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*nif*H homologs from soil metagenome

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Biological nitrogen fixation is an important process that allows nitrogen uptake into biological systems. But it is still unclear from where the first *nif* gene was formed or evolved.

Soil samples from different agro-climatic zones of western Indian Himalaya were analysed for the diversity of *nif*H in their metagenome. Metagenomic DNA was isolated followed by polymerase chain reaction (PCR) amplification and quantification. Soil of Pithoragarh (locality of temperate climatic region) was further used for shotgun meta-genomic library construction. Clones having similar insert were screened and finally 176 clones were used for colony hybridisation. All the clones were hybridised with two *nif*H probes, i. e. NB6 and BR1 and positive clones were sequenced. *In-silico* study of sequenced shotgun clones reveals *nif*H homology with several other genes which are not directly involved in nitrogen fixation but belong to bacterial genera which are known for nitrogen fixing ability. We are proposing a hypothesis that *nif* genes like *nif*H may evolve from their nearest genes or adjacent regions and in due course become specific in their functions.

Key words: nitrogenase, metagenomics, *nif*H, real time PCR

INTRODUCTION

Nitrogen fixation is an important process that facilitates nitrogen uptake into biological systems. The ability to fix nitrogen is widely distributed among phylogenetically diverse variety of bacterial species either culturable or unculturable but appears to be limited to the methanogens within *Archaea*. (Ruvkun et al., 1980; Sibold et al., 1985; Lobo et al., 1992; Young et al., 1992; Raymond et al., 2004). The dinitrogen reduction process is mediated by the enzyme complex nitrogenase. This complex comprises two different metalloproteins: Fe protein and Mo-Fe protein. The former is a homodimer made up of the *nif*H gene product while the latter is a $\alpha_2\beta_2$ heterotetramer containing the *nif*D, *nif*K genes products (Jasrotia, Ogram, 2008). Therefore, looking for any of the above mentioned genes, i. e. *nif*H or *nif*D or *nif*K, is an ideal tool for analyzing the nitrogen fixing community in soil (Wartiainen et al., 2008). Nitrogenase genes are the only known example of such highly conserved prokaryotic translated genes. These genes are either extraordinarily conserved in evolution or have been exchanged between different nitrogen-fixing species relatively recently in evolutionary time (Ruvkun, Ausubel, 1980). Till date it is unclear from where the first *nif* gene was formed or evolved. What were the conditions which forced bacteria to fix nitrogen and to start *nif* gene formation? What were the conditions which actually directed bacterial communities to fix nitrogen and to start *nif* gene formation?

The aim of the present work is to find out the sequences which are homologous to *nif*H but not directly involved in nitrogen fixation. However, the present research does not explain the above question but we hope that the work will give some clue regarding the evolution of *nif* genes.

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MATERIALS AND METHODS

Extraction of DNA from soil samples

Samples were collected from the upper layer (0 to 15 cm) of the studied soils, from different geographic locales, namely temperate regions of Ranichauri (78°30'E, 30°15'N, 1950 m) and Pithoragarh (80°2'E, 29°47'N, 1967 m), subtropical region of Chamoli (30°51'N, 79°4', 1 300 m) and Tarai region of Pantnagar (29°N, 243.8 m). Soil from at least five locations of each site was sampled, collected, composited and homogenized by sieving and stored at 4 °C till further use. Soil DNA was extracted using PowersoilTM DNA isolation kit (Mobio Lab. Inc., Carlsbad, CA, USA) as described by the manufacturer and quantified by ultraviolet (UV) spectrophotometry at 260 nm.

Quantification of *nif*H gene by qPCR

Extraction of genomic DNA of Bradyrhizobium japonicum USDA 6

The culture of *B. japonicum USDA 6* was provided by departmental culture collection at G. B. Pant University of Agr. & Tech., Pantnagar, India. Cells were harvested from 200 ml grown culture (0.6 OD) of *B. japonicum USDA*. The genomic DNA was isolated using one step RNA reagent (BioBasic Inc. Canada, also for DNA & Protein).

