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Haplotype Studies in Bacterial Species Surviving in Different Areas

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Pollution is one of the most serious environmental problems; it significantly changes the environmental conditions and profoundly influences the distribution of bacterial communities. Here, the influence of automobiles pollution on the community composition, diversity and evolution of bacteria in Rajkot (Gujarat, India) was investigated. Phylogenetic analysis of *16S rDNA* genes showed that the majority of bacterial sequences undergo evolutionary changes in order to adapt in harsh conditions. Bacteria of same genus and species surviving in different areas (Polluted and non-polluted) showed differences in clade and phylogenic relationships for *16S rDNA* gene sequences. The analysis of DNA polymorphisms in *16S rDNA* sequences by DnaSP 5.10 software showed that the bacterial species undergo haplotype variations which leads to mutation in *16S rDNA* gene at nucleotide level. These provided insights into the evolutionary forces acting on populations and species due to change in environmental condition by pollution.

Key words: Pollution; Bacteria; *16S rDNA;* Haplotype variations.

Ability of microorganisms to transform and degrade many types of pollutants in different matrixes (soil, water, sediments and air) has been widely recognized during the last decades (Saval 1998, Autry and Ellis 1992). They play an important role in degrading these pollutants and maintaining nutritional chains that are an important part of the biological balance (Madigan et al. 1998). Adapting several abilities, microorganisms have become an important influence on the ecological systems, making them necessary for superior organism's life in the planet. Microorganisms survive in contaminated habitat because they are metabolically capable of utilizing its resources and can occupy a suitable niche (Madigan et al. 1998). The microbial cell can be viewed as being in a dynamic state, adapting readily to shifts in environmental parameters by means of a wide variety of genotypic and phenotypic accomodations (Harder and Dukhizen 1983, Tempest and Neijssel 1978, Tempest et al. 1983).

To isolate and identify bacterial species surviving in soil containing hydrocarbons, soil samples were collected from the automobile's industrial area of Rajkot. Bacterial diversity of this industrial area was compared with the bacterial diversity of Saurashtra university campus. This was done to know the type of organisms capable of surviving in the polluted and non-polluted soils. Biodiversity of bacterial species surviving in both this area were studied by isolating and identifying them using biochemical tests and sequencing of *16S rDNA* gene. Population genetics is a branch of the evolutionary biology that tries to determine the level and distribution of genetic polymorphism in natural populations and also to detect the evolutionary forces (mutation, migration, selection and drift) that could determine the pattern of genetic variation observed in natural populations. Ideally, the best way to quantify genetic variation

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in natural populations is by comparison of DNA sequences (Kreitman 1983). Hence, DnaSP software was used to determine genetic variation in *16S rDNA* sequences of bacterial species due to hydrocarbon pollution. This was done to study genetic variation among the bacterial population in polluted and non-polluted environment. It had helped to study the change in conserved *16S rDNA* sequences due to adaptive capacity of microorganisms and variation in diversity of bacterial species at genetic level.

MATERIALS AND METHODS

Sample collection, Bacterial isolation and identification

Soil samples were collected from two sites viz. Saurashtra University campus and an industrial site near Aaji dam, Rajkot which were referred as non-polluted and polluted sites respectively. Appropriate dilutions of each of the samples were prepared which were further inoculated in N-broth for 24 h at 37°C. The cultures were further streaked on the agar plates and incubated for 24 h at 37 °C. The well isolated colonies observed on the next day, were subcultured and finally preserved at 4°C. Further, biochemical tests were performed from these colonies to identify bacterial species on the basis of Bergy's manual classification.

