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# Molecular identification and detection of genetic variability through RAPD-PCR technique among bacterial strains isolated from hot spring of MP

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## Abstract:

Microbial diversity in the natural environment is extensive. Methods for studying diversity vary and diversity can be studied at different levels. The rmophiles have been a subject of great interest as they are very important for production of industrially useful enzymes like protease, cellulose, cellulose free xylanase, amylase and also for their industrial application in dye, detergent , leather, pharmaceuticals, paper and pulp and other industries.

The present study aimed for molecular identification and determination of genetic diversity of bacterial strains using random amplified polymorphic DNA (RAPD), obtained from Chhoti Anhoni hot water spring, located at pachmarhi biosphere reserve of central India . A total of 15 bacterial isolates were isolated,purified and used for molecular and genetic variability analysis. Their DNA was isolated and amplified after using two sets of universal bacterial primers after optimization. To confirm the amplification, the PCR products were loaded on an agarose gel (1.2%) and electrophoresis was carried out. Bands were visualized under UV light and documented in the gel. Amplification product of the 16s rRNA were obtained from all DNA extracted, using the PCR primers, 9F and 1542 R, which has resulted in a single band of approximately 1500bp in length and In RAPD analysis total 71 fragments were generated, of which 68 were polymorphic with an average of 6.8 bands per primer. The size of the product varied from 141bp to 2670bp. The similarity index of the isolates within each group ranged from 0.128 to 0.552. Regardless of the oligonucleotide primer employed the 15 bacterial isolates studied were separated into three genetic group composed of HSM1, HSM3, HSM4 and HSM5 (group 1), HSM 6A, HSM6B, HSM 7, HSM8, HSM9 and CA1 (group 2) and CA2, CA4, CA5, CA6 and CA7 (group 3). The PIC value ranges from 0.339 to 0.499.

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**Keywords**: Hot spring, Bacteria, genetic diversity,16s rRNA ,PCR, Random amplified polymorphic DNA (RAPD).

## 1. Introduction:

Hot springs are considered as pious sources as they provide certain solutions to some medical ailments which usually lead to superstitions. The bacteria present in these hot springs are the main reason for these medical solutions but apart from their application in the medical industry ,these thermophilic bacteria are found useful for various other industries . The main purpose that they resolve in these industries are because of their novel enzymes which are the topic of limelight nowadays. Studies shows that these thermophilic bacteria have capability of degrading a lot of substances also , which after extensive research can be a new solution to degradation of certain pollutants .It was reported by geological survey of India that 340 hot springs are present in India (Chandrasekharam, 2005, Bisht et al, 2011) and grouped into 6 geothermal provinces on the basis of their geotectonic setup.There are many hot springs all over the world including India such as Badrinath and Gangotri in UP, Sohna in Haryana, Rajgir in Bihar, Barkeshwar in West Bengal, and Ganeshpuri in West Coast ,Maharashtra etc.Some of the hot springs which are present in Central India are Anhoni hot spring pipariya, Choti Anhoni Sohagpur, Fatehpur Hot spring, Dhuni pani, Babeha etc.The Choti anhoni comes under a most importatnt lineaments/ rifted (Son narmada lineament) structure of the subcontinent with long history of tectonic reactivation with most promising resource base in central India (Pal and Bhimasankaram, 1976; Shanker,1986).Geological survey of India has drilled a boreholes to a depth of approx. 635 meter for petroleum oil extraction and observed the presence of inflammable gases (Approx 80% methane) (Pandey and Negi 1995, Sarolkar , 2010, Vaidya , 2015).It is very important to study the microbial diversity in order to understand the distribution of organism, functional potential, regulation, sustainable management of disturbed, unknown and untapped microbial biodiversity of this region (Torsvik et al., 1996). Different conventional methods developed for analyzing the diversity of microbial communities in their natural environment based on their functional potential and molecular characterization(Fakruddin and Khanjada, 2013).

