APPLIED AND ENVIRONMENTAL MICROBIOLOGY, June 1993, p. 1702–1708 Vol. 59, No. 6
0099-2240/93/061702-07$02.00/0

Use of Repetitive Sequences and the Polymerase Chain Reaction Technique To Classify Genetically Related *Bradyrhizobium japonicum* Serocluster 123 Strains†

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Received 13 January 1993/Accepted 25 March 1993

We have determined that repetitive (repetitive extragenic palindromic [REP] and enterobacterial repetitive intergenic consensus [ERIC]) sequences used in conjunction with the polymerase chain reaction technique (REP and ERIC PCR) provide an effective means of differentiating between and classifying genetically related *Bradyrhizobium japonicum* serocluster 123 strains. Analysis of REP and ERIC PCR-generated dendrograms indicated that this technique can effectively differentiate between closely related strains which were indistinguishable by using other classification methods. To maximize the genomic differences detected by REP and ERIC PCR fingerprint patterns, the REP and the ERIC data sets were combined for statistical analyses. REP-plus-ERIC PCR fingerprints were also found to provide a method to differentiate between highly diverse strains of *Bradyrhizobium* spp., but they did not provide an effective means for classifying these strains because of the relatively low number of REP and ERIC consensus sequences found in some of the bradyrhizobia. Our results also suggest that there is a relationship between nodulation phenotypes and the distribution of REP and ERIC consensus sequences within the genomes of B. japonicum serogroup 123 and 127 strains. Results obtained by restriction fragment length polymorphism hybridization analyses were correlated with the phylogenetic classification of *B. japonicum* serocluster 123 strains obtained by using REP and ERIC PCR.

Members of the genus *Bradyrhizobium* are slow-growing, gram-negative, heterotrophic bacteria which have the ability to form root nodules on several leguminous plants. On the basis of DNA-DNA homology studies, physiological characteristics, and nodulation host-range phenotypes, these organisms have been divided into two groups, *Bradyrhizobium japonicum* and *Bradyrhizobium* spp. (4, 5, 9). The *B. japonicum* strains, which nodulate soybeans, have been further subdivided into two groups, I and IA, on the basis of DNA-DNA hybridization studies (4).

The fluorescent-antibody, immunoagglutination reaction, and enzyme-linked immunosorbent assay techniques have also been used to assign the bradyrhizobia to distinct serological groups on the basis of immunological reactions of their surface antigens (1, 13, 25). Several studies have indicated that the surface antigens of the bradyrhizobia are more strain specific than the flagellar or internal antigens (3, 25). Relative to the fast-growing rhizobia, *B. japonicum* strains show little or no serological cross-reactivity and are serologically distinct (13).

In the upper midwest United States, members of *B. japonicum* serocluster 123 are the dominant indigenous competitors for the nodulation of soybeans. The term serocluster 123 was suggested by Schmidt et al. (17) to describe the serological relatedness of strains in serogroups 123, 127, and 129. Genetic and biochemical analyses of serocluster 123 strains have indicated that there is a relationship between serogroup, nodulation phenotype, and physical organization of the genome (8, 14, 15). Several plant introduction (PI) genotypes, including PI 371607, that selectively inhibit nodulation by serocluster 123 strains have been found (8, 15). The current serological divisions, however, fail to adequately reflect the genetic and phenotypic diversity among member isolates (12, 14, 15, 17).

Several molecular methods have also been used to characterize closely related bradyrhizobial strains. They include DNA fingerprint and restriction fragment length polymorphism (RFLP) analyses (11, 15, 17, 21) and field inversion gel electrophoresis (19). Although these techniques are useful for identifying and categorizing genetically dissimilar organisms, they have not proven especially useful for characterizing near-isogenic bradyrhizobia.