DNA isolation, *16S rDNA* Amplification and Sequencing

Bacterial DNA was extracted according to the method given by Chudasama and Thaker (2012) followed by quality assessment using 1% agarose gel electrophoresis and measurement of concentration and purity using microplate reader (µ Quant, Bio-Tek instruments, USA). The amplification of 16S rDNA was performed in a 25-µL final volume containing 1 µL total DNA, 20 µM 8F primer (5'-AGAGTTTGATCCTGGCTCAG-3'), 0.2 µM 1517R primer (5'-ACGGCTACCTTGTTACGACTT -3'), 10 mM of dNTPs mix, 25mM $MgCl_2$ and 1 U Taq DNA polymerase (Genei-Merck). The reaction conditions were as follows: 95°C for 5 min followed by 30 cycles of denaturation at 95°C for 1.5 min., annealing at 54°C for 1 min and primer extension at 72°C for 3.5 min, followed by a final extension at 72°C for 7 min. The reaction products were separated by running 5 µL of the PCR mixture on a 2% (w/v) agarose gel and staining the bands with ethidium bromide (Sambrook et al., 1989). Subsequently, the PCR products were sequenced using the amplifying primers and BigDye Terminator v3.1 Cycle Sequencing chemistry (Applied Biosystems, Foster City, California, USA) as per manufacturer's protocol. After purification, the reaction products were analyzed on an ABI Genetic Analyzer 3130 (Applied Biosystems). Sequence editing was performed using Bioedit. All sequences were submitted to the NCBI Genbank database using the standalone submission tool Sequin.

Bioinformatics' analyses

Sequences obtained were analyzed by bioinformatics tools for accurate identification and mutation analysis of *16S rDNA* gene of bacteria surviving in polluted and non-polluted area.

NCBI Blast analysis

16S rDNA gene sequences obtained from the wet lab experimentation were compared with the 16S rDNA sequences present in the NCBI-Genbank database using the basic local alignment search tool (BLAST). Accuracy of results was determined by recording the query coverage and percent identity of sequences with sequences available in NCBI database. The basic local alignment search tool (BLAST) finds regions of local similarity between sequences. BLAST can also be used to infer functional and evolutionary relationships between sequences as well as to identify members of gene families.

Multiple Sequence Alignment and Phylogenetic analyses

Multiple sequence alignment of *16S rDNA* gene sequences obtained from polluted and non-polluted bacterial species was done with the sequences available in the NCBI database to study the genetic variation among the bacterial species. The multiple sequence alignment was carried out using ClustalX. The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model. The bootstrap consensus tree inferred from 2700 replicates was taken to represent the evolutionary history of the taxa analyzed, using the MEGA 5.05 software.

Nucleotide sequence polymorphism analyses

The nucleotide sequence polymorphism was calculated using DnaSP version 5.10 (Librado

and Rozas, 2009). Haplotype/Nucleotide Diversity was determined by the change in following parameters of DNA polymorphism.

1) The number of Segregating Sites (S)
2) The total number of mutations (Eta)
3) The number of haplotypes (NHap) (Nei 1987)
4) Haplotype (gene) diversity and its sampling variance (Nei 1987).
5) Nucleotide diversity (Pi), (Nei 1987) (Nei 1987)
6) The average number of nucleotide differences (k) (Tajima 1983).
7) Theta (mutation per gene or per site) from (Eta) or from (S) (Watterson 1975; Nei 1987)

Tajima's Test using DnaSP software

This feature of DnaSP v5.10 calculated the D test statistic proposed by Tajima (1989) for testing the hypothesis that all mutations are selectively neutral (Kimura 1983). The D test is based on the differences between the number of segregating sites and the average number of nucleotide differences.

RESULTS

Isolation and Identification of bacteria from soil samples using biochemical tests

Soil samples were collected from two different areas of Rajkot. Among them aaji dam area was considered as polluted area because of presence of various automobile industries, whereas Saurashtra University campus was consider as non-polluted area. From this soil samples, 42 morphologically distinct colonies were separated. Among these 27 bacterial colonies were isolated from polluted area and 15 bacterial colonies were isolated from non polluted area. From these bacterial samples, 18 bacteria were identified up to genus and species level using biochemical characteristics. Among them 4 bacterial species were found in polluted as well as non-polluted samples.

