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Genetic Characterization of Rhizobia Associated with Horse Gram [*Macrotyloma uniflorum* (Lam.) Verdc.] Based on RAPD and RFLP

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Authors' contributions

This work was carried out in collaboration between all authors. Authors PE and AJAM designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript and managed literature searches. Authors VRSGD, VZ and VMK managed the analyses of the study and literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aim: To genetically characterize rhizobia collected from the root nodules of Horse gram plants grown in different soil samples by using the methods RAPD and RFLP.
Place and Duration of Study: Root nodules were collected from the plants grown in soil samples collected from different regions in Andhra Pradesh and Telangana states, India.
Methodology: These isolates were characterized by two RAPD primers RPO4 and RPO5 of 10 nucleotide length. The PCR amplicons were purified by Qiaquick® PCR purification kit and digested by three restriction endonucleases i.e., *TaqI, HpaII* and *AluI*.
Results: The RAPD profiles and restriction analysis of 16S rRNA gene with the three

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endonucleases showed high polymorphism. RAPD and RFLP analysis of these 32 horse gram rhizobia showed that these isolates fall into four major clusters. The first clusters of both dendrograms from RAPD and RFLP contained the majority of the rhizobial isolates.
Conclusion: From this, it is clear that RAPD and RFLP may play an important role if applied, to know the genetic diversity of rhizobia. High genetic diversity was observed among the horse gram rhizobial population and very few of the bacteria were considered to be identical. It clearly shows that the horse gram rhizobia are phylogenetically distinct.

Keywords: Rhizobia; RAPD-PCR; PCR-RFLP; RDP; dendrogram; diversity.

1. INTRODUCTION

Horse gram [*Macrotyloma uniflorum* (Lam.) Verdc. = *Dolichos biflorus* (Linn.)] is an important pulse crop and it is extensively cultivated on light red and gravel soils of peninsular India. It derives its importance from its adaptability to poor and adverse climatic conditions which are unsuitable for other pulse crops. In India horse gram is cultivated in 1.1 million hectares during both kharif and rabi seasons. This grain legume is most extensively grown in the states viz., Tamil Nadu, Karnataka and Andhra Pradesh of South India. Being a legume it also fixes atmospheric nitrogen and improves soil fertility. It is widely cultivated as a grain legume and fodder crop in this country and constitutes an essential supplement for the cereal based balanced diet of low income populations [1]. Growth and yield of this legume crop were drastically increased when they were inoculated with nitrogen fixing rhizobia [2]. India has a long history of horse gram cultivation and it is considered one of the primary centers of origin and diversification of this legume [3]. The genetic diversity detected among the horse gram nodule isolates was comparable to that determined for rhizobia associated with legumes in their place of origin [4,5,6].

Little is known about the rhizobia associated with horse gram i.e. effect of sodium chloride [7] and salts on growth [8], exopolysaccharide production [9], mercuric chloride tolerance [10] and the effect of metal chlorides [11]. Some agronomically important activities like production of IAA [12], siderophore [13], solubilization of inorganic phosphate [14], HCN (Hydrocyanic acid) production [15] was also studied. There are very few studies on genetic diversity of the microsymbionts associated with horse gram [16]. *M. uniflorum* species were found to be nodulated with *Bradyrhizobium* spp. [17] but one author [18] reported for the first time that horse gram was nodulated by a member of the genus *Caulobacter* which belongs to the family *Caulobacteriaceae* in the order *Caulobacterales* of *Alphaproteobacteria*.

Legumes are usually nodulated by indigenous root nodule bacteria. The symbiosis formed through interaction between rhizobia and legume plants, such as horse gram can transform atmospheric N_2 to ammonia by supplying nitrogen to the plant and enhancing its ability to withstand stress even in arid environments [19]. Abundant types of indigenous rhizobia are widely distributed in various geographical and ecological areas of the world [20,21,22,23]. Nodule-forming bacteria have traditionally been assigned to taxonomic groups on the basis of their host specificity. Various phenotypic and genotypic methodologies are being used to identify and characterize bacteria [23]. Although phenotypic methods play a significant role in identification but the molecular methods are more reliable and authenticated for identification and to study genetic diversity of bacterial isolates. Major molecular techniques are PCR (polymerase chain reaction) - RAPD (randomly amplified polymorphic DNA), RFLP (restriction fragment length polymorphism), AFLP (amplified fragment length polymorphism), SSR (single sequence repeats) and 16S rRNA gene sequencing.

Therefore, in the present study an attempt was made to characterize the rhizobia associated with horse gram by using random amplified polymorphic DNA and RFLP analysis of PCR amplified 16S rRNA gene.