Recently, it had been shown that DNA primers corresponding to repetitive extragenic palindromic (REP) (22) and enterobacterial repetitive intergenic consensus (ERIC) (6) sequences, coupled with the polymerase chain reaction (PCR) technique (REP and ERIC PCR), can be used to fingerprint the genomes of a variety of gram-negative soil bacteria (2, 23). The REP and ERIC sequences contain highly conserved central inverted repeats, do not show significant homology to each other, and are normally found in intergenic transcribed, but not translated, regions. Their relative positions in the genome of a particular bacterial isolate appear to be conserved in closely related strains and are distinct in diverse species (genera) (10, 23, 24). The PCR amplification of genomic regions between REP or ERIC copies (24) produces a collection of distinct fragments (or typical fingerprint pattern) on an agarose gel (2, 23).

For several *Rhizobium meliloti* strains, de Bruijn (2) has shown that there is a correlation between data derived from REP and ERIC PCR analyses and phylogenetic data generated by multilocus enzyme electrophoresis. This author's results indicated that the REP and ERIC PCR technique is a useful additional tool for the classification of bacteria. The

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† Manuscript 20,445 in the University of Minnesota Agricultural Experiment Station series.

TABLE 1. Bacteria and PCR primers used and their sources

Strain or primer	Source[a]
B. japonicum serogroup 123	
USDA 123, USDA 162, USDA 432	1
IA5, IA23, IA35, IN34, IN79, MN9, OH6, OH9, OH13	2
B. japonicum serogroup 127	
USDA 127, USDA 171, USDA 185, USDA 424, USDA 425, USDA 430	1
IA44, IA67, IN56, IN64, PA3	2
WI3058	3
Becker 4N-18	4
B. japonicum serogroup 129	
USDA 129, USDA 426, USDA 427, USDA 429, USDA 434, USDA 435	1
AK1-3a, IA3H2-6, IN78, MS6-4a	2
B. japonicum (other serogroup strains)	
USDA 4, USDA 6, USDA 38, USDA 62, USDA 110, USDA 122, USDA 124, USDA 228, USDA 433	1
Bradyrhizobium spp. USDA 31, USDA 46, USDA 61, USDA 76, USDA 94, USDA 130	1
PCR primers[b]	
REP1R-I (3′-CGGICTACIGCIGCIIII-5′)	
REP2-I (5′-ICGICTTATCIGGCCTAC-3′)	
ERIC1R (3′-CACTTAGGGGTCCTCGAATGTA-5′)	
ERIC2 (5′-AAGTAAGTGACTGGGGTGAGCG-3′)	

[a] Sources: 1, U.S. Department of Agriculture, Beltsville, Md.; 2, M. Sadowsky, University of Minnesota, St. Paul; 3, B. Kamicker, University of Wisconsin, Madison; and 4, E. L. Schmidt, University of Minnesota, St. Paul.
[b] Abbreviations: G, guanine; A, adenine; C, cytosine; T, thymine; and I, inosine.

REP and ERIC PCR technique is particularly valuable when one considers its simplicity; purified genomic DNA is not required as a PCR template. Interpretable REP and ERIC PCR patterns can be generated from DNA obtained from a small number of intact cells, suspended in PCR buffer, and used directly as a template for PCR. This technique works equally well with bacteria obtained from liquid cultures and colonies from plates or from legume nodules and minimizes sample preparation time (2a).

In this article we describe the use of REP and ERIC PCR fingerprinting and RFLP analysis using a hyperreiterated DNA probe to classify serologically related and genetically similar *B. japonicum* serocluster 123 strains. In addition, we present the comparison of 15 serologically distinct *B. japonicum* and *Bradyrhizobia* spp. strains by using REP and ERIC PCR fingerprint analysis.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Strains used in this study and their sources are listed in Table 1. *B. japonicum* strains were maintained on AG medium at 30°C (15).

DNA manipulations. Total bacterial genomic DNA was isolated as described previously (15). For hybridizations, genomic DNA was digested to completion with *Hin*dIII, separated by horizontal electrophoresis on 0.8% agarose gels, and transferred to nylon filters as described previously (15, 16). The hyperreiterated DNA probe, pFR2508, was eluted from agarose gels, ^{32}P labelled by the random primer method, and hybridized to filters as described previously (12, 15).