Bacterial species identified from the polluted soil samples were *Bacillus azotoformans, Bacillus alvei, Bacillus subtilis, Lactobacillus casei, Corynebacterium xerosis, Corynebacterium kutsceri, Lactobacillus delbrueckii, Enterobacter munditii, Micrococcus varians, Staphylococcus aureus, Staphylococcus*

Table 1. Comparison of DNA polymorphism in *16S rDNA* gene sequence of same bacterial species found in polluted and non-polluted site

Name of Bacteria	Number of Segregating Sites, S	Total number of mutations, (Eta)	Number of haplotype (s)	Haplotype (gene) diversity	Nucleotide diversity, (Pi)	Average number of nucleotide differences, (k)	Mutation per gene (Theta from Eta (h)) or from S
Bacillus megaterium	251	251	2	1.00	0.583	251	0.583
Bacillus sphaericus	165	165	2	1.00	0.481	165	0.481
Staphylococcus aureus	202	202	2	1.00	0.541	202	0.541
Staphylococcus saprophyticus	130	130	2	1.00	0.384	130	0.384

Table 2. Comparison of DNA Polymorphism in 16s rDNA gene sequences of bacterial species of Polluted and Non-polluted site with sequences available in the NCBI database

Name of Bacteria	Number of Segregating Sites, S	Total number of mutations, (Eta)	Number of haplotype (s)	Haplotype (gene) diversity	Nucleotide diversity, (Pi)	Average number of nucleotide differences, (k)	Mutation per gene (Theta from Eta (h))	Mutation per site from (s)
Bacillus megaterium (non-polluted)	303	350	10	0.506	0.07435	32.865	0.19366	0.16766
Bacillus megaterium (polluted)	-	-	-	-	-	-	-	-
Bacillus sphericus (polluted)	163	213	10	0.784	0.32754	65.836	0.30320	0.23202
Bacillus sphaericus (non-polluted)	178	249	12	0.895	0.33467	74.632	0.31947	0.22838
Staphylococcus saprophyticus (non-polluted)	0	0	1	0.000	0.00	-	-	-
Staphylococcus saprophyticus (polluted)	0	0	1	0.000	0.00	-	-	-
Staphylococcus aureus (non-polluted)	0	0	1	0.000	0.00	-	-	-
Staphylococcus aureus (polluted)	0	0	1	0.000	0.00	-	-	-

Table 3. Tajima's test

Name of bacteria	Total number of segregating sites S	Number of nucleotide sequences n	Average number of nucleotide differences between pairs of sequences k	Statistical significance	Tajima's D
Bacillus megaterium (non-polluted)	303	34	32.86453	P < 0.05	2.12373
Bacillus megaterium (polluted)	-	-	-	-	0.00
Bacillus sphaericus (non-polluted)	178	19	74.63158	P > 0.05	1.95009
Bacillus sphaericus (polluted)	163	19	65.83626	P > 0.05	1.72307
Staphylococcus aureus (non -polluted)	0	30	0	0	0.00
Staphylococcus aureus (polluted)	0	30	0	0	0.00
Staphylococcus saprophyticus (non -polluted)	-	-	-	-	0.00
Staphylococcus saprophyticus (polluted)	-	-	-	-	0.00

epidermidis, Staphylococcus saprophyticus, Bacillus megaterium, Bacillus sphericus, Enterococcus acidominimus, Streptococcus mitis, Enterococcus faecium. Bacterial species identified from non-polluted soil samples were *Bacillus azotoformans, Lactobacillus fermantii, Staphylococcus aureus, Staphylococcus epidermidis, Staphylococcus saprophyticus, Bacillus sphaericans, Enterococcus faecium.*

Identification of bacteria using *16S rDNA* gene

For molecular identification of these bacterial species, *16S rDNA* gene sequence analysis was carried out. DNA was isolated from all the bacterial species for accurate amplification of *16S rDNA* gene. Sequencing analysis of 16S gene was done using ABI 3130 genetic analyzer.

These sequences were further analysed by bioinformatics tools for accurate identification of *16S rDNA* gene. NCBI BLAST tool was used to confirm the percent identity of *16S rDNA* gene sequence with relative bacterial sequence available in the database. Multiple sequence analysis was done to study the phylogenetic relationship of *16S rDNA* gene sequence with the gene sequences available in the database.

