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Molecular characterization among strains of chickpea root nodule bacteria isolated from different areas of middle Gujarat

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ABSTRACT

Eighteen strains of Root nodule bacteria were collected from the chickpea plant, grown in different areas of middle Gujarat, *viz.*, Anand, Dahod, Thasara, Arnej and Dhanduka. These strains were confirmed as Rhizobium by using different biochemical test and maintained their pure culture. Molecular characterization based on repetitive DNA sequence especially, ERIC sequence (Enterobacterial Repetitive Intergeneric Consensus) were done together with two known Rhizobium strains, one commercial culture (GSFC, Vadodara), five standard strains of Rhizobium and one standard strain of *Agribacterium tumefacinus*. The total of 320 no of amplicons was generated by using ERIC primer pair. The strain MTCC 4188 (*Mesorhizobium ciceri*) produced highest no of amplicons while strain MTCC 120 (*Bradyrhizobium japonicum*) showed a less no of amplicons. Data analysis of ERIC fingerprinting pattern clustered all RNB strains and standard strains into four major clusters as per their phylogenetic relationship. Majority of RNB strains (65 per cent) were closely related to the genus *Mesorhizobium ciceri* species and *Mesorhizobium loti*, while remaining 40 per cent RNB strains showed similarity to *Rhizobium leguminosarum* (MTCC 99) and *Agrobacterium tumefaciens* (MTCC 431). The ERIC-PCR fingerprinting could become a powerful tool for depicting the genetic diversity among eighteen RNB strains and standard strains. The data based on ERIC fingerprinting pattern could help to determine phylogenetic relationships among these RNB strains and will be helpful for development of diagnostic primer for identification of efficient strains of Chickpea Root Nodulating Bacteria.

Key words : Chickpea, *Rhizobium*, Root nodule bacteria, Phylogenetic

INTRODUCTION

Chickpea (*Cicer arietinum* L.), belongs to the family *Leguminosae*, sub family *Papiliondiae*, tribe *cicerae*, is third most widely grown, self pollinated grain legume in the world. India is a premier chickpea growing country accounting for 67 per cent of total area and production of the world (Ali *et al.*, 2005). In Gujarat, the area of cultivation under chickpea is 170.0 thousand hectares, producing 140.0 thousand tonnes with productivity of 850 kg ha^{-1} (Source http://agricoop.nic.in/Agristatistics.htm).

Besides wide nutritional and agriculture importance, Chickpea also plays an important role in improving soil fertility through the process of Biological Nitrogen Fixation (BNF). *Rhizobium* spp. mainly from the genus *Mesorhizobium* form nodules on chickpea root and fix atmospheric nitrogen symbiotically. Rainfed chickpea growing areas fall under Bhal and Coastal Agro climatic zone (zoneVIII), where the soils are poor in drainage and saline, sodic or saline-sodic in nature and alkaline in reaction. The legume *Rhizobium* Symbiosis is affected by salinity, as it affects the growth of *Rhizobium*, nutrient uptake of plant and nodule formation mechanism. The yield and productivity of chickpea in these areas is affected due to environmental conditions such as drought and salinity. The chickpea variety GG-2 is mainly grown in Bhal area. There is a tremendous scope to increase yield and production of chickpea in this area through improved efficiency of biological nitrogen fixation, which in turn will also reduce the requirement of nitrogenous fertilizer and ultimately cost the of production.

Diverse bacterial population is observed in root nodules, such as Pseudomonas, Rhodopseudomonas, Agromyces, Bacillus, Microbacterium, Phyllobacterium etc. (Zakhia *et al*., 2006). Chickpea root nodules may contain some other endophytic bacteria, which help nodulation and nitrogen fixation. These bacteria (mainly *Bacillus* and *Pseudomonas*) promote nodulation by native Rhizobia and co-inoculation of such bacteria with effective Rhizobium strains of chickpea increase nodulation and nitrogen fixation significantly (Parmar *et al*., 1999). These-helping bacteria may also promote the plant growth by solubilization of minerals such as phosphorus, production of siderophores, which solublize and sequester iron or production of plant growth regulators (Hormones) (Tilak *et al.*, 2005). The regions of middle Gujarat may contain strains of Rhizobium, well adapted to varying soil and environmental conditions. The Rhizobium strains isolated from these areas will be very effective for evaluating as a chickpea inoculant in saline areas of middle Gujarat, due to possessing capacity to survive or persist