Oligonucleotide primers and PCR conditions. Oligonucleotide PCR primers and their sequences are listed in Table 1. Primers were synthesized with a DNA synthesizer (model 380B; Applied Biosystems, Foster City, Calif.) by the Macromolecular Structure, Sequence, and Synthesis Facility at Michigan State University. The PCRs were performed as described previously (2), except that 35 cycles instead of 30 were used. PCR products were separated by horizontal electrophoresis on 1.5% agarose gels, stained with ethidium bromide, and photographed.

Data analysis. REP and ERIC PCR fingerprints were converted to a two-dimensional binary matrix (1, presence of a PCR product; 0, absence of a PCR product) and analyzed by using the biostatistical analysis program NTSYS-pc (Applied Biostatistics, Inc.). Except where noted, all comparisons were done on fingerprints generated on the same gel. For data scoring, photographs of gels were enlarged (to 8 by 10 in. [ca. 20 by 25 cm]) and the PCR products were analyzed by using visual pair-wise comparisons of adjacent lanes. All bands were scored regardless of their intensity. The enlargements allowed a resolution of about 0.5 mm (bands in different lanes that varied in height by more than 0.5 mm were not considered the same). Lanes (strains) were compared by reading horizontally across the gel, from the bottom to the top; if a band was present, it was assigned a value of 1 at that location, whereas if it was absent, it was assigned a value of 0. The presence of single, dominant bands in each lane allowed alignment of PCR products across noncontiguous lanes. Dendrograms were generated by using the SIMQUAL (Similarity for Qualitative [20] data) and SAHN (Sequential Agglomerative Hierarchical and Nested [18]) clustering subroutines of NTSYS-pc.

RESULTS AND DISCUSSION

REP and ERIC PCR classification of *B. japonicum* serogroup 123 and serogroup 127 strains. Total genomic DNAs from 11 *B. japonicum* serogroup 123 strains, 12 serogroup 127 strains, 1 serogroup 129 strain, and 2 serogroup-undefined serocluster 123 strains were used as a template for PCR by using either the REP (REP1R-I and REP2-I) or ERIC (ERIC1R and ERIC2) primers. Products from the PCR were separated on 1.5% agarose gels and stained with ethidium bromide. Both sets of primers yielded multiple DNA products ranging in size from approximately 0.1 to 4.5 kb. Results of the PCR with the ERIC primers are shown in Fig. 1. Similar results were obtained with the REP primers (data not shown).

To examine the reproducibility of the system, the REP and ERIC PCRs were repeated at least twice. PCR products generated from the second set of reactions yielded fragments with a mobility nearly identical to that of fragments seen the first time, indicating that the patterns generated are reproducible.

To classify the serocluster 123 strains, we converted the results from the stained agarose gels into a two-dimensional binary matrix. All strains which had a PCR product of a particular size were scored positive at that location in the matrix, while strains not having a product of that particular size were scored negative. Except where indicated, all comparisons between strains were made within the same gel. Data was analyzed by using the commercial biostatistics program NTSYS-pc. The SIMQUAL and SAHN cluster analyses were used to generate dendrograms representing the products from REP and ERIC PCRs.