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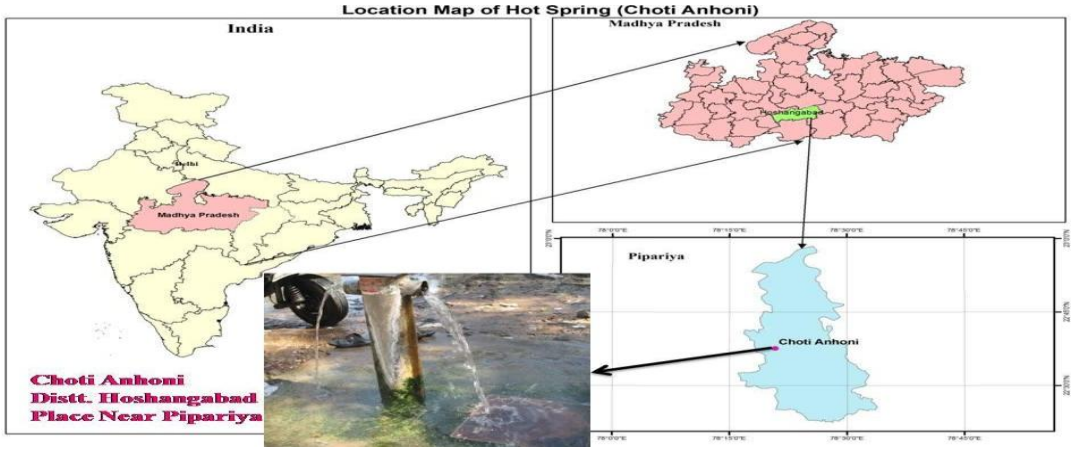
## 2.Materials and Methods

### Study area and sample collection

A water and green mat sample were collected in winter season in the month of January 2019 from the hot water spring, Choti Anhoni (**22$^0$ 37' 39.9'' N**, **0.78$^0$ 20' 51.4"**E) located in Chhindwara district of Central India in pachmarhi biosphere reserves (**Fig. 1**). Geological survey of India has drilled boreholes to a depth of appx 635 mts for petroleum oil extraction and observed the presence of inflammable gases Approx 80% methane (Pandey and Negi 1995, Sarolkar , 2010, Vaidya , 2015). The hot spring water was also checked for the presence




ofinflammablegases at the time of sampling as mentioned in previous studies. Samples were collected in a sterile plastic container which were disinfected with 0.05% bleach solution and labeled properly and placed in an ice box and immediately brought to the laboratory for analysis within 18-24 hrs. The rest of the samples were stored at 4$^0$C for further analysis. The temperature and pH of the water were analyzed at the site of the collection using the digital pH meter and a temperature recorder.

**Fig. 1 Location Map of Sample collection (Choti Anhoni district Hoshangabad)**

### Isolation and pure culture development

Bacteria were isolated using standard serial dilution techniques (Johnson and Curl, 1972). The water sample was spread on tryptone yeast extract dextrose agar supplemented with 0.125% dipotassium hydrogen phosphate (pH-7.5+ 2) 0.5% extra agar and incubated at 50$^0$C in dark for 48 hrs. Individual colonies which are differentiated in shape, size and color were picked up and purified on agar plates using successive streaking methods and finally a

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single colony obtained from the mixed cultures were used for molecular analysis.

