ELSEVIER

FEMS Microbiology Letters 205 (2001) 171–178

FEMS MICROBIOLOGY Letters

www.fems-microbiology.org

Molecular diversity of the plasmid genotypes among *Rhizobium* gene pools of sesbanias from different habitats of a semi-arid region (Delhi)

Asif Mohmmed [a,*], Radhey Shyam Sharma [a,b], Sher Ali [c], Cherukuri Raghavendra Babu [a,b]

[a] *Department of Botany, University of Delhi, Delhi-110 007, India*
[b] *Centre for Environmental Management of Degraded Ecosystems, University of Delhi, Delhi-110 007, India*
[c] *National Institute of Immunology, Aruna Asaf Ali Marg, New Delhi-110 067, India*

Received 6 February 2001; received in revised form 9 September 2001; accepted 21 September 2001

First published online 8 November 2001

Abstract

Plasmid genotypes of root nodulating rhizobial isolates of *Sesbania*, sampled from six ecologically distinct habitats, were characterized. Plasmid profile analysis revealed nine different plasmid types having molecular masses ranging from 30 to 300 MDa, distributed among six profile types that grouped the isolates into six plasmid classes. The six plasmid profiles were diverged from each other and lack many common plasmid types among them. Variation in number and types of symbiotic (*Sym*) plasmid was assessed by hybridization of plasmid profiles with *sym* gene probes. Relatedness among different plasmid types was assessed by hybridization of total DNAs as well as plasmid profiles of different isolates with labelled intact plasmid. Plasticity of plasmid genotype and possible recombination between different plasmid types is suggested from the results obtained. Structural diversity among *sym* plasmids was assessed by PCR amplified product profiles using primer corresponding to the reiterated *nif* promoter consensus element (NPC-PCR). A total of 26 NPC-PCR profile types were recognized. Genetic diversity among *sym* plasmids of isolates belonging to the same plasmid class and having similar *sym* plasmid suggested recombinations and rearrangements of sequences within the *sym* plasmids. Cluster analysis based upon similarity among profile types sorted the isolates across the ecological gradient. We suggest that habitat heterogeneity and plasticity of plasmid genotype together contribute for the generation of genetic diversity leading to strainal differentiation in rhizobia.

Keywords: *Rhizobium* gene pool; *Sesbania* species; Ecological diversity; Plasmid; Molecular polymorphism; Genome plasticity

1. Introduction

Bacterial genomes harbour extra chromosomal elements such as plasmid and transposable elements. Plasmids, in fact, constitute 25–50% of the total DNA in some bacteria including *Rhizobium* [1]. A number of analyses at the molecular level have revealed fine differences among rhizobia and led to the characterization of plasmid genotype and hence refinement of lineages [2–4]. However, it did not explain the genetic differentiation of plasmid genotype in natural populations under the influence of habitat factors.

Sesbanias inhabit a wide range of ecological niches; therefore the *Rhizobium* gene pools nodulating sesbanias are ideal for experimentation to understand the evolution of genomic diversity in response to habitat variation. Root nodulating bacteria of sesbanias have been characterized phenotypically and at the molecular level [5–8]. These studies suggested that they belong to distinct species of *Rhizobium* or *Sinorhizobium*. Studies on the molecular diversity of this unique group of rhizobia from different ecological backgrounds are lacking. In the present study plasmid genotypes of rhizobial isolates from ecologically different habitats were characterized. The diversity in plasmid profiles, the homology among different plasmids and the structural diversity among *sym* plasmids of these isolates were studied to understand the role of habitat het-

* Corresponding author. Present address: International Centre for Genetic Engineering and Biotechnology, P.O. Box 10504, Aruna Asaf Ali Marg, New Delhi-110 067, India. Tel.: +91 (11) 618 9358.
E-mail address: amohd@icgeb.res.in (A. Mohmmed).

0378-1097/01/$20.00
PII: S0378-1097(01)00463-3

erogeneity in genetic differentiation leading to divergence in rhizobia.

2. Materials and methods

2.1. Sampling sites

Six sampling sites from different ecological habitats were selected from the Delhi region (28°24′–28°53′N; 76°50′–77°20′E) for the collection of root nodules. The locations of these sites are shown in Fig. 1. The sites were well differentiated from each other with respect to soil characteristics, land use pattern and other ecological factors (Table 1). *Sesbania* species found on each site and their mode of inhabitance are given in Table 2.