Amplification of *nif*H gene from *B. japonicum USDA 6* and standard curve preparation

The *nif*H gene was amplified using the previously reported primers set (Pol F & Pol R) by Poly et al., 2001. PCR products were cleaned using a QIA quick purification kit (Qiagen Inc., Chatsworth, CA) and cloned using Qiagen PCR cloning plus kit (Qiagen Inc., Chatsworth, CA) and sequenced. Standard curve was prepared using reamplified DNA from *B. japonicum USDA 6* clone**.** Tenfold dilution series (starting from 50 ng) were used as an external standard for quantification by real time PCR. With the help of standard curve the amount of DNA was quantified by software provided by the manufacturer (Bio-Rad Lab, Hercules, CA, USA).

qPCR of *nif*H gene from soil samples

The primers set Pol F & Pol R was also used to perform the real time PCR quantification of *nif*H gene from soil DNA. The RT-PCR mix contains SYBR green super-mix (Bio-Rad Labs, Hercules, CA, USA), Pol F, Pol R, soil DNA, DNA (*B. japonicum*) and triple distilled water in ratio of 12.5:1:1:1:0:6.5 (for standard reaction in µl) and 12.5:1:1:0:1:6.5 (for sample reaction in µl), respectively. The reaction was run in a pTC-150 mini cycler PCR machine (MJ-Research, USA which now merged in Bio-Rad) for 35 cycles (94 °C for 1 min, 58 °C for 2 min) after initial denaturation at 94 °C for 3 min.

Shotgun library preparation

Concentrating the DNA

The extracted soil DNA was further eluted by adding 5 ml of solution C6 using Power Max soil-™ DNA isolation kit (Mobio Lab. Inc., Carlsbad, CA, USA). The final volume of eluted DNA (1.5–2 µg) was then concentrated by adding 0.2 ml of 5 M NaCl and mixed by inversion. After that 10.4 ml of 100% cold ethanol was added and mixed by inversion. Finally, this was centrifuged at 2500x g for 30 min at room temperature. Pellet was washed with 70% ethanol and air dried. Washed DNA was finally resuspended in 100 µl autoclaved ultrapure water and stored at –20 °C.

Restriction digestion of soil DNA

Soil DNA (1 µg) was restricted using BfuC1 (New England Biolab, Inc., MA, USA) tetra cutter enzyme. The reaction mixture was incubated at 37 °C for 10 min. The total volume (100 µl) of restricted product was loaded on 1.5% agarose gel and DNA of size 500 bp-2kb was eluted (20 µl) from the gel by Auprep gel extraction kit (Life Technology, India). It was further ligated with dephosphorylated BamHI restricted pUC19 vector DNA by preparing competent cells of *E. coli* DH5α strain and then transformation of recombinant DNA into *E. coli* DH5α strain.

Screening of the clones

Isolation and restriction digestion of recombinant DNA from clones

Recombinant plasmid DNA was extracted using Hi PurA Plasmid Mini Kit (Hi Media, Mumbai, India) according to manufacturer protocol and subjected to double digestion with EcoRI and HindIII restriction enzymes (Fermentas; Life Sciences, Sahney Kirkwood Compound, Mumbai, India) and reaction mixture was incubated at 37 °C for 1 h, run on 1% agarose gel and photographed by Gel Documentation System (Bio-Rad Lab, Hercules, CA, USA).

Colony hybridisation for *nif*H positive clones

Protocol for colony hybridisation was adapted from Sambrook et al., 1989. Further, for hybridisation all the shotgun clones were picked and patched into LB agar plate containing ampicillin (100 µg/ml) sequentially. Maximum 16 clones were patched on one plate. The *nif*H positive clone which was used for probe preparation was used as positive control and clone containing only pUC19 DNA (without insert) was taken as negative control on each plate. Furthermore, reamplified *nif*H gene insert from two selected clones, one from soil DNA and the other from *B. japonicum USDA 6*, were used as probe and radiolabeled with ^{32}P-dATP using DNA labelling kit (Amersum GE Healthcare, UK). Positive clones were selected on the basis of their insert size. The clones having less than 2kb fragment were sequenced by M13F and M13R sequencing primers via primer walking method at Delhi University South Campus, New Delhi. All the sequences of selected clones were submitted to NCBI-GenBank (Table 1).