2. MATERIALS AND METHODS

2.1 Bacterial Strains

Root nodules were collected from the plants grown in soil samples collected from different regions in Andhra Pradesh and Telangana states, India (Fig. 1). In the process, nodules were separated from the roots and washed with 0.01% mercuric chloride. The nodules were repeatedly washed with sterilized distilled water

to get rid of the sterilizing agent. Following serial dilution agar plate technique [24] using yeast extract mannitol agar (YEMA) medium containing 0.0025% congo red dye [25], bacterial isolations was carried out. After that these plates were incubated at 28±1℃ and observed daily. Bacterial colonies appeared after 2-3 days were picked up and streaked on YEMA plates. Pure cultures were obtained with one or more further sub culturing steps. Root nodulating ability of these isolates was determined by nodulation test [26]. Furthermore, all the isolates were subjected to authentication test before performing all the experiments. They were designated as HGR-1 (Horse Gram Rhizobia) to HGR-32.

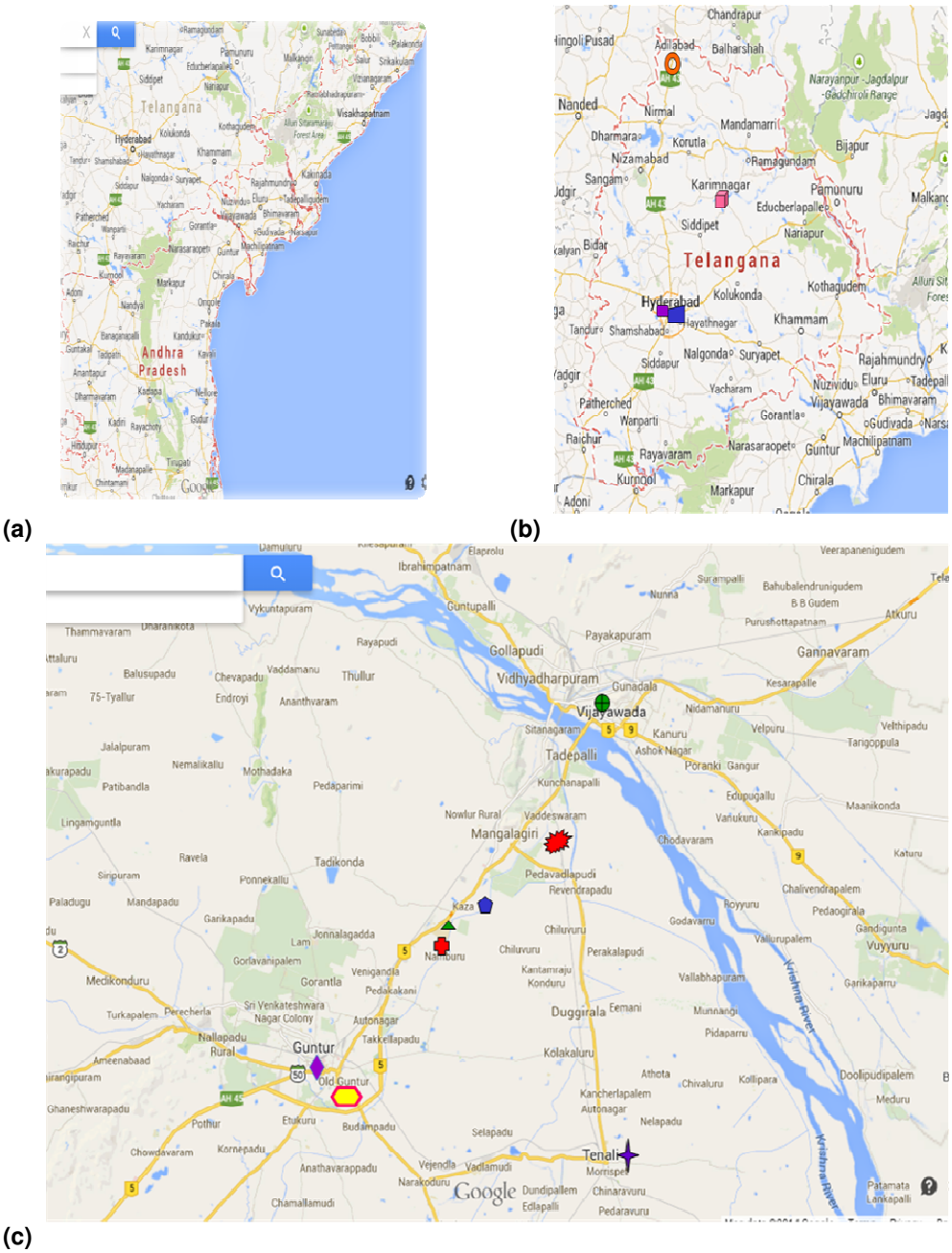

Fig. 1. Soil sample collection sites from Telangana and Andhra Pradesh states in India. (a) Andhra Pradesh state including both Telangana and Andhra Pradesh (b) Telangana (c) Andhra Pradesh

2.2 Sample Preparation for Direct Amplification of DNA

Pure cultures were maintained and genomic DNA was isolated [27] from each of them, concentration of the DNA was determined by using Nanodrop® spectrophotometry.

