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Full Length Research Paper

Genotypic characterization of indigenous *Sinorhizobium meliloti* and *Rhizobium sullae* by rep-PCR, RAPD and ARDRA analyses

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The rhizobia, *Sinorhizobium meliloti* and *Rhizobium sullae,* which fix nitrogen in root nodules of alfalfa (*Medicago sativa* L.) and sulla (*Hedysarum* sp.) forage legumes, respectively, were isolated from root nodules and soils from Morocco. We used three PCR-based techniques namely, rep-PCR, RAPD and ARDRA techniques for genotypic characterization of 10 isolates of *S. meliloti* and *R. sullae*, in order to identify rapid and reliable techniques for applications in population genetic analysis of these species. The analysis revealed characteristic banding patterns for *S. meliloti* and *R. sullae* isolates by all the three techniques, even though the isolates are from a narrow geographic region in Morocco. Furthermore, the results showed that the rep-PCR with REP and ERIC primers was more efficient than RAPD and ARDRA technique for genotyping *S. meliloti* isolates; and rep-PCR with REP primers and the ARDRA technique with restriction enzyme *Hin*fI, were more efficient than the other rep-PCR and RAPD-PCR techniques for genotyping *R. sullae* isolates.

Key words: *Sinorhizobium meliloti*, *Rhizobium sullae*, rep-PCR, ARDRA, RAPD, genetic diversity.

INTRODUCTION

Among the soil bacteria, rhizobia are of particular interest in agriculture due to the formation of root nodules on leguminous plants where atmospheric nitrogen is being fixed. *Sinorhizobium meliloti* and *Rhizobium sullae* are rhizobia which fix nitrogen in root nodules of alfalfa (*Medicago sativa* L.) and sulla (*Hedysarum* spp.) forage legumes, respectively. These two forage legume species are important part of Mediterranean agriculture, especially in the North Africa region. Due to the reduced need for application of nitrogenous fertilizers, the rhizobia have a great agriculture value and play an important role in improving soil fertility in farming systems. Inoculation of these legumes with efficient strains of the rhizobia has significant economical and ecological benefits. However, the presence of indigenous soil strains, those are usually highly competitive and well adapted to certain environment, can reduce the inoculation benefits even with highly efficient strains. Therefore, there is a need to estimate biological diversity exists in the native rhizobia populations, in order to understand the population dynamics of these rhizobia in the natural populations and to perform selection of suitable efficient nitrogen fixing strains for inoculation to the crops.

Several authors have used molecular techniques for genotypic characterization and diversity analysis of bacteria including rhizobia. These investigations initially concentrated on estimating diversity at conserved regions of genomes like 16S rRNA genes using technique called amplified rDNA restriction analysis (ARDRA) (Laguerre et al., 1994; Taghavi et al., 1996; Pandey et al., 2004). Subsequently genomic fingerprinting by PCR amplification with random primers, termed RAPDs (after Random Amplified Polymorphic DNA) Williams et al. (1990) has gained popularity as a useful technique for comparative

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Table 1. Sampling of *S. meliloti* and *R. sullae* isolates.

Isolate	Date of collection (Year/Month/Date)	Sampling site in Morocco	Host cultivar[a]	Type of sample collected
S. meliloti				
1	2005/03/10	Demnate	Demnate population	Nodules
2	2005/03/10	Demnate	Demnate population	Nodules
3	2005/03/10	Demnate	Demnate population	Nodules
4	2005/03/10	Demnate	Demnate population	Soil
5	2005/03/03	Ziz	Ziz population	Soil
6	2005/03/16	Erfoud	Erfoud population	Soil
7	2005/03/16	Erfoud	Erfoud population	Soil
8	2005/03/16	Erfoud	Erfoud population	Soil
9	2005/03/16	Erfoud	Erfoud population	Soil
10	2005/03/16	Erfoud	Erfoud population	Soil
R. sullae				
1	2007/04/07	Tanger	Grimaldi	Nodules
2	2007/04/07	Tanger	Ecotype 1	Nodules
3	2007/04/07	Tanger	Ecotype 2	Nodules
4	2007/04/07	Tanger	Ecotype 3	Nodules
5	2007/04/07	Tanger	Ecotype 4	Nodules
6	2007/04/07	Tanger	Ecotype 5	Nodules
7	2007/04/07	Tanger	Ecotype 6	Nodules
8	2007/04/07	Tanger	Ecotype 7	Nodules
9	2007/04/07	Tanger	Ecotype 8	Nodules
10	2007/04/07	Tanger	Ecotype 9	Nodules