Multiple Sequence Alignment and Phylogenetic analyses

Phylogenetic tree was constructed from the multiple sequence analysis data to gain information about organism's evolutionary relationships. *Bacillus megaterium* of polluted and non-polluted site were in same clade but *Bacillus megaterium* of non-polluted site was more closely related with sequence having accession no.HE578782.1 (Fig: 1). However in *Bacillus sphaericus* and *Staphylococcus aureus* polluted and non polluted site showed an evolutionary relatedness with each other. Sequences of *Bacillus sphaericus* were evolutionary closely related with sequences having accession no. JQ292902.1, JQ319536.1 and JF905442.1 (Fig: 2, 3). Sequences of *Staphylococcus aureus* was closely related with JN315148.1, JF281742.1, and HQ012011.1 (Fig: 3). S. saprophyticus of polluted and non-polluted site were closely related with each other. They were in same clade and one cluster. *S.saprophyticus* of

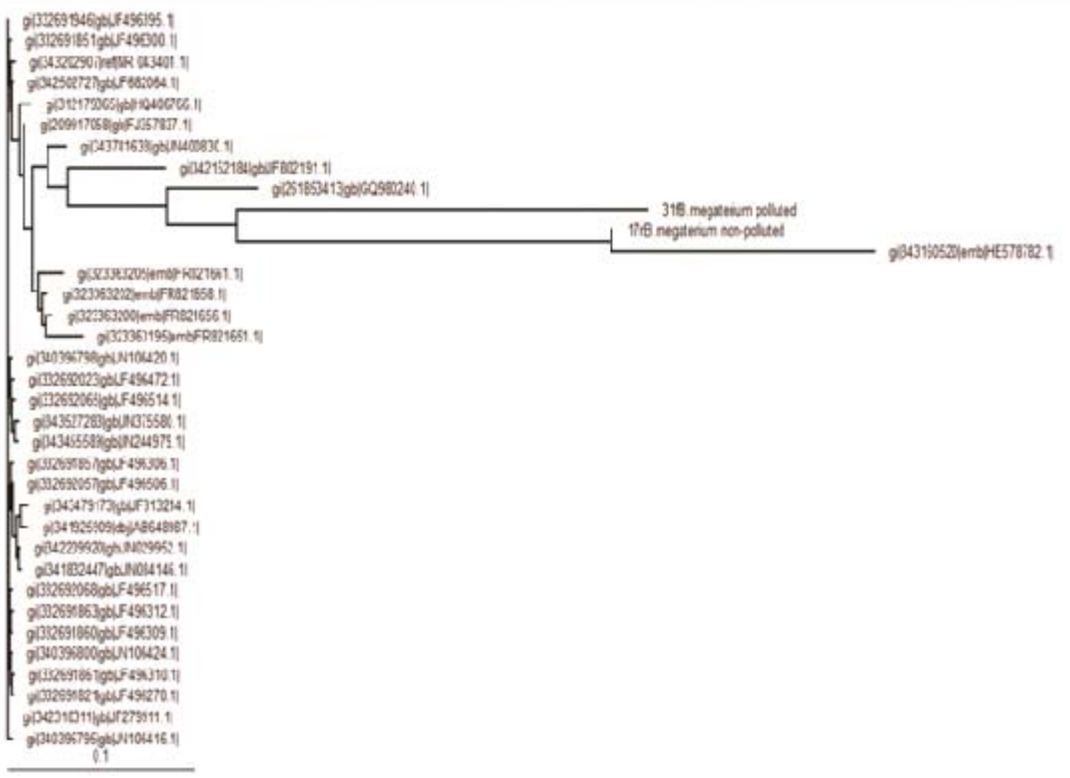

Fig. 1. Phylogenetic tress showing evolutionary relationship of polluted and non-polluted site *B. megaterium* with 16s rDNA sequences available in the NCBl database

non-poluted site was evolutionary closely related with HM854828.1, JF784014.1 and JF718435.1 (Fig: 4).