2.3 PCR-RAPD Analysis

Two reported primers [28] of 10 nucleotide length, RPO4 and RPO5 (Table 1) were synthesized at Bioserve Biotechnologies Pvt. Ltd., Hyderabad, India.

The PCR-RAPD amplification reactions were carried [28] in 50 µl reaction mix consisting of 1XTaq buffer (750 mM), Tris HCl, (pH 8.8), 200 mM $(NH_4)_2SO_4$, 0.1% Tween 20, 0.2 mM of dNTPs, 20 pM of primer, 1.5 mM $MgCl_2$, 30 ng of DNA template; 1.25 units of Taq DNA polymerase (Fermentas, USA) and Milli Q water to a final volume of 50 µl. The thermal cycling conditions were carried out in a Corbett Research Thermal cycler with initial denaturation at 94℃ for 8 min, followed by 5 cycles of denaturation at 94℃ for 30 s, annealing at 40℃ for 2 min and an extension at 72℃ for 1 min, followed by 35 cycles of 94℃ for 5 s, 45℃ for 25 s and 72℃ for 90 s; followed by a final cycle of 94℃ for 10 s, 45℃ for 20 s and 72℃ for 5 min and a final extension at 72℃ for 10 min. The obtained amplicons were analysed on 1% agarose gels along with two markers Gene Ruler™ DNA Ladder Mix (Fermentas, USA) and λ EcoRI + HindIII double digest marker (Fermentas, USA). The image was photographed using gel documentation unit (Alpha innotech, USA).

2.4 PCR-RFLP Analysis

16S rRNA gene of all 32 isolates tested were amplified [29] using the primers 16F27 and 16R1525XP (Table 1) synthesized at Bioserve Biotechnologies Pvt. Limited, Hyderabad, India.

All the horse gram rhizobia produced a single band of about 1500 bp. This size corresponded to the expected length of the 16S rRNA genes of rhizobia. PCR amplification of the 16S rRNA gene was carried in 50 µl reaction mix consisting of 1XTaq buffer [750 mM Tris HCl, pH 8.8, 200 mM $(NH_4)_2SO_4$, 0.1% Tween 20], 0.2 mM of dNTPs, 10 pM of each of the primers, 1.5 mM $MgCl_2$, 30 ng of DNA template; 1.25 units of Taq DNA polymerase (Fermentas, USA) and Milli Q water to a final volume of 50 µl. The thermal cycling conditions were carried out in a Corbett Research Thermal cycler with initial denaturation at 95℃ for 1 min, followed by 35 cycles of denaturation at 94℃ for 60 s, annealing at 55℃ for 1 min and an extension at 72℃ for 1 min, followed by a final extension at 72℃ for 10 min. The obtained amplicons were analysed on 1% agarose along with two markers Gene Ruler™ DNA Ladder Mix (Fermentas, USA) and λ-pUC Mix, 4 (Fermentas, USA).

The PCR amplicons were purified by Qiaquick® PCR purification kit (Qiagen) and digested by the restriction endonucleases i.e., *TaqI, HpaII* and *AluI* (Fermentas, USA) according to the manufacturer's recommendations. Restriction digestions were performed in 15 µl reaction volume mixes with recommended units of enzyme and appropriate buffers at 37℃ for overnight. The products were analysed on 1% agarose gel with two reference markers Gene Ruler™ DNA Ladder Mix (Fermentas, USA) and 50 bp DNA ladder (Fermentas, USA) were separated on 1% agarose gels. The image was photographed using gel documentation unit (Alpha innotech, USA).

The sizes of RAPD and RFLP fragments were estimated by comparison with markers. RAPD and RFLP data was recorded in a scoring pattern where the presence of a band or fragment was given a score 1 and absence of a band was given a score 0. Dendrograms based on UPGMA method were produced using Free Tree Package V.0.9.1.50 and dendrograms were viewed using Tree Explorer Tool V.2.12. Distance matrices were calculated using Free Tree package from data of RAPD and RFLP as described [30].

