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Molecular Characterization of *nif* genes of diazotrophs from different rice production systems in Tamil Nadu

S. Kanimoli*, M. Karthik and K. Kumar

Department of Agricultural Microbiology, TNAU, Coimbatore - 641003, India.

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Heterotrophic diazotrophs were isolated from rhizosphere of rice grown in different locations in Tamil Nadu. The selected diazotrophic bacterial colonies were characterized by cell shape, morphology, Gram reaction and other biochemical tests for their tentative identification. A total of hundred and ten isolates obtained were subjected to acetylene reduction assay (ARA) and ninety eight isolates recorded significant amount of nitrogenase activity.The authenticated diazotrophic isolates from rice rhizosphere were screened for the presence of *nif* H gene by detection of partial amplification of *nif* H gene. The universal primers *nif* Hb1 and CDHP *nif* 723R, which could amplify 550 bp, were used for screening the isolates by PCR amplification.The *nif* H amplification could be clearly detected in all the 9 isolates from rice rhizosphere *viz.*, *De*LTP$_2$, *Ab*LTP$_2$, *As*LC$_1$I, *Ab*SKN, *Ps*SAK$_2$, *Be*SC$_2$S$_2$, *Ab*AA$_1$A$_2$, *Ps*AKK$_3$, *De*ATM$_3$.

Key words: Diazotrophs, Acetylene Reduction Assay, *nif* H, rice rhizosphere, PCR.

Biological Nitrogen Fixation (BNF) has become important in rice farming systems because this process diminishes the need for expensive chemical fertilizers which have been associated with numerous health and environmental problems. The extensive exploitation of BNF would provide economic benefits to small farmers avoiding the influence of chemical fertilizers.

Wetland rice receives significant proportion of its nitrogen requirement from biological nitrogen fixation. However, rice yields in these systems are very low and must be increased by about 50% in order to meet the projected demands in 2020 (Ladha and Reddy, 2000). This could necessitate a doubling in the use of N – fertilization, which is neither desirable nor sustainable. A potential alternative is to increase the contribution made by N-fixation. Malarvizhi and Latha (1999) described the associative N fixation is however highly variable ranging from 0 to 36 % of total N demand from air depending on the rice variety. It has been suggested that associative N_2 fixation, unlikely to be affected by genotype and environment interactions, since the diazotrophs are loosely associated with the plants and thus are more valuable to changes in the environment. It has been shown that the rhizosphere of rice is particularly abundant in species of *Azospirillum, Pseudomonas* and in the members of Enterobacteriaceae. In addition various other genera *Agromonas, Alcaligenes, Azotobacter, Burkholderia, Clostridium, Flavobacterium, Xanthobacter and Zoogloea* have also been isolated from paddy field soil. Ueda *et al*. (1995) concluded recent investigation has shown the large and highly diverse population of culturable diazotrophs is also associated with the rice.

Symbiotic N_2 fixation by legumes is generally expected to be the dominant source of

* To whom all correspondence should be addressed.
E-mail: kanimicro76@gmail.com

biological of N input in the earth. In recent years, N balance and ^{15}N techniques have provided convincing evidence that non-legumes such as wetland rice can, under certain conditions, derive a considerable amount of N from associated N_2 fixing bacteria in the plant rhizosphere (Chalk, 1991; Mark and Craswell, 1992). In non-legume agro systems, rice fields are considered to be ideal niches for biological nitrogen fixation because of their characteristic ecological conditions. It is well known that a remarkable diversity of N_2 fixing bacteria are naturally associated with field grown rice (Balandreau, 1986). The free living heterotrophic N_2 fixers are a potentially important source of N_2 fixation in rice fields (Boddey *et al*., 1995, Mahadevappa and Shenoy, 2000).