Table 1. ***In-silico*** **analysis of the clone sequences selected by shotgun method**

Clone / Accession No.	Length	ORF	Protein	E-value	Function	Organism
S108/ HM172515	1220	2–802	Hypothetical protein Acid_3175	3e–164	Unknown (bankit1317925)	*Solibacter usitatus Ellin6076*
S138/ HM172516	784	266–676	Conserved/hypothetical protein A2cp1_2855	1e–27	unknown	*Anaeromyxobacter dehalogenans 2CP-1*
S142/ HM172517	535	1–516	Transporter, hydrophobe / amphiphile efflux-1 family(AcrB)	7e–71	transport	*Acidobacterium capsulatum ATCC 51196*
S146/ HM172518	617	3–575	(Nodb)de-N-acetylase family protein(PIG-L superfamily)	2e–75	synthesis of lipooligosaccharide signal molecule	*Acidobacterium capsulatum ATCC 51196*
S158/ HM172519	951	–	197 bp at 5' side: conserved hypothetical protein	0.36	–	*Chlorobium chlorochromatii CaD3, Propionibacterium freudenreichii*
S58/ HM172520	1057	659–1056	Twin-arginine translocation pathway signal	2e–27	legume symbiosis	*Mesorhizobium* sp. *BNC1*
S160/ HM172521	856	160–540	Sigma-70 factor	4e–26	Transcriptional regulator of a group of genes those are essential for aromatic amino acid biosynthesis and transport	*Rhodopirellula baltica SH 1*
S162/ HM172522	730		Conserved membrane protein; Rpal _1374/ vnfA	7e–68	V nitrogenise transcriptional regulator	Rhodopseudomonas palustris *TIE-1*
S55/ HM172523	1057	87–494	Diaminopimelate decarboxylase	4e–80	meso-2,6-diaminoheptanedioate $\rightleftarrows$ L-lysine + CO_2	*Rhodopseudomonas palustris BisB5*
S154/ HM172524	708	452–707	Type I secretion outer membrane protein, TolC	1e–09	an ABC transporter system for protein secretion	*Methylovorus* sp. *SIP3-4*

***In-silico* analysis**

The sequenced clones were subjected to NCBI-ORF finder tool (www.ncbi.nlm.nih.gov/projects/gorf) to find the coding ORFs in the sequences. The ORFs found are then queried in NCBI-BLAST (www.ncbi.nlm.nih.gov/blast.cgi) to find their respective homology with the known sequences in the database.

RESULTS AND DISCUSSION

Quantification of *nif*H gene from soil

Quantification of the *nif*H DNA from the used soils was carried out using the standard curve drawn on the basis of the known concentration of DNA of *B. japonicum USDA 6*. Quantification showed a linear relation ($R^2 = 1.0$) between log values of bacterial genomic DNA and real time PCR threshold cycles over the range of the examined DNA concentrations (data not shown). The copy no. of gene was calculated using formula: number of copies = (amount × 6.022×10^{23}/ (length × $10^9 \times 650$) assuming one copy of ribosomal gene per genome (Kabir et al., 2003). The results from the real time quantification show that Pithoragarh soil has the highest gene copy number, i. e. 3.22×10^{10}/µl of DNA followed by Pantnagar soil (9.29×10^9/µl of DNA). However, a slight difference has been observed within Pantnagar, Ranichauri (8.34×10^9/µl of DNA) and Chamoli (7.85×10^9/µl of DNA) soils. The least copy number of *nif*H gene was reported in Glacier soil (2.29×10^9/µl of DNA) whereas the copy number of Badrinath soil is 4.63×10^9/µl of DNA, respectively (Table 2).

Shotgun library screening

Since temperate soil of Pithoragarh has the highest *nif*H gene copy number, this soil sample was further used for shotgun meta-genomic library construction. The soil DNA was digested with BfuCI endonuclease (Fig. 1) and the restricted fragments were ligated with pUC19 vector and transformed into *E. coli* DH5α cells. More than 500 clones having insert between 0.5 to 2kb were screened for functional analysis of *nif*H gene.