Table 1. Primers used for RAPD and RFLP analysis

RAPD Primers	
RPO4	5' GGAAGTCGCC-3'
RPO5	5' AGTCGTCCCC-3'
RFLP primers (Universal Eubacterial-specific primers)	
16F27	5'-CCAGAGTTTGATCMTGGCTCTG-3'
16R1525 XP	5'-TTCTGCAGTCTAGAAGGAGGTGWTCCAGCC-3'

3. RESULTS

The rhizobial cells were Gram negative non spore forming rods, the size of the cells was 2 to

2.3 µm long with 0.5 - to 1 µm width. The sizes of the colonies were 6-8 mm in diameter after 72 h on YEMA medium at room temperature. The optimum pH was in the range of 7-7.5. These strains grow at a temperature between 10 to 40ºC. Lower concentrations of NaCl favoured growth of these rhizobia. All the isolates were resistant to ampicillin and rifampicin. The isolates HGR-11, 22 and 23 showed resistance to most of the antibiotics tested, where as the isolate HGR-4 showed susceptibility towards most of the antibiotics. All the isolates are positive for citrase, nitrate reductase, tryptophanase, asparginase, catalase and the production of ammonia. Four strains (which showed more positive characters among all the isolates in their previous characterization tests) were selected for 16S rRNA sequence and were submitted to the NCBI GenBank under the accession numbers GQ483457, GQ483458, GQ483459 and GQ483460.

The RAPD marker has been effectively used to detect the genetic diversity among 32 isolates collected from various parts in Andhra Pradesh and Telangana states, India. The primer RPO4 yielded fragments ranging from 3500 bp to 100 bp and 1-9 fragments were considered for analysis. These rhizobia showed high polymorphism with the primer RPO5. The primer RPO5 amplified the DNA up to eleven fragments per strain (4000 bp to 100 bp) and these bands were included in the cluster analysis. The dendrogram constructed using 61 bands scored from the RAPD amplified DNA fragment data (Fig. 2) showed two major clusters. The first cluster comprised about 40% of the rhizobia while the second cluster contained 37% of the strains. Seven isolates were differentiated with the remaining isolates.

The results of this study indicate that RAPD provides a high degree of discrimination between horse gram rhizobia. Genomic DNA fingerprinting using random amplification of polymorphic DNA (RAPD) has been found to be useful in differentiation between closely related bacteria. Total genomic DNAs from 32 rhizobia were used as templates for RAPD fingerprinting. The primers RPO4 and RPO5 produced different and informative banding patterns for all the isolates. Rhizobial isolates of cluster I (HGR-29,30,31,32,**4**,8,5,14,7,10,11 and **13**) is genetically distant from the cluster II (HGR-15,18,**6**,9,23,22,3,17,21,28,20 and 27). The isolates HGR-26,1,2,16,19,24 and **25** showed more divergence with the remaining isolates. This result implies that *Rhizobium* isolates belonging to cluster I and those from cluster II are significantly diverse.

The RFLP analysis with restriction endonucleases *Taq1*, *HpaII* and *AluI* revealed a considerable degree of genomic heterogeneity among the 32 *Rhizobium* strains tested. These strains showed wide variation in their banding pattern with the enzyme *Taq1*. Restriction analysis of 16S rRNA gene with this enzyme yielded six different patterns i.e. 1000 bp to 1500 bp. Eighty one fragments were considered for the phylogenetic analysis. The dendrogram showed that these isolates fall into four major clusters. The first cluster contains 28% strains, second cluster comprised about 15% of the rhizobia. Third cluster contains 12% strains. The fourth cluster comprises of 28%, whereas the isolates HGR-3, 8, 14, 25 and 31 grouped separately. On analysis of the dendrogram (Fig. 3) it was observed that the *Rhizobium* isolates clustered according to their geographical location. The gels representing RAPD and RFLP of the DNA samples are shown in Figs. 4 and 5 respectively.

The data of this study indicated that from 0-6 distinct patterns were detected with each of the three endonucleases and the isolates used. The number of restriction patterns obtained with each of the three restriction enzymes were as follows *TaqI* (0-6 patterns), *HpaII* (0-6 patterns), *AluI* (0-5 patterns). The reason why there was no digestion of amplified product from some strains by *TaqI, HpaII* and *AluI* could be due to absence of the restriction sites.

4. DISCUSSION

The RAPD is the most reliable, rapid and practical method [31] used for phylogenetic relationships among and within closely related species [32]. The genetic characterization by RAPD-PCR was applied to study the status of *Sinorhizobium meliloti* [33], *Bradyrhizobium* [34] and nodule isolates of *Acacia* sp. [35]. The PCR-RFLP method used in this study appears to be a rapid tool for the differentiation and estimation of genetic relationship between *Rhizobium* 16S rRNA genes at the species and higher levels.