2.2. Collection of root nodules and isolation of nodule bacteria

Root nodules from five randomly selected healthy phenotypes of *Sesbania* species found on each site were sampled. Isolation, maintenance and authentication of bacteria from root nodules were done following standard procedures outlined by Vincent [9]. All the isolates were designated by a three-letter code followed by a three-digit number. Details of the isolates used in the study and their codes are given in Table 2.

2.3. Separation of plasmids

Plasmids profiles of rhizobial isolates were analysed by the 'in well lysis' method of Eckhardt [10] with few modifications, as outlined by Plazinski et al. [11]. The molecular masses of the plasmids were determined by calibrating against the relative mobility of plasmids of known molecular masses.

2.4. Isolation, and slot blotting of total DNAs

Total genomic DNAs were isolated from all the rhizobial isolates following the procedure outlined by Wilson [12]. Aliquots of 200 ng of total genomic DNA samples from different isolates were diluted in sterile DW to a final volume of 100 μl, and denatured by heating at 100°C for 10 min in a water bath. These samples were then loaded in slots of a slot blot apparatus and blotted on a nylon membrane by applying a vacuum of 200 mmHg.

2.5. Hybridization of gels and membrane bound DNA

Plasmid DNA separated on agarose gels was denatured

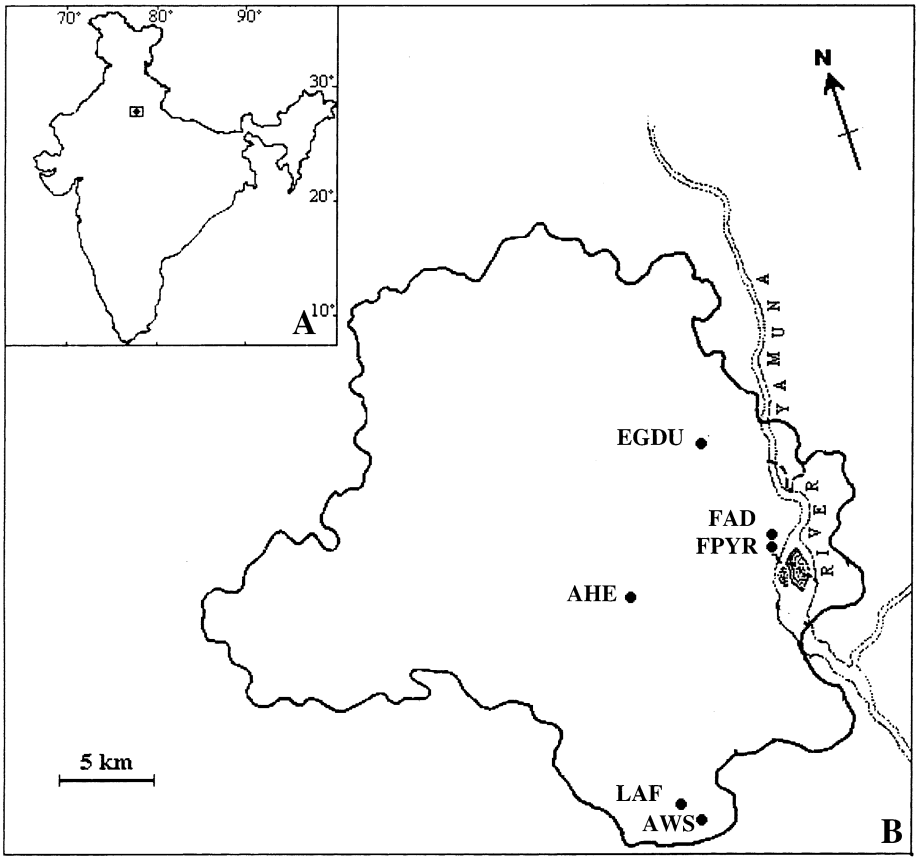

Fig. 1. Map showing the location of the Delhi region in India (A), and locations of sampling sites (●) in the Delhi region (B).