[a]Host in case of *S. meliloti* is alfalfa and in case *R. sullae* is sulla.

genome analysis (Harrison et al., 1992; Neimann et al., 1997; Hungria et al., 2000). More recently, PCR amplification with primers specific to the repetitive genetic elements REP (for Repetitive Extragenic Pallindromic), ERIC (Enterobacterial Repetitive Intergenic Consensus) and BOX (composed of the box A, B and C subunits) Martin et al. (1992), collectively known as rep-PCR, has been used for genomic fingerprinting of Gram-negative bacteria (Louws et al., 1994). These repetitive elements, located in the intergenic regions of many bacterial genomes, are considered to be highly conserved (Martin et al., 1992) and as such are useful for elucidating relationships within and between bacterial species including rhizobia (de Bruijn, 1992; Vinuesa et al., 1998; Chen et al., 2000).

The estimation of diversity within natural population of *S. meliloti* and *R. sullae* requires fast and accurate technique that can effectively differentiate strains. Our objective in this study is to compare the efficiencies of RAPD, rep-PCR and Amplified rDNA restriction analysis (ARDRA) techniques to detect polymorphism within *S. meliloti* and *R. sullae* species collected from natural populations and to identify suitable PCR-based techniques for studying population genetics and diversity analysis of these rhizobia.

MATERIALS AND METHODS

Isolate sampling

All isolates were obtained from different sites located in the most important alfalfa and sulla production areas in Morocco. The rhizobia isolates used in this study were isolated either from the collected nodules in field or from the root nodules of young plants of alfalfa grown on soil samples (isolated by a trapping method; only in case of *S. meliloti*). The origin and designation of rhizobia isolates used in this study are presented in Table 1.

DNA extraction

Bacterial DNA of 10 isolates of *S. meliloti* and of *R. sullae* was extracted by simple boiling method modified after Sambrook et al. (1989). Bacteria were grown in TY agar Petri dishes at 28°C for 2 days. A loop full of cells was suspended in 25 µl of sterile distillated water and followed an addition of 25 µl of freshly prepared lysis buffer containing 0.1 N NaOH and 0.5% SDS. The mixture was boiled in a water bath for 15 min. Two hundred µl of TE (10 mM Tris-HCl pH 8.0, 0.1 mM EDTA) was added to the mixture which was then subjected to centrifugation for 15 min at 13,000 rpm. The supernatant formed by the aqueous phase that contains clear and suspended DNA was transferred to new sterile tubes. The extracted DNA was further used for the following PCR assays and each assay was repeated at least three times. Only consistent results were recorded and used for data analysis.