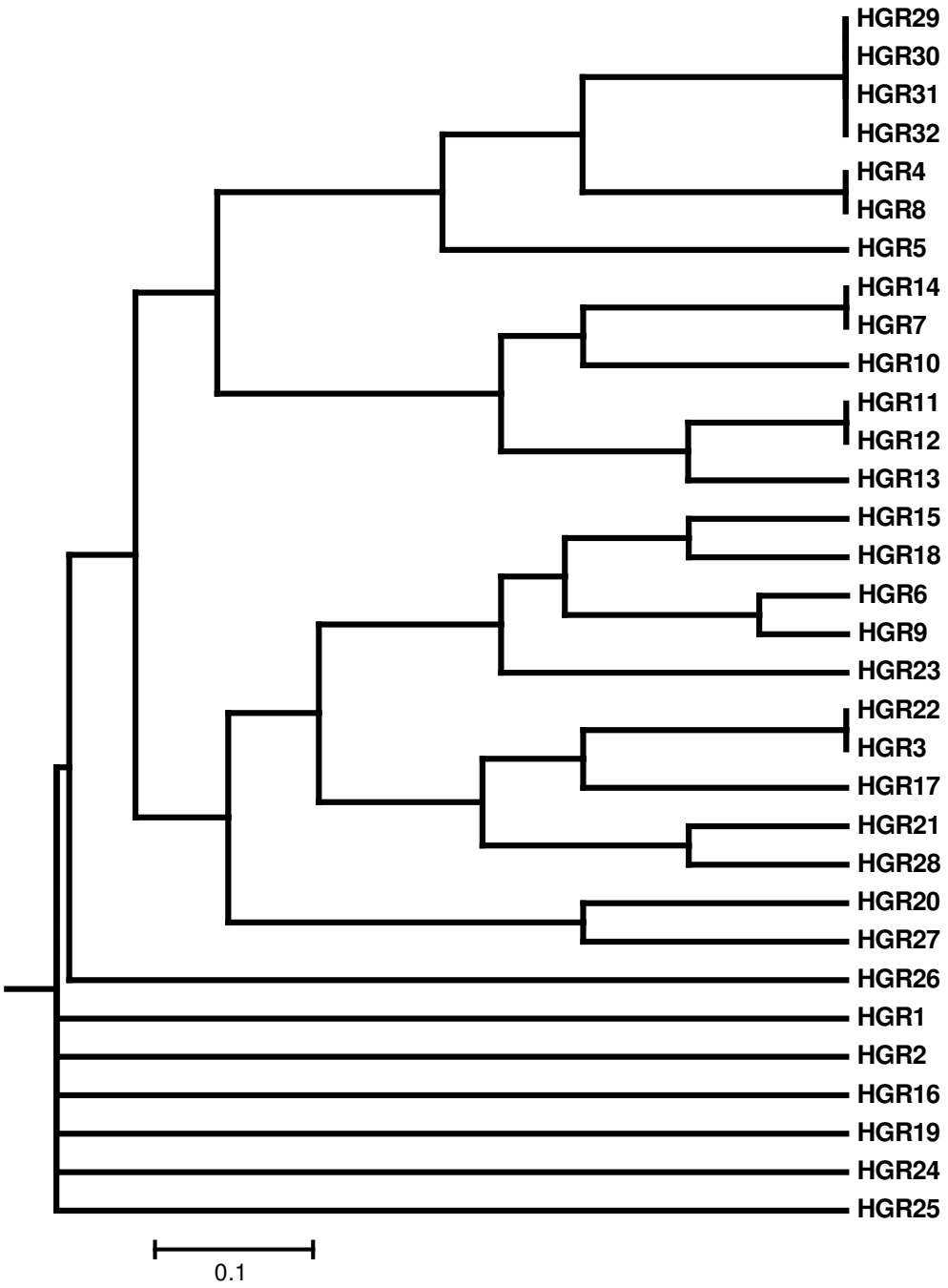

Fig. 2. RPO5 primer PCR- RAPD dendrogram showing genetic relationships among the *Rhizobium* isolates used in the present study constructed by using UPGMA method, Free Tree Package V.0.9.1.50 and dendrograms were viewed using Tree Explorer Tool V.2.12

The method will also be very useful in clarifying taxonomic status of rhizobia that nodulate less extensively studied leguminous plants and in detecting potential new taxa [36,37,38]. A considerable level of genetic diversity was determined among 42% of peanut rhizobia using PCR-RFLP of 16S-23S rDNA [39] and it was also applied in the identification of rhizobia [40]. Rhizobia nodulating *Leucaena leucocephala*, *Mimosa affinis* and *Sesbania herbaceae* analysed through PCR-RFLP of 16S rRNA genes and observed that isolates from a single legume

species were dispersed under various species of the same genus or different genera [41].

The RAPD profiles with the primer RPO4 and RPO5 and restriction analysis of 16S rRNA gene with the enzyme *Taq1*, *HpaII* and *AluI* showed high polymorphism. RAPD and RFLP analysis of these 32 horse gram rhizobia showed that these isolates fall into four major clusters. The first clusters of both dendrograms from RAPD and RFLP contained the majority of the rhizobial isolates. Among the 32 isolates of horse gram the isolates HGR-4, 6, 13 and 25 fall into separate clusters with the primer RPO5. Restriction digestion with the enzyme *Taq*1 also shows that the isolates HGR-4 and 13 showed close similarity and the isolate HGR-6 is grouped into separate cluster whereas the isolate HGR-25 grouped separately.

