Nepal Journal of Science and Technology 1 (1999) 91-98

Genetic Study of NOD X + and NOD X - Containing *Rhizobium leguminosarum* biovar *viciae* from Nepal and China

K. D. Yami[1], A. Tempelman-Bobbink[2] and T. A. Lie[2]

[1]*Royal Nepal Academy of Science and Technology, P. O. Box 3323, Kathmandu, Nepal*
[2]*Microbiology Laboratory, Department of Biomolecular Sciences, Wageningen Agricultural University, 6703 CT Wageningen, The Netherlands*

Received October 1998; accepted February 1999

Abstract

Seventy eight isolates of *Rhizobium leguminosarum* biovar *viciae (Rlv.)* from Central Nepal and five isolates of *Rlv.* from North East China were made from the nodules of cultivars (cv.) Afganisthan, Nepal and Rondo inoculated with soil samples collected from diverse parts of these two countries. This is the first report that NOD X containing *Rlv.* bacteria similar to TOM primitive Rhyzobium strain of Afganistan pea are present in soil of Nepal and China. Among the soil samples tested those from valleys of Nepal mostly did not contain NOD X *Rlv.* whereas such bacteria were prevalent more in the mountains. Amplification of 16S and 16-23S of isolates of *Rlv.* bacteria by polymerase chain reactions (PCR) were carried out and the products were subjected to Amplified Ribosomal DNA Restriction Analysis (ARDRA). The resulting restriction fragment patterns when analysed with Molecular Analyst Software Fingerprinting the dendrograms showed two diverse groups of bacterial genotypes within the locations of Nepal and China from where the soil samples have been collected. The groupings were irrespective of whether they contain NOD X or not.

Introduction

Importance of *R. leguminosarum* bacteria lies in its ability to form Nitrogen fixing nodules in peas, broadbean, lentil etc. which are widely grown in Asian subcontinent because they are among the cheapest sources of protein. Genetic variation within cultivated pea lines, with regard to symbiotic response to *Rhizobium*, is very low (Lie *et al.* 1976). In contrast, large variation was detected in wild and primitive plants, and several lines were found which have a defect either in nodule formation or in Nitrogen fixation (Lie *et al.* 1987). It has been found that *Rlv.* strain TOM from Middle East is capable of nodulating a primitive pea line from Afganisthan (Lie 1978). NOD X is the gene whose presence in TOM makes it capable of nodulating pea cv. Afganisthan (Davis *et al.*1988). The main function of NOD X is acetylation of nod factor. In this study primitive pea like Afganisthan, small seeded Nepalese pea and European cultivar Rondo were used as host plants for trapping *Rlv* bacteria from the collected soil samples. Since NOD X genes are located in plasmids of the bacteria which could be easily transmissible to other *Rlv.* or other rhizobia genetic variation of the chromosomal background of the *Rlv.* isolates also has been studied to see the biodiversity.

Due to the ubiquity of ribosomal RNA molecules in all cellular life forms, comparative analysis of their sequences has been used widely to study diversity of microorganisms in natural environments (Head *et al.* 1998). 16Sr RNA gene is widely used for identification of bacterial species since they are highly conserved. In contrast the sequence of 16-23S spacer region shows considerable variation even within species in both the length and the sequence of this region. Although less discreminating, PCR-RFLP analysis of intergenic spacer between genes coding for 16S and 23SrRNA (16S and 23SrDNA) yields intraspecific polymorphism (Laguerre *et al.* 1996). In this study ribosomal genes like 16-23S and 16S genes were amplified and subjected to ristriction endonuclease digestion which is called Amplified Ribosomal DNA Restriction Analysis (ARDRA) (Massol-Deya *et al.* 1995). The resulting restriction fragment patterns were then used as a fingerprints for the identification of bacterial genomes. This method

is based upon the princ!ple that the restriction sites on the RNA operon are conserved according to phylogenetic patterns.

Methodology

Collection of soil samples. Eleven soil samples from Central Nepal and three soil samples from North East China were collected. Detail of the locations are given in Table 1.