Nucleotide sequence polymorphism analyses

Four identical bacterial species (*B. megaterium*, *B. sphaericus*, *S. aureus*, and *S. saprophyticus*) were obtained from polluted and non-polluted area. Pairwise alignment of *16S rDNA* sequences obtained from these bacterial species was done. These aligned sequences data was loaded in DnaSP 5.10 software and various parameters mentioned above were calculated. Mutational analysis showed that there were 251, 165, 202, and 130 mutations in *B. megaterium*, *B. sphaericus*, *S. aureus* and *S. saprophyticus* respectively which were isolated from polluted area. Nucleotide diversity in *B. megaterium* was 0.58372, *B. sphaericus* was 0.48105, *S. aureus* was 0.54155, and *S. saprophyticus* was 0.38462 (Table:1). Hence, it was observed that there was remarkable change in the *16S rDNA* sequences at nucleotide level.

Further, multiple sequence alignment of all bacterial species identified was done with the sequences available in the NCBI database and file was loaded in the DnaSP 5.10 software and DNA polymorphism was studied. Bacterial species from non-polluted and polluted area showed a remarkable change in segregating sites (S), total number of mutations (Eta), number of haplotypes, haplotype diversity, nucleotide diversity (Pi), average number of nucleotide differences (k), mutation per gene, and mutation per site was observed (Table: 2)

Tajima's Test using DnaSP software

A positive value of Tajima's D indicated that there has been 'balancing selection' and the data showed a few divergent haplotypes, whereas a negative value suggested that 'purifying selection' have occurred and the data reveal an excess of singletons (Table: 3).

DISCUSSION

Different bacterial genera have been characterized from hydrocarbon polluted soils in different geographical and ecological contexts (Van Hamme et al. 2003, Maila et al. 2004, Maila and Cloete 2005, Maila et al. 2006, Hamamura et al. 2006). Although experimental and climatic conditions differed considerably in each study, some

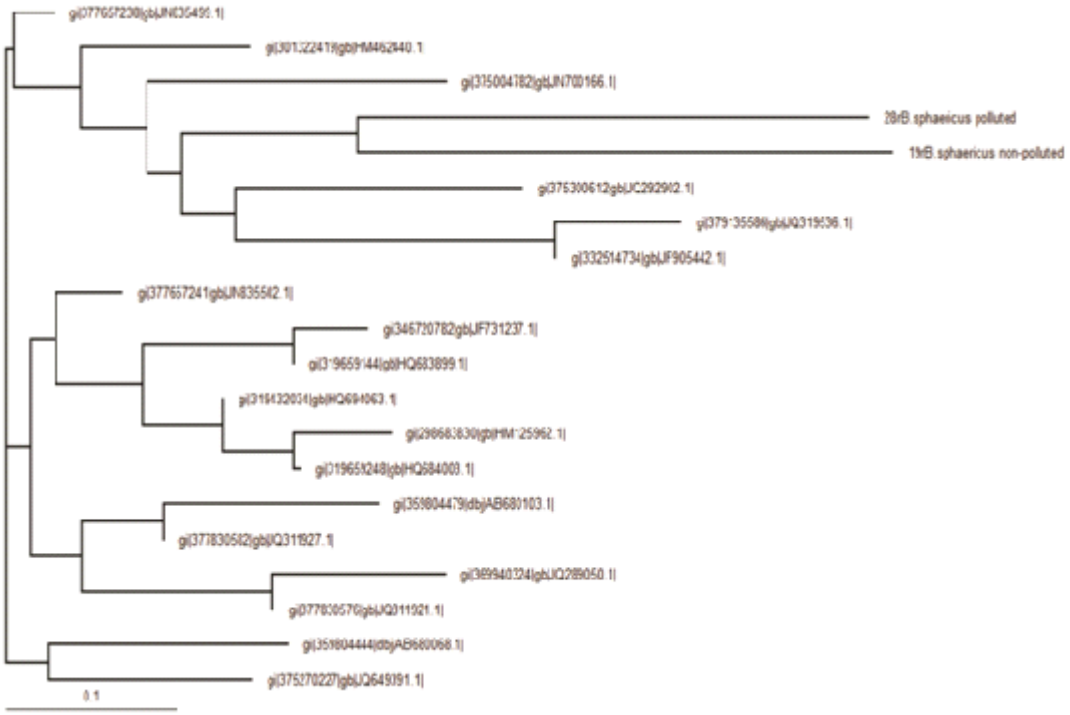

Fig. 2. Phylogenetic tress showing evolutionary relationship of polluted and non-polluted site *B. sphaericus* with 16s rDNA sequences available in the NCBl database

