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# *nifH* gene of reference being the source to study the marine *Azotobacter* sp.

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## Abstract

The nitrogenase enzyme is present in diverse lineages of prokaryotes and is generally believed to be within the *nif* gene, *nifH* gene which encodes the Fe protein component of nitrogenase enzyme. In the present study, around 11 *Azotobacter* strains were isolated and identified using routine microbiological techniques. Different *nifH* primers were used for the study of *Azotobacter* sp from the marine region by PCR technique. The genomic DNA of the *Azotobacter* isolates and reference were extracted and assayed by biophotometer. It was observed that the DNA samples were pure and their concentrations ranged from 0.705 to 0.88 mg / ml. It was revealed that the *nifH* gene was amplified by specific and universal primers whereas non-specific primer does not amplify the gene. The intensity of the amplified product was more when universal primer was used in strains TA1 and MA 378 than other isolates at the range of 370 bp. The genus specific primer (Primer 3) produced band at the range of 390bp for all the isolates. This showed 100% homogeneity with *nifH* genes among marine *Azotobacter* sp. recorded. Yet another genus specific primer (Primer 4) was more specific and amplified at the range of 370 bp for the most of the isolates. It was analogous to that of Primer 3 by producing clear identical bands; however, synthesis of sequences (370bp) resembled that of Primer 2. It was clearly showing a high degree of homogeneity among the strains

**Keywords:** amplification, *Azotobacter*, homogenneity, nifH gene, primers

## INTRODUCTION

The genome of the *Azotobacter* has the gene coding for nitrogenase enzyme, involved in the nitrogen fixation. DNA-based studies mainly provide information on community structure, and provide additional information on activities of specific populations (Greer *et al.*, 2001). The *nifH* sequences in soil indicate a great diversity and great variation of diazotroph community compositions in different soils. Molecular methods based on universal PCR detection of *nifH* marker genes have been successfully applied to describe diazotroph population (Burgmann *et al.*, 2003; Musat *et al.*, 2006). The PCR method used, which led to a rapid identification of the *Azotobacter* and other isolates, was previously reported by de Bruijn (1992) and Vinuesa *et al.* (1998) to be highly specific for each isolate and useful for classification of bacterial strains. The present investigation was aimed to study the *Azotobacter* population from the seagrass ecosystem, Thondi coast of Tamilnadu, India by molecular techniques. The study included determination of the gene size, diversity and specificity of *nifH* primers for the gene of *Azotobacter* sp.

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## MATERIALS AND METHODS

### Strains

The strains (marine isolates from water and sediments) were isolated by routine microbiological methods. The isolates were selected by primary screening methods and then the isolates were subjected for the identification of *nifH* gene by PCR method using different primers. The strains used are presented in Table 1.

### Extraction of DNA

The strains listed in (Table 1) were subjected for the DNA extraction by CTAB method. Then the Extracted DNA samples (20µl) were assayed by using Biophotometer (eppendorf) for the purity and concentration of the DNA. The results were further confirmed by agarose gel electrophoresis.

### PCR analysis

Different primers were selected from earlier references for the study of marine diazotropic *Azotobacter* sp. The existence and homogeneity of the isolates (marine diazotropic *Azotobacter* sp) were analysed by 4 different primers obtained from Operon Biotechnologies, Germany.

**(a) Primers**

Primer 1- Non specific

Forward-5'-GGAATTCCTGYGAYCCNAARGCCNA-3'

**Plate 1.** Strains of Azotobacter isolated / used

| Strain | Description |
|---|---|
| TA 1 | *Azotobacter chroococcum* (MTCC 446) |
| MA 14 | *Azotobacter* sp. |
| MA 20 | *Azotobacter* sp. |
| MA 45 | *Azotobacter* sp. |
| MA 64 | *Azotobacter* sp. |
| MA 102 | *Azotobacter* sp |
| MA 147 | *Azotobacter* sp. |
| MA 212 | *Azotobacter* sp. |
| MA 326 | *Azotobacter* sp. |
| MA 378 | *Azotobacter* sp. |
| MA 452 | *Azotobacter* sp. |
| MA 532 | *Azotobacter* sp. |

MTCC – *Microbial Type Culture Collection, Chandigarh, India.*
MA - *Marine Azotobacter*, TA – *Type culture Azotobacter.*

Reverse – 5′-CGGATCCGDNGCCATCATYTCNCC-3′

It was from nifH of *Anabaena* sp. the product size was about 324bp (Burlage *et al.*, 1998).