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in critical situation for a long time in the rhizosphere of the chickpea to compete and/or complement the native microflora. To achieve the maximum biological nitrogen fixation in these areas it is necessary to identify and characterize root nodulating bacteria before releasing them as commercial bioinoculant. The molecular marker *viz*., REP, ERIC and BOX are very efficient to characterize these Chickpea root nodule bacteria. This three families of repetitive sequences have been identified, *viz*. the 35-40 bp repetitive extragenic palindromic (REP) sequence, the 124-127 bp enterobacterial repetitive intergenic consensus (ERIC) sequence, and the 154 bp BOX elements. The repetitive elements may be present in both orientations, and oligonucleotide primers have been designed to prime DNA synthesis outward from the inverted repeats in REP and ERIC, and from the boxA subunit of BOX, in the polymerase chain reaction (De Bruijn 1992 and Prakash *et al.*, 2006). The use of these primer (s) leads to the selective amplification of distinct genomic regions located between REP, ERIC or BOX elements. The corresponding protocols are referred to as REP-PCR, ERIC-PCR and BOX-PCR genomic fingerprinting respectively, and rep-PCR genomic fingerprinting collectively (De Bruijn, 1992). Out of these three primers, the larger 126 bp ERIC elements contain a highly conserved central inverted repeat and are located in extragenic regions. There is no any report of molecular characterization of chickpea root nodule bacteria from middle Gujarat, therefore an attempt has been made to undertake the research on molecular characterization of chickpea root nodule bacteria using ERIC PCR fingerprinting.

MATERIALS AND METHODS

Isolation, purification and confirmation of Rhizobium:

The root nodules were isolated from various cultivars of chickpea from different areas of middle Gujarat, sp. Dhandhuka, Arnej, Anand, Thasara and Dahod (Table 1). The standard strains of *Rhizobium* and *Agrobacterium* were procured from IMTECH, Chandigarh. The chickpea *Mesorhizobium* strain IC2002 was brought from Department of Microbiology, ICRISAT, Patancheru, Andhra Pradesh, India.

Isolation of root nodule bacteria was done on YEMA media as described by Somasegaran and Hoben (1985). Pure cultures of bacteria were obtained by continuous subculturing of typical rhizobial colony (Jordan *et al.*, 1984) on YEMA media and incubated at 28° C for 2-3 days. Bacterial isolates were confirmed as *Rhizobium* using different biochemical test (Allen and Allen 1950). Similarly the phenotypic characteristics such as pH tolerance, salt tolerance, intrinsic antibiotic resistance, carbohydrate utilization pattern and its symbiotic infectivity as well as efficiency were studied.

Genomic DNA isolation:

The genomic DNA was extracted from overnight grown cultures at 28-30°C in Yeast extract mannitol (YEM) medium. The pellet were obtained by centrifuging overnight grown culture at 7000 rpm for 5 min and washed twice with 2 ml of 0.7 % NaCl. The pellets were resuspended with 2 ml of TEN buffer (50mM Tris, pH 7.5; 50mM EDTA, pH 8.0; 150mM, NaCl) and repeated the same procedure in order to remove the exo-polysaccharides (EPS). Then pellet were suspended in 1ml of TE buffer (10mM Tris, pH 8.0; 1mM EDTA, pH 8.0) in addition, 100µl of 10 % SDS, and 6 µl proteinase K was added then incubated the reaction at 37°C for1 hour, followed by addition of 360 µl of 5M NaCl and 300 µl CTAB NaCl solutions (4.1gm NaCl and 10gm CTAB in 100ml Sterile water) and samples were again incubated at 65°C for 20 minutes. The aqueous phase was pooled from tube in a 1.5 ml microfuge tube up to a volume of 750 µl from treatment of equal volume of Phenol/$CHCl_3$/ isoamyl alcohol (25:24:1). The aqueous phase was treated

Table 1 : The RNB isolates of chickpea collected from middle Gujarat region and standard/ reference strains