Nitrogenase, the enzyme highly essential for reducing nitrogen to ammonia, is composed of Fe (dinitrogenase) and Mo-Fe protein (dinitrogenase reductase) is encoded by *nif* gene. A substantial molecular diversity of N_2 fixing bacteria has been detected in field grown rice based on retrieval of *nif* H or *nif* D gene fragments from root DNA (da Rocha *et al*., 1986). Since the *nif* H gene only occurs in nitrogen fixing microorganisms, it has been used to monitor the presence of these diazotrophs, for example, in pure cultures (Frank *et al*., 1998), in soil (Widmer *et al*., 1999) and plants. In view of the above, this study was aimed to identify the diazotrophic nature of bacteria isolated from rice rhizosphere soil of different locations of Tamil Nadu, India, using partial amplification of PCR with *nif* H primer.

MATERIALS AND METHODS

Isolation of diazotrophic bacteria from rhizosphere soil

Diazotrophic bacteria were isolated by following standard plate count method (Allen, 1953). The different N free media used were Waksman No 77 medium for *Azotobacter*, Becking' s medium (Becking,1961) for *Beijerinckia*, Nitrogen- free glucose mineral medium for *Derxia* (Becking,1961) and King's B medium (King *et al*., 1954) for *Pseudomonas*. In case of *Azospirillum* Most Probable Number (MPN) technique (Cochran, 1950) was followed by using Nitrogen free malic acid semi solid medium (Dobereiner, 1980).

One gram of soil from each sample was aseptically weighed, transferred to 100 ml sterile water blank and shaken (120 rpm) for 30 min to get 10^{-2} dilution. Likewise, the sample was diluted serially with 9 ml water blanks until the appropriate dilution was obtained. Aliquots (1ml) from the serially diluted samples (10^{-3} to 10^{-6}) were added to five different N-free media in Petri plates. The plates were incubated for 7 days at 30^0C and morphologically different colonies appearing on the medium were isolated and subcultured for further analysis.

Purification and maintenance of cultures

Single, well separated and morphologically different colonies growing on the plate were picked and purified by streak plate method. The purified colonies were transferred into agar slants and mass multiplied in the respective medium.

Diazotrophy of the isolates

Diazotrophy of the isolates were determined on the basis of *nif* H gene amplification by Polymerase Chain Reaction (PCR).

Identification of the nif genes of diazotrophs using nif H primer

The genomic DNA from the isolates *viz*., *De*LTP$_2$ (Derxia,lowland, Trichy, Poovalur) *Ab*LTP$_2$(Azotobacter, lowland, Trichy, Poovalur), *As*LC$_1$I (Azospirillum, lowland, Coimbatore (Thondamuthur), Ikaraipoluvampatty,), *Ab*SKN (Azotobacter, SRI,Killikulam, Naanalkadu), *Ps*SAK$_2$ (Pseudomonas, SRI, Kelamaruthuvakudi), *Be*SC$_2$S$_2$ (Beijerinckia,SRI, Coimbatore (Pollachi), Somandurai), *Ab*AA$_1$A$_2$ (Azotobacter,Aerobic, Aduthurai, Avainyapuram,), *Ps*AKK$_3$ (Pseudomonas, Aerobic, Killikulam, Kongaraiyarkuruchi), *De*ATM$_3$ (Derxia, Aerobic, Trichy, Maandurai) which exhibited moderate to high nitrogenase activity was isolated using the standard protocol of hexadecyl-trimethyl ammonium bromide (CTAB) method given by Melody (1997) with slight modifications. Actively grown culture of 25 ml quantity was taken in a centrifuge tube and centrifuged at 6,000 rpm for 5 min at 4^0 C. The supernatant was removed, the pellet was suspended in 1 ml TE buffer, added with 0.5 ml of 1-butanol, vortexed well to mix with the cells (to remove extracellular materials), centrifuged at 5000 rpm for 5 min at 4^0C, the supernatant was

