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PLASMID PROFILING AND RAPD MARKER ANALYSIS OF PAKISTANI GROWTH PROMOTING BACTERIA

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ABSTRACT

Nineteen chickpea (*Cicer arietin*um L.) and eleven green pea (*Pisum sativum* L.) root nodule rhizobia collected from different localities of Pakistan were characterized for plasmid profiles and genomic diversity. Plasmid profile analysis and RAPD finger printing revealed genetic diversity among studied chickpea and green pea rhizobia. Cluster analysis of plasmid categorized chickpea in four groups and green pea into five groups at the distance of 0.3 and 1.5 respectively. Plasmid numbers varied from 1 to 3, while size of plasmid varied from 2.3kb to 33kb for chickpea isolates. Plasmid number for green pea rhizobia varied from 1 to 2 and size from 9.4kb to 33kb. Soil isolates nodulating both crops showed more variations for plasmid size than plasmid number. Chickpea rhizobia were less diverse for plasmid number and size than green pea rhizobia. Cluster analysis of RAPD banding patterns revealed three clusters for chickpea and six clusters for green pea. Therefore RAPD analysis is an efficient discriminatory method for genetic diversity estimation of *Rhizobium* strains.

Key words: *Cicer arietin*um L., *Pisum sativum* L., *Rhizobium, Plasmid, RAPD Analysis*

INTRODUCTION

Members of the genus *Rhizobium* form nodules and fix atmospheric nitrogen in the roots of legumes. According to previous findings genes for nodulation and nitrogen fixation are plasmid-borne in *Rhizobium* (Higashi, 1967; Johnston *et al.*, 1978; Zurkowski and Lorkiewicz, 1978; Brewin *et al.*, 1982). Plasmids are considered important for saprophytic characteristics and sodium chloride tolerance (Loccoz and Weaver, 1996). Plasmid number varies from 0 to 11 and size from 150 to 1,683 kb in family *Rhizobeaceae* (Romero and Broom, 2004). The data for plasmid number and size in Pakistan is far behind. Therefore study of plasmid number and size is considered important for selection of efficient symbionts.

It is also necessary to characterize and identify rhizobia at molecular level before they are made comercially available as Nitrogen biofertilizer (Sahgal and Johri, 2003). Assessment of genetic diversity within rhizobia has received attention in past two decades (Ando and Yokoyama, 1999; Satyaprakash and Annapurna,

2006; Kücük and Kivanc, 2008). The discovery of molecular markers revolted the entire scenario of biology. DNA-based molecular markers have acted as versatile tools to study genomic diversity. Randomly ampli-fied DNA analysis (RAPD) is one of the most reliable fingerprinting method for phylogenetic grouping of rhizobial strains (Labes *et al*., 1996; Oliveira *et al*., 2000; Kumar *et al*., 2006; Sajjad *et al*., 2008; Rajasundari *et al*., 2009). Chickpea and Green pea are good protein source and most utilized food legumes in Pakistan. Some research reported beneficial uses of inoculums in maize and actinorhizial plants (Selim *et al*., 2010; Sughra *et al*., 2010). Lot of work is available on the genetic diversity of these pulses (Ghafoor *et al*., 2005; Ali *et al*., 2007; Jannatabadi *et al*., 2013; *Gixhari et al.*, 2014) but genetic diversity of their symbiotic partner and use of biofertilizer is neglected in Pakistan. Biofertilizer are not only cheap but it also increases productivity and nutrient status of soil. In order to improve production and protein quality of chickpea and green pea crops new candidate strains of Pakistan to be used as biofertilizer must be screened for genetic diversity. Therefore, 19 chickpea and 11 green pea rhizobial isolates obtained from Pakistani soils were characterized for their plasmid size, number and genomic polymorphism.

MATERIALS AND METHODS

Isolation of Rhizobia from soil and nodulation test: In order to isolate rhizobia 27 localities of all over Pakistan were selected. About one Kilogram of 8–22 cm deep soil was used. First stones were separated than soils were ground in pistil mortar and finally sieved to store in plastic jars.

Seed samples of chickpea (NCS 0530) and green pea (Meteor) were obtained from National Agriculture Research Centre, Islamabad (NARC) for isolation of soil rhizobia. Commercial strain of rhizobia for chickpea and green pea was isolated from biofertilizer (Bio-power) through root nodules.