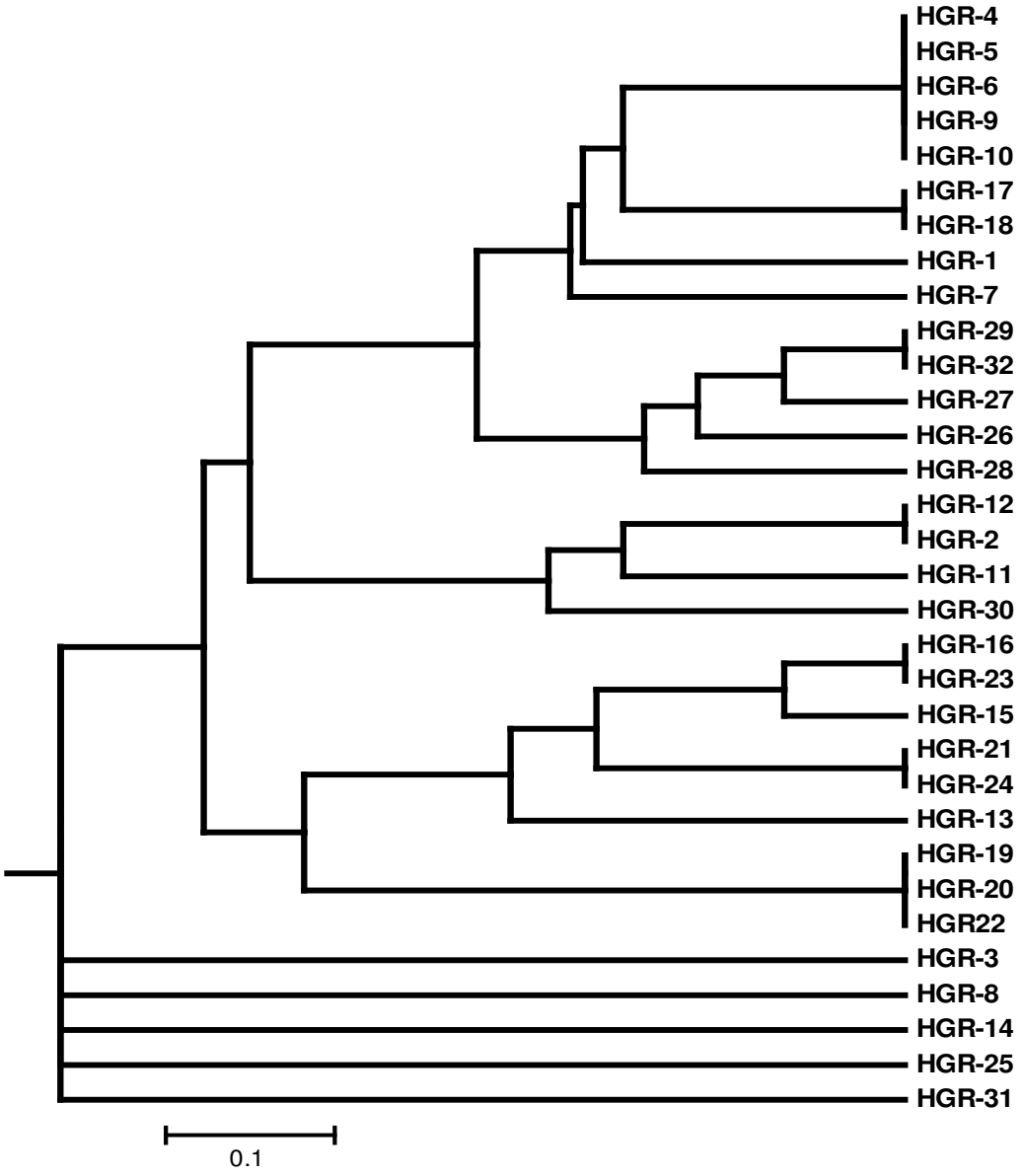

Fig. 3. *Taq*I PCR-RFLP analysis dendrogram showing genetic relationships among the isolates of *Rhizobium* in the present study constructed by UPGMA method method, Free Tree Package V.0.9.1.50 and dendrograms were viewed using Tree Explorer Tool V.2.12