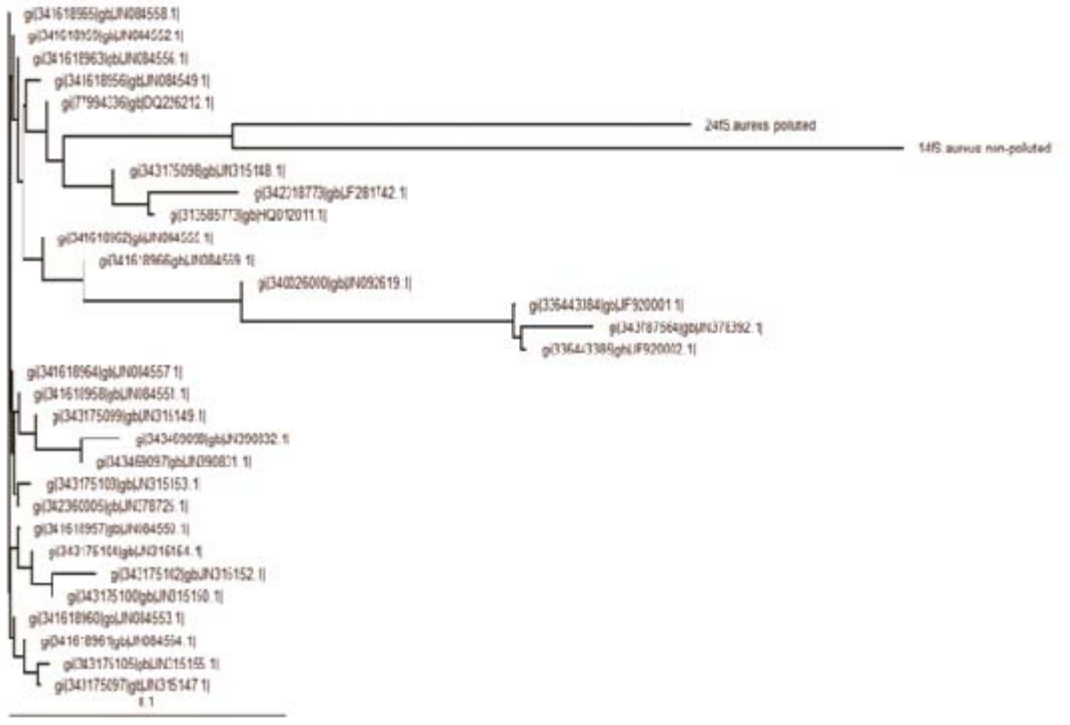

Fig. 3. Phylogenetic tress showing evolutionary relationship of polluted and non-polluted site *S. aureus* with 16s rDNA sequences available in the NCBl database