When the dendrograms derived from the REP and the ERIC PCR data were compared, we found that they did not yield identical results (data not shown). Although there was not a difference in the major grouping of strains, there was some variation in the grouping of closely related strains

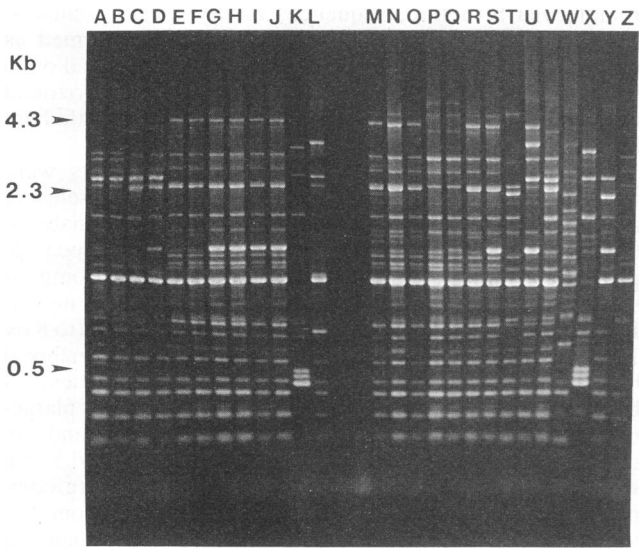

FIG. 1. ERIC PCR fingerprint patterns of genomic DNA from *B. japonicum* serogroup 123 and 127 strains. The PCR product patterns were generated by using the ERIC primers listed in Table 1. Lanes: A, IA23; B, USDA 432; C, OH6; D, OH9; E, USDA 162; F, IA35; G, IN79; H, OH13; I, USDA 123; J, IN34; K, USDA 228; L, MN9; M, WI3058; N, PA3; O, USDA 127; P, IA67; Q, IN56; R, IA44; S, USDA 123; T, Becker 4N-18; U, USDA 171; V, IN64; W, USDA 129 (serogroup 129); X, USDA 185; Y, USDA 430; Z, USDA 424.

between the REP and the ERIC PCR-generated dendrograms. This result suggested that one set of primers detected differences in the genome of a strain which was not evidenced when the other set of primers was used. To maximize the specificity of the REP- and the ERIC-derived PCR patterns, we generated a combined matrix composed of REP and ERIC PCR product patterns and termed these REP-plus-ERIC data sets.

The dendrogram generated from the combined matrices show that REP and ERIC PCR fingerprinting can differentiate between serologically related *B. japonicum* serocluster 123 strains (Fig. 2). The serogroup 123 strains showed a high degree of relative genetic similarity and were placed in one major subgroup. Members of the 123 subgroup had relative genetic similarities of approximately 0.90 or greater as determined by using REP-plus-ERIC data sets. One exception to this group, however, was strain MN9, a serogroup 123 strain which appears genetically distinct from other isolates. This high degree of relative genetic similarity was not unexpected since the majority of serogroup 123 strains exhibit the same nodulation host-range phenotypes (8, 15) and have been shown to be related by biochemical, physiological, serological, and hybridization analyses (8, 14, 15, 17).

With the serogroup 127 strains, however, there was sufficient variation in the REP and ERIC PCR patterns to prevent classification of these strains into one major subgroup. The results in Fig. 2 indicate that several serogroup 127 strains (IA44, IA67, IN56, and IN64) are genetically more similar to serogroup 123 strains than to other members of serogroup 127. This result is in agreement with our previous studies which have shown that there is substantial genetic diversity among serogroup 127 strains, as determined by DNA hybridization analysis (14). Our results, coupled with the previously established serological interrelatedness of serogroup 123 and 127 strains (17), suggest that serogroup 123 and 127 may have evolved from a common ancestral progenitor.