Table 1
Sampling sites for collection of root nodules and their soil characteristics

Site	Land use and site characteristics	Soil characteristics					
		Soil type	OM (%)	PO_4-P (ppm)	NO_3-N (ppm)	K^+ (ppm)	pH
I. Experimental garden of Department of Botany, University of Delhi (EGDU)	A number of plant species are introduced and maintained in different plots of the garden for experimental purposes; anthropogenic activities are high	sandy loam of Yamuna alluvium	9.6	97.9	69.9	115.8	6.5
II. Aravalli hills extension in Delhi (AHE)	Open degraded area with large number of invasive plant species	skeletal	0.72	21.7	23.5	54.5	6.0
III. Asola Wildlife Sanctuary (AWS)	Desertified and xeric flat lands with loose soil and scattered xerophytic trees with some grass species; grazing by cattle is common	skeletal	0.72	4.89	15.7	33.8	7.6
IV. Low lying agricultural fields near site III (LAF)	Artificially irrigated fields used for cultivation of cereals and vegetables; sesbanias were cultivated as green manure	sandy loam	0.93	5.85	23.2	19.3	5.5
V. Flood plains of Yamuna river (FPYR)	Mostly covered with water in monsoon, otherwise used for cultivation of cereals	sandy	1.86	12.7	73.7	56.2	6.0
VI. Fly ash dumps of Indraprastha thermal power station (FAD)	Dumps of fine ash left after burning of coal; usually barren except for cultivated sesbanias	fine ash	2.7	29.2	24.8	31.0	7.2

by soaking gels in 0.5 M NaOH for 20 min and then neutralized in 1.0 M ammonium acetate, 0.02 M NaOH solution. Gels were then dried at 60°C under vacuum. Hybridization of the gels with α-^{32}P labelled probe was done according to Hynes et al. [13]. Hybridization of nylon membrane bound DNA was performed as described by Young and Wexler [4].

2.6. PCR using nifH promoter consensus element primer (NPC-PCR)

Variation in *sym* plasmid was detected by PCR using *nif* directed primer [14]. The sequence of the 20 nucleotide length *nif* promoter consensus primer (NPC) used was 5′-AAT TTT CAA GCG TCG TGC CA-3′. PCR amplification was done in 10 μl reaction mix following Richardson et al. [14]. The amplified DNA products were separated on 1.5% agarose gel.

2.7. Description and preparation of hybridization probes

Two plasmids were used as *sym* probes, plasmid pIJ1246 containing *nod* E, F, D, A, B and C genes [15] and plasmid pIJ1247 containing inserts of *nif* H and D [4] from *sym* plasmid pRL1JI of *Rhizobium leguminosarum* biovar *viciae* strain 248. Plasmids of the rhizobial isolates to be used as probes were electroeluted from Eckhardt's gel.

2.8. Data analysis

The PCR product profiles were converted into two-dimensional binary matrices. The lanes were compared by reading horizontally across the gel; the presence of PCR product of a particular size was assigned a value of 1 and its absence a value of 0. Similarity matrices were constructed between pairs of profile types following Dice's coefficient and analysed by UPGMA (unweighted pair group method using arithmetic averages) cluster analysis [16] using biostatistical analysis programme NTSYS-PC (Applied Biostatistics, Inc.).

3. Results

3.1. Plasmid profiles of the Rhizobium isolates

All the isolates harboured one to three plasmids. The molecular masses of these plasmids showed marked diversity among the isolates and varied from 30 MDa to 300

Table 2
Sesbania species found on different sampling sites, their mode of inhabitance and *Rhizobium* isolates (designated by codes) cultured and maintained from root nodules sampled from them

Sampling site	Host plant species found	Mode of inhabitance of host plant	Codes used for *Rhizobium* isolates
EGDU	*S. sesban*	Spontaneous	SSR301, SSR302, SSR304, SSR305, SSR306, SSR312, SSR313
	S. aegyptica	Spontaneous	SAR605, SAR608, SAR610, SAR611
	Sesbania rostrata	Spontaneous as well as cultivated annually (introduced through seeds in 1990)	SRR915, SRR916, SRR917
	Sesbania cannabina	Spontaneous	SCR690[a]
AHE	*S. sesban*	Spontaneous	SSR353, SSR354, SSR355, SSR356, SSR357, SSR358, SSR359, SSR360, SSR361, SSR362
AWS	*S. aegyptica*	Spontaneous	SAR615, SAR617, SAR620, SAR621
LAF	*S. sesban*	Cultivated annually	SSR321, SSR322, SSR323, SSR324, SSR325, SSR326, SSR327, SSR328, SSR329, SSR330
FPYR	*S. sesban*	Spontaneous as well as cultivated	SSR338, SSR339, SSR340, SSR341, SSR342, SSR343, SSR344, SSR345, SSR346, SSR347
FAD	*S. sesban*	Cultivated annually (introduced through seeds in 1990)	SSR335, SSR336, SSR337

[a]Isolate obtained from germ plasm maintained at the University of Delhi, originally collected from EGDU [6].