Positive clones were subjected to double digestion with EcoRI and HindIII restriction enzymes (Fig. 2). Clones having similar insert were removed and finally 176 clones were selected for colony hybridisation. All the clones were

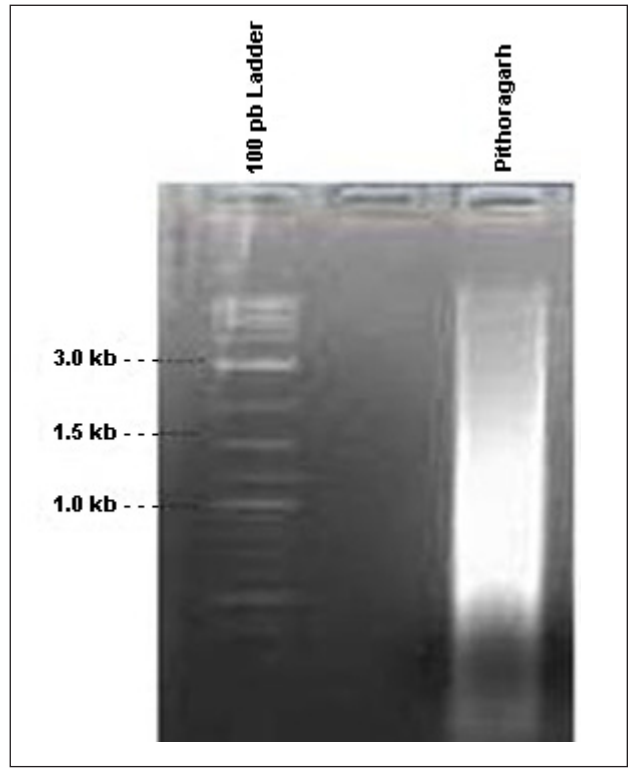

Fig. 1. Restriction digestion of Pithoragarh soil DNA with BfuCI endonuclease

Table 2. **Quantification of nifH gene from different soil samples using real time PCR**

Sample site	Concentration of amplified *nif*H gene (ng/µl of DNA)	Copy no. of *nif*H gene[a]
Pantnagar	3.61 ± 1.51	9.29×10^9
Chamoli	3.05 ± 0.15	7.85×10^9
Ranichauri	3.24 ± 0.53	8.34×10^9
Pithoragarh	12.5 ± 1.07	3.22×10^{10}
Badrinaath	1.8 ± 0.23	4.63×10^9
Glacier	0.89 ± 0.16	2.29×10^9

[a] The formula used for copy no. calculation is: number of copies = (amount *N / (length * 1×10^9 * m)
where m = average weight of a base pair (bp) is 650 Daltons, N = Avogadro's number (6.022×10^{23} molecules / mole)

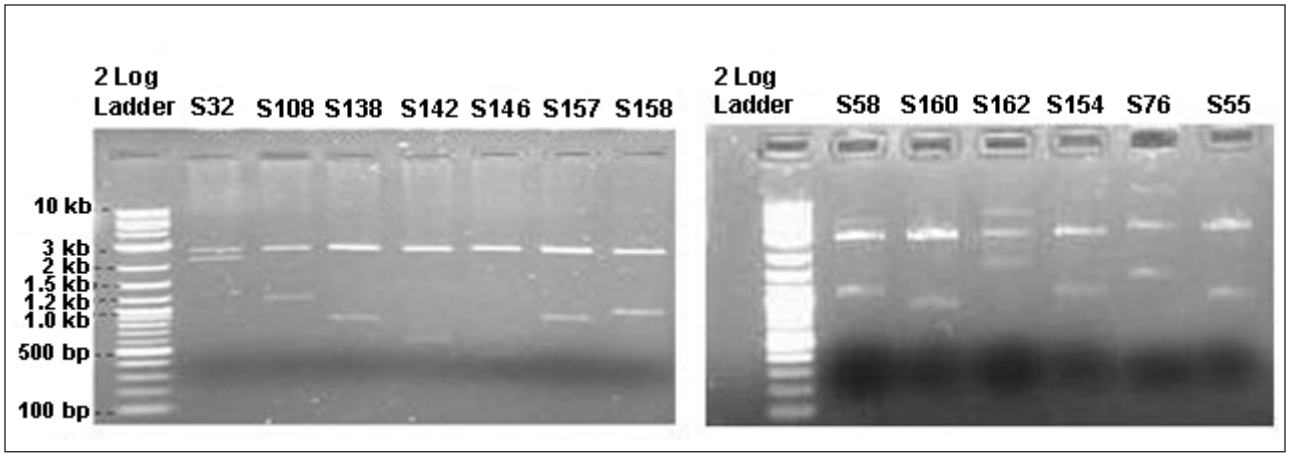

Fig. 2. Double digestion (EcoRI / HindIII) profile of selected shotgun clones which were screened by colony hybridisation results and sequenced, subsequently