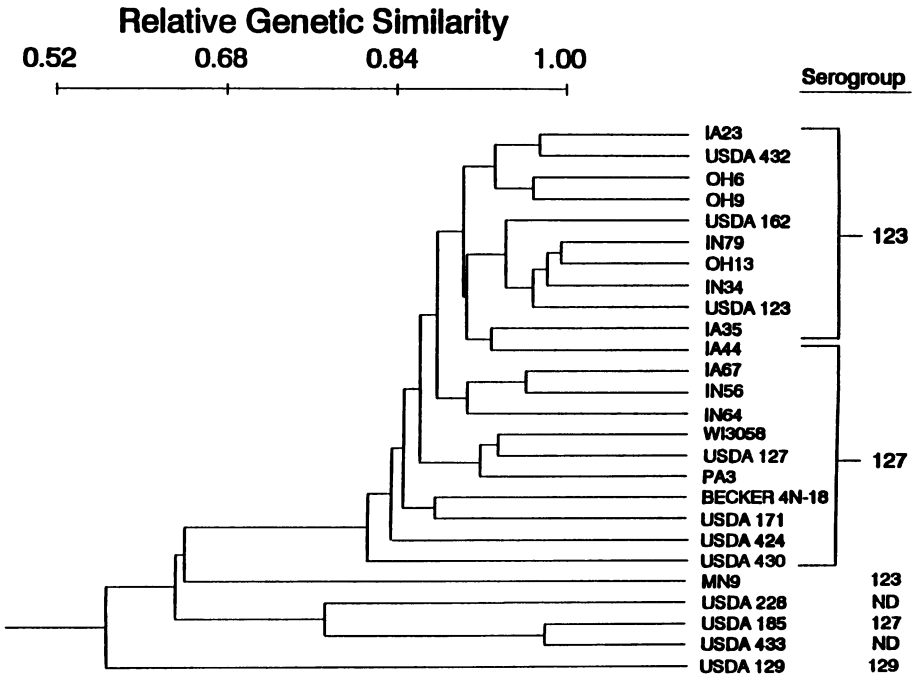

FIG. 2. Dendrogram of *B. japonicum* serogroup 123 and 127 strains derived from PCR fingerprints generated from REP and ERIC PCR primers. The dendrogram was generated from combined REP and ERIC data sets. The PCR fingerprint patterns for USDA 433 were determined from two separate sets of agarose gels.

The results in Fig. 2 also show a grouping of three strains (USDA 185, USDA 228, and USDA 433) which have PCR product patterns which do not resemble those of any of the other strains. Two of these strains, USDA 185 and USDA 228, were isolated from soils obtained in the People's Republic of China, while USDA 433 was isolated from a Mississippi field soil. Although all three strains are in serocluster 123, only one strain, USDA 185, can be serologically classified by using serogroup-specific antisera (serogroup 127) (8).

The results shown in Fig. 2 also indicate that there is a near-perfect correlation between nodulation phenotype on PI 371607 (8, 13, 15) and relative genetic similarity of these strains as determined by using REP-plus-ERIC data sets. For example, the serogroup 127 strains USDA 171, USDA 424, USDA 430, and Becker N4-18 were grouped together by REP and ERIC PCR, and all were found to be unrestricted for nodulation on PI 371607. A similar result was also seen for strains USDA 185, USDA 228, and USDA 433. In addition, all serogroup 123 strains, except for strain USDA 432, were found to be restricted for nodulation on PI 371607 and were grouped together by REP and ERIC PCR. The similarity grouped serogroup 127 strains, USDA 127, WI3058, and PA3, were also found to be restricted for nodulation by PI 371607. These results suggest that there is a relationship between nodulation phenotype on soybean genotypes and the distribution of REP and ERIC consensus sequences within the genomes of serogroup 123 and 127 strains.

REP and ERIC PCR classification of *B. japonicum* serogroup 129 strains. Genomic DNA isolated from nine *B. japonicum* serogroup 129 strains was used as a template for PCR with REP and ERIC primers as described above. The results of the REP and ERIC PCR method with REP primers are presented in Fig. 3. Individual serogroup 129 dendrograms generated from REP and ERIC PCR products showed minor differences in the grouping of very closely related strains. Consequently, the REP and the ERIC PCR patterns were combined and a REP and ERIC PCR-derived dendrogram was generated for these nine strains (Fig. 4). The combined dendrogram clearly shows that the strains examined can be divided into two distinct groups. The predominant group consists of eight closely related strains which show very little similarity to the other serogroup 129 strain tested. Previous studies have indicated that strains from the predominant group have similar host-range phenotypes for nodulation of the PI genotypes (8, 15). Although there is a high degree of relative genetic similarity between these strains, REP and ERIC PCR patterns can be used to differentiate several of these strains (Fig. 3). In previous studies, we were unable to show differences among these closely related serogroup 129 strains (15).