discarded and the pellet was resuspended in 2 ml of TE buffer and centrifuged again to remove all traces of butanol. The pellet was resuspended in 1 ml TE buffer added with 100 ml lysozyme (10 mg ml^{-1} freshly prepared) and incubated at room temperature for 5 min. After incubation, 100 ml of 10 per cent SDS and 25 ml of 100 mg ml^{-1} proteinase K were added, mixed well and incubated at 37 ^{0} C for 1 h. To this, 200 ml of 5 M NaCl was added and mixed well. CTAB solution in 150 µl quantity was added, mixed well and incubated at 65^0 C for 10 min. The mixture was extracted with 1 ml of phenol:chloroform mixture, mixed well and centrifuged at 6000 rpm for 15 min at 4^{0}C. The aqueous layer was transferred carefully to a 2 ml microfuge tube and DNA was precipitated by adding 0.6 volume of ice cold isopropanol, incubated 1h to overnight at-20^{0}C. The DNA was pelletized by centrifugationat12000 rpmfor15min at 4^{0}C. The pellet was washed with 70 per cent ethanol, dried under vacuum for 10 min and resuspended in 50 ml of TE buffer. One ml DNAse free RNAse (10mg ml^{-1}) was also added by swirling and incubated at 37^{0}C for 30 min. The purity of the genomic DNA was assessed from the A260/A280 and A260/A230 extinction ratios. The DNA was stored at -20^{0}C for further use.

Agarose gel electrophoresis was performed based on the method given by Sambrook *et al.* (1989) to check the quality of DNA and also to separate the products amplified through polymerase chain reaction. 1X TAE tank buffer in 500 ml quantity was prepared to fill the electrophoresis tank and to prepare the gel. In a separate conical flask, agarose (0.8 per cent for genomic DNA and 1.5 per cent for PCR product) was added to 1X TAE buffer, boiled till the agarose dissolved completely and cooled to lukewarm temperature. Ethidium bromide was added at the rate of 5 ml 100 ml^{-1} to agarose solution and was allowed to mix completely. It was then poured into the gel mould and the comb was placed properly, allowed to solidify for half an h at room temperature. After solidification the comb was removed carefully. The caste gel was placed in the electrophoresis tank containing 1X TAE buffer with the well near the cathode and submerged to a depth of 1 cm. Fifteen ml of the PCR product was mixed with 3ml of 6X tracking dye and mixed well by pipetting in and out for 3 times. The mixture was loaded into the wells with the help of the micropipette. Two ml of 1 kb DNA ladder (Fermentas, USA) was loaded in one of the wells as a standard marker. The cathode and anode were connected to power pack using power cord and the gel was run at a constant voltage of 60 volts. The negatively charged DNA molecules move towards the anode and get separated according to their molecular weight. The power was turned off when the marker reached the anode end and the gel was viewed in an UV trans illuminator and the banding pattern was analyzed.

After the separation of the PCR products with 1 per cent agarose gel, it was viewed and photographed using Alpha imager TM1200 documentation and analysis system.

Amplification of 16S rDNA

Full-length 16S rDNAs (1500 bp) were amplified from the isolates by PCR using the universal forward primer fd1 (5' AGAGTTTG ATCGTGGCTCAG 3') and the universal reverse primer rp2 (5' ACGGCTACCTT GTTACCACTT 3'). The 45 µl PCR reaction mixture consisted of DNA template 50 ng, 1XTaq buffer, 0.2 mM of each of dNTP mixture, 1 µM of each primers, 1.5mM $MgCl_2$ and 2U of Taq DNA polymerase (Bangalore Genei, India). PCR amplification was performed in a thermocycler (Eppendorf Master cycler, German) using the following conditions: initial denaturation at 95° C for 1 min, 35 cycles consisting of 94°C for 1 min (denaturation), 60°C for 1 min (annealing), 72°C for 1 min (primer extension) and final extension at 72°C for 5 min (Weisberg *et al.*, 1991).

Sequencing reactions were performed using ABI prism terminator cycle sequencing ready reaction kit and electrophoresis of the products were carried out on an Applied Biosystems (Model 3100) automated sequencer at Yaazh Genomics, India.