MDa. Based upon the molecular mass, a total of nine plasmid types were recognized. Based upon the distribution pattern of these plasmid types, six profile types, A–F, were recognized (Table 3). Each plasmid type was designated by code pSR- ('p' stands for plasmid, 'S' for *Sesbania* and 'R' for *Rhizobium*) followed by a suffix representing the molecular mass of the plasmid and profile type. All the isolates were grouped into six plasmid profile classes, I–VI, based upon the plasmid profile types shown by them (Table 3).

3.2. Identification of sym plasmids

The plasmid types pSR250A, pSR180B, pSR180C, pSR220D, pSR110E, pSR180F and pSR90F hybridized with both *nif* and *nod* gene probes. In the plasmid profiles of the isolates of *Sesbania aegyptica* from EGDU (SAR605, SAR608, SAR610 and SAR611), the *nod* gene probe hybridized with both pSR110D and pSR220D in contrast to hybridization with only pSR220D in other isolates (Table 3).

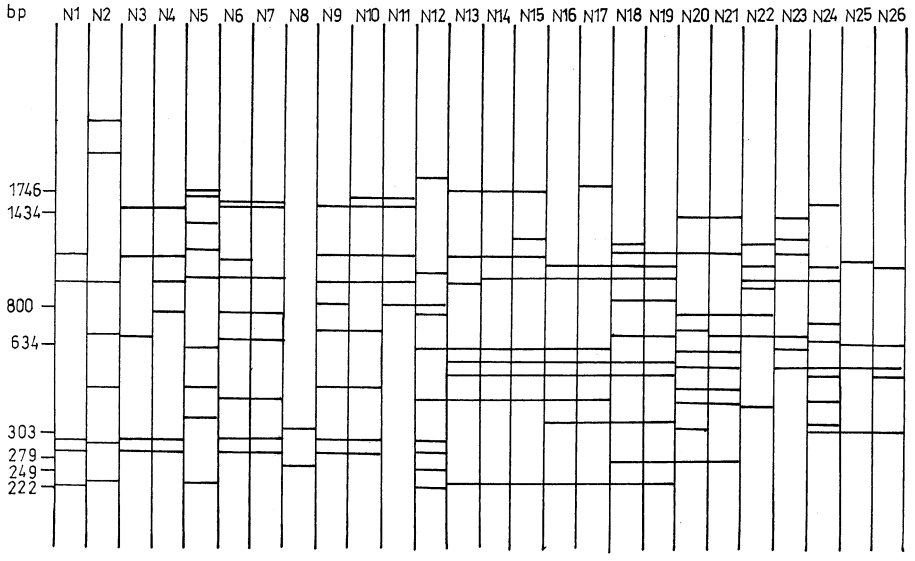

Fig. 2. Schematic representation of the NPC-PCR profile types (designated N1 to N26). The relative locations of the molecular mass marker restriction fragments and their sizes expressed in base pairs are indicated in the left hand margin.

Table 3
Plasmid profile types and NPC-PCR amplified DNA product profiles shown by *Rhizobium* isolates and grouping of *Rhizobium* isolates into plasmid profile classes

Plasmid profile type	No. of plasmids harboured	Mol. mass of plasmids (MDa)	Plasmid profile class	*Rhizobium* isolates	Sampling site	NPC-PCR profile type
A	1	250[a]	I	SSR301	EGDU	N1
B	2	180[a], 60	II	SSR302	EGDU	N2
C	3	180[a], 90, 30	III	SCR690	EGDU	N8
D	2	220[a,c],110[b,c]	IV	SSR304, SSR305, SR306	EGDU	N3
				SSR312, SSR313	EGDU	N4
				SAR605, SAR608	EGDU	N9
				SAR610	EGDU	N10
				SAR611	EGDU	N11
				SAR615, SAR617	AWS	N12
				SAR620, SAR621	AWS	N12
				SRR915, SRR917	EGDU	N6
				SRR916	EGDU	N7
E	2	300[c], 110[a,c]	V	SSR321, SSR322, SSR323	LAF	N13
				SSR324, SSR325, SSR326	LAF	N14
				SSR327, SSR329, SSR330	LAF	N14
				SSR328	LAF	N15
				SSR338	FPYR	N20
				SSR339	FPYR	N21
				SSR340	FPYR	N22
				SSR341	FPYR	N23
				SSR342, SSR343	FPYR	N24
				SSR344, SSR345	FPYR	N24
				SSR346	FPYR	N25
				SSR347	FPYR	N26
				SSR353, SSR356, SSR357	AHE	N16
				SSR354, SSR355	AHE	N17
				SSR358, SSR359, SSR362	AHE	N18
				SSR360, SSR361	AHE	N19
F	3	180[a], 90[a], 70	VI	SSR335, SSR336, SSR337	FAD	N5

[a]Hybridized with both *nif* and *nod* gene probes.
[b]Hybridized only with *nod* gene probes in *S. aegyptica* isolates from EGDU.
[c]Hybridized with pSR300E plasmid probe.