Rep-PCR

Polymerase chain reaction (PCR) targeting repetitive DNA sequences (rep-PCR) such as repetitive extragenic palindromic sequences (REP) Versalovic et al. (1991), enterobacterial repetitive intergenic consensus sequences (ERIC) Hulton et al. (1991) and BOX-PCR based on primers targeting the highly conserved repetitive DNA sequences of the Box A subunit of the BOX element of *Streptococcus pneumoniae* (Versalovic et al., 1991) were performed according to the methodology of de Bruijn (1992). The amplification was carried out in tubes containing 25 µl of final reaction volume. The reaction mixture contained 2.5 µl of DMSO (100%), 14.65 µl of sterile distilled water, 2.5 µl of 10x PCR buffer (Roche, Germany), 1.25 µl of dNTPs (2 mM), 0.55 µl of REP primer (Versalovic et al., 1991) (Rep1 5' IIIICGICGICATCIGGC 3' and Rep2 5' ICGICTTATCIGGCCTAC 3'; 0.3 µg each), 0.44 µl of ERIC primer (Versalovic et al., 1991) (Eric1 5' ATGTAAGCTCCTGGGGATTCAC 3' and Eric2 5' AAGTAAGTGACTGGGGTGAGCG 3'; 0.3 µg each), 0.44 µl of BOX primer (5' CTACGGCAAGGCGACGCTGACG 3') and 0.4 µl (2 U) of *Taq* DNA polymerase. After the addition of 2 µl (50 ng) of DNA, the reaction mix was placed on a thermocycler (Master cycler, Eppendorf, Germany) and subjected to PCR cycles: 95°C for 7 min, followed by 35 cycles of 94°C for 1 min, 53°C for 1 min and 65°C for 8 min, and followed by the final elongation at 65°C for 8 min. PCR amplified fragments were electrophoresed in an agarose gel (1.5%) and were visualized using ethidium bromide staining.

RAPD analysis

Four RAPD primers namely OPY-04 (5' AAGGCTCGAC 3'), OPY-07 (5'GACCGTCTGT 3'), OPW-05 (5' CTGCTTCGAG 3') and OPW-18 (5' GGCGCAACTG 3') were used. RAPD reactions were carried out in 20 µl volumes containing 2 µl of 10x PCR buffer (Roche, Germany), 1.25 µl of 2 mM dNTPs, 3 µl of RAPD primer (10 pmole) and 0.25 µl (1.25 U) of *Taq* DNA polymerase. After the addition of 2 µl (50 ng) of DNA, the reaction mix was placed on a thermocycler (Master cycler, Eppendorf, Germany) and subjected to PCR cycles: 94°C for 3 min, followed by 30 cycles of 94°C for 1 min, 36°C for 1 min and 72°C for 2 min, and followed by the final elongation at 72°C for 6 min. PCR amplified fragments were electrophoresed in an agarose gel (1.5%) and were visualized under UV light after staining with ethidium bromide, and the photographed.

Amplified rDNA restriction analysis (ARDRA)

Two primers FGPS1490-72 (5'-TGCGGCTGGATCCCCTCCTT-3') (Normand et al., 1996), and FGPL132-38 (5'-CCGGGTTTCCCCATTCGG-3') (Ponsonnet and Nesme, 1994) were used for PCR amplification. A 25 µl reaction mixture included 2 µl (50 ng) of bacterial DNA as template, 0.5 µM each of primer, 1.5 U of Taq polymerase, 2.5 µl of dNTPs (2 mM) and 2.5 µl of 10x PCR buffer (Roche, Germany). The reaction conditions were: initial denaturation of 3 min at 95°C followed by 35 cycles 95°C for 30 s, 55°C for 1 min and 72°C for 1 min, followed by a final extension of 3 min at 72°C. Amplified DNA was visualized in 8% native polyacrylamide gels. An aliquot of 6 µl of the PCR reaction product was used for restriction digestion with *Hae*III and *Hin*fI at 37°C for 4 h in a final volume of 20 µl. The digested products were analysed electrophoretically on 8% native polyacrylamide gel and were visualized under UV light after staining with ethidium bromide.

Data analysis

The comparison of amplified DNA profiles for each of the primers was performed on the basis of the presence (1) or absence (0) of fragments. Genetic parameters namely number of polymorphic loci, number of genotypes detected, major allele frequency, polymorphic information content (PIC) and pair-wise shared allele genetic distance were estimated using the software program PowerMarker version 3.25 (Liu and Muse, 2005). The pair-wise genetic distance matrix was used for constructing the dendrogram using the UPGMA (Unweighted Pair Group Method with Arithmetic Averages) algorithm on Power Marker.