Phylogenetic analysis

The cultures were identified at species level by performing a similarity search against the GenBank database (website:http://www.ncbi.nih.gov/BLAST). The phylogenetic tree was constructed with sequences of 16S rDNA from different eubacteria, which was obtained from NCBI. Sequences were aligned using the CLUSTAL X software (Thompson *et al.*, 1997). The evolutionary distances were calculated by neighbor-joining method of Saitou and Nei (1987)

using MEGA 4.0 software and the tree file was analyzed using tree view (Tamura *et al.*, 2007).

The identification of *nif* H gene for the 9 diazotrophic isolates selected was done based on PCR amplification of *nif* H gene using specific universal primer.

Presence of *nif* H gene in the isolates was detected by partial amplification of the gene using specific universal primers. The forward primer nifHb1 (5'GGCTGCGATC CCAAGGCTGA3') and reverse primer CDHP nif723R (5'GATGTTCGC GCGGCACGAADT 3') were used for amplification of *nif* H gene (550 bp). The 45 µl PCR reaction mixture contain DNA template 50 ng, 1XTaq buffer, 0.2 mM of each of dNTP mixture, 1 µM of each primer, 1.5mM $MgCl_2$ and 2U of Taq DNA polymerase (Yaazh Genomics, India). PCR amplification was performed in a thermocycler (Eppendorf Master cycler, German) using conditions: initial denaturation at 95°C for 5 min, 35 cycles consisting of 94°C for 1 min (denaturation), 60°C for 1 min (annealing), 72°C for 1 min (primer extension) and final extension at 72°C for 5 min.

RESULTS AND DISCUSSION

Several factors *viz*., root morphology, the stage of plant growth, root exudates and physical and chemical properties of the soil are reported to influence the occurrence and distribution of microbial communities in the soil and rhizosphere. Previous isolations of nitrogen fixing bacteria have revealed a broad diversity of diazotrophs to inhabit the crop rhizosphere (Vessey, 2003) and this study surveyed the rhizosphere soil of rice grown in different regions of Tamil Nadu for the presence of nitrogen fixing bacteria. A total of hundred and ten nitrogen fixing bacterial isolates were isolated from different locations in Tamil Nadu and subjected to Acetylene Reduction Assay (ARA) and ninety eight isolates recorded significant amount of nitrogenase activity. Based on maximum nitrogenase activity nine isolates were screened for *nif* H gene analysis.

Authentication of diazotrophic isolates from rhizosphere soil of rice from different locations of Tamil Nadu by 16S rRNA gene sequence homology and phylogeny construction

The total genomic DNA of all the 9 isolates from rice rhizosphere were extracted to amplify the 16S rDNA. High concentration of good quality genomic DNA was obtained.

In order to gain insights about the bacterial identity, the full length DNA amplification and sequencing of 16S rRNA gene was performed. The 16S rRNA gene of all the 9 isolates was amplified using universal primers FD1 and RP2. All amplified products produced a single band with approximately 1500 bp length and the differences among them were not visible in 1 per cent agarose gel.

Comparative BLAST analysis which includes the closest species and per cent homology of full length 16S rDNA revealed the presence of wide diversity of alpha, beta and gamma proteobacteria. Out of 9 diazotrophic isolates, 2 isolates represented the alpha proteobacterial group, 2 isolates represented beta proteobacterial group, 5 isolates represented gamma proteobacterial group.

The isolate *Ab*LTP_2 showed close similarity to *Azotobacter paspali*, *Azotobacter salinestris* and *Pseudomonas* sp. The isolate *Ab*SKN showed high resemblance to *Azotobacter beijerinckii* while the isolates *Ps*$SAK_2$and *Ps*AKK_3 were closely related to *Pseudomonas fluorescence* and *Pseudomonas* sp. respectively. The isolates *De*LTP_2 and *De*ATM_3 were found to be closely comparable to *Methylocella silvestri* and *Derxia* sp. respectively. The alpha proteobacterial isolates *As*LC_1I and *Be*SC_2S_2 highly resembled *Azospirillum* sp, *Azospirillum brasilense* and *Azospirillum zeae*. The isolate *Ab*AA_1A_2 belonging to gamma proteobacteria greatly comparable to *Azorhizophilus paspali* and *Beijerinckia indica*. The percentage similarity of all the isolates to the closest sequence in the Genbank ranged from 96-100 per cent (Fig.1).