Primer 2 - Universal primer

Forward 5′- GGITGYGAYCCNAAVGCNGA -3′

Reverse 5′ – GCRTAIABNGCCATCATYTC′- 3′

A data base of 137 *nifH* DNA sequences of cultivated diazotrophs published in GenBank was assembled and manually aligned by using Bio-Edit version 5.0.9 (Hall, 1999). The DNA sequences of the primer sites were defined by the *nifH*-universal primer set (Widmer *et al.*, 1999). Degeneracy is indicated by standard conventions: Y, C/T; N, A/C/G/T; V, A/C/G; R, A/G; B, C/G/T.

I (inosine) is used to replace N at the 5′ portion of the degenerate primers. The expected size of the PCR products was 370 bp for all strains.

Primer 3 – Specific primer

5′- GCIWTYTAYGGIAARGGIGG-3′

5′- AAICCRCCRCAIACIACRTC-3′

It was from *Azotobacter vinelandii* - M20568, the product size was 390 bp. Where, I-Inosine, R - A or G, W - A or T, and Y - C or T (Ueda *et al.*, 1995).

Primer 4 – Specific primer

Forward 5′-GGTTGTGACCCGAAAGCTGA-3′

Reverse 5′-GCGTACATGGCCATCATCTC-3′

Primer set *nifH*-g1 targets *Azotobacter* spp. *nifH* sequences (GenBank accession numbers M11579, M20568, X13519, M73020, X03916). Amplification of DNA with this primer was expected to yield a fragment of approximately 370 bp for all strains. (Burgmann *et al.*, 2004).

**(b) PCR Reactions**

PCR reactions were done based on a modified protocol proposed by Burgmann *et al.* (2004). 100 ng of each DNA sample was used for amplification. About 15 ml of DNA quality water was used to make a final volume up to 25 ml. Amplification was performed using thermal cycler (Eppendorf). The amplification conditions were denaturation at 94 °C for 1 min, annealing at 56 °C for 1 min, elongation at 72 °C for 1 min. The steps 2-4 were repeated 35 times, and the final extension was at 72 °C for 10 min and then 4 °C holding. The amplified products (10 ml) were resolved by electrophoresis in 1.5 % (w/v) agarose gels containing ethidium bromide (1.6 mg $ml^{-1}$) at 50 V in TBE buffer. The sizes of the amplified products were determined by comparison with 100bp DNA ladder (Genei, India).

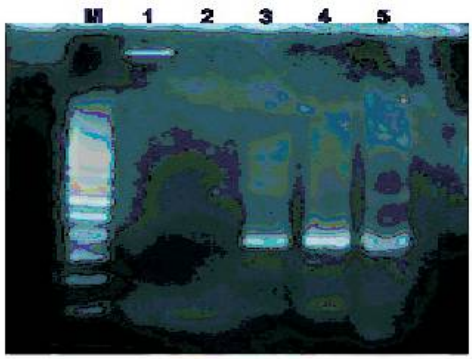
## RESULTS

In the present study, 12 strains were selected for the analysis of *nifH* gene, for which the genomic DNA were isolated from the isolates, with the MTCC culture as a standard. The DNA was assayed using biophotometer for the purity and the concentrations. The DNA samples 260/280 values were between the 1.8 and 2.0 and the concentration of DNA ranged from 0.705 to 0.88 mg / ml.