3.3. Relatedness among plasmids

The total DNAs of the isolates of classes I, II, III and VI did not show a hybridization signal with the labelled pSR300E. However, total DNAs of the isolates of class IV showed hybridization with the probe. For further assessing the homologies among plasmids of isolates of classes IV and V, the Eckhardt's gels with plasmid profiles were hybridized with labelled pSR300E probe. The plasmids of both class IV and class V isolates showed hybridization with the probe.

3.4. NPC-PCR amplification profiles

The *sym* plasmids were further characterized by PCR amplified DNA product pattern using *nif* directed primer containing nitrogen fixation promoter consensus element (NPC-PCR). The sequence of the primer used corresponds to the conserved region located in the *nif* H region of *R. leguminosarum* biovar *trifolii* strain ANU843. This region forms a part of the repeated sequence which is present in 5–10 copies on the symbiotic plasmid of the species [17].

The number of bands in the amplification profiles varied from two to 11 among the isolates and their length varied from 200 bp to 2600 bp (Fig. 2). A total of 52 fragments of different lengths were amplified from different isolates. Based on the diversity in length and number of amplified fragments the different patterns were grouped into 26 types, designated as N1 to N26 (Fig. 1). Some of the profiles were isolate specific, whereas others were shared by 2–6 isolates (Table 3). A high level of polymorphism among the profiles was reflected in the dendrogram (Fig. 3) based on the similarity matrix (not shown) constructed using these profiles. Isolates of *Sesbania sesban* from the same site showed a higher percentage of similarity among themselves as compared to isolates from other sites. Pro-