Screening of diazotrophic isolates for the presence of nitrogenase iron protein gene (*nif* H)

The authenticated diazotrophic isolates from rice rhizosphere were screened for the presence of *nif* H gene by detection of partial amplification of *nif* H gene. The universal primers nifHb1 and CDHPnif 723R, which could amplify 550 bp, were used for screening the isolates by PCR amplification.The *nif* H amplification could be clearly detected in all the 9 isolates from rice rhizosphere *viz.*, *De*LTP_2, *Ab*LTP_2, *As*LC_1I, *Ab*SKN,

*Ps*SAK$_2$, *Be*SC$_2$S$_2$, *Ab*AA$_1$A$_2$, *Ps*AKK$_3$, *De*ATM$_3$.
The identification of 9 diazotrophic isolates from rhizosphere soil of rice by 16S rRNA gene sequencing showed the prevelance of various genera of alpha, beta and gamma proteobacteria . By and large, it was observed that majority (50 per cent) of the isolates belonged to the Gamma proteobacterial group and was distributed among various species of the genera *Azotobacter* (*Ab*LTP$_2$, *Ab*SKN, *Ab*AA$_1$A$_2$), *Pseudomonas* (*Ps*SAK$_2$ and *Ps*AKK$_3$).The beta proteobacteria was represented by *Derxia* (*De*LTP$_2$ and *De*ATM$_3$). The diazotrophic alpha proteobacteria was represented by *Azospirillum* sp. (*As*LC$_1$I) and *Beijerinckia* sp. (*Be*SC$_2$S$_2$).

These findings in the present study was highly in accordance with the findings of Chowdhury *et al.* (2007), who established the occurrence of a diversity of nitrogen fixing bacteria including *Azospirillum*, *Azotobacter*, *Pseudomonas* and *Variovorax paradoxus* in the rhizosphere of perennial desert grass, *Lasiurus sindicus*.

NifH gene diversity

According to Chien and Zinder (1996), *nifH* topology can be divided into four major clusters and that all *nifH* genes fall into one of four clusters: cluster I includes standard molybdenum nitrogenase from cyanobacteria and proteobacteria (alpha, beta and gamma), as well as gamma