**Molecular Characterization:**

**Genomic DNA extraction:**

Genomic DNA from pure culture was extracted using a phenol chloroform extraction method with minor modifications. Bacterial cells were grown in Tryptone yeast broth (10 ml) at 50$^0$C in a shaking incubator for 24 hrs and harvested by centrifugation at 5000g for 5 min. Pellets were transferred to 2ml fresh centrifuged tubes, washed in phosphate buffer saline (8g NaCl, 0.2g KCl, 1.15g, $Na_2H_2PO_4$ and 2 g $KH_2PO_4$ pH- 7.4) and resuspend the pellet in 450 µl of TE buffer (10 mm Tris HCL pH-8, 1 mm EDTA, pH-8), supplemented with 45 µl of 10% SDS and 5 µl of20mg/ml protein aseK. The mixture was in cub ate dat37$^0$C for 1hourinwaterbathwithshaking the sample sat an interval of 15 minutes. The resulting mixture was allowed to cool for 1-2 minutes and thereafter 500 µl of phenol chloroform solution were added and mixed by gentle inversion until the three phases were completely mixed. After centrifugation (10,000 rpm for 10 minutes at room temperature) the supernatant (aqueous phase) was transferred to a sterile microcentrifuge tube. Again the phase was extracted using the phenol chloroform and centrifuged at 10000 rpm for 5 min. Thereafter, 50 µl of 3M sodium acetate (pH 5.2) and 300 µl of isopropanol were added and were kept overnight in a freezer at -20$^0$C to precipitate DNA. Centrifugation at 12000 rpm for 30 minutes was done at 4$^0$C, and the DNA pellet was washed with ice cold ethanol (70%), The extracted DNA was air dried at RT and finally, dissolved in 50 µl of TE buffer (10 mM Tris HCL pH-8, 1 mM EDTA, pH-8) DNA samples were processed either immediately after extraction or kept at 4$^0$C until required.

Purity of total genomic DNA was checked using a UV Spectrophotometer (ND-1000) by calculating the A260/280 ratio (Sambrook et al. 1989) and running the samples on 0.8% agarose gel and visualized based on the intensities of the band when compared with Lambda DNA marker (100bp ladder, used to determine concentration) by ethidium bromide staining under UV light in Gel Documentation System.

**Optimisation of PCR with 16Sr RNA primers:**

**Primers:** 9F( 5'AGAAAGGAGGTGATCGAGCC3'), 1542R(5' GAGTTTGATCCTGGCTCAG3')(white el al 2012). Primers were used for this study 0.19 mg of 9F primer and 0.20 mg of 1542R primers were provided by

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Imperial Life Sciences (P) Limited in lyophilized condition.The concentration of DNA required in the mixture for the PCR process to obtain better amplification lies between 50 -100 ŋg/µl but the amount of DNA obtained per µl is not uniform hence it is diluted to obtain uniform concentration according to the quantity of the DNA isolated.

**RAPD-PCR Analysis**

RAPD profiles were generated using the PCR amplification of DNA extracted from 15 bacterial isolates, with 10 single decamer random oligonucleotide primers (Bangalore Genei, India). Each amplification reaction mixture of 25 µl contained 1 µl of genomic template DNA (50 ng), 12.5 µl of 2x red dye master mix, 1 µl of primer, (Bangalore Genei Pvt. Ltd., India) and 10.5 µl deionized water (RNAse, DNAse free). In -ve control's template DNA was replaced with nuclease-free water. The amplification reactions were performed in a Gradient Automatic Thermal Cycler (Eppendorf MasterCycler Gradient, Hamburg, Germany) in the following cycles: holding at 94$^0$C for 5 min, followed by 8 cycles of 94$^0$C for 45 Sec, 55$^0$C for 1 min and 72$^0$C for 1.5 min and 35 cycles of 94$^0$C for 45 Sec, 55$^0$C for 1 min and 72$^0$C for 1 min and a final additional extension at 72$^0$C for 10 min and a holding temperature of 4$^0$C. Amplified PCR products were electrophoretically separated in 1.2% (w/v) agarose gels and stained with ethidium bromide (0.5 µg/ml). Gels with amplification fragments were visualized and photographed under UV light using Gel Documentation System (Alpha Innotech). The size of the polymorphic fragments was analyzed using lambda DNA (EcoR1-HindIII double digests) . A 100 bp DNA ladder was used as size standard molecular markers. Raw gel images were recorded through Alpha View Software. All the experiments were repeated thrice to ensure reproducibility. The best gels of the replicates were used for band scoring.