Seeds were surface sterilized with 70% alcohol for 2–3 minutes. To isolate commercial strain seeds were moistened first with distilled water followed by dipping in commercial biofertilizer (Bio-power). Sterile soils were used for seedling growth and nodulation. The pots were kept in green house. Inoculated pots were watered when ever needed while commercial strains (Bio-power) in addition were given nitrogen free nutrient media once a week.

Four to six healthy nodules were removed from 6 week old plants and washed with distill water. Surface sterilization was performed with 95% ethanol followed by 5.25% sodium hypochlorite according to Weaver and Graham (1994).

Nodules were cured in sterilized water with glass rod for streaking on rhizobial selective media (yeast extract manitol agar containing 0.0025% (w/v) Congo red (Vincent, 1970). The rhizobia that absorb little or no Congo red in

the dark were finally were sub cultured to obtain pure strains at 28°C.

For confirmation as gram-negative rods Gram's staining was performed. Final cultures were stored at –70°C in 50% glycerol solution and YEM broth in 1:1 ratio until used.

Plasmid DNA extraction: Plasmid DNA was extracted using Innu PREP Plasmid mini kit (Analytik gena), catalog # 845-KS-504005. Extraction procedure was performed according to manufacturer's protocol. Ten ml rhizobial broths were used to isolate plasmid DNA. λ DNA was used as a molecular weight marker. Electrophoresis was carried out in 1X Tris borate buffer, using 1% agarose gel at 100V. Gels were stained in Ethidium bromide and photographed with Ingenius Syngene Bio-imaging System (Japan).

Genomic DNA extraction: Genomic DNA was extracted using Innu PREP Bacteria DNA kit (Analytik gena) catalog # 845-KS-6000050.

RAPD analysis studies: A modified RAPD method based on de Oliveira *et al.* (2000) was used with PCR thermal cycler model 9700 (Applied Biosystems, USA). After standardization of PCR, 25 µl reaction mixture containing 1x PCR buffer (10mmol Tris HCl (pH 8.3), 50mM KCl, 1.5mM $MgCl_2$, 200µM each deoxynucleotide triphosphate (dNTP), 2.5 nM of 10-mer primer (e-oligo, Gene Link, 140 Old Mill River Road, Hawthrone, NY 10532), 1 unit Ampli-Taq Gold DNA polymerase (Applied Biosystems, Japan) and approximately 20ng of template DNA was found optimum for the amplification of bacterial genomic DNA. The thermal cycler was programmed to complete 45 cycles of 1 minute at 94°C for denaturation, 1 minute at 36°C for annealing, 2 minutes at 72°C for primer extension and 1 cycle of 7 min at 72°C for final extension.

Primer selection: Out of 34 used primers, two primers (B8, B10) for chickpea and three primers (A5, E4, F8) for green pea nodulating rhizobia were eventually chosen (generated at least one band for all samples) to examine the genetic diversity of *Rhizobium* strains.

Electrophoresis of amplified products: After amplification, 3 µl dye and10 µl of amplification products were then loaded in 1.5% agarose gels for electrophoresis in 1 x TBE (10mM Tris-Borate, 1mM EDTA) buffer and run at 100V for 40 min. Hundred base pair ladder and λ DNA were used as molecular weight markers. The gels were stained with Ethidium bromide for 40 min and photographed under UV light.

Statistical analysis: The data subjected to computer cluster analysis, while phonogram was constructed by the unweighted pair-group method with arithmetic average (Statistica 6.0).

RESULTS AND DISCUSSION

Plasmid profile: Electrophoretic screeening of plasmid is important parameter contributing bacterial diversity. The plasmid profile of chickpea rhizobia is given in (Figure-1). Plasmid numbers varied from 1 to 3 for chickpea rhizobia, while size of plasmid varied from 2.3kb to 33kb.