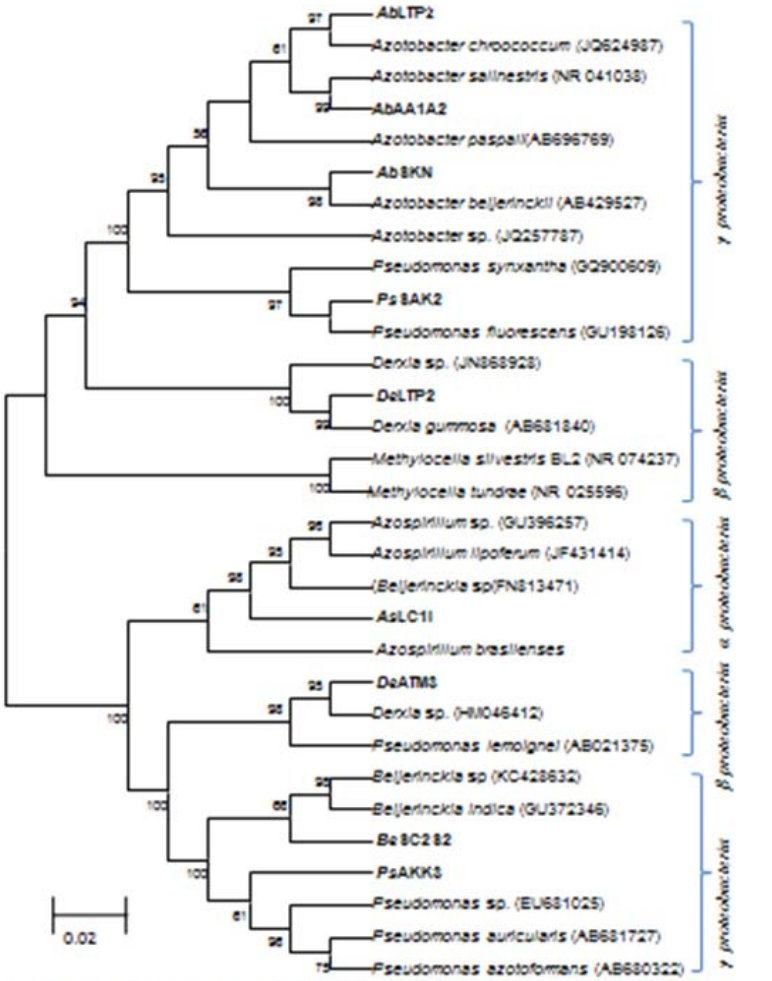

Fig.1 Phylogenetic tree based on neighbour joining method showing the position of 16S rDNA fragments amplified from pure cultures of the diazotrophic isolates. Bar scale indicates number of base pair divergence during evolution

Fig. 1.

proteobacterial *nif*H, and cluster II includes methanogen nitrogenase and bacterial *nif* H. Cluster III includes nitrogenase from diverse anaerobic bacteria such as clostridia (lowG+C, Grampositive) and sulfate reducers delta proteobacteria). Cluster IV includes divergent nitrogenase from archaea.

In the present study, the amplification of approximately 550 bp long *nifH* fragment was carried out using universal primers nifH-b1 and CDHPnif723R. The *nifH* genes could be amplified from 9 isolates obtained from rhizosphere soil of rice. The isolates, which carried the *nifH* genes were *Azotobacter chroococcum* (*Ab*LTP_2), *Azotobacter* sp. (*Ab*SKN, *Ab*AA_1A_2), *Pseudomonas fluorescence* (*Ps*SAK_2) and *Pseudomonas* sp. (*Ps*AKK_3), *Azospirillum lipoferum* (*As*LC_1I), *Beijerinckia indica* (*Be*SC_2S_2) and *Derxia gummosa* (*De*LTP_2) and *Derxia* sp. (*De*ATM_3). The genera belonging to *Azospirillum, Azotobacter, Beijerinckia,Derxia and Pseudomonas* are well known nitrogen fixers and the presence of iron protein gene has already been confirmed by Park *et al*. 2005.

In the present study, *nifH* genes amplified from all the 9 isolates which produced highly reproducible single bands. A BLASTn search of these sequences with the most similar *nif H* gene sequences of the GenBank database (http://www.ncbi.nlm.nih.gov) revealed the closest *nif H* sequence identities from the sequence database. The results indicated that the *nif H* sequences of 9 isolates were 95 to 99 per cent homologous with the *nif H* sequence of *Azospirillum brasilense* (X51500) respectively.