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**Data analysis and Scoring**

In RAPD analysis, each fragment/band produced was treated as a unit character. Only the primers that displayed reproducible, clear and intense bands were scored and their molecular weight was calculated using Alpha view software. For all accessions, amplified DNA fragments were converted into binary character matrices as (1) when present or (0) when absent. Polymorphic information content (PIC) values were calculated for RAPD primer employing the formula of Roldan Ruiz et al., (2001):

PIC = 2fi (1-fi), Where fi = Frequency of amplified allele (present band) and (1-fi) = frequency of the null allele (absent band) of marker (I).

Marker index was calculated according to the formula MI= Product of PIC and the number of polymorphic bands per assay unit (Powell et al., 1996).

The data obtained were combined in a single matrix and evaluation of fragmentation patterns was carried out using a similarity index. The Similarity index (SI) values between the RAPD profile of any two individuals were calculated using the Nei genetic similarity index (Nei and Li 1979). Using dice coefficients, a similarity matrix involving 15 accessions was generated by PAST (Paleontological statistics) software (Hammer 2001). The similarity matrix data were subjected to an unweighted pair group method for arithmetic average (UPGMA) cluster analysis to generate a dendrogram using the average linkage procedure. The results were analyzed based on the principle that a band is considered to be 'polymorphic' if it is present in some individuals and absent in others, and 'Monomorphic' if present in all the individuals or accessions. Amplifications were repeated twice to confirm the results.

In addition, principal coordinate analysis was also performed using PAST Software.

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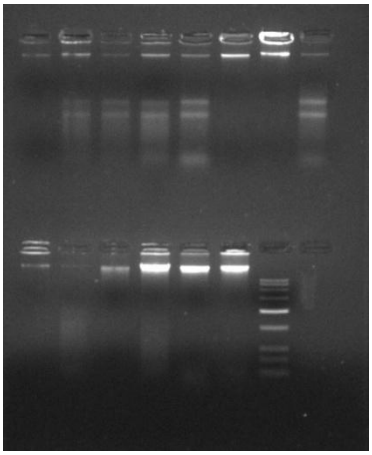
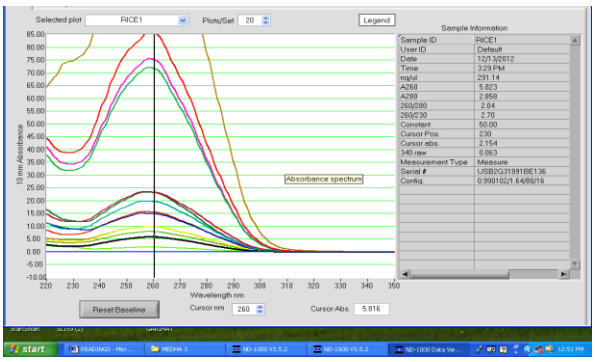
## 3. Results:

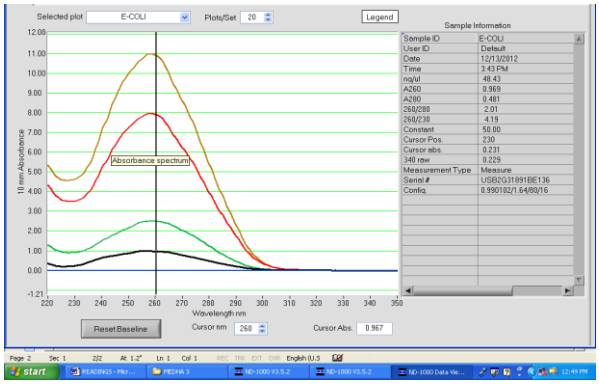
### Molecular characterisation:

**Isolation of DNA:**Genomic DNA from all 15 strains were isolated by the conventional method.The DNA obtained from all the isolates was between 1.6 to 2.04 where some contamination of protein was observed.