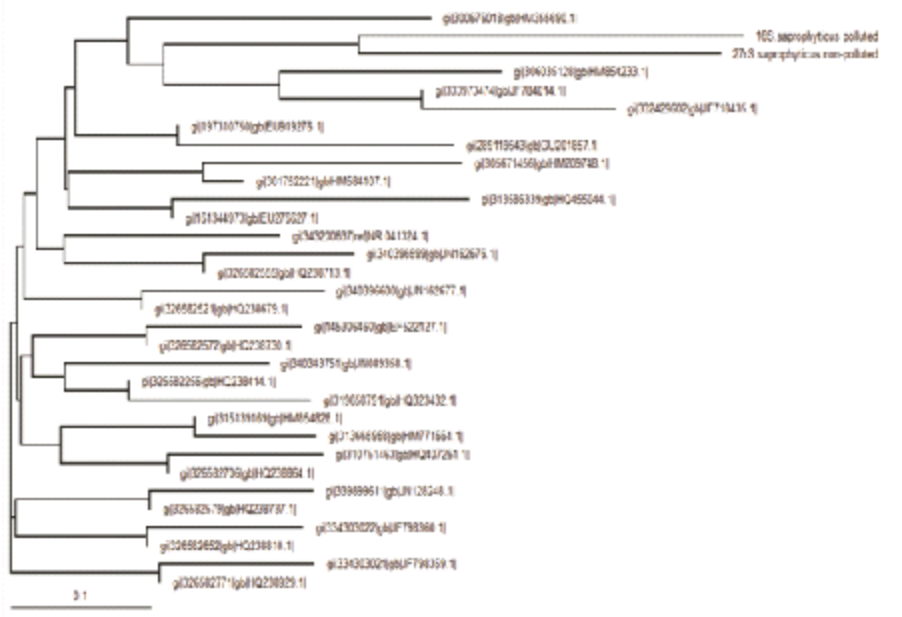

Fig. 4. Phylogenetic tress showing evolutionary relationship of polluted and non-polluted site *S. saprophyticus* with 16s rDNA sequences available in the NCBl database

organisms tend to be common habitants of the hydrocarbon polluted soils. In the present studies, Bacterial species identified from the polluted soil samples were *Bacillus azotoformans, Bacillus larvae, Bacillus alvei, Bacillus coagulans, Lactobacillus casei, Corynebacterium xerosis, Corynebacterium kutsceri, Enterobacter munditii, Staphylococcus aureus, Staphylococcus epidermidis, Staphylococcus saprophyticus, Bacillus marinus, Enterococcus hirae, Enterococcus faecium.* These types of organisms were also isolated and identified from the previous studies done by scientists. Although places differ in the geographical area, bacteria remained almost similar. Vasileva-Tonkova and Gesheva (2003) had shown the presence of *Corynebacterium sp.* in antartic soils polluted with hydrocarbons. Bahig et al. (2008) showed the presence of species of *Bacillus, Micrococcus, Staphylococcus, Pseudomonas, Eschershia, Shigella, Xanthomonas, Acetobacter, Citrobacter, Enterobacter, Moraxella* and *Methylococcus in* agriculture soil irrigated with waste water from industries at egypt. Manoharan et al. (2010) also found the presence of *Staphylococcus sp., Micrococcus sp. and Pseudomonas sp.* in indigenous soils contaminated with petroleum. Das and Chandran (2010) showed nine bacterial strains, namely, *Pseudomonas fluorescens, P. aeruginosa, Bacillus subtilis, Bacillus* sp., *Alcaligenes* sp., *Acinetobacter lwoffi, Flavobacterium sp., Micrococcus roseus, and Corynebacterium* sp. are capable of degrading crude oil. Okerentugba and Ezeronye (2003) also showed the potential of *Chromobacterium, Flavobacterium, Bacillus, Vibro, Citrobacter, Enterobacter, Micrococcus, Klebsiella, Planococcus, Pseudomonas* and *Camplobacter* species in degradation of petroleum. Austin et al. (1977) examined 99 strains of petroleum degrading bacteria, isolated from Chesapeake Bay water and sediment, by numerical taxonomy procedures. Eighty-five percent of the petroleum degrading bacteria examined in this study were defined at the 80 to 85% similarity level within 14 phenetic groups. This groups were identified as *actinomycetes* (mycelial forms, four clusters), *coryneforms*, *Enterobacteriaceae*, *Klebsiella aerogenes*, *Micrococcus* sp. (two clusters), *Nocardia* sp. (two clusters), *Pseudomonas* sp. (two clusters), and *Sphaerotilus natans.*

This similarity in bacterial species surviving in different geographical environment suggests that it is the nutrition availability which makes bacterial species to thrive in particular environment. Contaminants are often potential energy sources for bacteria. They survive in contaminated habitat because they are metabolically capable of utilizing its resources and can occupy a suitable niche. They have broad range of enzymes that enable them to degrade many chemicals (Madigan et al. 1998). They are omnipresent, and are capable of rapid growth when provided with nutrients and conditions favorable for metabolism and cell division. They are involved in catalysis and synthesis of organic matter in the aquatic and terrestrial environments. Many substances, such as lignin, cellulose, chitin, pectin, agar, hydrocarbons, phenols, and other organic chemicals, are degraded by them. The rate of decomposition of organic compounds depends upon their chemical structure and complexity and upon environmental conditions (Madigan et al. 1998). Hence, in present studies bacterial species found in automobiles polluted soil were more than that of Saurashtra University campus soil. This result suggests that increase in pollutants of soil is directly proportional to the bacterial population.