Isolations of R. leguminosarum biovar viciae. Ten soil samples from Nepal and three soil samples from China were used for inoculating different pea cultivars from Nepal, Afganisthan and Europe. One gram each of collected soil sample was inoculated in each pea seedling grown in 300 ml. Erlemeyer,s flask containing Hoaglans Nitrogen free nutrient solution (Winarno and Lie 1979). All the plants were grown in aseptic condition in growth room at 20°C. The plants were harvested after five weeks of growth. Rlv. bacteria were isolated from nodules of each plant. Several isolates were also made from *Vicia faba*, *Lens culinaris*, pea cv Nepal, pea cv. Sikkim all grown in the same garden soil of Koteshwor, Kathmandu. In order to study the inoculation response of pure culture isolates from both pea cv. Afganisthan and Nepal on both the cultivars plant experiments were set up as described before.

Polymerase Chain Reactions (PCR). Each bacterial isolate was grown in Yeast Extract Mannitol broth for 24 hours at 28°C. The cells were harvested by centrifuging, washing and final resuspension in sterile nano pure water. 5 μl of the cell suspension (10^3-10^4) was used directly as DNA template for each PCR reaction (Harrison *et al.* 1992). All primers used for the PCR reactions were from Pharmacia Biotech Benelux, P. O. Box 1386, NL-4700 BJ Roosendaal, The Netherlands.

NOD X PCR. Amplification of NODX DNA fragment was carried out as described by Kozik et. Al. (1995) in 25 μl reaction mixture containing 10 x PCR buffer, 50mM $MgCl_2$, 1% W1 Detergent, 5 mMdNTPs, 50 ng each of NodX I and Nod X II primers, 1 unit of Taq polymerase and 5 μl of fresh harvested bacterial suspension. Two oligo,s spanning the coding region designed for the Nod X gene of Rlv strain TOM [Davis *et al.* 1988] were:

NOD X I : 5' - TTCGGCGGCGGCTAATGAGA-3', 220 bp upstream of the translation start and NOD X II : 5'- GGGCTGTGGTGTCTGGGATG-3', 60 bp downstream of the translation stop.

The PCR reaction was carried out in Perkin Elmer DNA Thermal Cycler 2400. The thermo cycle profile of the PCR reaction was : initial denaturation of bacterial cells at 94°C for 7 minutes followed by 35 cycles of denaturation at 94°C for 1 minute, annealing at 58°C for 1 minute and extension at 72°C for 3 minutes, followed by final extension period of 7 minutes at 72°C.

16-23s PCR. This PCR reaction was carried out by slight modification of Massol-Deya *et al.* method (1995). The PCR mixture contained 1 x PCR buffer, 2.5 mM $MgCl_2$, 1% W1 Detergent, 0.25 mM dNTPs, 100 ng of each primers pHr and p23SROI per reaction, 1.25 units of Taq polymerase and 5μ l of bacterial suspension. For this 16-23Sr DNA intergenic region, amplification was carried out by using a pair of highly conserved 16S and 23S ribosomal primers:

Primer	Position	Sequence
pHr (rev)	1518 - 1541	TGCGGCTGG ATCACCTCCTT
p23SRO1	1069 - 1052	GGCTGCTTCT AAGCCAAC
16S	23S	5S
8	pHr 1510	p23SRO1

The thermocycle program was carried out in Amplitron PCR machine and the program consisted of : initial denaturation at 95°C for 3 minutes followed by 35 cycles of denaturation at 94°C for 45 seconds, annealing at 50°C for 30 seconds and extension at 68°C for 1minute 30 seconds followed by final extension period of 4 minutes at 72°C.

16S PCR. Amplification of 16S r DNA was carried with some selected pea isolates as this PCR does not show much variation. 100μ l of a PCR mixture contained 1x PCR Buffer, 50 mM $MgCl_2$, 5 mM dNTPs, 1% W1 Detergent, 10μ M of primer 8, 10μ M of primer 1510, 1 unit of Taq polymerase and 5μ l of bacterial suspension.