The purified DNA of strain TA 1 was used for amplification of *nifH* gene with specific (Primer 3 and 4), non-specific (primer 1) and universal (primer 2) primers. It revealed that the *nifH* gene was amplified by specific and universal primers whereas non-specific primer was unable to amplify (Plate 1).

When the Universal primer (primer 2) was used against all the selected strains, it scored clear amplified products at 370bp in all the lanes except lanes 5, 9 and 10. These three lanes were clear immediately after amplification and later became diffused. Strain MA 378 (lane 11) scored a high intense band as like TA 1 (lane 2), whereas all other strains showed identical bands at 370bp (Plate 2).

Specific primer (Primer 3) was used for the same template DNA samples and it was recorded that all the strains scored clear visible bands at 390bp region. Strain MA 64 (Lane 5) showed a faint band and MA 532 (Lane 13) showed a secondary diffused band at 600bp. The *nifH* gene amplification by the primer 3 was specific, clear and identical in all the strains with amplified fragment at 390bp range. It showed 100% homogeneity with *nifH* genes of marine *Azotobacter* sp (Plate 3).




M - DNA (100bp) Marker
1 - Template DNA
2 - *nif H* gene by Primer 1
3 - *nif H* gene by Primer 2
4 - *nif H* gene by Primer 3
5 - *nif H* gene by Primer 4

**Plate 1.** PCR amplification of *nifH* gene (TA1) using different primers

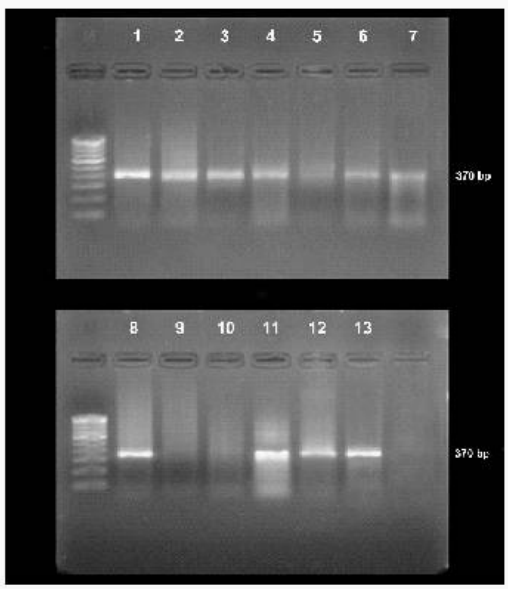



Plate 2. *NifH* gene by PCR using Primer 2

| Lane | Isolates |
|---|---|
| M | Marker 100 bp DNA marker |
| 1 | TA 1 |
| 2 | MA 14 |
| 3 | MA 20 |
| 4 | MA 45 |
| 5 | MA 64 |
| 6 | MA 102 |
| 7 | MA 147 |
| 8 | TA 1 |
| 9 | MA 212 |
| 10 | MA 326 |
| 11 | MA 378 |
| 12 | MA 452 |
| 13 | MA 532 |