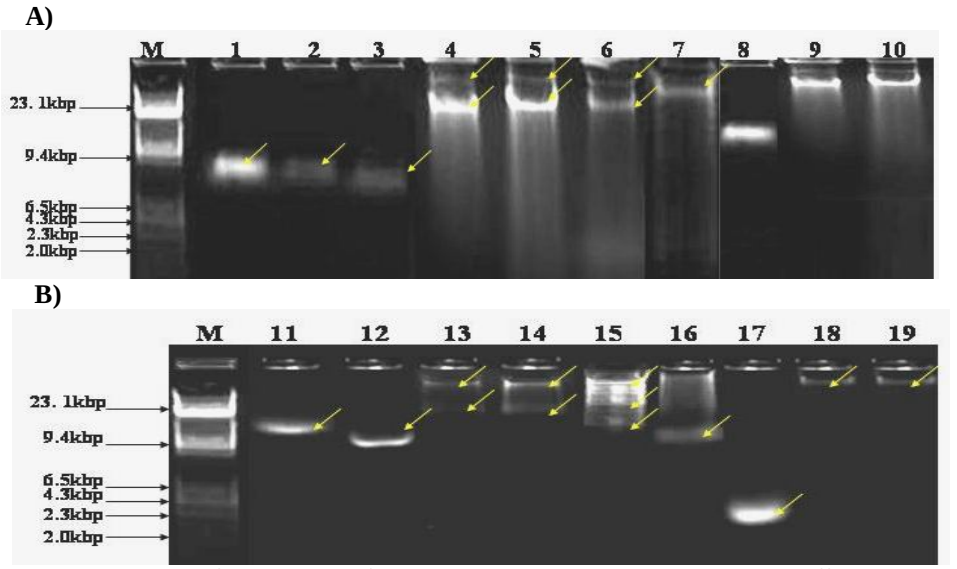


Figure-1: Plasmid profiles (a and b) of 19 chickpea root nodule rhizobia, showing different plasmid groups. M = Molecular weight marker λ DNA; Lane 1. 2cp, Lane 2. 3cp, Lane 3. 5cp, Lane 4. 6cp, Lane 5. 7cp, Lane 6. 8cp, Lane 7. 9cp, Lane 8. 11cp, Lane 9. 12cp, Lane 10. 13cp, Lane 11. 14cp, Lane 12.16cp, Lane 13. 19cp, Lane 14. 21cp, Lane 15. 22cp, Lane 16. 24cp, Lane 17. 25cp, Lane 18. 26cp, Lane 19. 27cp

It is clear from dandogram constructed on distances that over all chickpea soil isolates showed close association (Figure-2). At the distance of 0.3 four clusters were evident. Cluster 1, contained 5 isolates and check strain (BPcp) sharing 33kb plasmid; cluster 2 included 3 isolates having 23.1kb plasmid in common. The 3rd cluster contents 2 isolates having 23.4kb plasmid and largest 4th cluster encompassed 9 isolates that possess only one plasmid band in the range of 2.3kb to 16.3kb.

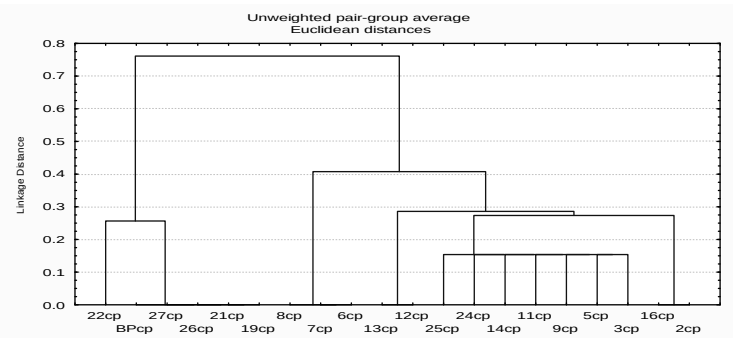


Figure-2: Dandogram of 19 chickpea isolates and commercial strain derived from plasmid profile analysis

The plasmid profile of green pea rhizobia is shown in Figure-3. Plasmid numbers varied from 1 to 2 and size of plasmid varied from 9.4kb to 33kb. Data of Clusters analysis is presented in Figure-4 showing five main clusters at the distance of 1.5. Cluster 1 contained check strain (BPcp) alone having 22 and 24kb plasmids. Cluster 2 included four isolates sharing 20kb plasmid and 3rd cluster encompassed three isolates possessing 11 and 23.1kb plasmids, while one isolate constitute cluster 4 that have 12kb plasmid and last cluster again contains three isolates having 13kb plasmid. Over all chickpea and green pea isolates showed close association for plasmid profiles; although existence of diversity is evident from presence of four and five clusters at the distance of .3 and 1.5 respectively.