Primer sequences for the 16Sr DNA gene amplification:

Primer	Position	Sequence (5' - 3')
8	8 - 37	CACGGAT CCAGAGTTT GAT(C/T) (A/C)TGG

		CTCAG
15101537 -	1510	GTGCTGCAGG GTTA CTTGTT ACGACT

This PCR reaction was also carried out in Amplitron PCR machine. The thermocycle program for the reaction was set up as follows : initial denaturation at 94°C for 5 minutes followed by 35 cycles of denaturation at 94°C for 1 minute, annealing at 54°C for 2 minutes and extension at 72°C for 2 minutes followed by final extension period of 7 minutes at 72°C.

All the PCR products obtained by DNA amplifications were quickly observed in 1% Agarose MP (Gmbh, Germany) gel by horizontal electrophoresis in 1 % TAE buffer at 70 volt for half an hour. The amount of amplified DNA was quantified by Image Quant software. All the images were stored as TIFF files in computer.

ARDRA. The 16 -23S rDNA and 16S r DNA PCR products of the pea isolates were all subjected to Amplified rDNA Restriction Analysis (ARDRA) with 3 units each of three restriction endonuclease enzymes CfoI, HaeIII and MspI with their respective reaction buffers. The digestion was carried out at 37°C for at least one and half hour in water bath. Thus digested products were then run in 3% Agarose gel with Ehidium Bromide in a horizontal gel electrophoresis in 1% TAE buffer at 100 volt for two hours. The gel image was then observed under UV light and stored as TIFF files. All the TIFF files of ARDRA were then converted, normalized and analysed with Molecular Analyst Software Fingerprinting Version 1.12 Applied Maths (Bio Rad). Finally dendrograms based on a combination of the three restriction patterns were constructed.

Results and Discussions

Nodulation Experiments. Nodulation assay (Table 2) to examine host range showed that the soil samples of Kathmandu valley produced mostly ineffective nodules in pea cv. Afganisthan and Nepal whereas the soil samples from outside the valley showed good effective nodulation except Tatopani soil which in both cultivars showed nodulation delayed by about a week than others. In case of all Rondo the inoculated soil samples showed good nodulation except Tatopani soil which did not produce any nodules. Seventy eight isolates of *Rlv.* were made from the nodules of the three pea cultivars inoculated with Nepalese soil samples. Similarly five isolates were made from nodules of pea cv. Afganisthan and Rondo inoculated with Chinese soil samples (Table 3). Nodulation study with pure culture isolates (Table 4 and 5) showed majority of the isolates produced similar nodulation behaviour. When tested on pea cv. Afganisthan majority of Nepalese pea isolates produced either thick rooting system or few ineffective root nodules except for the isolates N80, N90 and N111. On the other hand Afgan pea isolates when tested on pea cv. Nepal produced effective nodules by all isolates except A10, A40, A60 and A70. The results suggest that the *Rlv.* strains compatible with Dutch pea cv Rondo are widespread in soils of Nepal. This is in accordance with the experience in The Netherlands that effective *Rhizobium* strains for the pea plants are present in most cultivated soils (Lie and Goktan 1984). However, *Rhizobium* strains compatible with primitive pea cv. Afganisthan are few and most of the strains were found to be non nodulating, ineffective strains. As reported by Lie and Goktan in 1984 a casual transfer of a limited number of *Rhizobium* genotypes may have taken place, probably as soil contaminants on seeds or pods.

NOD X PCR. As obvious isolates taken from pea cv. Afganisthan inoculated with Nepalese and Chinese soil samples all showed NOD X fragment similar to that of TOM (Table 3). The oligo's, designed from the NOD X gene from Rlv. strain TOM, not only amplified the NOD X gene of *Rlv.* strain TOM, but were also able to amplify NOD X homologous sequences in other *Rlv.* bacteria originating from Nepal, China, Tibet, Syria and Turkey (Tempelman-Bobbink *et al.* 1998). The tested *Rlv.* isolates all gave a 1.3 kb NOD X PCR product. This result indicates that the NOD X gene is widely spread throughout Asia. However in case of isolates taken from pea cv. Nepal those only from Kathmandu valley did not show NOD X. The absence of NOD X from this valley may be due to the geographical isolations from other places. In Rondo, isolates from Ramshah Path and Nagarjun showed NOD X.