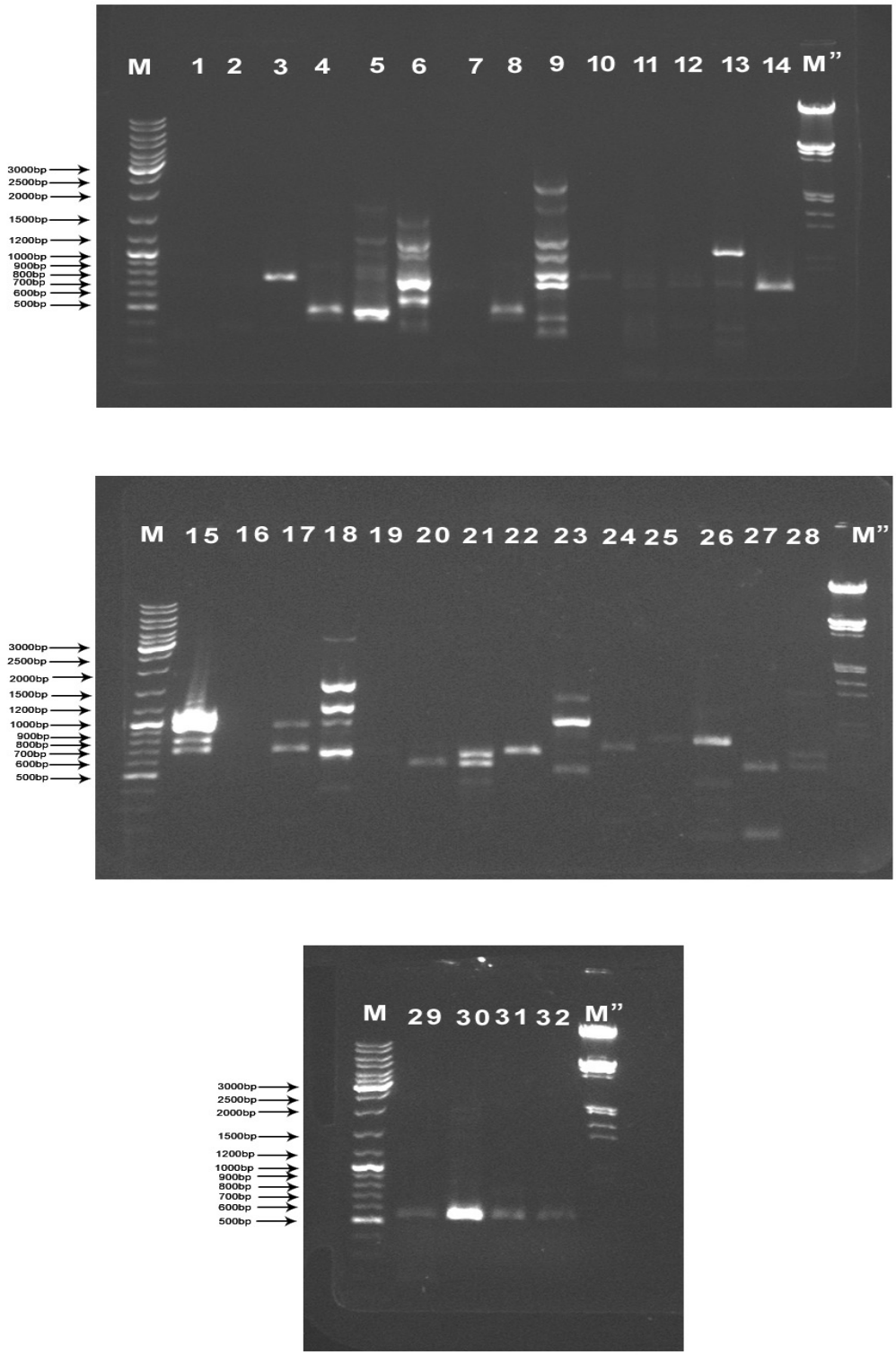

Fig. 4. RAPD banding pattern of 32 Horse gram rhizobia using RP05 primer

M: Marker lane - Gene Ruler DNA Ladder Mix (Fermentas, USA) M” Marker 2: Fermentas λ EcoRI HindIII double digest