RNB Isolates	
Locations	RNB isoaltes (Host genotype)
Arnej	AR-1(ICCV-2), AR-2 (GG-2), AR-3 (K-902), AR-4(GG-1), AR-5(Chaffa), AR-6(GG-2), AR-7(GG-2), AR-8(GG-2), AR-9(Chaffa)
Dhandhuka	Dan-1(GG-2)
Anand	An-1(GG2), An-2(ICC-4), An-3 (Chaffa), An-4(Dahod Yellow), An-5(GG-2)
Dahod	Daho-1 (Dahod Yellow)
Thasara	Tha-1(GG-2), Tha-2(GG-2)
Reference strains	
Source	Name of strain
GSFC (Vadodara)	GSFC(Commercial culture)
UAS,Dharawad	Dha-1(Chickpea Rhizobium)
IARI, New Delhi	SP-6 and SP-7 (Chickpea Rhizobium)
IMTECH, Chandigarh	MTCC 2378 (*Mesorhizobium loti),* MTCC 99(*Rhizobium leguminosarum),* MTCC 120 (*Bradyrhizobium japonicum*), MTCC 431(*Agrobacterium tumefaciens*), MTCC 4188(*Mesorhizobium ciceri*)
ICRISAT, Patancheru	IC2002 (*Mesorhizobium ciceri*)

twice with chloroform: Isoamyl alcohol (24:1). The DNA was precipitated by adding 0.1 volume of 3M sodium acetate and 2 volume of chilled ethanol, kept at -20^{0}C for two hour/overnight. The DNA pellet was washed twice with 70 % ethanol, air dried briefly and dissolved in 100 µl of sterile TE. The DNA samples were treated with RNase A (20 µg/ml) for 2 hour at 37^{0}C. The enzyme was deactivated at 62^{0}C for 10 minutes.

ERIC PCR:

PCR amplification of ERIC sequences for all RNB isolates was conducted in 25 µl reaction volumes using conserved ERIC F and ERIC R primers. Each reaction consisted of 50 ng/µl DNA template, 1X Gitschier Buffer (83 mM $(NH_4)_2SO_4$, 335 mM Tris-HCl pH 8.8, 33.5 mM $MgCl_2$, 33.5 µM EDTA, 150 mM ß-mercapto-ethanol), 1X BSA, 10 % DMSO, 0.25 mM dNTPs, 1U Taq DNA polymerase, each of 10 pmol primers ERIC F (ATG TAA GCT CCT GGG GAT TCA C) and ERIC R (AAG TAA GTG ACT GGG TGA GCG), and remaining sterile distilled water. The PCR protocol was standardised to amplify ERIC sequences from different RNB isolates. The standardised protocol had cycling parameters of initial denaturation at 94^{0}C for 5min followed by 40 cycles of denaturation at 94^0 C for 1 min, annealing at 52^{0}C for 1.5 min and extension at 65^{o}C for 8 min. A final extension at 65^{0}C for 16 min was done at the end of amplification. Negative controls were used to test for false priming and amplification.

A 12 µl PCR amplification product for each of the isolate was visualized in a 1.5% agarose gel and viewed under UV light following staining with ethidium bromide. The presence and absence of bands were observed and scoring was done as 1 or 0, respectively. The data were analysed using NTSYSpc software and UPGMA algorithm.

RESULTS AND DISCUSSION

The genomic DNA fingerprinting of RNB isolates and standard strains by using ERIC primers, revealed multiple distinct DNA amplicons of size, ranging from approximately 100 to 4500 bp (Fig. 1 and 2). The total number of amplicons generated by ERIC primer is 320. The standard strain MTCC 120 amplified a unique amplicons of size 600 bp. While the strain MTCC 4188 could shown maximum of 20 ERIC amplicons. The *Rhizobium* strains used in this study could show a common fragment of 300 bp in almost all strains except strain Daho 1 and MTCC120.

Based on ERIC-PCR fingerprint pattern, dendrogram was generated, which clustered all RNB strains and standard strains into four major groups (Fig. 3). The first group comprised nine RNB strains and two standard strains, six RNB strians from Arnej (AR-1, AR-2, AR-3, AR-6, AR-8, AR-9), a one strain from Anand (An-1), Dhandhuka (Dan-1), and Dharwad (Dha-1), and two standard strains of *Mesorhizobium ciceri* (MTCC 4188 and IC2002). RNB strains of group I have shown 25 per cent similarity to the standard strains of *Mesorhizobium ciceri* (MTCC 4188 and IC2000).

Group II comprised five RNB strains and one standard strain of *Mesorhizobium loti* (MTCC 2378). The strains of group II separated from group I at 80 per cent dissimilarity; RNB strains AR-7, An-4, An-5, Tha-2 and GSFC have shown close identity to *Mesorhizobium loti* with similarity index of 33.6 per cent. Out of five RNB strains of this group, strain Tha-2 and GSFC were closely related, showing almost similar banding pattern and 85 per cent similarity value.