2(a)




2(b)

**Fig 2a and b shows Qualitative and Quantitative estimation of DNA extracted from Bacterial isolates.**

### PCR Optimisation for 16s rRNA

An optimized PCR amplification produces a single, bright band on a gel as seen in fig 3 and Electrophoresis of PCR amplicons demonstrating 16s rRNA patterns of bacterial isolates showing band of 1500bp with ladder(shown in fig 4).

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**Elution of PCR product**

Amplification product of the 16s rRNA were obtained from all DNA extracted, using the PCR primers, 9F and 1542 R, which has resulted in a single band of approximately 1500bp in length as shown in fig.5 ,which indicates that the primer synthesised was used by all the bacterial strains isolated and the specific 1500bp bands was further processed for sequencing.

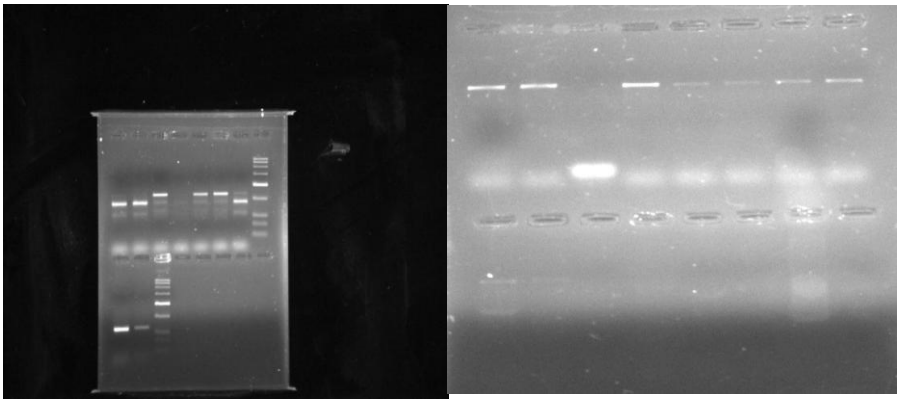

**Fig.3 Optimisation of PCR amplification with single, bright band on a gel**.

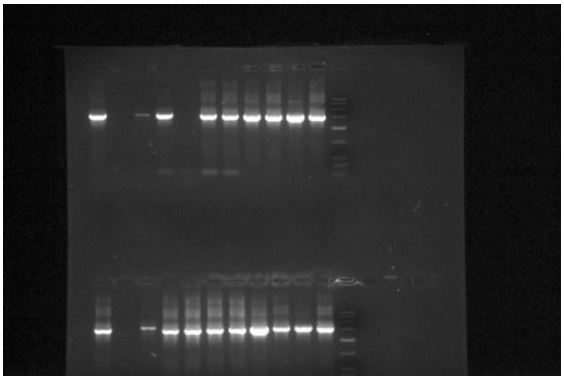

**fig.4 Electrophoresis of PCR amplicons demonstrating 16s rRNA patterns of bacterial isolates showing band of 1500bp with ladder.**

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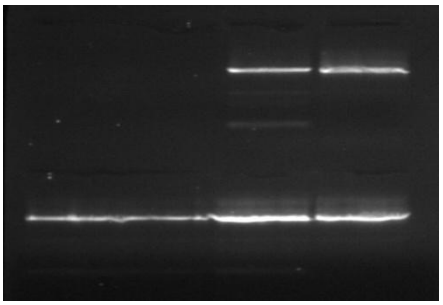

**Fig 5:Elution of specific 1500bp DNA fragment from agarose gel**

## Genetic variability analysis through RAPD PCR

In the present study the binary matrix was generated based on a clear and well defined band with each primer. The RAPD data was recorded , attributing '1' to the presence and '0' to the absence of the band .Total 71 fragments were generated, of which 68 were polymorphic with an average of 6.8 bands per primer. The size of the product varied from 141bp to 2670bp. The similarity index of the isolates within each group, estimated on the basis of jaccard's similarity coefficient ranged from 0.128 to 0.552. Our findings also indicate that primer RBa-7 produced the maximum number of fragments whereas the minimum number of fragments was produced with primer RBa-10.