**Plate 2.** *nifH* gene by PCR using primer 2

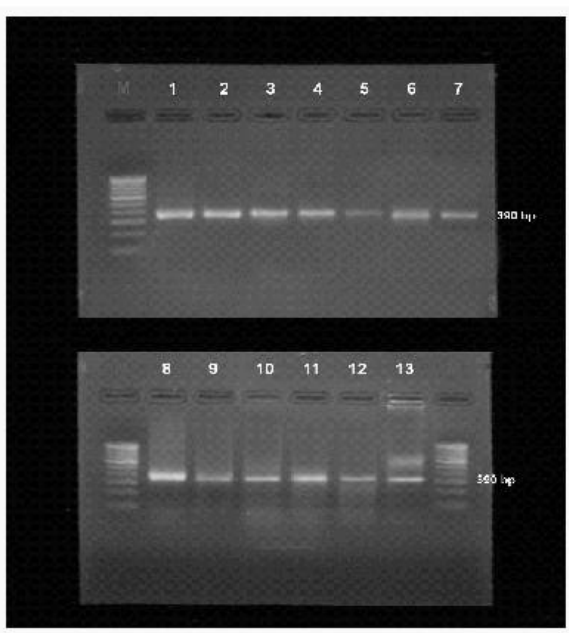



Plate 3. *NifH* gene by PCR using Primer 3

| Lane | Isolates |
|---|---|
| M | Marker 100 bp DNA marker |
| 1 | TA 1 |
| 2 | MA 14 |
| 3 | MA 20 |
| 4 | MA 45 |
| 5 | MA 64 |
| 6 | MA 102 |
| 7 | MA 147 |
| 8 | TA 1 |
| 9 | MA 212 |
| 10 | MA 326 |
| 11 | MA 378 |
| 12 | MA 452 |
| 13 | MA 532 |

**Plate 3.** *nifH* gene by PCR using primer 3

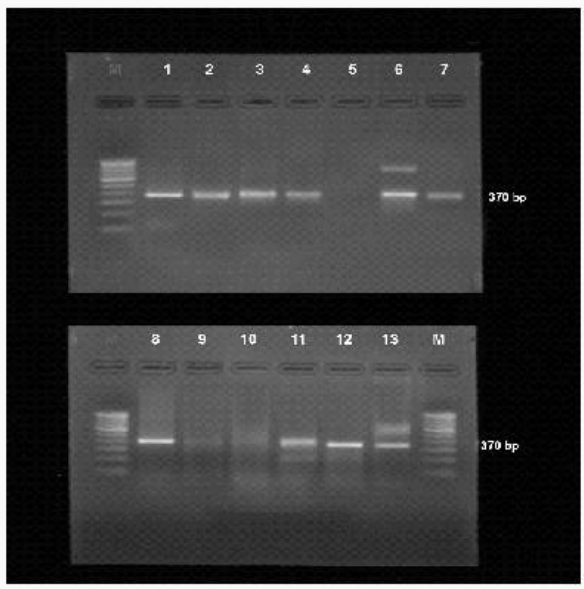



Plate 4. *NifH* gene by PCR using Primer 4

| Lane | Isolates |
|---|---|
| M | Marker 100 bp DNA marker |
| 1 | TA 1 |
| 2 | MA 14 |
| 3 | MA 20 |
| 4 | MA 45 |
| 5 | MA 64 |
| 6 | MA 102 |
| 7 | MA 147 |
| 8 | TA 1 |
| 9 | MA 212 |
| 10 | MA 326 |
| 11 | MA 376 |
| 12 | MA 452 |
| 13 | MA 532 |

**Plate 4.** ***nifH*** **gene by PCR using primer 4**

Specific primer (Primer 4) was also used to amplify the template DNAs of the marine isolates. It scored a clear identical band in TA 1 and some marine isolates. In contrast, the strain MA 64 was not amplified and the strain MA 212 and MA 326 did not produce clear bands when primer 4 was used, though it produced a faint band of 600 bp, above the *nifH* gene band. It revealed that the primer was more specific and amplifying only the 370 bp *nifH* sequences of *Azotobacter* sp. It was analogous to that of Primer 3 by producing clear identical bands. However synthesis of sequences (370bp) resembled that of Primer 2 (Plate 4).

Bands were scored as present or absent. A high degree of homeogeneity was demonstrated in all the isolates based on the presence of *nifH* gene. The DNA was clearly amplified by the primers which showed bands of 370 and 390 bp. It encodes the *nifH* gene responsible for the Fe protein of the nitrogenase enzyme. The results of PCR with primer 1 showed no banding pattern in agarose gel, where as primer 2 and primer 4 showed the amplification of *nifH* DNA products of 370 bp. (Plate 2 and 4). The *nifH* gene was amplified by primer 3 which was a 390 bp fragment. Clear identical bands were observed in all the marine isolates. It was also observed that there was no substantial difference between the banding pattern among the marine isolates and TA1 strain. As such the strains exhibited homogeneity to a greater extent (Plate 3).