Remaining eight RNB strains and two standard strains *i.e. Rhizobium leguminosarum* (MTCC 99) and *Agrobacterium tumefaciens* (MTCC 431) were clustered in group III. The RNB strains, An-3, Tha-1, SP-6, SP-7 and Dha-1 have shown 25 per cent similarity to each

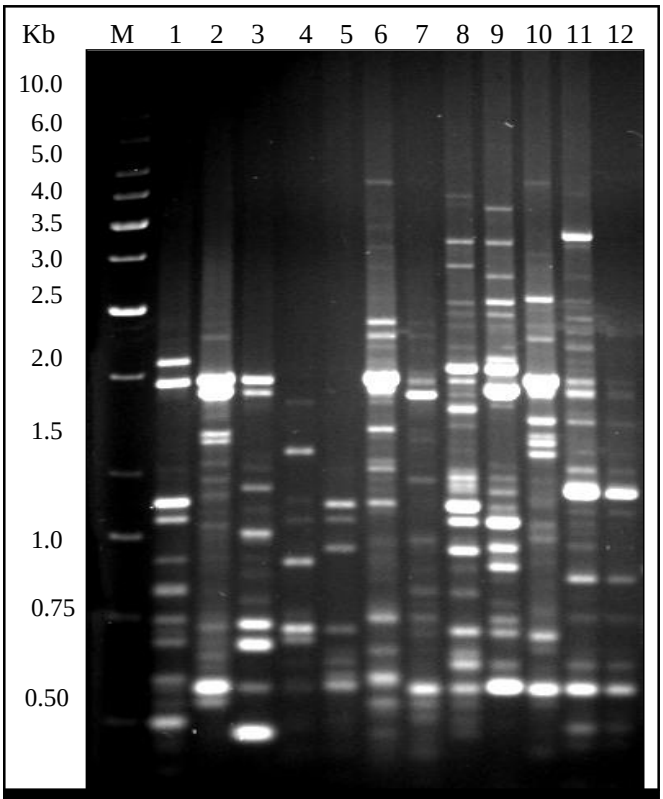

Fig. 1 : ERIC-PCR fingerprint of RNB isolates. M-1 Kb DNA Ladder, 1- AR-1, 2- AR-2, 3- AR-3, 4- AR-4, 5- AR-5, 6- AR-6, 7- AR-7, 8- AR-8, 9- AR-9, 10- Dan-1, 11- An-1, 12- An-2

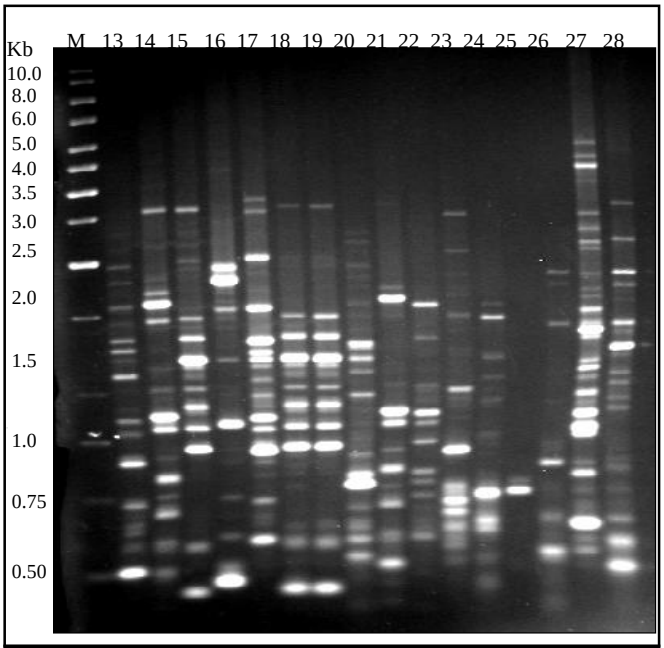

Fig. 2 : ERIC-PCR fingerprint of RNB isolates and standard strains. M-1 Kb DNA ladder, 13- An-3, 14- An-4, 15- An-5, 16- Daho-1, 17- Tha-1, 18- Tha-2, 19- GSFC, 20- Dha-1, 21- SP-6, 22- SP-7, 23- MTCC 2378, 24- MTCC 99, 25- MTCC 120, 26- MTCC 431, 27- MTCC 4188, 28- IC2002