| **Primer** | **Accessions** | **Total no. of bands (*a*)** | **Total no. of polymorphic bands (*b*)** | **Total no. of monomorphic bands (*b*)** | **Polymorphism (*b*/*a* × 100)** | **Fragment size (bp)** | **PIC Value** | **MI** |
|---|---|---|---|---|---|---|---|---|
| **RBa-1** | AM911690 | 10 | 10 | 0 | 100% | 155-2250 | 0.339 | 3.99 |
| **RBa-2** | AM773311 | 8 | 8 | 0 | 100% | 164-2074 | 0.499 | 3.99 |
| **RBa-3** | AM773772 | 7 | 7 | 0 | 100% | 181-2175 | 0.419 | 2.93 |
| **RBa-4** | AM911679 | 5 | 3 | 2 | 60% | 258-1820 | 0.339 | 1.02 |
| **RBa-5** | AM911680 | 9 | 8 | 1 | 88.8% | 141-2335 | 0.499 | 3.99 |
| **RBa-6** | AM773778 | 5 | 5 | 0 | 100% | 311-1826 | 0.423 | 2.11 |
| **RBa-7** | AM773318 | 13 | 13 | 0 | 100% | 370-1672 | 0.407 | 5.29 |
| **RBa-8** | AM911681 | 5 | 5 | 0 | 100% | 377-1666 | 0.429 | 2.14 |

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|---|---|---|---|---|---|---|---|---|
| **RBa-9** | AM911682 | 8 | 8 | 0 | 100% | 270-2670 | 0.437 | 3.50 |
| **RBa-10** | AM911683 | 4 | 4 | 0 | 100% | 252-1316 | 0.351 | 1.40 |
| **Total** | **10 Primers** | **71** | **68** | **03** | **94.8** | | **4.17** | **30.36** |
| **Average** | | **7.1** | **6.8** | **0.3** | **94.9** | | **0.417** | **3.063** |

**Table 1:** RAPD primer with number of amplified bands, polymorphic bands, % polymorphism, fragment size, PIC Value and MI .

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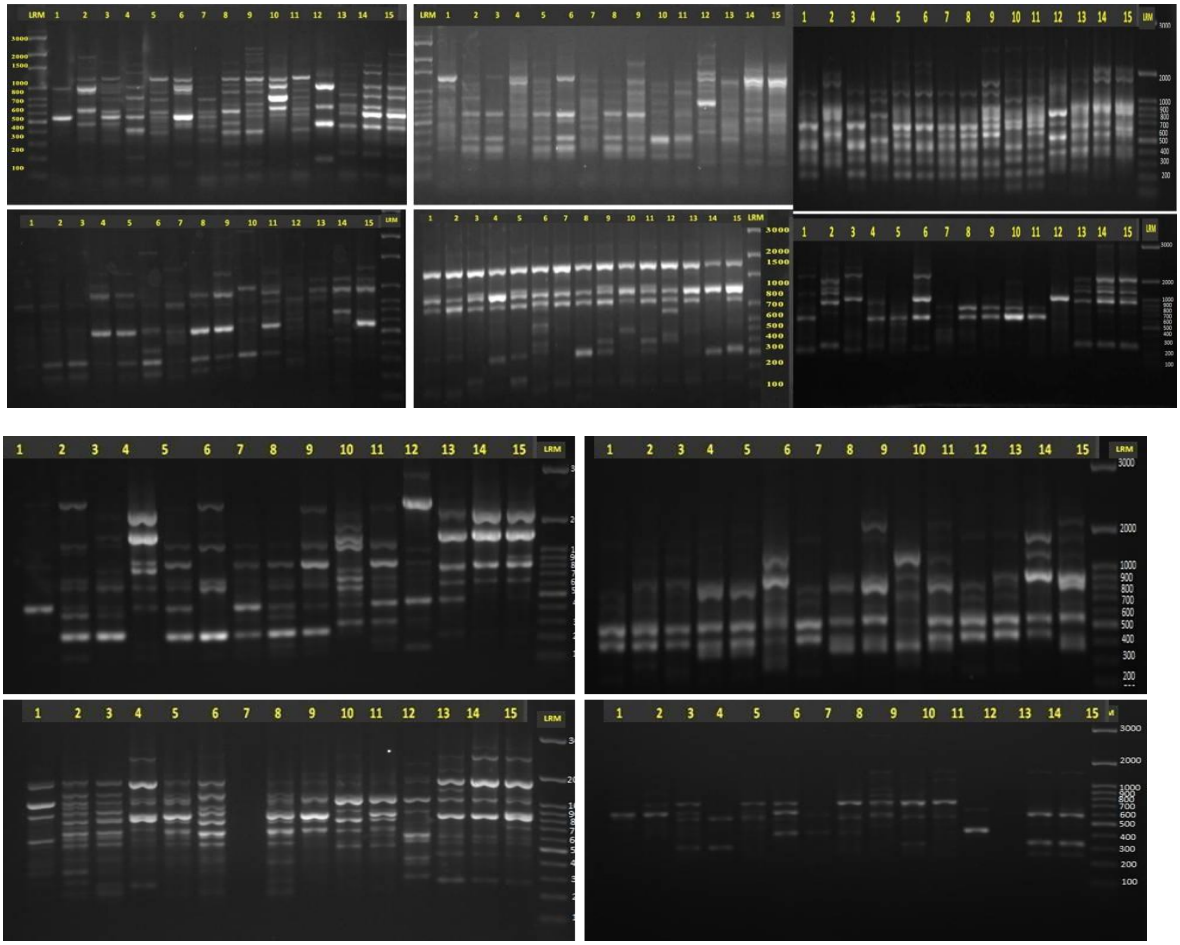