REP and ERIC PCR classification of *B. japonicum* serogroup and *Bradyrhizobium* spp. strains. To determine the utility of the REP and ERIC PCR for differentiating between other *Bradyrhizobium* strains, we used PCRs with REP and ERIC primers on genomic DNAs from strains in DNA homology groups I, IA, and II (4). The results in Fig. 5 show that PCR with REP primers yields a variety of different PCR product patterns for each of the strains examined. Interestingly, many of the strains examined had relatively simple PCR product patterns (Fig. 5, lanes A, C, F, and G), indicating that REP and ERIC sequences are not well conserved among the *Bradyrhizobium* strains tested. Similar results were also seen with the PCR fingerprints generated with ERIC primers (data not shown). The majority of the

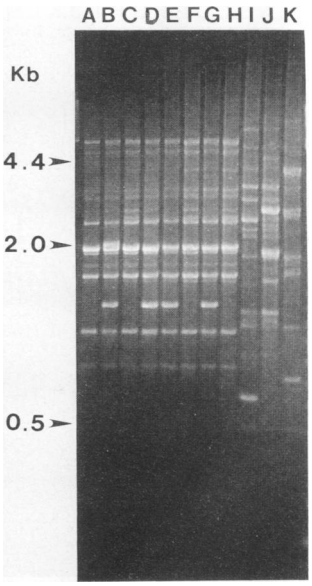

FIG. 3. REP PCR fingerprint patterns of genomic DNA from *B. japonicum* serogroup 129 strains. The pattern of PCR products was generated by using the REP primers listed in Table 1. Lanes: A, USDA 422; B, USDA 434; C, USDA 435; D, USDA 427; E, USDA 426; F, USDA 129; G, USDA 429; H, USDA 436; I, USDA 425 (serogroup 127); J, IN78; K, USDA 433.

REP and ERIC PCR patterns were distinct, however, and could be used to differentiate between strains.

To determine whether the REP and ERIC PCR technique can be used to classify serologically distinct *Bradyrhizobium* strains, we combined REP and ERIC PCR fingerprint data and generated a REP and ERIC PCR-derived dendrogram for the 15 strains examined (Fig. 6). REP and ERIC PCR effectively grouped together strains which have been shown to be serologically interrelated (strains USDA 123 and 127 and strains USDA 122 and 129). However, REP and ERIC PCR grouped the remaining strains in a manner which did not correlate with previous classifications based on serological analyses (25), DNA-DNA homology studies (4), or fatty acid composition (9). This was most likely due to the limited number of REP and ERIC sequences in the genomes of some

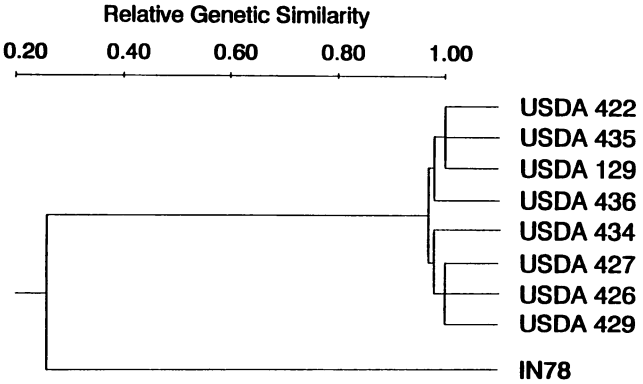

FIG. 4. Dendrogram of *B. japonicum* serogroup 129 strains derived from REP and ERIC PCR fingerprints generated by using both REP and ERIC primers. The dendrogram was generated from combined REP and ERIC data sets.