IGS ARDRA of 16-23S and 16S PCR products. Analysis of IGS ARDRA of 16-23S PCR products by Molecular Analyst Software Fingerprinting version 1.12 Applied Maths showed two major distinct groups:

Table 1. Description of Locations of Collected Soil Samples

Soil ample numbers	Location	Type of location	Type of land	Crop
1.	Surya Binayak,Bhaktapur district, Nepal	Base of a mountain	Cultivated	Potato
2.	Sano Thimi, Bhaktapur district, Nepal	Terrace land	Cultivated	*Vicia faba*
3.	Nagarjun, Ktm. Valley, Nepal	Mountain	Ploughed	None
4.	Chamati, Ktm. Valley, Nepal	Kathmandu valley	Cultivated	Wheat
5.	Tripureshwor, Ktm. Valley, Nepal	Kathmandu valley	Cultivated	*V. faba*
6.	Ramshah Path, Ktm. Valley, Nepal	Kathmandu valley	Cultivated	*V. faba*
7.	Harisidhhi, Lalitpur district, Nepal	Terrace land, Ktm valley	Cultivated	*V. faba*
8.	Tatopani, Dolakha district near Tibet	Base of a mountain	Cultivated	Wheat
9.	Dolalghat, Sindhu -palchok district, Nepal	Mountain	Fallow	None
10.	Panchkhal, Kabre district, Nepal	A small valley	Ploughed	None
11.	Dhulikhel, Kabre district, Nepal	Mountain	Fallow	None
12.	Koteshwor, Ktm., Nepal	Kathmandu valley	Cultivated	Pea, *V. faba* & Lentil
13.	Wenchun, HLJ, China	Mudanjiang city	Fallow	None
14.	Linsheng Xiang, Jilin province, China	Dunhua city	Fallow	None
15.	Xiaopuchaihe, Jilin province, China	Dunhua city	Fallow	None

Table 2. Comparative studies of nodulation of three pea cultivars inoculated with different soil samples

Soil samples	Av. no of nodules in pea cv. Afganisthan	Av. no. of nodules in pea cv. Nepal	Av. no. of nodules in pea cv. Rondo
1.	2.00, ineffective	2.00, ineffective	52.00, effective
2.	70.00 mostly ineffective	Few white swellings	Not inoculated
3.	45.50, mostly effective	7.00, mostly effective	53.50, effective
4.	39.50, mostly ineffective	1.00, ineffective, many white swellings	54.00, effective
6.	48.00, mostly ineffective	37.00, mostly effective	41.00, effective
7.	61.20 mostly ineffective	9.00, ineffective	49.50, effective
8.	48.00, mostly ineffective, delayed nodulation	5.00, ineffective, delayed nodulation	No nodulation
9.	> 100, effective	15.00, effective	Not inoculated
10.	> 100, effective	18.00 effective	60.00, effective
11.	> 100, effective	11.00 effective	75.00, effective
13.	> 50, effective	Not inoculated	> 100, effective
14.	12.50, ineffective	Not inoculated	> 100, mostly ineffective
15.	12.00, effective	Not inoculated	> 100, mostly ineffective

Note : Effective means green plant having majority of pink nodules and ineffective means yellow dry plant with majority of white nodules.

Table 3. *R. leguminosarum* biovar *viciae* isolates of different pea cultivars inoculated with different soil samples and presence or absence of NOD X