hybridised with two probes, i. e. NB6 and BR1 (Fig. 3) which codes the partial *nif*H gene of uncultured bacterium and *B. japonicum USDA 6*, respectively. From colony hybridisation with NB6 probe DNA, 52 clones were found to be positive. However, 32 clones were positive in colony hybridisation with probe BR1. The blot intensity in case of hybridisation with probe BR1 is higher than the hybridisation with NB6. Thirteen clones (S32, S55, S76, S108, S138, S142, S146, S154, S157 & S158 selected by NB6 probe whereas S58, S160 & S162 from BR1 probe) having less than 2kb insert (Figs. 4 and 5) were sequenced by primer walking method using M13R & M13F sequencing primers and submitted to NCBI database.

In-silico analysis

The open reading frame (ORF) coded by respective sequences is listed in Table 1. Clones S32 and S157 do not have any ORF of suitable length due to its short length since both are smaller

>probe NB6
CCATCATCTCGCCGGAGCAGACGATATAGATCTCTTCGGCCTTGC
CTTCGCGGATCGGCATGGCAAAGCCCCCGCAGACAACGTCACCG
AGTACGTCGTAGAAGACAAAGTCGAGATCAGGAGTGTAGGCGCCG
TTTTCTTCCAGGAAGTTGATGGCGGTGATGACACCCCGGCCGGCA
CAGCCGACGCCCGGCTCAGGACCGCCGGACTCGACGCACTTCAC
ATAGCCATAACCGACCTTCATGACATCCTGCAGTTCCAGATCCTCG
ACCGTGCCCAGCTCGCGCACCAGGTCCACGACCGTATTCTGTGCC
TTGGCATGCAGTATCAAACGGGTCGAGTCAGCCTTCGGGTCGCAG
CCGACGATCATGACCTTTTTGCCCAGTGATGCCAAACCTGCCACC
GTGTTCTGTGTGGTGGTTGATTTGCCGATGCCACCTTT

>probe BR1
TGCGATCCGAAACGCTGACTCGACTCGCCTTATTCTGCACGCCAAGGC
TCAAGACACGATTTTGAGTCTTGCCGCGAGCGCCGGCAGCGTGGAGG
ATCTAGAGCTCGAGGACGTAATGAAGGTTGGCTACCAGGACATTCGCT
GCGTTGAGTCCGGTGGCCCTGAGCCAGGTGTCGGCTGCGCCGGCCG
CGGTGTCATCACCTCGATCAATTTTCTTGAAGAGAACGGAGCCTACGA
GAACATTGACTATGTTTCTTACGATGTGCTTGGCGACGTTGTTTGCGGT
GGCTTTGCGATGCCAATCCGCGAAAACAAGGCGCAGGAGATCTACATC
GTGATGTCCGGGGAAATGATGGC