## DISCUSSION

Nitrogenase gene (*nifH*) sequences amplified directly from oceanic waters showed that the open ocean contains more diverse diazotrophic microbial populations and more diverse habitats for nitrogen fixers than previously observed by classical microbiological techniques (Zehr *et al.*, 1998).

More recently, functional genes (e.g., the *nifH* gene as a marker for $N_2$ fixation) have also been used for this purpose (Zehr and McReynolds, 1989; Widmer *et al.*, 1999; Poly *et al.*, 2001a). DNA-based studies have been reported to provide more information on community structure (Burgmann *et al.*, 2003).

Due to the physiological diversity of diazotrophs and the documented unculturability of many prokaryotes (Pace, 1996; Hugenholtz *et al.*, 1998), cultivation-based strategies have severe limitations for the description of the diversity of free-living soil diazotrophs. Therefore, molecular approaches have been developed and successfully applied to describe diazotroph communities in different ecosystems (Widmer *et al.*, 1999; Piceno *et al.*, 1999; Lovell *et al.*, 2000; Poly *et al.*, 2001; Rosch *et al.*, 2002; Hamelin *et al.*, 2002). The molecular approach to study the diversity of diazotroph organisms is primarily based on PCR amplification of a marker gene (*nifH*) for $N_2$ fixation (Bairoch, 1993; Musat *et al.*, 2006a).

In the present investigation, 12 strains of *Azotobacter* were selected and the genomic DNA was extracted and assayed by biophotometer. The DNA was pure and had the concentration of 0.705 to 0.88 mg / ml for the isolates

Based on the analysis of the *nifH* gene nucleotide sequences from GenBank, a system of primers was developed that makes it possible to obtain 370 and 390 bp PCR fragments of the *nifH* gene of nitrogen-fixing bacteria and archaea. The effectiveness of the proposed system for revealing the presence of *nifH* genes was demonstrated by PCR on the DNA isolated from nitrogen-fixing prokaryotes and belonging to different taxonomic groups for which the primary structure of these genes is known. The *nifH* gene sequences of nitrogen-fixing prokaryotes of the genera *Xanthobacter*, *Beijerinckia*, and *Methanosarcina*, for which

the capacity for nitrogen fixation was demonstrated earlier (Enkh-Amgalan *et al.*, 2005; Fisher *et al.*, 2007).

Based on previous reports, in the present investigation, four different primers have been selected for the amplification of *nifH* gene. Among them, primer 3 gave the best amplification of *nifH* in all the selected strains. The maximum molecular weight of the amplified product observed was 390 bp except for 2 strains. Amplification by primer 4 resulted in 370 bp fragment. Similar results were obtained with primer 2.

Based on the similarity in the size of amplified products in 2 different experiments in which primer 2 and 4 were used, it can be speculated that the specific and universal primer anneal to the *nifH* gene region of the genome in close proximity. Similarly, in a study conducted by Marin *et al.* (2003), with *Burkholderia* genus, the *glnB* and *nifH* gene fragments were amplified by PCR using universal degenerated primers and 100% similarity for the *nifH* genes was observed in two strains of diazotrophs in natural environments.

Universal primers have been designed and successfully used for the amplification of *nifH* gene among diazotrophs (Zehr *et al.*, 1998; Widmer *et al.*, 1999; Poly *et al.*, 2001a; Rosch *et al.*, 2002). In the present study, in order to achieve universal amplification, these primers have been designed to target *nifH* gene regions encoding highly conserved amino acid sequences. Primer 3, which is a genus specific primer, resulted in expected size of amplified product. This clearly indicates that genus / species specific primers have wide application on the amplification, than universal primers.