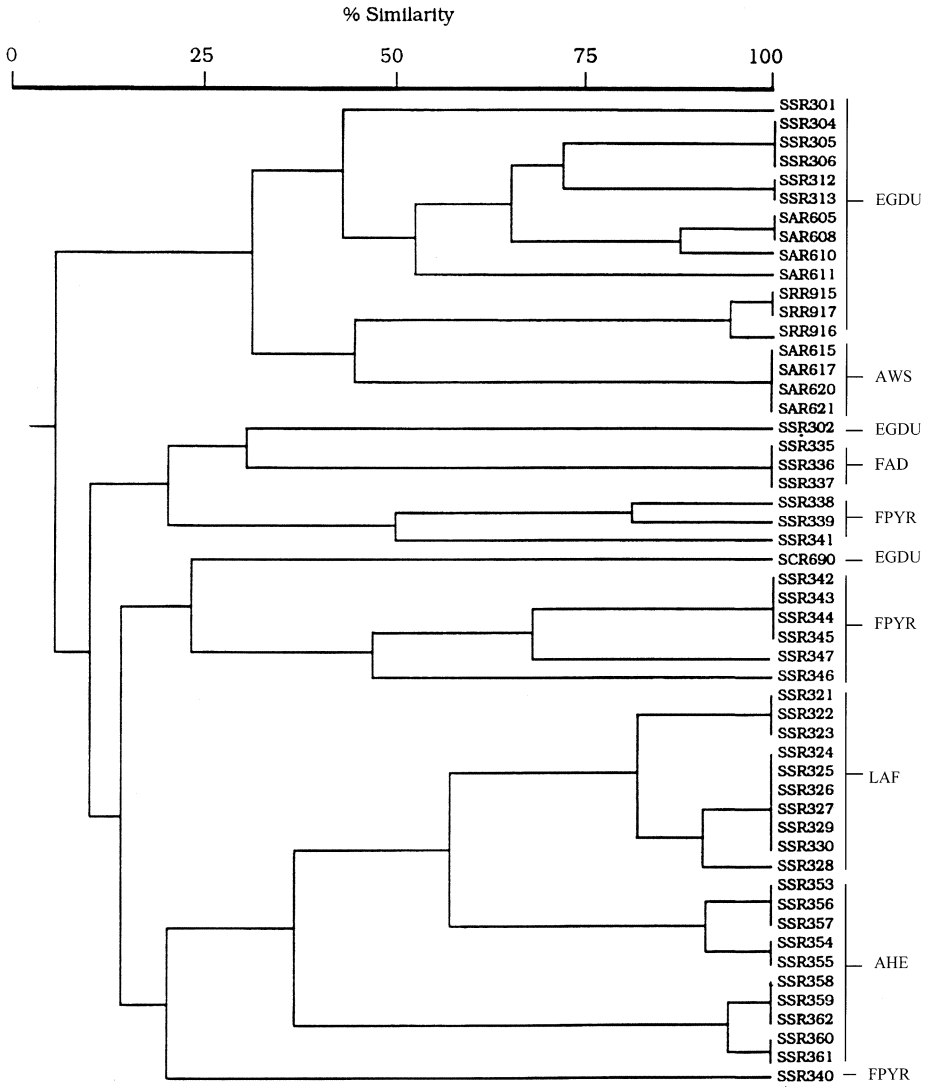

Fig. 3. Dendrogram showing the relationships among *Rhizobium* isolates at different levels of similarity. The dendrogram was constructed using UPGMA based on similarity matrix constructed using profiles of NPC-PCR amplified DNA products. The collection sites of the isolates are indicated in the right hand margin.

files of *S. aegyptica* isolates from EGDU showed higher similarity (62–72%) with profiles of *S. sesban* isolates from the same site and formed one compound cluster with them, but they showed low similarity (13–33%) with profiles of other isolates of *S. aegyptica* from AWS.

4. Discussion

The six sites selected in the present study for sampling of root nodule bacteria were ecologically distinct from each other. Consequently *Rhizobium* populations that nodulate *Sesbania* species inhabiting the ecologically distinct sites within an area of 1500 km^2 are ideal to study the molecular polymorphism exhibited in their plasmid genotype in response to ecological diversity of the habitat.

In terms of kind and number of plasmids, the diversity observed among different *Rhizobium* isolates of *Sesbania* species is rather narrow as compared to the diversity observed in other species of *Rhizobium* [3,18]. Although the range of variation in the number of plasmid types and profiles was rather narrow, the profiles share a very few common plasmid types. This is contrary to the observations made by earlier workers [3,18] and suggests that the isolates are genetically diverged with respect to plasmid genotype. The observed patterns of variation in plasmid profiles may be due to differential environmental selection pressures operating on the rhizobia inhabiting soils having different edaphic profiles and not influenced by host genotype. For example, profiles of isolates from site I (EGDU) are different from most of the other isolates, whereas some of the isolates from different host plants showed similar profiles. Similarly, isolates of *S. sesban* from the markedly distinct habitat FAD showed a unique

profile type. Within the isolates of site I marked diversity was observed, probably because the habitat is highly mosaic and disturbed. Palmer and Young [19] also showed higher diversity in the rhizobial population inhabiting disturbed cultivated sites as compared to those inhabiting undisturbed grass lands.

The presence of two *sym* plasmids of molecular masses 90 and 180 MDa in *S. sesban* isolates from site FAD suggests that either the entire *sym* plasmid might have reiterated twice or there is duplication and exchange of *sym* genes between the plasmids. Similarly, in some of the isolates of *S. aegyptica*, *nod* genes were localized on two plasmids. These observations suggest that recombinations among plasmids are rampant. Similar observations have also been made by earlier workers, suggesting that deletion, cointegration, duplication or recombination arising from interaction between multiple *sym* plasmids may occur [20].

The fact that total DNAs of isolates belonging to plasmid classes I, II, III and VI did not hybridize with the plasmid pSR300E (non-*sym* plasmid of profile E) probe suggests that the sequences of the plasmid genotypes of these isolates are diverged from class IV and V isolates. The probe hybridized with total DNAs of the isolates of classes IV and V, suggesting that the isolates from three *Sesbania* species from different habitats share some common sequences of non-*sym* plasmid. Further, the probe hybridized with both the *sym* and non-*sym* plasmids of these isolates, suggesting a possible recombination between *sym* and non-*sym* plasmids. This indicates that the observed diversity in plasmid profiles is not only due to interaction between different *sym* plasmids but also to interaction between *sym* and non-*sym* plasmids. The diversity observed in the number and types of *sym* plasmids, variation in the number of *sym* plasmids among isolates of the same plasmid class, homologies among plasmids of different profile types and homology among *sym* and non-*sym* plasmids suggest that the plasmid genotype is in a state of flux and contributes to genome plasticity. These results corroborated the results obtained by other workers [20]. Such a flux might be due to the addition or deletion of certain sequences and/or recombination or cointegration of plasmids in response to selection pressures.