Fig. 3. Nucleotide sequences of probe NB6 and BR1

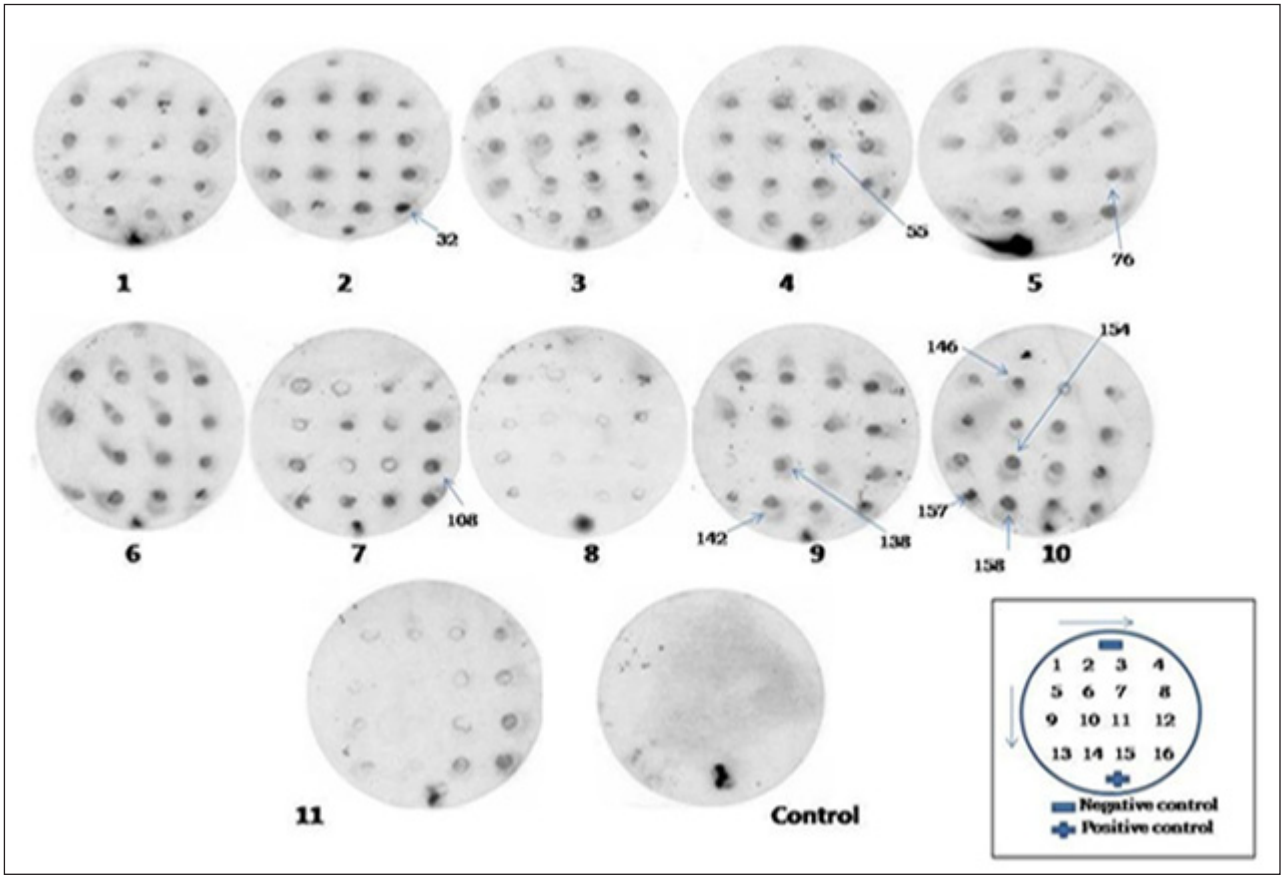

Fig. 4. Colony hybridisation of shotgun clones with probe NB6. Where E. coli DH5α transformed with pUC 19 plasmid was used as negative control and clone NB6 was taken as positive control in each plate individually as depicted in box