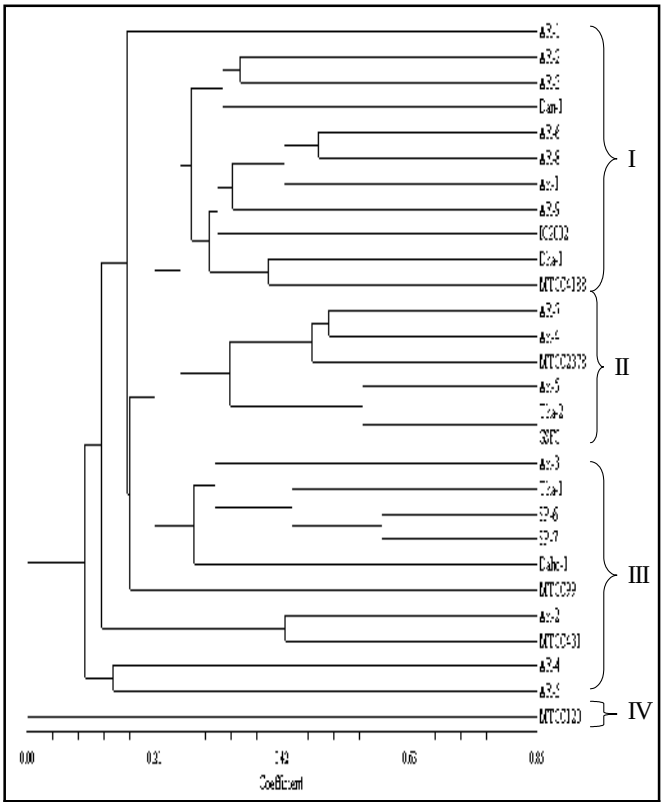

Fig 3 : Dendrogram based on ERIC PCR fingerprint analysis depicting the relationship among eighteen RNB isolates and six standard strains

other. The standard strain, *Rhizobium leguminosarum* (MTCC 99) appeared unique in this group and shown 16.8 per cent similarity with other RNB strains of this group and standard strains. Although RNB strain An-3 shown 42 per cent similarity with *Agrobacterium tumefaciens* (MTCC 431), both were distinct from others strains of this group at 88.4 per cent dissimilarity. Similarly the two strains from Arnej *viz*., AR-4 and AR-5 were also clustered in group III, but differ from all the other RNB strains as well as standard strains at 91.6 per cent dissimilarity.

The *Bradyrhizobium japonicum* (MTCC 120) was different, maintained its unique identity and categorized into separate group IV.

The ERIC- PCR analysis of chickpea RNB isolates and standard strains classified all the standard strains as per their taxonomic groups. Both the species of *Mesorhizobium ciceri* came under the group I, followed by *Mesorhizobium loti* (MTCC 2378) in group II, *Rhizobium leguminosarum* (MTCC 99) and *Agrobacterium tumefaciens* (MTCC 431) in group III, *Bradyrhizobium japonicum* (MTCC 120) in group IV. Similar phylogenetic classification was reported previously, based on the 16S rDNA sequencing and other molecular techniques (Eardly *et al.,* 1992, Gaunt *et al.,* 2001, Wang *et al.,* 1998, Kwon *et al.,* 2005 and Vinusa *et al.,* 1998)

In the present investigation, majority of the RNB isolates (65 per cent) were closely related to *Mesorhizobium ciceri* species and *Mesorhizobium loti*, while remaining 40 per cent showed closeness to *Rhizobium leguminosarum* (MTCC 99) and *Agrobacterium tumefaciens* (MTCC 431). None of the isolates was related to *Bradyrhizobium japonicum* (MTCC 120). These results were also consistent with the previous reports, which indicated that chickpea rhizobia are more closely related to *Mesorhizobium species* (Nour *et al.,* 1994, Jarvis *et al.,* 1997 and Maatallah *et al.,* 2002). The results presented herewith clearly established that ERIC like sequences (elements) are present in genomes of gram negative soil bacteria, such as Rhizobia and Agrobacteria, and there by support, the data presented by De Brujn *et al.* (1992) on the ubiquitous nature of these elements in bacteria. The present results also support the conclusion of Versalovic *et al.* (1991) and De Brujn *et al.* (1992) that the ERIC-PCR could become a powerful tool for the molecular genetic analysis of bacteria and for bacterial taxonomy, as it allows the fingerprinting of individual genera, species and strains and could help to determine phylogenetic relationships.

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