)	S(--)
Colistin (Cl) 50 µg	R	S(-)	R
Ceftriaxone (Ci) 30 µg	S(--)	S(--)	S(--)

The isolates Grk2 and Grk4 could grow over a broader range of pH, ranging from pH 5-9, while Grk5 grew well between the pH 5-7 (data not shown). The temperature optima of all the isolates were in the range of 30-40 °C (Fig. 2.11).

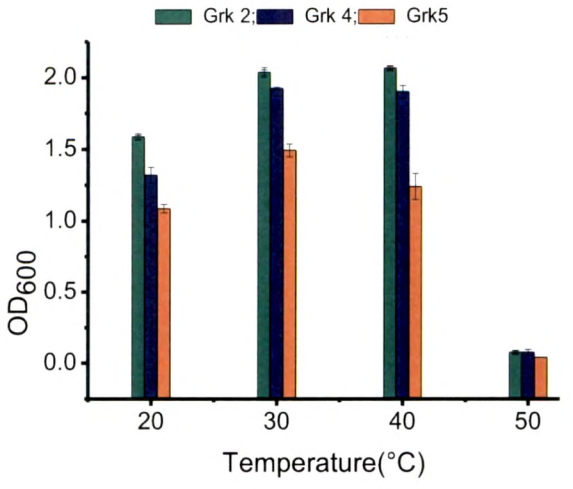


Fig. 2.11: Growth of radiation resistant bacterial isolates at different temperatures.

2.4 Conclusion

Majority of studies characterizing deinococci from environments involve enrichment of samples by a high dose of radiation, a step which obliterates information about species abundance and diversity and gives no clue about the uncultivable deinoccocci in the environment. This work presents for the first time a comprehensive study of the diversity of deinococci by using culture independent approach by a newly developed hemi-nested 16S rRNA gene based PCR protocol. The application of this protocol was demonstrated (i) for ascertaining presence of deinococci from several diverse environmental samples, as they are rare members of natural communities; (ii) for confirmation of radiation resistant isolates as deinococci by a simple PCR tool; (iii) for studying deinococcal diversity by using multiple approaches such as clone library construction and fingerprinting technique such as DGGE; and (iv) for obtaining valuable information about species richness and abundance, since enrichment and isolation of bacteria by radiation treatment is not necessary for detection and quantification of deinococci by this method. The protocol developed has the advantage that it is sensitive enough to detect deinococci directly from community DNA samples without exposing the native population to the strong selective pressure imposed by radiation. In an interesting approach adopted by Suen et al., (2007) of using genome sequences to define ecological niche, the authors have placed *Deinococcus* species in the same niche along with several phylogenetically unrelated members, many of which show radiation resistance and xeric tolerance. The

protocol developed in the current study will help experimentally confirm the predictions of these authors, since many environments can be easily sampled. It should also be possible to use the hemi-nested PCR approach for *in situ* hybridization and for quantitative PCR.