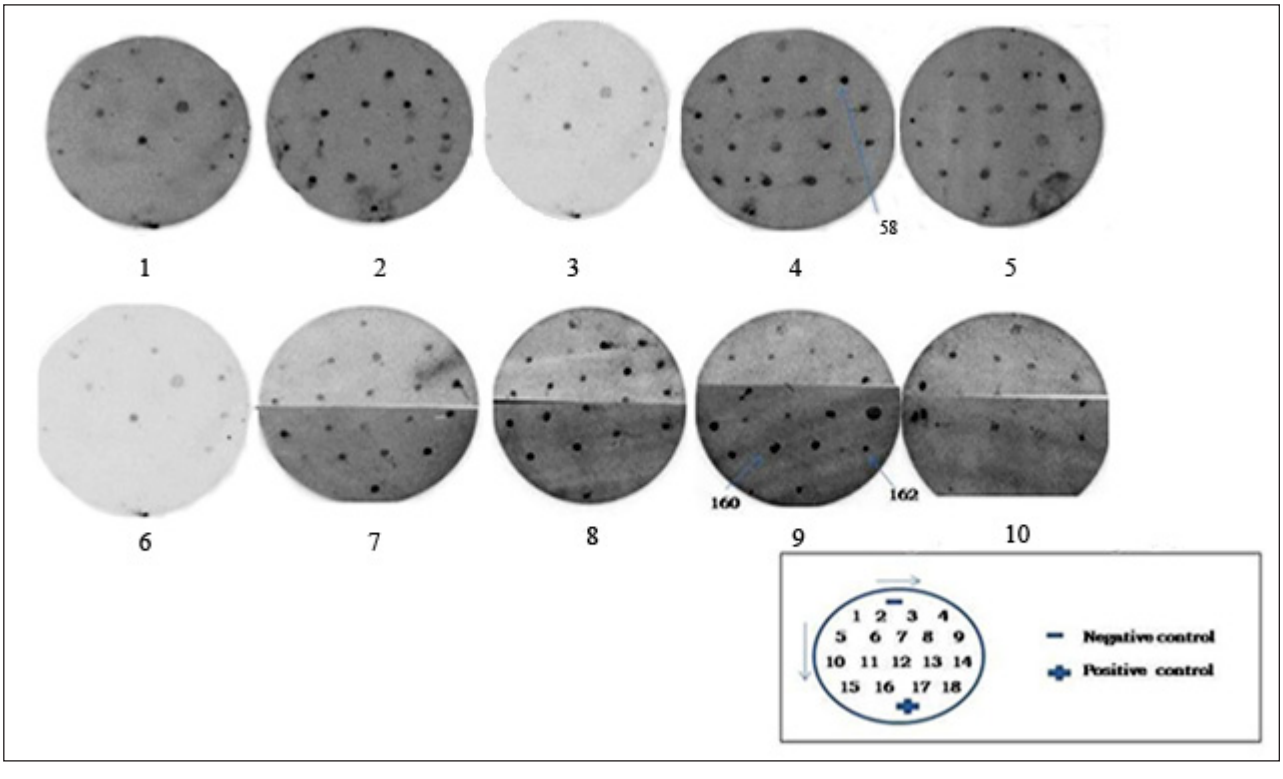

Fig. 5. Colony hybridisation results of shotgun clones with probe BR1. Where E. coli DH5α transformed with pUC 19 plasmid was used as negative control and clone BR1 was taken as positive control in each plate individually as depicted in box

than 300 bp. All clone sequences are either showing their link to nitrogen fixating gene cluster, or genes which express with nitrogenase (EC 1.18.6.1) gene cluster, or other genes homologous to *nif*H/vnfh/anfH gene sequences. All the sequences have some conserved nucleotides. However, none of them directly show homology with *nif*H gene. Moreover, their link with nitrogenase gene cluster or other adjacent genes can be established considering the fact that maximum clones sequences show the homology with protein reported in nitrogen fixing community (Table 1).

Clones S58, S16O and S162 (all are screened by hybridisation with probe BR1) show more than significant homology with genes involved in nitrogen fixation. Clone S162 has the ORF which code for the gene vnfA of *Rhodopseudomonas palustris* (RPA No. 1374). *R. palustris* is a purple facultatively photosynthetic bacterium that is an attractive organism to develop as a biocatalyst for hydrogen production by means of nitrogen fixation because it can generate ATP from light and reductant from acetate and green plant-derived aromatic compounds to drive this process. The *vnf*A gene product is the V nitrogenase transcriptional regulator, a part of clusters (Oda et al., 2005). Further, S58 and S160 show the presence of twin arginine translocation (Tat) gene of *Mesorhizobium* sp. BNC1 and sigma 70 factor of *Rhodospirellula baltica* SH1, respectively. Both organisms are well established for nitrogen fixation. The former is a well known symbiotic nitrogen fixer while the other is reported as free living nitrogen fixing bacteria (Sant'Anna et al., 2009). The gene encoded by clone S58 is closely related to nitrogen fixation. This Tat system is essential for symbiotic nitrogen fixation by *B. japonicum* (Thony-Meyer et al., 1989) and *Rhizobium leguminosarum* (Wu et al., 1996) and also for hydrogenase activity and cytochrome *c*-dependent respiration.