NPC-PCR amplification profiles revealed a high degree of polymorphism not only in length but also in number of amplified fragments. This suggests that the *nif* H promoter sequence is reiterated and dispersed on the *sym* plasmid. Reiteration the *nif* HDK promoter region has been demonstrated in another study [17]. Reiteration of *nif* H gene promoter sequences, their non-random pattern of distribution in the genome, mutations and recombination between reiterated sequences are probably involved in the origin and evolution of the observed diversity in NPC-PCR amplification profiles. The NPC-PCR amplification profiles revealed fine differences among the isolates as compared to plasmid typing. Within a single plasmid class, several isolates can be easily discriminated from each other by strain specific profiles. This suggest that *sym* plasmids may undergo recombination or rearrangements changing the pattern of distribution of repeat, that grouping based upon plasmid profile is unrealistic and that there is a cryptic genetic diversity within the same plasmid class. It has also been proposed by earlier workers [21] that frequent rearrangements in symbiotic plasmid might occur because of interaction between repeated DNA sequences.

In fact relationships established based on NPC-PCR reflect sorting of isolates across the ecological diversity. For example isolates of *S. aegyptica* from EGDU showed profiles which are markedly different from that of *S. aegyptica* isolates from AWS having a low percentage of similarity. Similarly, isolates of *S. sesban* from fly ash showed a profile distinct from all other isolates of *S. sesban*; class V isolates of *S. sesban* from a swampy habitat (FPYR) and a dry habitat (AHE) showed a distinct profile type having a low percentage of similarity. Similar results were obtained for these isolates with restriction fragment length polymorphism analyses using intact *sym* plasmid and *sym* gene probes (Mohmmed et al., unpublished data).

A number of isolates from different host plants grouped into the same cluster, suggesting that the origin from a particular host plant might not influence the differentiation among these isolates, which is expected due to the fact that all the isolates were found to be promiscuous among the four *Sesbania* species (Mohmmed et al., unpublished data). It has also been reported by other workers that rhizobial isolates from different species of sesbanias can cross-nodulate [7].

It is important to note the higher resolution level achieved by NPC-PCR in the discrimination of individual genotypes and its efficacy in the refinement of grouping of the isolates as compared to plasmid profile analysis. The degree of diversity attained by the plasmid profile analysis still masks most of the habitat driven diversity that is finely revealed by NPC-PCR. In this respect it is interesting to note that a number of isolates from three different sites (LAF, FPYR and AHE) showed plasmid profile type V, but that they showed diversity among them by NPC-PCR, which sorted them into ecologically predictable groups matching their origin. This shows how, within the always-plastic rhizobial genome, there is a hierarchical stability between the plasmid and plasmid borne genes, therefore promoter duplication, rearrangements and gene size insertional events occur at a much faster rate than do phenomena as plasmid deletion, curing and acquisition.

Our results suggest that the plasmid genotype of the *Rhizobium* gene pool that nodulate sesbanias is plastic in nature and is in a state of flux. The plasmid genotype can reshuffle in response to spatio-temporal environmental changes, resulting in generation of variability. Such a variability in the plasmid genotype enables the organism to

adapt rapidly to habitat heterogeneity. In other words, habitat heterogeneity contributes to the origin and evolution of diversity in the plasmid genotype of rhizobia.

Acknowledgements

We thank J.P.W. Young for his advice on this work and for providing us pIJ1247 and pIJ1246. This work was supported by project grants (Nos. BT/R and D/12/15/92) to CEMDE and a core grant to NII by DBT. A.M. and R.S.S. were supported by UGC and CSIR research fellowships, respectively.