MacGregor *et al.* (2001) amplified both cyanobacterial and non-cyanobacterial *nifH* gene sequences (encoding the nitrogenase iron protein) from lakewater samples, which showed evidence for the presence of bacteria capable of nitrogen fixation. Whereas in the present study, the same *Anabaena* primer (primer 1) was used and no amplification has been observed. This may be due to the non-specificity of the primer for *Azotobacter* sp. In conclusion, primer 3, which yielded 390bp fragment, can be used as the most suitable primer for the amplification of *nifH* gene and molecular profiling of marine isolates of *Azotobacter* sp.

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## REFERENCE

Amann, R. I., W. Ludwig and K. Schleifer, 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiol. Rev.*, 59:143–169.

Bairoch, A. 1993. The ENZYME data bank. *Nucleic Acids Res.*, 21:3155–3156.

Burgmann, H., F. Widmer, W. V. Sigler and J. Zeyer. 2003. mRNA Extraction and Reverse Transcription-PCR Protocol for Detection of nifH Gene Expression by Azotobacter vinelandii in Soil. *Appl. Environ. Microbiol.*, 69:1928-1935.

Burgmann, H., F. Widmer, W. Von Sigler, and J. Zeyer. 2004. New Molecular Screening Tools for Analysis of Free-Living Diazotrophs in Soil. *Appl. Environ. Microbiol.*, 70:240-247.

Burlage, R. S., R. Atlas, D. Stahl, G. Geesey and G. Sayler. 1998. Techniques in microbial ecology. Oxford University Press, New York, N.Y. (*ed.*), (Kelly, D. P., and A. P. Wood. Microbes of the sulfur cycle). P.31-57

de Bruijn, F. J. 1992. Use of repetitive (repetitive extragenic palindromic and enterobacterial repetitive intergeneric consensus) sequences and the polymerase chain reaction to fingerprint the genomes of *Rhizobium meliloti* isolates and other soil bacteria. *Appl. Environ. Microbiol.*, 58:2180–2187.

Enkh-Amgalan, J., Hiroko Kawasaki and Tatsuji Seki, 2005. *NifH* and *NifD* sequences of heliobacteria: a new lineage in the nitrogenase phylogeny. *FEMS Microbiol. Lett.*, 243: 73–79.

Fisher, K., Lowe, D. J., Tavares, P., Pereira, A. S., Huynh, B. H., Edmondson, D. and Newton, W. E. 2007. Conformations Generated during Turnover of the Azotobacter vinelandii Nitrogenase MoFe Protein and Their Relationship to Physiological Function, *J. Inorg. Biochem.*, 10: 10-16.

Greer, C. W., Whyte, L. G., Lawrence, J. R., Masson, L. and Brousseau, R. 2001. Genomics technologies for environmental science. *Environ. Sci. Technol.*, 35:364A-370A.

Hall, T. A. 1999. Bio-Edit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp. Ser.*, 41:95–98.

Hamelin, J., Fromin, N., Tarnawski, S., Teyssier-Cuvelle, S. and Aragno, M. 2002. *nifH* gene diversity in the bacterial community associated with the rhizosphere of *Molinia coerulea*, an oligonitrophilic perennial grass. *Environ. Microbiol.*, 4:477–481.

Hugenholtz, P., Goebel, B. M. and Pace, N. R. 1998. Impact of culture independent studies on the emerging phylogenetic view of bacterial diversity. *J. Bacteriol.*, 180:4765–4774.

Jones, R., Woodley, P., Birkmann-Zinoni, A. and Robson, R. L. 1993. The nifH gene encoding the Fe protein

component of the molybdenum nitrogenase from Azotobacter chroococcum. *Environ. Microbiol.*, 123:145-6.

Lovell, C. R., Piceno, Y. M., Quattro, J. M. and Bagwell, C. E. 2000. Molecular analysis of diazotroph diversity in the rhizosphere of the smooth cordgrass, *Spartina alterniflora*. *Appl. Environ. Microbiol.*, 66:3814–3822.