Further, clones sequenced after colony hybridisation using NB6 are S32, S55, S76, S108, S138, S142, S146, S154, S157 and S158. *In-silico* sequence analysis of all the clones show that clones S55, S142, S146 and S154 have the homology with diaminopimelate decarboxylase of *R. palustris BisB5*, amphiphile efflux-1 family protein (AcrB) of *A. capsulatum ATCC 51196*, Nod b (de-N-acetylase family protein) of *Acidobacterium capsulatum ATCC 51196* and type I secretion outer membrane protein (TolC) of *Methylovorus* sp. *SIP3-4*, respectively. Whereas clones S108, S138 and S158 have the homology with hypothetical proteins of *Solibacter usitatus Ellin6076, Anaeromyxobacter dehalogenans 2CP-1* and *Chlorobium chlorochromatii CaD3*, respectively. Most of the proteins coded by these clones are present in bacteria which are reported for nitrogen fixing ability (Table 1). However, clones S32, S76 and S157 do not show significant homology with any protein. Further, clones S142 and S154 code for transporter proteins. Type I secretion outer membrane protein is an ABC transporter system for protein secretion. It is reported that various ABC and other transporter systems are adjacent to nitrogenase gene clusters whose expression increases with nitrogenase expression (Oda et al., 2005). Clone 146 shows the homology with Nodb gene which indicates that this clone contains the fragment from bacterium which forms nodulation in plants and may be involved in nitrogen fixation. This Nodb gene is involved in the synthesis of lipooligosaccharide signal molecule during nodule formation (John et al., 1993). Reports are also available that various hypothetical proteins are available under nitrogenase gene cluster (Oda et al., 2005). The previous study conducted by Oda et al., 2005 reports that hypothetical or conserved hypothetical or proteins of unknown function are present in vanadium and molybdenum nitrogenase gene cluster. However, iron nitrogenase cluster does not have any hypothetical protein or conserved hypothetical or proteins of unknown function. This study also supports the presence of hypothetical protein or conserved hypothetical protein adjacent to vanadium and iron nitrogenase gene clusters.

It is concluded that the temperate soil of Pithoragarh (temperate region) has dominance of *nif*H gene. In general, *in-silico* study of sequenced shotgun clones reveals that all the ORFs present in respective clone sequences have the homology with genes involved in nitrogen fixation directly or indirectly. We are proposing a hypothesis that *nif* genes like *nif*H may evolve from their nearest genes or adjacent regions and in due course become specific in their functions.

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*nif*H HOMOLOGAI IŠ DIRVOŽEMIO METAGENOMO

S a n t r a u k a

Biologinė azoto fiksacija yra svarbus procesas, kurio dėka azotas patenka į biologines sistemas. Tačiau iki šiol nėra aišku, kaip susiformavo ar evoliucionavo pirmasis *nif* genas. *nif*H genų įvairovė jų metagenome buvo analizuota dirvožemio mėginiuose iš įvairių agrarinių klimatinių zonų Indijos Himalajuose. Išskirta metagenominė DNR ir kiekybiškai įvertintas *nif*H genas naudojant polimerazinę grandininę reakciją (PGR). Kuriant metagenomo biblioteką buvo naudojamas dirvožemis iš Pithoragarho vietovės (vidutinio klimato zona). Tikrinti panašius intarpus turintys klonai, o 176 klonai buvo naudojami kolonijos hibridizacijai. Visi klonai buvo hibridizuoti su dviem *nif*H zondais – NB6 ir BR1, teigiami klonai buvo sekvenuoti. Sekvenuotų klonų *in-silico* tyrimas atskleidė *nif*H homologiją su keletu kitų genų, tiesiogiai nedalyvaujančių azoto fiksacijoje, bet priklausančių bakterijų genčiai, kuri geba fiksuoti azotą. Mūsų hipotezė: *nif* genai, pavyzdžiui, *nif*H, evoliucionavo iš artimiausių genų arba gretimų regionų ir tinkamu laiku jų funkcijos tapo specifinės.

Raktažodžiai: nitrogenazė, metagenomika, *nif*H, tikro laiko PGR