RESULTS

Rep-PCR analysis

The total genomic DNA from the isolates was used as a template for rep-PCR amplification with REP, ERIC and BOX primers to produce DNA fingerprints. The reproducible and specific polymorphic banding pattern was obtained with REP and ERIC primers for *S. meliloti*, whereas BOX primer did not reveal any polymorphism. The DNAs of *S. meliloti* isolates amplified with specific REP-PCR primers produced an average of 12 bands and distinguished all the 10 isolates into 10 genotypes with genetic distance ranged from 0.0833- 0.8333 (Table 2). However, in *R. sullae* DNA amplification with REP primers produced only 5 polymorphic bands and distinguished the 10 isolates into 7 genotypes with genetic distance ranged from 0.00 - 0.80. In *S. meliloti*, ERIC primers amplified 15 polymorphic bands (Figure 1) resulted in grouping of 10 isolates into 8 genotypes, whereas it did not reveal any polymorphism in *R. sullae.* Although, BOX primers failed to detect polymorphisms in *S. meliloti*, it amplified 2 polymorphic bands in *R. sullae*, which enabled to group the isolates into 3 genotypes. Similarly, polymorphic information content (PIC) values were also differed among primers tested and between the two species, and were highest for REP primers in both *S. melioti* and *R. sullae* (Table 2). The major allele frequency was also lowest in rep-PCR with REP primers in both the species.

RAPD analysis

Among the 4 RAPD primers tested, only two primers (OPY-04 and OPY-07) revealed polymorphisms within *S. meliloti* and *R. sullae* (Table 2). The primer OPY-04 detected 6 polymorphic bands in *S. meliloti* and distinguished isolates into 6 genotypes with PIC value of 0.2675 and genetic distance ranged from 0.0000- 0.8333. Though, this primer amplified only one polymorphic band in *R. sullae*, PIC values were high when compared to *S. meliloti*. The primer OPY-07 amplified only one polymorphic band in the both species and discriminated the isolates into 2 genotypes, with PIC value comparatively higher in *R. sullae.*

ARDRA analysis

When the 16S rDNA regions were amplified and electro-

Table 2. Number of polymorphic bands, number of genotypes and polymorphic information content detected by different DNA marker techniques in *S. meliloti* and *R. sullae.*

DNA Marker techniques	Number of polymorphic bands	Number of genotypes	Polymorphic information content (PIC)[a]	Genetic distance (Range)[b]	Major allele frequency
S. meliloti					
REP	12	10	0.2911	0.0833- 0.8333	0.7417
ERIC	15	8	0.2622	0.0000- 0.6250	0.7625
BOX	0	0	0	-	-
RAPD (OPY-04)	6	6	0.2675	0.0000- 0.8333	0.7500
RAPD (OPY-07)	1	2	0.1638	0.0000- 1.0000	0.9000
Amplified rDNA	1	2	0.2741	0,0000- 1.0000	0.7750
ARDRA with *Hae*III	4	4	0.2741	0.0000- 1.0000	0.7750
ARDRA with *Hin*fI	2	2	0.1638	0.0000-1.0000	0.9000
R. sullae					
REP	5	7	0.2586	0.0000- 0.8000	0.7800
ERIC	0	0	0	-	-
BOX	2	3	0.2478	0.0000- 1.0000	0.8000
RAPD (OPY-04)	1	2	0.5274	0.0000- 1.0000	0.5000
RAPD (OPY-07)	1	2	0.2688	0.0000- 1.0000	0.8000
Amplified rDNA	5	4	0.2816	0.0000- 0.8000	0.7400
ARDRA with *Hae*III	2	2	0.1638	0.0000- 0.8000	0.9000
ARDRA with *Hin*fI	3	6	0.3362	0.0000- 1.0000	0.6333

[a]Polymorphism information content (PIC) was estimated according to Botstein et al. (1980).
[b]Shared allele distance was estimated according to Chakraborty and Jin (1993).