Soil samples	Isolates from					
	pea cv. Afganistan	pea cv. Nepal	pea cv. Rondo	pea cv. Sikkim	*V. faba*	*L. culinaris*
1. Surya Binayak	A 10, A 11 +	N 10, N 11, N13 -	R 10, R 11, R13 -			
2. Sano Thimi	A 20, A 21, A22	+ N 20 -				
3. Nagarjun	A 30, A 31, A32 +	N 31, N 32 -	R 30, R 31, R32, R 34 -			
4. Chamati	A 40, A 41 +	N 40 -	R 40, R 41, R 42 -			
6. Ramshah Path	A 60, A 61 +	N 60, N 61, N62 -	R 60, R 61 +			
7. Harisidhhi	A 70, A 71 +	N 70, N 71, N72, N 74	-R 70, R 71 -			
8. Tatopani	A 80 +	N 80, N 81, N82 +				
9. Dolalghat	A 90, A 91, A92 +	N 90, N 92 +				
10. Panchkhal	A 101, A 102 +	N 100, N 101, N 102 +	R 100, R 101, R103,			
11. Dhulikhel	A 110, A 111 +	N 110, N 111, N 112 +	R 104 +, R 110, R 111 -			
12. Koteshwor		Kp 1, Kp 2, Kp 3, Kp 4 +		Skpn 1 Skpn 2 +	Kv2, Kv4	Kl 1 Kl2, K13-
13. Wenchun, China	W2Af +		W2Rh -			
14. Linshen-gxiang, China	W7Af +		W7Rh -			
15. Xiao-puchaihe, China			W8Rh +			

+ Presence of NOD X; - Absence of NOD X

Table 4. Nodulation study of Afgan pea isolates on pea cv. Afganisthan and Nepal

Afgan pea isolates	Nodules in pea cv. Afganisthan	Av. fresh shoot weight (g) of pea cv. Afganisthan	Nodules in pea cv. Nepal	Av. fresh shoot weight (g) of pea cv. Nepal
TOM*	92, effective	3.11	17,effective	1.27
PF2**	No nodulation	0.87	36,effective	1.80
A 10.	14,ineffective	1.17	14, ineffective	0.87
A 30.	17, mostly effective	2.25	19, mostly effective	1.70
A 40.	4, ineffective	1.26	1, ineffective	0.90
A 60.	Only white nodules	1.08	6, ineffective	0.62
A 70	32, ineffective	1.86	13, ineffective	0.98
A 80.	24, effective	3.26	23, effective	1.68
A 90.	48, effective	3.51	14, effective	1.14
A 101.	54, effective	1.99	22, effective	2.42
A 110.	42, effective	2.63	43, effective	1.85

* and ** (Nod X +) Winaro and Lie (1979)