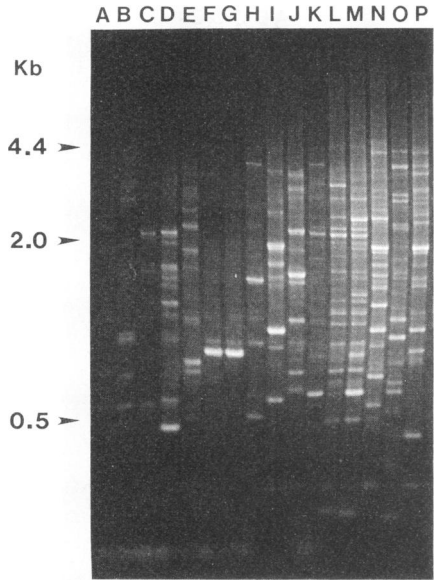

FIG. 5. REP PCR fingerprint patterns of genomic DNA from *B. japonicum* and *Bradyrhizobium* spp. strains. Lanes: A, USDA 6; B, USDA 94; C, USDA 124; D, USDA 61; E, USDA 38; F, USDA 46; G, USDA 110; H, USDA 31; I, USDA 122; J, USDA 4; K, USDA 76; L, USDA 123; M, USDA 127; N, USDA 129; O, USDA 130; P, USDA 62.

of the strains tested. This result suggests that although REP and ERIC PCR provides a way to differentiate between these strains, it does not provide an effective means for classifying genetically divergent *Bradyrhizobium* strains.

Use of repeated-sequence RFLP analysis to classify serocluster 123 strains. To determine whether other repeated sequences could be used to classify *B. japonicum* serogroup

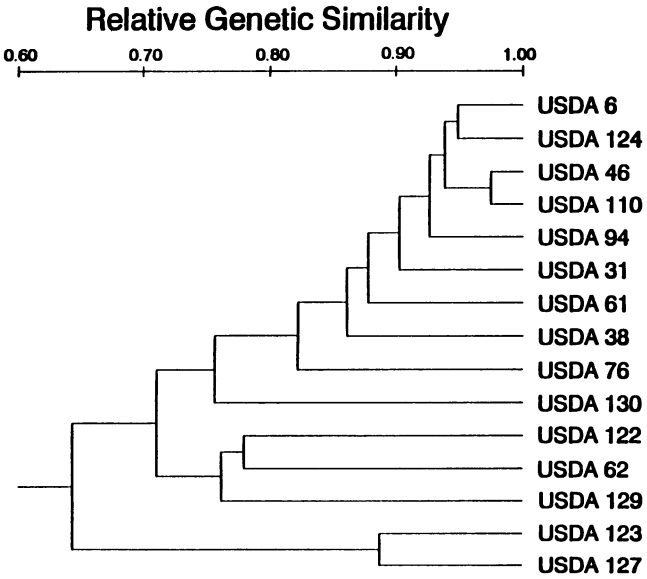

FIG. 6. Dendrogram of *B. japonicum* and *Bradyrhizobium* spp. strains derived from PCR fingerprints generated from REP and ERIC PCR primers. The dendrogram was generated from combined REP and ERIC data sets.

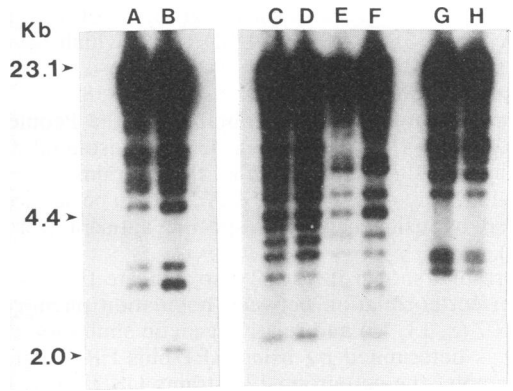

FIG. 7. Southern hybridization of pFR2508 probe to *Hin*dIII-digested genomic DNA from *B. japonicum* serogroup 123 and 127 strains. Lanes A to D contain DNAs from serogroup 123 strains, and lanes E to H contain DNAs from serogroup 127 strains. Lanes: A, IA23; B, USDA 432; C, OH13; D, USDA 123; E, USDA 127; F, PA3; G, IA67; H, IN56.