MacGregor, B.J., Van Mooy, B., Baker, B.J., Mellon, M., Moisander, P.H., Paerl, H.W., Zehr, J., Hollander, D. and Stahl, D.A. 2001. Microbiological, molecular biological and stable isotopic evidence for nitrogen fixation in the open waters of Lake Michigan. *Environ. Microbiol.*, 3:205-19.

Marin, V. A., Teixeira, K. R. and Baldani, J. I. 2003. Characterization of amplified polymerase chain reaction glnB and nifH gene fragments of nitrogen-fixing Burkholderia species. *Lett. Appl. Microbiol.*, 36:77-82.

Michener, C. D. and Sokal, R. R. 1957. A quantitative approach to a problem in classification. Evolution., 11:130–162.

Musat, F., Harder, J. and Widdel, F. 2006. Study of nitrogen fixation in microbial communities of oil-contaminated marine sediment microcosms. *Systematic Appl. Microbiol.*, 8:1834-43.

Musat, N., Ursula Werner, Katrin Knittel, Steffen Kolb, Tanja Dodenhof, Justus, E. E. van Beusekom, Dirk de Beer, Nicole Dubilier and Rudolf Amann. 2006a. Microbial community structure of sandy intertidal sediments in the North Sea, Sylt-Romo Basin, Wadden Sea. *Systematic Appl. Microbiol.*, 29:333–348.

Pace, N. R. 1996. New perspective on the natural microbial world: *Mol. Microb. Ecol.*, 62:463–470.

Piceno, Y. M., Noble, P. A. and Lovell, C. R. 1999. Spatial and temporal assessment of diazotroph assemblage composition in vegetated salt marsh sediments using denaturing gradient gel electrophoresis analysis. *Microb. Ecol.*, 38:157–167.

Poly, F., Lionel Ranjard, Sylvie Nazaret, François Gourbière, and Lucile Jocteur Monrozier. 2001. Comparison of nifH Gene Pools in Soils and Soil Microenvironments with Contrasting Properties. *Appl. Environ. Microbiol.*, 67:2255-2262.

Poly, F., Monrozier, L. J. and Bally, R. 2001a. Improvement in the RFLP procedure for studying the diversity of *nifH* genes in communities of nitrogen fixers in soil. *Res. Microbiol.*, 152:95–103.

Rosch, C., Mergel, A. and Bothe, H. 2002. Biodiversity of denitrifying and dinitrogen-fixing bacteria in an acid forest soil. *Appl. Environ. Microbiol.*, 68:3818–3829.

Vinuesa, P., Rademaker, J. L. W., de Bruijn, F. J. and Werner, D. 1998. Genotypic characterization of *Bradyrhizobium* strains nodulating endemic woody legumes of the Canary islands by PCR-restriction fragment length polymorphism analysis of genes encoding 16S rDNA (16S rDNA) and 16S-23S rDNA intergenic spacers, repetitive extragenic palindromic PCR gemomic fingerprint, and partial 16S rDNA sequencing. *Appl. Environ.Microbiol.*, 64:2096–2104.

Widmer, F., Shaffer, B. T., Porteous, L. A. and Seidler, R. J. 1999. Analysis of *nifH* gene pool complexity in soil and litter at a Douglas fir forest site in the Oregon Cascade Mountain Range. *Appl. Environ. Microbiol.*, 65:374–380.

Zehr, J. P. and Capone, D. G. 1996. Problems and promises of assaying the genetic potential for nitrogen fixation in the marine environment. *Microb. Ecol.*, 32:263–281.

Zehr, J. P., Mellon, M. T. and Zani, S. 1998. New nitrogen-fixing microorganisms detected in oligotrophic oceans by amplification of nitrogenase (*nifH*) genes. *Appl. Environ. Microbiol.*, 64:3444–3450.

Zehr, J. P. and McReynolds, L. A. 1989. Use of degenerate oligonucleotides for amplification of the *nifH* gene from the marine cyanobacterium *Trichodesmium thiebautii*. *Appl. Environ. Microbiol.*, 55:2522–2526.