** strain (Nod X -) from European cultivar Rondo

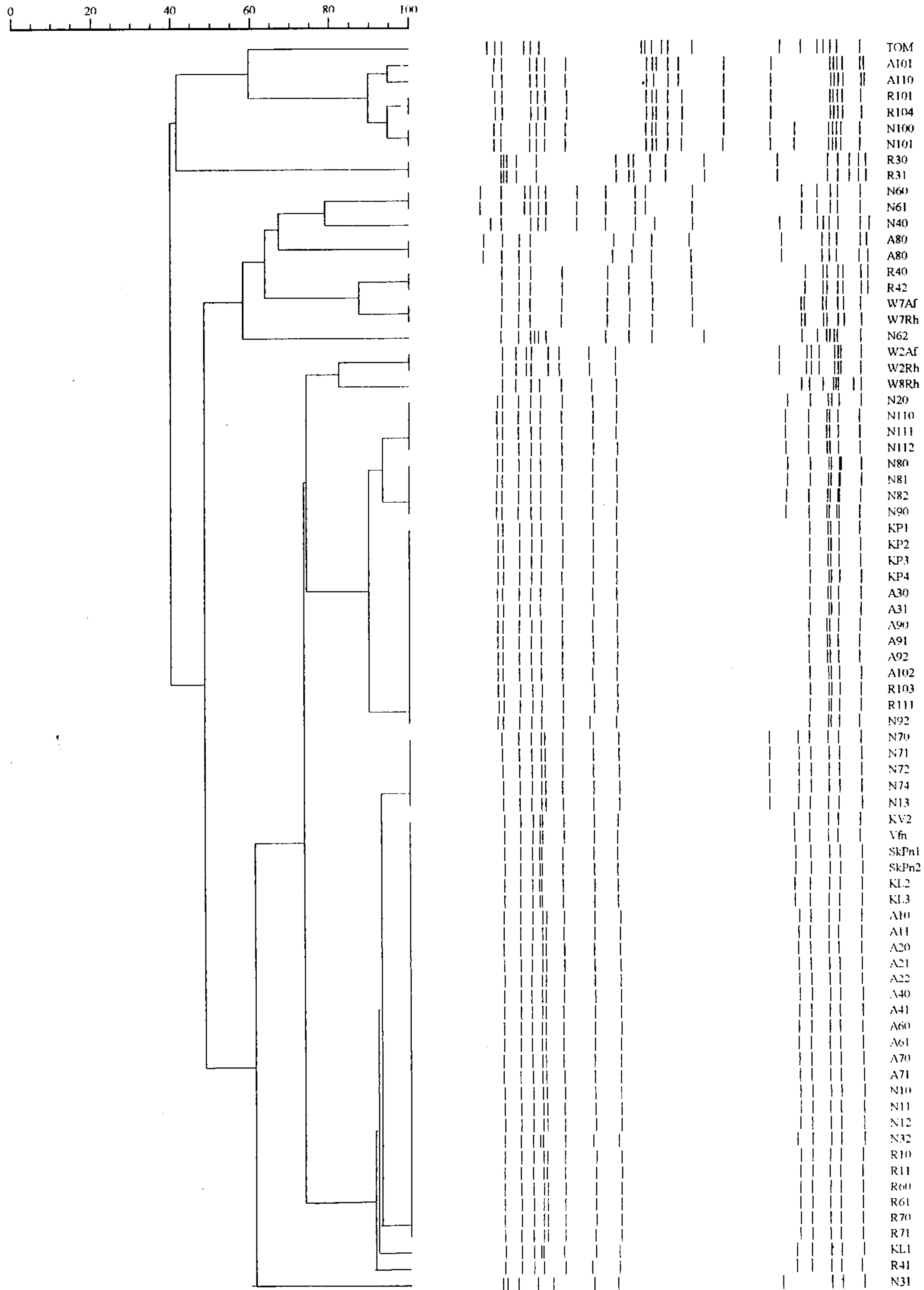

Figure 1. Ribosomal RNA operon showing approximate location of priming binding sites for PCR amplification

Table 5. Nodulation study of Nepalese pea isolates on pea cv. Nepal and Afganisthan

Nepalese pea isolates	Nodules in pea cv. Nepal	Av. fresh shoot weight of pea cv. Nepal	Nodules in pea cv. Afganisthan	Av. shoot weight of pea cv. Afganisthan
N 10.	5, ineffective	2.00	Roots thickened	0.96
N 30.	7, ineffective	1.21	Roots thickened	1.15
N 40.	9, ineffective	1.81	1, ineffective	1.04
N 61.	6, mostly ineffective	1.99	Roots thickened	1.19
N 70.	12 ineffective	1.47	16 mostly ineffective	1.42
N 80.	11, mostly ineffective	1.42	46, effective	3.12
N 90	2, ineffective	1.17	76 effective	2.80
N 100	12, effective	2.55	Roots thickened	0.87
N 111	14, effective	2.20	90, effective	2.94

Note : An isolate was scored as effective if it could form majority of pink nodules and if the plant is healthy and green.