Majority of genetic evolutionary/ taxonomic work is based on aligning DNA sequences and then reconstructing the phylogeny based on predefined evolutionary models (Woese 1987, Ludwig et al. 1998, Kolaczkowski and Thornton 2004). Many conserved genes like *gyrase-II, pthA, rpo, nuoB, petC* and *16S rDNA* are used as phylogenetic markers for identification and isolation of bacterial populations (Palmieri et al 2010). Among them the use of *16S rDNA* gene was done to study bacterial phylogeny and taxonomy was done because it is the most common housekeeping genetic marker used for a number of reasons. These reasons include (i) its presence in almost all bacteria, often existing as a multigene family, or operons; (ii) the function of the *16S rDNA* gene over time has not changed, suggesting that random sequence changes are a more accurate measure of time (evolution); and (iii) the *16S rDNA* gene (1,500 bp) is large enough for informatics purposes. It has properties, which predestine it as a universal phylogenetic marker (Patel 2001). Hence, multiple sequence alignment of *16S rDNA* gene

sequences was done with the sequences available in the NCBI data base and phylogenetic trees were generated. Remarkable changes in the clade of neibour-joining tree of bacterial species found in polluted and non-polluted area helped to study the evolutionary pattern among the bacterial species due to change in environmental condition by pollution. Further, pairwise alignment between *16S rDNA* sequences of bacterial species isolated from polluted and non-polluted area helped to study the nucleotide diversity among the species using DnaSP software. Nucleotide diversity is a measure of genetic variation. It is usually associated with other statistical measures of population diversity and is similar to expected heterozygosity. This can be calculated by examining the DNA sequences directly or may be estimated from molecular marker data. Once the nucleotide diversity has been estimated, it can be found out whether adaptation has potentially played any role in influencing these sequence changes.

Tajima's test, or D test statistic (Tajima, 1989) test is the neutral theory of molecular evolution (Kimura 1983). This was done to study the evolutionary changes among the organisms due to adaptation at molecular level. This test suggests that vast majority of molecular differences that arise through spontaneous mutation do not influence the fitness of the individual. A corollary to this theory is that the genomes evolve primarily through the process of genetic drift. This statistic is used to monitor diversity within or between ecological populations, to examine the genetic variation in related species or to determine evolutionary relationships. Tajima's D statistic compared the difference between two estimates of the amount of nucleotide variation, one being simply the number of segregating sites (Watterson, 1975) and the other one being the average number of pairwise differences (Nei and Li, 1979; Tajima, 1983).

From present studies it was concluded that different bacterial species showed wide variations among the polluted and non-polluted samples studied from the selected area than that of closely related sequences derived from the database. Nucleotide variations and significant changes in the ¸ and D values of the sequences in comparison with the related sequences derived from the nucleotide database suggested that the pollution might have lead bacterial species towards adaptation at morphological and genetic level. The same genus and species surviving in the different areas (polluted and non-polluted) showed differences in clade and phylogenic relationships for *16S rDNA* gene sequences. The analysis of DNA polymorphisms of *16S rDNA* sequences provided insights into the evolutionary forces acting on populations and species due to change in environmental condition by pollution. Nucleotide diversity in the *16S rDNA* gene sequence of bacterial species isolated from polluted and non-polluted soil samples was determined (Table: 1). Although sequences data for the *16S rDNA* gene is highly conserved in different microorganisms, it has been shown to be very accurate for identification of bacteria at genus and species level. However, it showed variation at genetic level in bacterial species surviving in different environmental conditions. Previous studies has shown that the regions on the *16S rDNA* are quite conserved and others are variable (Kullen et al. 2000). These variable regions might help microorganisms to adapt the environmental changes due to pollution. However, further analysis is required to prove this fact.

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