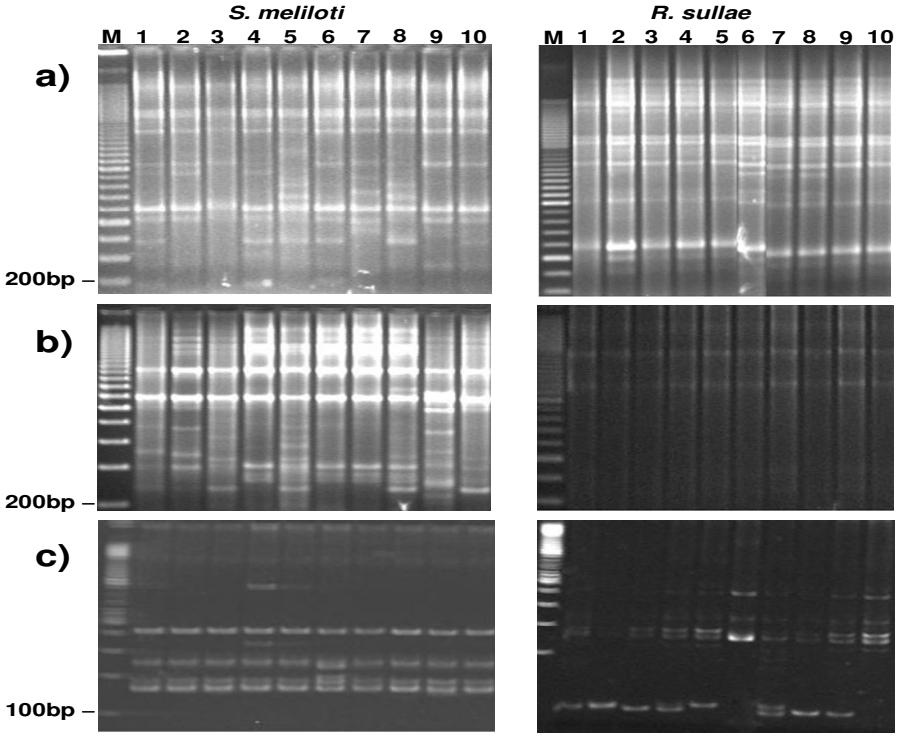

Figure 1. PCR-based DNA marker analysis using (a) REP primers, (b) ERIC primers and (c) ARDRA with *Hin*fI enzyme in *S. meliloti* and *R. sullae*. Lane M contains 100 bp marker.

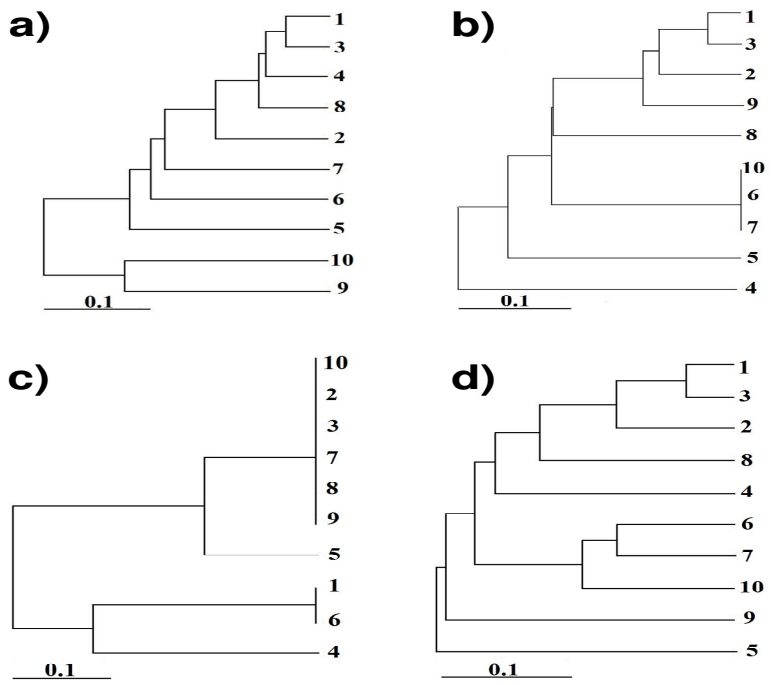

Figure 2. Dendrograms constructed using the UPGMA algorithm based on the shared allele genetic distance estimated by various PCR-based DNA marker techniques in *S. meliloti*. The dendrograms were constructed based on polymorphic data obtained from (a) PCR with REP primers, (b) PCR with ERIC primers, (c) ARDRA with *Hae*III enzyme, and (d) PCR with REP and ERIC primers, separately.