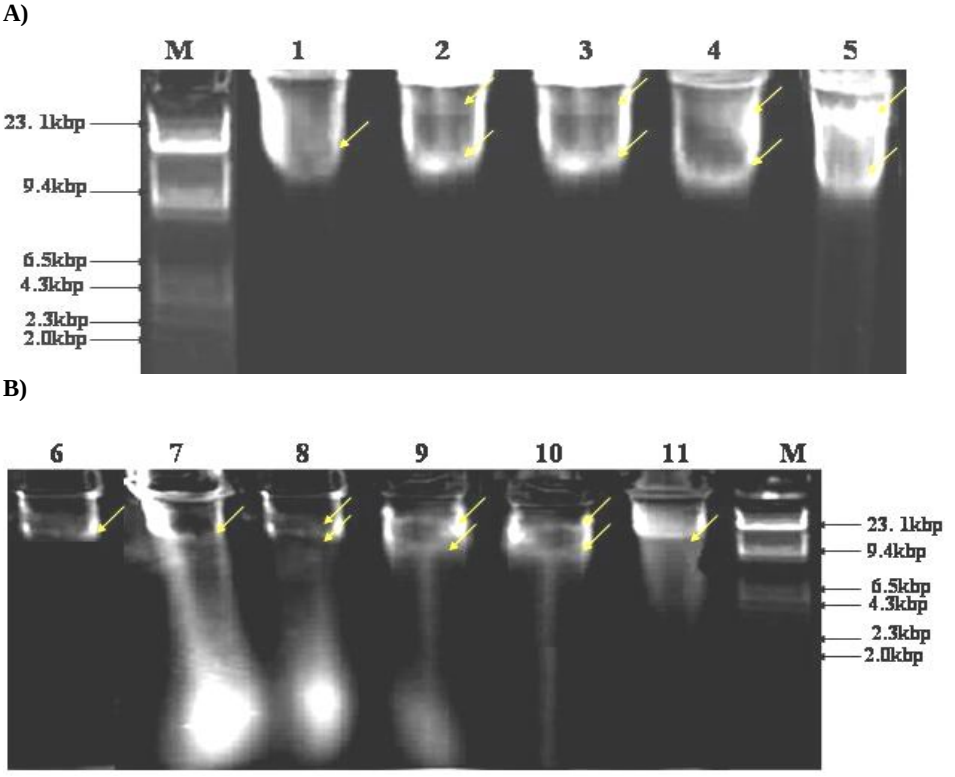


Figure-3: Plasmid profiles (a and b) of 11 green pea root nodule rhizobia, showing different plasmid groups. M = Molecular weight marker λ DNA; Lane 1. 3gp, Lane 2. 7gp, Lane 3. 8gp, Lane 4. 9gp, Lane 5. 12gp, Lane 6. 18gp, Lane 7. 19gp, Lane 8. 23gp, Lane 9. 24gp, Lane 10. 26gp, Lane 11. 27gp,