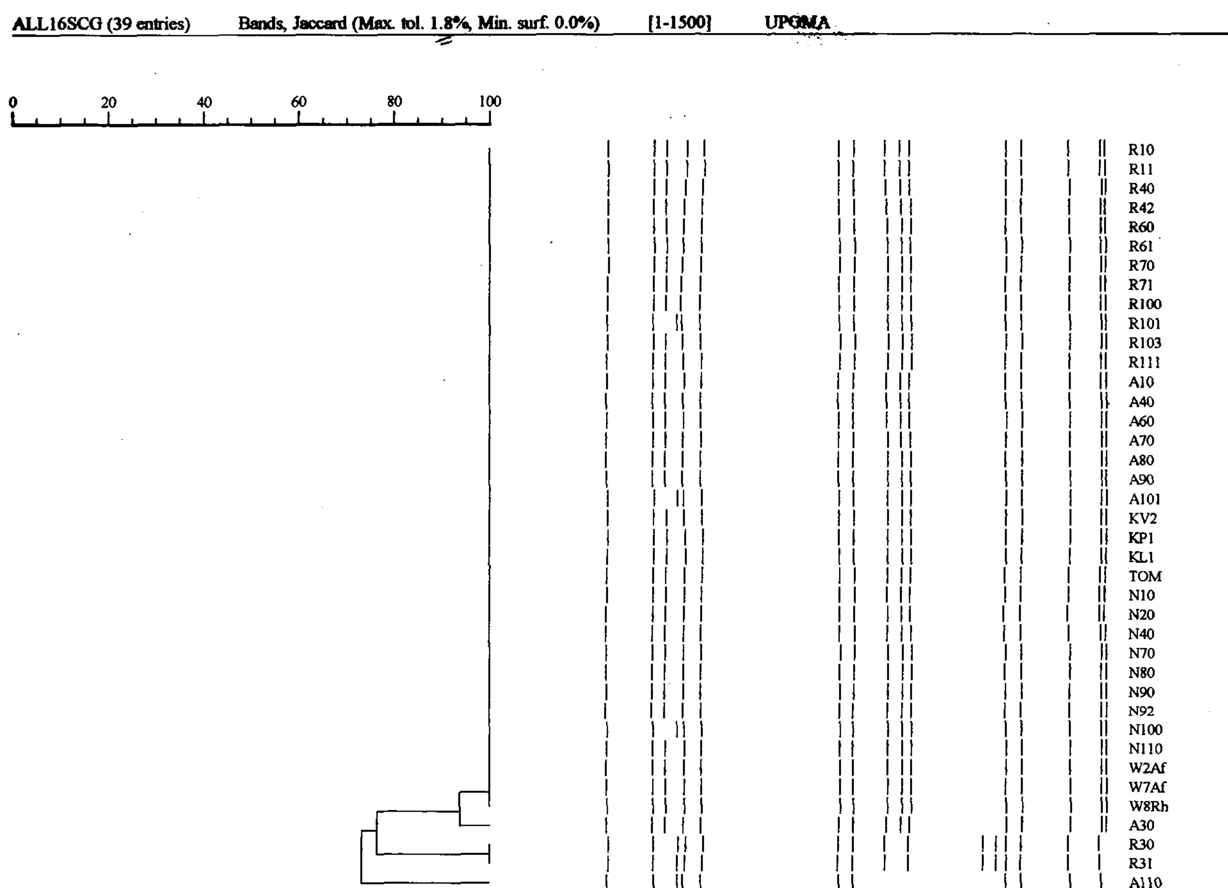

Figure 2. 16SrDNA molecular finger printing of 39 isolates

I Group : Similar to Afganistan strain TOM in containing NOD X fragement and in having similar 16S and 16-23S DNA fingerprinting. This group consists of strains A 101, A 110, R 101, R 104, N 100 and N 101, all from soil samples collected from Panchkhal and Dhulikhel which are from the same district Sindhupalchok.

II Group : It is the largest group with all the rest of the isolates of Nepal and China.

This group could be divided into two subdivisions :

i. Isolates all from China, W2Af, W2Rh and W8Rh, and isolates from Tatopani, A 80, and isolates from Kathmandu, N 40, N 60, N 61, R 40, R 42.

ii. Rest of the isolates from soils collected from Nepal.

The 16-23SrDNA intergenic spacer region proved to be useful part of the chromosomal DNA to be used for identification of closely related RIv isolates. However most of the *Riv.* isolates could not be used for identification using 16SrDNA genes. Analysis of 16S IGS ARDRA finger printing did not show substantial difference between the isolates except for the two Rondo isolates from Nagarjun mountain which are quite different from others.

Conclusion

For the first time NOD X has been found in soil of Nepal and China. As obvious all the Afgan isolates contained NOD X irrespective of locations from where the soil samples have been collected. However in case of pea cv. Nepal and Rondo NOD X containing bacteria was found exclusively only outside Kathmandu valley of Nepal. One of the reasons for absence of NOD X from the isolates of the the valley could be the excessive cultivation of cultivated pea and vicia in this valley which do not require NOD X for nodulation in their host plants.

Acknowledgements

I wish to thank Dr. T. A. Lie, Microbiology Laboratory of Department of Biomolecular Sciences, Wageningen Agriculture University, The Netherlands for providing me fellowship to carry out this project at the same laboratory.

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