phoressed on native polyacrylamide gels, one and 5 polymorphic DNA bands were detected in *S. meliloti* and *R. sullae*, respectively (Table 2). However, PIC values were almost similar in both the species. Further digestion of the PCR products with *Hae*III and *Hin*fI restriction enzymes, resulted in increased number of polymorphic bands in both the species. Number of genotypes detected and PIC value were higher when the amplified rDNA region of *S. meliloti* digested with *Hae*III compared to *Hin*fI, whereas both parameters were higher in *R. sullae* when digested with *Hin*fI.

DISCUSSION

The characterization of the indigenous rhizobia population plays an important role in better understanding of soil biodiversity and biological nitrogen fixation. In this study, we used three PCR-based techniques namely, rep-PCR, RAPD and ARDRA techniques to characterize genetic diversity of *S. meliloti* and *R. sullae*, in order to identify rapid and reliable techniques for their applications in genetic characterization and population genetic analysis of these species. Characteristic banding patterns were obtained for *S. meliloti* and *R. sullae* isolates by using all the three techniques, even though the isolates are from a narrow geographic region in Morocco.

The chromosomal location of rep sequences in *Rhizobium* species indicates genetic relationships among different strains and represents a relatively simple but efficient tool for genotyping (Labes et al., 1996). In our study, the number of polymorphic bands detected and PIC varied depending upon the species and the rep-PCR primers used for PCR amplification. Rep-PCR primers such as REP and ERIC detected higher number of polymorphic bands in case of *S. meliloti*, compared to RAPDs and ARDRA, and classified the isolates into 10 and 8 genotypes, respectively. By combining both REP and ERIC-PCR fragment profiles, further increases in detection of genomic differences and clear branching tree were observed in *S. meliloti* cluster analysis (Figure 2). In addition, the DNA fingerprints observed by REP and ERIC primers were also highly reproducible compared to RAPDs. Therefore, REP and ERIC primer based PCR detection techniques are very useful for fast, large scale and reliable genetic characterization of *S. meliloti* populations.

In *R. sullae*, REP- and BOX-fingerprinting techniques alone could differentiate only 7 and 3 genotypes, respectively, or together differentiated 9 genotypes out of 10 isolates tested. Even though RAPDs were suggested to amplify characteristic fragments (Sikora et al., 1997; 2002), in this study they were not efficient in fingerprinting *R. sullae*, as they amplify less number of polymorphic