**Fig 6 : Polymorphism pattern demonstration by RAPD primers (1-10) in 15 different Bacterial isolates.**

| | HSM1 | HSM3 | HSM 4 | HSM 5 | HSM6 A | HSM6 B | HSM7 | HSM 8 | HSM 9 | HSM CA1 | HS M CA2 | HS MC A4 | HSM CA5 | HS M CA6 | HSM CA7 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **HSM-1** | 1 | | | | | | | | | | | | | | |
| **HSM-3** | 0.339 | 1 | | | | | | | | | | | | | |
| **HSM-4** | 0.298 | 0.318 | 1 | | | | | | | | | | | | |

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|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **HSM-5** | 0.22 | 0.328 | 0.382 | 1 | | | | | | | | | | | |
| **HSM-6A** | 0.298 | 0.299 | 0.393 | 0.333 | 1 | | | | | | | | | | |
| **HSM-6B** | 0.305 | 0.268 | 0.35 | 0.197 | 0.35 | 1 | | | | | | | | | |
| **HSM-7** | 0.231 | 0.208 | 0.235 | 0.188 | 0.355 | 0.338 | 1 | | | | | | | | |
| **HSM-8** | 0.246 | 0.141 | 0.27 | 0.238 | 0.379 | 0.537 | 0.344 | 1 | | | | | | | |
| **HSM-9** | 0.184 | 0.212 | 0.175 | 0.211 | 0.253 | 0.347 | 0.299 | 0.391 | 1 | | | | | | |
| **HSM-CA1** | 0.157 | 0.161 | 0.197 | 0.169 | 0.25 | 0.333 | 0.213 | 0.299 | 0.329 | 1 | | | | | |
| **HSM-CA2** | 0.192 | 0.191 | 0.212 | 0.131 | 0.276 | 0.316 | 0.256 | 0.253 | 0.378 | 0.387 | 1 | | | | |
| **HSM-CA4** | 0.239 | 0.2 | 0.145 | 0.181 | 0.192 | 0.184 | 0.24 | 0.156 | 0.198 | 0.175 | 0.413 | 1 | | | |
| **HSM-CA5** | 0.214 | 0.324 | 0.187 | 0.261 | 0.203 | 0.211 | 0.25 | 0.197 | 0.281 | 0.28 | 0.403 | 0.508 | 1 | | |
| **HSM-CA6** | 0.225 | 0.266 | 0.247 | 0.391 | 0.282 | 0.253 | 0.198 | 0.24 | 0.321 | 0.21 | 0.341 | 0.351 | 0.457 | 1 | |
| **HSM-CA7** | 0.16 | 0.266 | 0.213 | 0.271 | 0.182 | 0.19 | **0.128** | 0.134 | 0.274 | 0.21 | 0.341 | 0.299 | 0.457 | **0.552** | 1 |