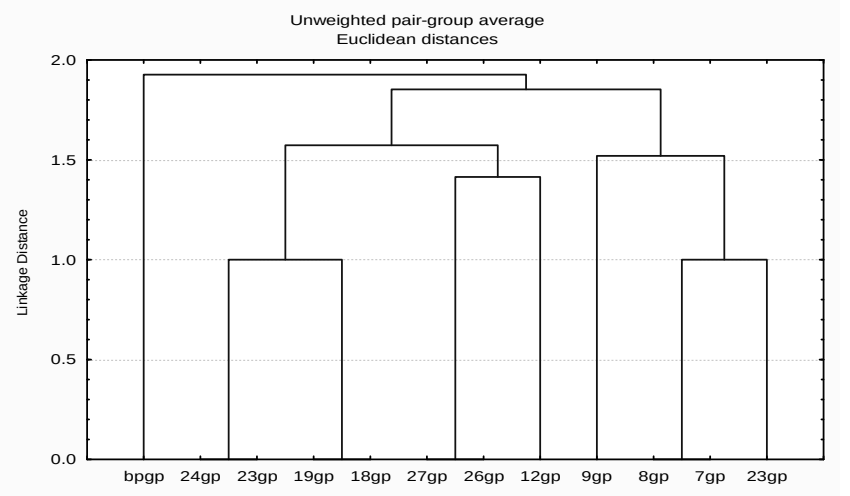
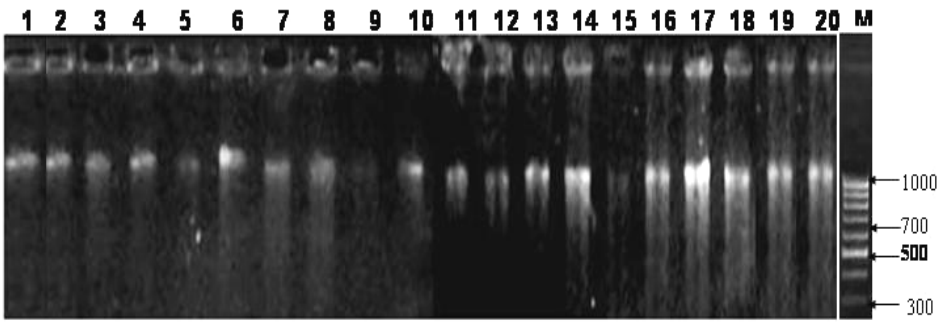


Figure-4: Dandogram of 11 green pea isolates and commercial strain derived from plasmid profile analysis

It is obvious from the results that chickpea rhizobia are less diverse for plasmid number and size than green pea rhizobia. Soil isolates nodulating both crops showed more variations for plasmid size than plasmid number. Studied rhizobia showed less number of plasmids than previous findings of Vessey and Cheminging'wa (2006), who reported one to five plasmid bands in *Rhizobium legueminosarum* bv *viciae* strains and Romero and Broom (2004) from 0 to 11 in family *Rhizobeaceae*.

RAPD analysis: Genomic DNA fingerprinting using random amplification of polymorphic DNA (RAPD) is a useful technique that not only discriminate bacterial strains, but also explore genetic diversity within them. The RAPD banding profile of chickpea rhizobia generated by primers B8 and B10 is given in Figure -5.

A)



B)

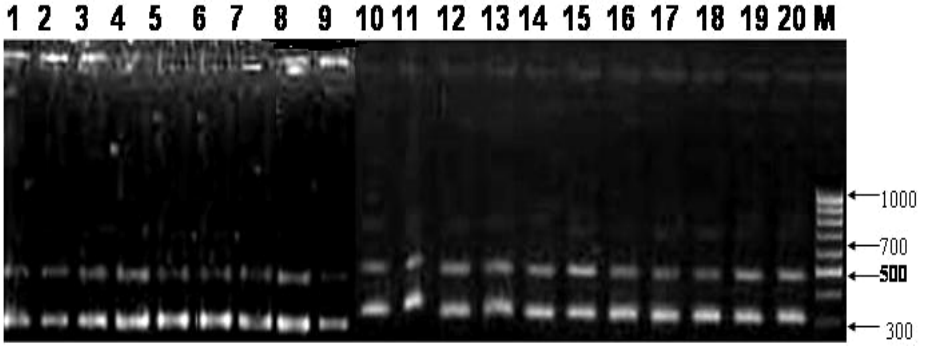


Figure-5: RAPD banding pattern of chickpea soil isolates with RAPD primer (A) B8(B) B10 (C). Lane 1. 2cp, Lane 2. 3cp, Lane 3. 5cp, Lane 4. 6cp, Lane 5. 7cp, Lane 6. 8cp, Lane 7. 9cp, Lane 8. 11cp, Lane 9. 12cp, Lane 10. 13cp, Lane 11. 14cp, Lane 12.16cp, Lane 13. 19cp, Lane 14. 21cp, Lane 15. 22cp, Lane 16. 24cp, Lane 17. 25cp, Lane 18. 26cp, Lane 19. 27cp Lane, 20.BPcp Lane M = Molecular weight marker λ DNA

At the distance of 1.5, three major clusters were evident (Figure-6). Cluster 1 included six isolates and BPcp, majority of them shared 320bp and 1100bp segment. Cluster 2 included six isolates that have 340bp segment in common. Third cluster encompassed seven isolates, majority of them possessing 300bp and 1200bp segment. 500 bp segment was shared by all the isolates except 9cp.