REFERENCES

1. Chalk, P.M. The contribution of associative and symbiotic nitrogen fixation to the nitrogen nutrition of non legumes. *Plant Soil*., 1991; **132**: 29- 39.
2. Boddey. R.M., de Oliveira, O.C., Urquiaga, S. Biological nitrogen fixation associated with sugarcane and rice: contributions and prospects for improvement. *Plant soil*., 1995;**174**: 195-209.
3. Mahadevappa, M., Shenoy,V.V. Towards nitrogen fixing rice (*Oryza sativa*). *Adv. Agric. Res. India*., 2000;**13**: 131-139.
4. da Rocha A, Ohki, S.T, Hiruki, C. Detection of mycoplasm like organisms *in situ* by indirect immunofluorescence microscopy. *Phytopathology.*, 1986; **76**: 864-868.
5. Frank, I.H., Fegan, M., Hayward, A.C. Nucleotide sequence of the *nif* H gene coding for nitrogen reduction in the acetic acid bacterium *Acetobacter diazotrophicus*. *Lett. Appl. Microbiol*., 1998; **26**: 12-16.
6. Widmer, F., Shaffer, B.T., Porteous, L.A., Seidler, R.J. Analysis of *nif* H gene pool complexity in soil and litter at a Douglas fir Forest site in the Oregan Cascade Mountain Range. *Appl. Envion. Microbiol.*, 1999; **65**:374-380.
7. Becking, J.H.Studies on nitrogen – fixing bacteria of the genus *Beijerinckia* -Geographical and ecological distribution in soils. *Plant Soil.*, 1961; **14**: 49-81.
8. Cochran, W.G. Estimation of bacterial densities by means of the most probable number. *Biometrics*., 1950; **6**: 105-116.
9. Dobereiner, J. Forage grasses and grain crops. In: Methods for Evaluation of Biological Nitrogen Fixation. (Eds.) J. F. J. Bergersen and Chichester, Wiley Publications 1980; 535–555.
10. King, J. W., Ward, M..K., Raney, D.E.. Two simple media for the demonstration of pyocyanin and fluorescein. *J. Lab. Clin. Med.*, 1954 ; **44:** 301-307.
11. Burris, R.H.Biochemical genetics of nitrogenase. In:Biology of Nitrogen Fixation. (Ed.) A. Quispel. North Holland Publishing Co., 1974; 3- 42.
12. Sambrook, J., Fritsch E. F., Maniatis, J. Molecular cloning - A laboratory manual. 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, USA. 1989; 345-350.
13. Weisberg.W.G., Barns, S.M., Pelletier, D.A., Lane,D.J. 16S ribosomal DNA amplification for phylogenetic study. *J. Bacteriol.*,1991;**173**: 697-703.
14. Thompson,J.D., Gibson,T.J., Plewniak,F., Jeanmougin,F., Higgins,H.G., The clustalx windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res*., 1997;**24**: 4876-4882.
15. Saitou, N., Nei, M. The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol. Biol. Evol*., 1987; **4**: 406-425.
16. Tamura, K., Dudley, J., Nei, M., Kumar,S. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol. Biol. Evol.*, 2007; **24**: 1596-1599.
17. Chien,Y.T., Zinder,S.H. Cloning, functional organization, transcript studies, and phylogenetic

analysis of the complete nitrogenase structural genes (*nif*HDK2) and associated genes in the archaeon *Methanosarcina barkeri* 227. *J. Bacteriol.,* 1996; **178**: 143-148.

18. Chowdhury,S.P., Schmid, M., Hartmann, A., Tripathi, A.K.. Identification of diazotrophs in the culturable bacterial community associated with roots of *Lasiurus sindicus*, a perennial grass of Thar desert, India. *Microb. Ecol.,* 2007; **54**: 82-90.
19. Ladha, J. K., Reddy, P.M. Steps toward nitrogen fixation in rice. In: The Quest for Nitrogen Fixation in Rice. (Eds.) J K Ladha and PM Reddy. International Rice Research Institute, Makati City, Philippines. 2000; 33 - 46.
20. Malarvizhi, P., Ladha, J. K. Influence of available nitrogen and rice genotype on associative dinitrogen fixation. *Soil Sci. Soc.Am. J.*,1999., **63**: 93-99.
21. Park, M., Kin, C.,Yang,J., Lee,H., Shin,W., Kim,S., Sa,T. Isolation and characterization of diazotrophic growth promoting bacteria from rhizosphere of agricultural crops of Korea. *Microbiol. Res.,* 2005; **160**: 127-133.
22. Vessey, J.K. Plant growth promoting rhizobacteria as biofertilizers. *Plant Soil*., 2003; **255**: 571 -586.
23. Ueda, T., Suga,Y., Yahiro, N., Matsuguchi T. Remarkable N_2-fixing bacterial diversity detected in rice roots by molecular evolutionary analysis of *nif* H gene sequences. *J. Bacteriol*., 1995; **177**: 1414–1417.