References

[1] Martinez, E., Romero, D. and Palacios, R. (1990) The Rhizobium genome. Crit. Rev. Plant Sci. 9, 59–93.
[2] Frieberg, C., Fellay, R., Bairoch, A., Broughton, W.J., Rosenta, A. and Pesset, X. (1997) Molecular basis of symbiosis between *Rhizobium* and legumes. Nature 387, 394–401.
[3] Bromfield, E.S.P., Therman, N.P., Whitwill, S.T. and Barran, L.R. (1987) Plasmids and symbiotic effectiveness of representative phage types from two indigenous populations of *Rhizobium meliloti*. J. Gen. Microbiol. 133, 3457–3466.
[4] Young, J.P.W. and Wexler, M. (1988) *Sym* plasmid and chromosomal genotypes are correlated in field populations of *Rhizobium leguminosarum*. J. Gen. Microbiol. 134, 2731–2739.
[5] Kishore, L., Natrajan, K. and Babu, C.R. (1996) Total soluble proteins and membrane lipopoly-saccharide profiles in differentiating *Rhizobium* isolates. Microbios 86, 143–156.
[6] Nair, S. (1988) *Rhizobium* Gene Pools from Wild Legumes and their Potential Applications in Ecosystem Management. Ph.D. Thesis, University of Delhi.
[7] de Lajudie, P., Willems, A., Pot, B., Dewettinck, D., Maestrojuan, G., Neyra, M., Collins, M.P., Dreyfus, B., Kersters, K. and Gillis, M. (1994) Polyphasic taxonomy of rhizobia: emendation of the genus *Sinorhizobium* and description of *Sinorhizobium saheli* sp. nov. and *Sinorhizobium teranga* sp. nov. Int. J. Syst. Bacteriol. 44, 715–733.
[8] Rana, D. and Krishanan, H.B. (1995) A new root nodulating symbiont of the tropical legume, *Sesbania*, *Rhizobium* sp. SIN-1, is closely related to *R. galegae* a species that nodulate temperate legumes. FEMS Microbiol. Lett. 134, 19–25.
[9] Vincent, J.M. (1970) A Manual for the Practical Study of the Root-nodule Bacteria, IBP Hand Book No. 15, 159 pp. Blackwell Scientific Publications, Oxford.
[10] Eckhardt, T. (1978) A rapid method for the identification of plasmid deoxyribonucleic acid in bacteria. Plasmid 1, 584–588.
[11] Plazinski, J., Chen, Y.H. and Rolfe, B.G. (1985) General method for the identification of plasmid species in fast growing soil micro-organisms. Appl. Environ. Microbiol. 48, 1001–1003.
[12] Wilson, K. (1988) Large-scale preparation of bacterial genomic DNA. In: Current Protocols in Molecular Biology, Vol. 1 (Ausbel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K., Eds.), pp. 60–63. John Wiley and Sons, New York.
[13] Hynes, M.F., Simon, R. and Puhler, A. (1985) The development of plasmid-free strains of *Agrobacterium tumefaciens* by using incompatibility with a *Rhizobium meliloti* plasmid to eliminate pAtC58. Plasmid 13, 99–105.
[14] Richardson, A.E., Viccars, L.A., Watson, J.M. and Gibson, A.H. (1995) Differentiation of *Rhizobium* strains using the polymerase chain reaction with random and directed primers. Soil Biol. Biochem. 27, 515–524.
[15] Downie, J.A., Ha, Q.S., Knight, C.D., Hombrecher, G. and Johnston, A.W.B. (1983) Cloning the symbiotic region of *Rhizobium leguminosarum*: the nodulation genes are between nitrogenase and a *nif* A-like gene. EMBO J. 2, 947–952.
[16] Sneath, P.H.A. and Sokal, R.R. (1973) Numerical Taxonomy; the Principles and Practice of Numerical Classification, 573 pp. W.H. Freeman and Co., San Francisco, CA.
[17] Watson, J.M. and Schofield, P.R. (1985) Species-specific, symbiotic plasmid-located repeated DNA sequences in *Rhizobium trifolii*. Mol. Gen. Genet. 199, 279–289.
[18] Laguerre, G., Mazurier, S.I. and Amarger, N. (1992) Plasmid profiles and restriction fragment length polymorphism of *Rhizobium leguminosarum* bv. *viciae* in field populations. FEMS Microbiol. Ecol. 101, 17–26.
[19] Palmer, K.M. and Young, J.P.W. (2000) Higher diversity of *Rhizobium leguminosarum* biovar *viciae* populations in arable soils than in grass soils. Appl. Environ. Microbiol. 66, 2445–2450.
[20] Soberon-Chavez, G., Najera, R., Olivera, H. and Segovia, L. (1986) Genetic rearrangements of a *Rhizobium phaseoli* plasmid. J. Bacteriol. 167, 487–491.
[21] Palacios, R., Castillo, M., Flores, M., Hernandez, G., Mavingui, P. and Romero, D. (1995) Dynamics of Rhizobium genome. In: Nirtogen Fixation: Fundamental and Applications (Tikhonovich, I.A., Promorov, N.A., Romanov, V.I. and Newton, W.E., Eds.), pp. 365–370. Kluwer Academic Publishers, Dordrecht.