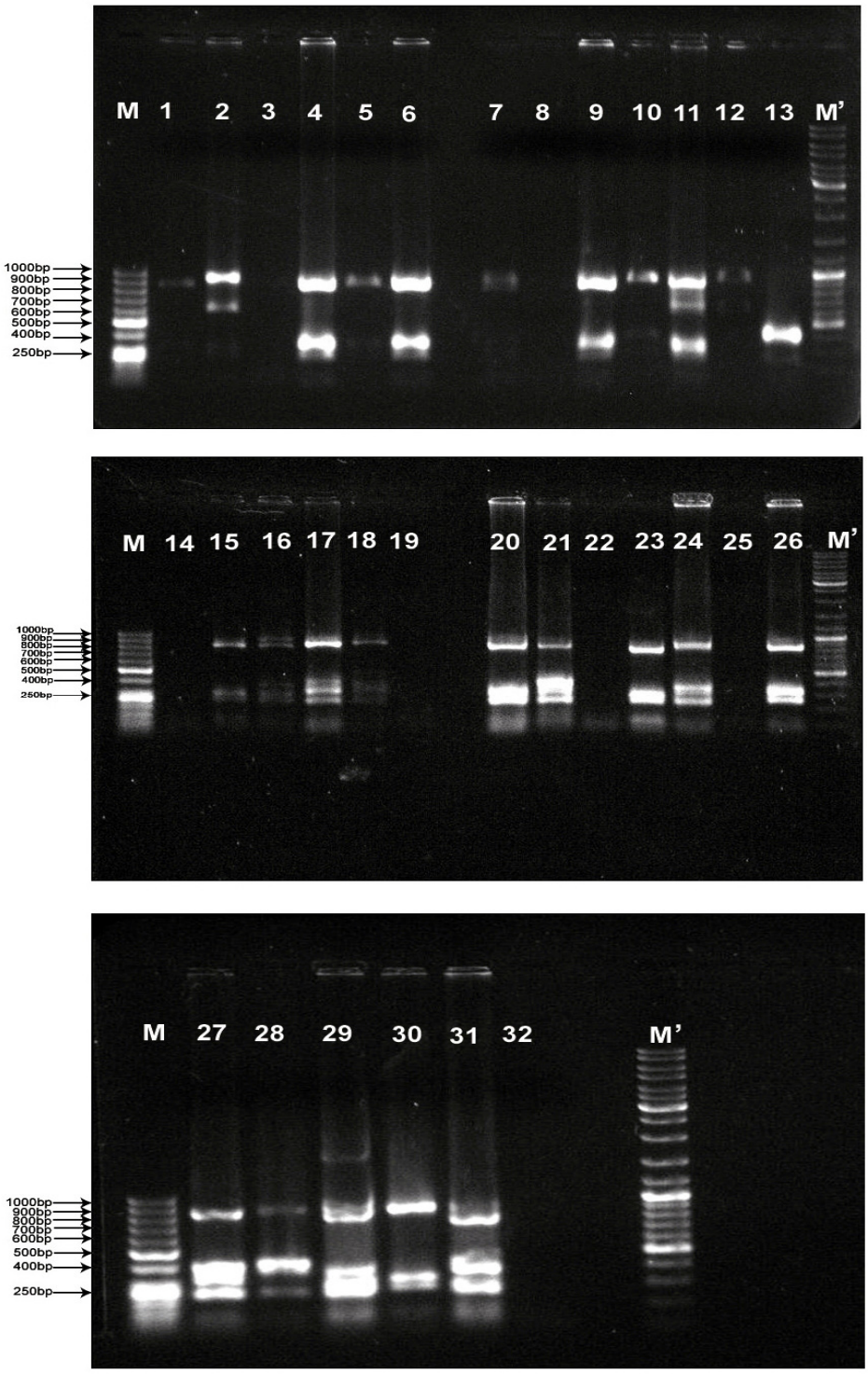

Fig. 5. RFLP banding pattern of 32 Horse gram rhizobia using the enzyme *Taq*1

M: Marker Lane - Gene Ruler^{TM} 50 bp DNA Ladder (Fermentas, USA)M': Marker 2 : Gene Ruler DNA Ladder Mix (Fermentas, USA)

This result is correlated with the study of previous workers [18]. They reported for the first time that a legume was nodulated by a member of the genus *Caulobacter* (HGR-25) and the rhizobia nodulating horse gram were phylogenetically distinct. Among the selected four representative isolates for 16S rRNA sequence, the isolates HGR-4, 6 and 13 showed more than 99% homology between them and they were grouped with *Rhizobium* reference strains where as the isolate HGR-25 showed 87.1, 87.4 and 87.2% homology with the isolates HGR-4, 6 and 13 respectively and were grouped with reference strains for *Caulobacter*. It revealed that the rhizobia nodulating the horse gram showed diversity and they may belong to different rhizobial groups and also nodulated by different genera.

From this, it is clear that RAPD and RFLP may play an important role if applied, to know the genetic diversity of rhizobia. High genetic diversity was observed among the horse gram rhizobial population and very few of the bacteria were considered to be identical. It clearly shows that the horse gram rhizobia are phylogenetically distinct.

5. CONCLUSION

The present study gives a picture of genetic variability among the 32 different isolates of Rhizobia collected from various places in and around Andhra Pradesh and Telangana, India. RAPD and PCR-RFLP were efficiently utilized to differentiate the four clusters of phylogenetic relationships among the isolates. The information could be efficiently used for future studies on Rhizobial isolates in these areas as well as in world scenario.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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