**Table 2: Jaccard's similarity coefficient among different bacterial isolates through RAPD.**

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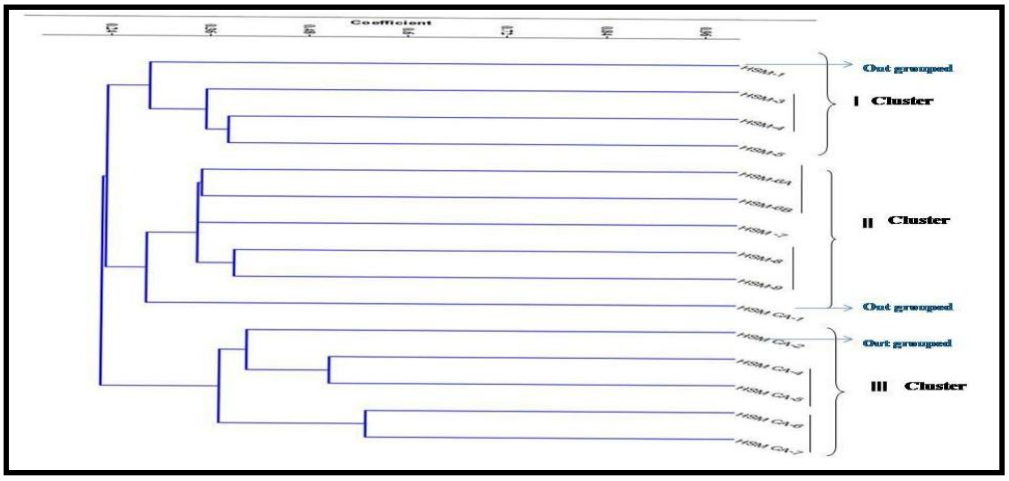


**Fig 7: Paired group Jaccard coefficient matrix of *Bacterial isolates* based on RAPD marker.**

## 4. Discussion

Anhoni hot springs are reported as important geothermal resources and located in the margins of Gondwana coalfields of India (Pandey and Negi, 1995).. The Bacteria were isolated from the water(Labelled as HSMCA) and microbial mat(Labelled as HSM) obtained from hot spring on tryptone yeast extract dextrose agar at $50^0$C. Total of 15 distinct bacterial isolates were obtained as pure culture. Bacterial isolates were molecularly characterized qualtitatively and quantitatively and PCR was optimised for 16srRNAanalysis**(Fig 2-5)**. Genetic variability was identified based on RAPD analysis, results summarized in **Table -2.**Dendrogram was constructed from the data obtained from RAPD analysis. The resulted dendrogram separated the isolates into three main clusters. First cluster consisted of four isolates HSM1, HSM3, HSM4 and HSM5, Cluster II was the largest cluster consisted of six isolates HSM 6A, HSM6B, HSM 7, HSM8, HSM9 and HSM-CA1 , that formed two sub clusters. HSM- CA-1 was clustered with group II with high divergence, while the rest of isolates lay in third cluster CA2, CA4, CA5, CA6 and CA7 **(Figure-7).** which shows the isolates are significantly divergent. The amplification pattern revealed a high level of polymorphism, suggesting that RAPD possesses the ability to resolve enhanced genetic variation in the studied bacterial isolates.

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