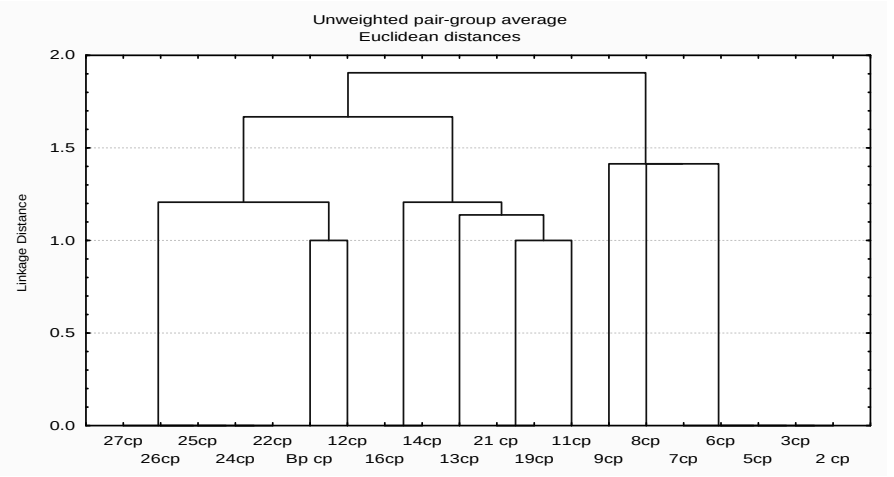


Figure-6: Dandogram of 19 chickpea isolates derived from RAPD fingerprinting generated by two primers

The RAPD banding profile of green pea rhizobia produced by primers A5, E4 and F8 is shown in Figure-7. Hierarchal clusters of distance matrix for green pea nodulating rhizobia are presented in the form of dandogram (Figure-8). At the distance of 1, six clusters were obvious. Cluster 1 included single strain (24gp) possessing unique fragment of 490 bps. Cluster 2 included two isolates that have 200bp and 380bp segments in common. Third cluster again encompassed two isolates possessing 200bp, 380bp and 450bp segments. Cluster 4 possessed two isolates that shared 450bp and 1000bp fragments. Cluster 5 encompassed two isolates and check strain (BPgp) having 200bp and 450bp in common. Last cluster consist of single strain (3gp) that possessed unique band of 500bp.