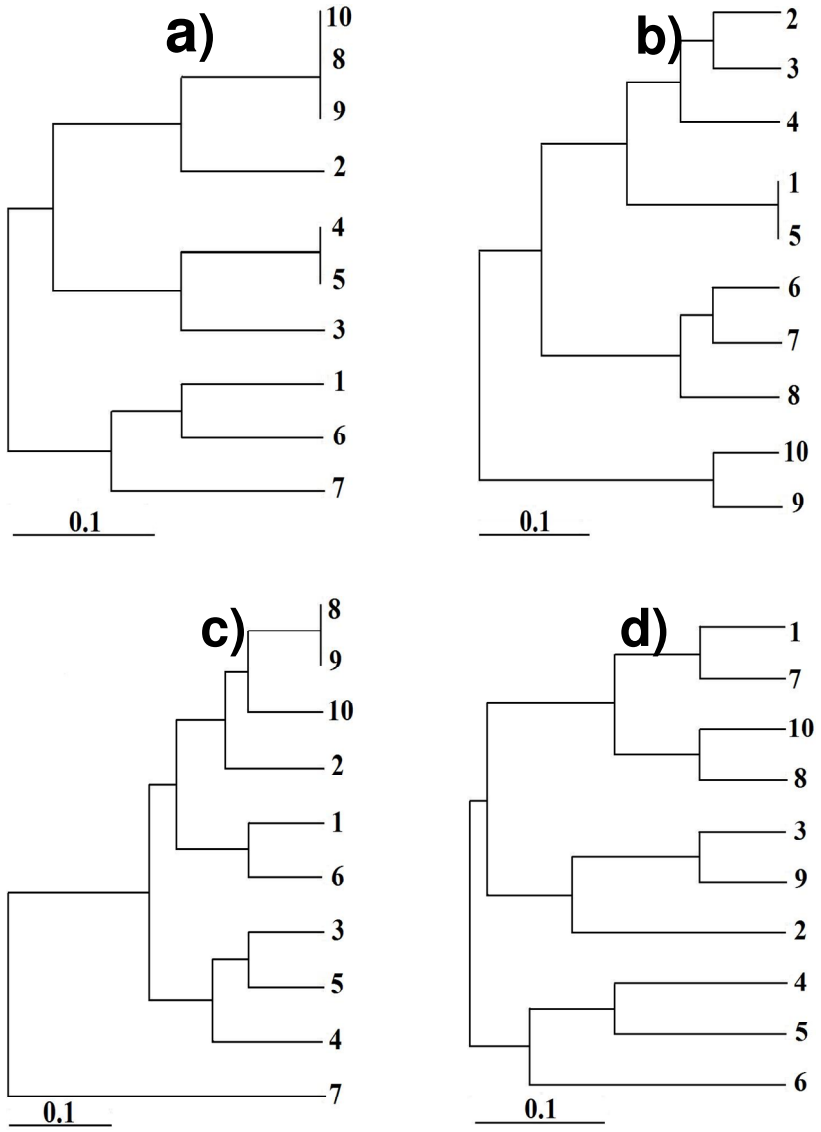

Figure 3. Dendrograms constructed using the UPGMA algorithm based on the shared allele genetic distance estimated by various PCR-based DNA marker techniques in *R. sullae*. The dendrograms were constructed based on polymorphic data obtained from (a) PCR with REP primers, (b) ARDRA with *Hin*fI enzyme, (c) PCR with REP and BOX primers separately, and (d) PCR with REP primers and ARDRA with *Hin*fI enzyme separately.

bands, which reduces the ability to distinguish genotypes. Furthermore, RAPDs are not readily reproducible. On the other hand, ARDRA with *Hin*fI enhanced genotypic differentiation. When we combined the data of REP-PCR and ARDRA with *Hin*fI, further improvement in the resolution and differentiation of genotypes were observed, classifying the 10 isolates into 10 genotypes (Figure 3). Therefore, the combined analysis of PCR with REP primer and ARDRA with *Hin*fI efficiently resolved genotypic diversity in *R. sullae*.

Perusal of literatures suggests that ability to detect polymorphisms varies with different DNA fingerprinting techniques. For example, in *Bradyrhizobium japonicum,* REP and ERIC primer based fingerprinting were efficient in the detection of genetic differences (Judd et al., 1993; Hungria et al., 2000; Sikora et al., 2002), supporting our observations in *S. meliloti*. The sequence of the 16S rRNA gene had been widely used as a phylogenetic marker to study genetic relationships between different species of bacteria (phylogeny). The analysis of this gene can therefore be considered a standard method for the identification of bacteria at the family, genus and species

levels, including rhizobia (Weisburg et al., 1991; Jeng et al., 2001; Sikora and Redzepović, 2003). However, in this study they were efficient in detecting polymorphism in *R. sullae* than in *S. meliloti*.

In conclusion, the result of this study showed that the rep-PCR with REP and ERIC primers was more efficient than RAPD and ARDRA technique for genotyping *S. meliloti* isolates. For *R. sullae*, rep-PCR with REP primers and the ARDRA technique with restriction enzyme *Hin*fI, were more efficient than the other rep-PCR and RAPD-PCR techniques.

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