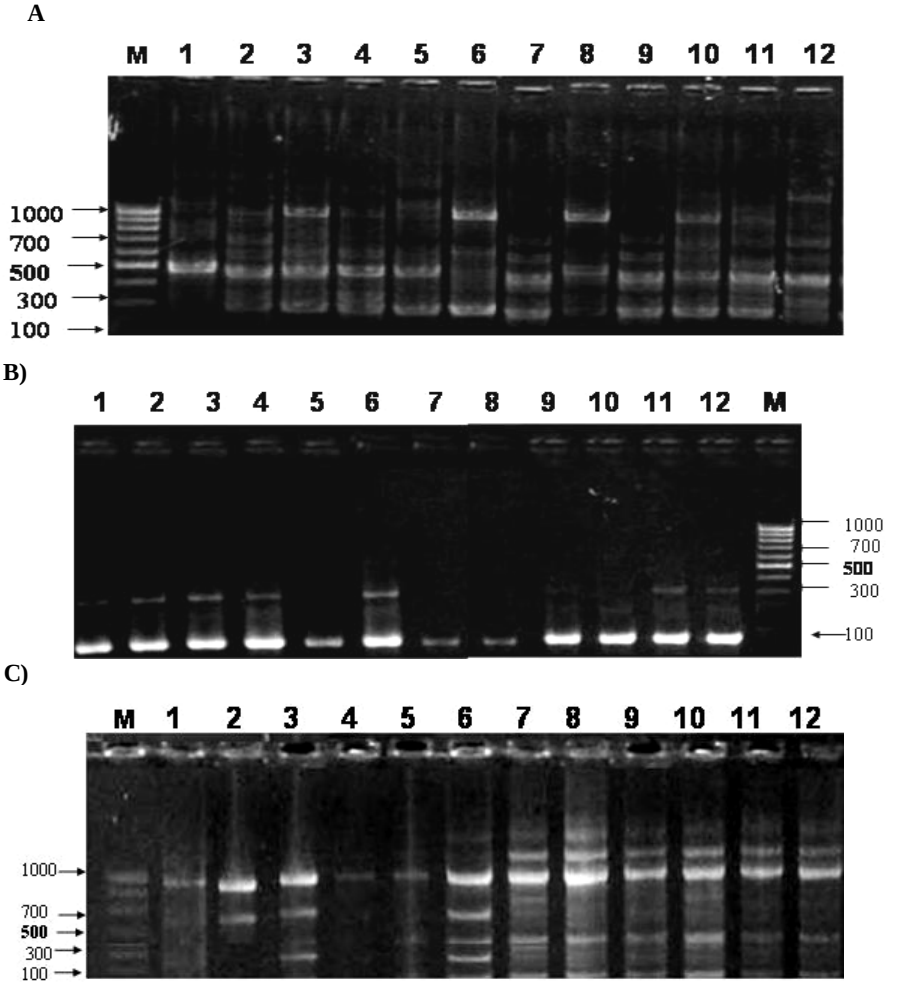


Figure-7: RAPD banding pattern of green pea soil isolates with RAPD primer (A) A5(B) E4 (C) F8. M = 100bp DNA ladder; Lane 1. 3gp, Lane 2. 7gp, Lane 3. 8gp, Lane 4. 9gp, Lane 5. 12gp, Lane 6. 18gp, Lane 7. 19gp, Lane 8. 23gp, Lane 9. 24gp, Lane 10. 26gp, Lane 11. 27gp, Lane 12. BPgp

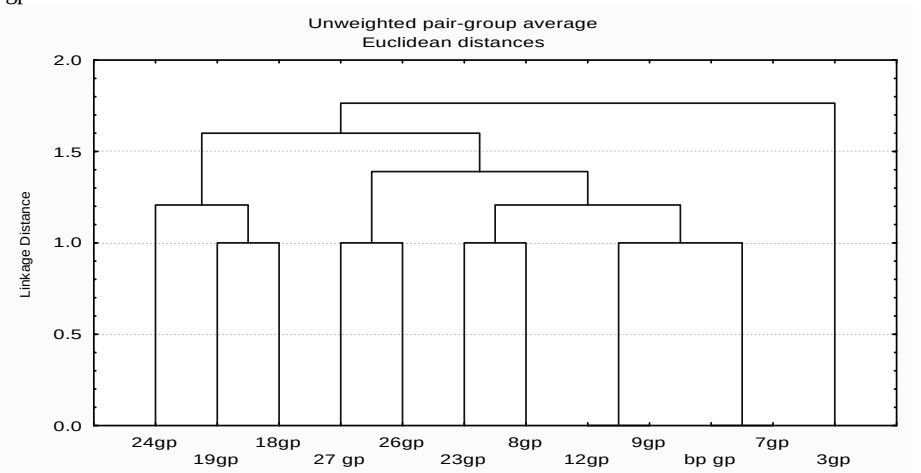


Figure-8: Dandogram of 11 green pea isolates and commercial strain derived from RAPD finger printing generated by three primers

Present results are consistent with the findings of de Oleveria *et al*. (2000); Priefer *et al*., (2001); Sajjad *et al*., (2008), who reported genetic polymorphism using RAPD banding patterns with 10-mer primer.

Cluster analysis conformed plasmid profile results that chickpea rhizobia were less diverse than green pea rhizobia evident from occurrence of randomly amplified polymorphic banding patterns. RAPD analysis efficiently discriminate *Rhizobium* strains.

Present results support findings of Kumar *et al*., (2006), Rajasundari *et al*., (2009), Bejarano *et al.,* (2014) and Hewedy *et al.,* (2014)

CONLUSION

The occurrence of two phenotypic groups suggests that different genomic species might exist in Pakistani soils. Plasmid profiling and RAPD finger-printing analysis are efficient discriminative methods for differentiating and studying genetic diversity of *Rhizobium* strains.

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