123 and 127 strains, we hybridized a ^{32}P-labelled DNA probe, pFR2508, to *Hin*dIII-digested genomic DNA from four pairs of *B. japonicum* serogroup 123 and 127 strains having a relative genetic similarity of greater than 0.95, as determined by using REP-plus-ERIC data sets. Probe pFR2508, which contains a 0.4-kb internal *Sal*I fragment from the *B. japonicum* serocluster 123-specific hyperreiterated DNA sequence HRS1, has been shown to be useful in discriminating among serologically related serocluster 123 strains (12). The results in Fig. 7 show that the hybridization patterns within each pair of strains appear nearly identical, indicating that REP and ERIC PCR effectively grouped these closely related strains. It should be noted, however, that all of the hybridizing bands cannot be read from the autoradiogram because of inherent problems associated with this type of analysis.

In an analogous manner, DNA from strains showing a relative similarity of less than 0.96 were digested with *Hin*dIII and hybridized to pFR2508. Although there was some relationship between the hybridization patterns, distinct differences were seen (data not shown). As the relative genetic similarity of strains determined by REP and ERIC PCR decreased, so did the similarity of the HRS1 hybridization patterns. This result indicates that REP and ERIC PCR fingerprints and repeated-sequence RFLP analysis provide an efficient means of classifying closely related *B. japonicum* serogroup 123 and serogroup 127 strains.

Similar results were seen with RFLP analysis of *Hin*dIII-digested genomic DNA from *B. japonicum* serocluster 129 strains by using pFR2508. The results shown in Fig. 8 indicate that closely related serogroup 129 strains have similar but distinct hybridization patterns. Strain IN78, however, had an RFLP profile distinctly different from those of the rest of the strains, indicating a high degree of genetic divergence. It is interesting to note, however, that the hybridization patterns of the strains which had a relative similarity of about 1.0, as determined by using REP-plus-ERIC data sets (USDA 422, USDA 435, and USDA 129; and USDA 426, USDA 427, and USDA 429), were found not to be identical when HRS1 RFLP analysis was used. This suggests that REP and ERIC PCR effectively grouped these strains but may have overestimated their relative genetic similarity.

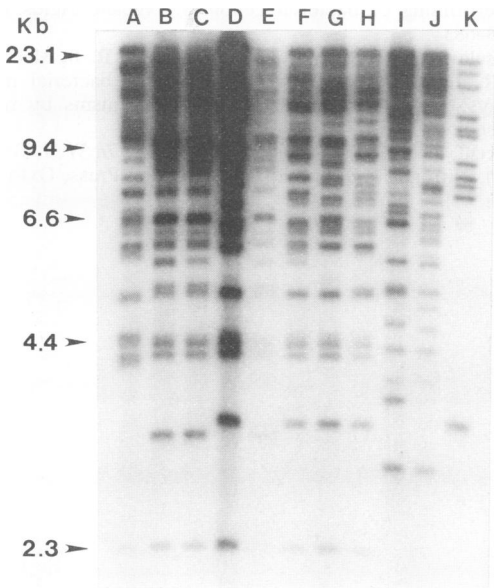

FIG. 8. Southern hybridization of pFR2508 probe to *Hin*dIII-digested genomic DNA from *B. japonicum* serogroup 129 strains. Lanes: A, USDA 436; B, USDA 435; C, USDA 129; D, USDA 427; E, USDA 422; F, USDA 434; G, USDA 426; H, USDA 429; I, USDA 426 (serogroup 127); J, IN78; K, USDA 122 (serogroup 122).

Another interesting result from the pFR2508 hybridization was seen with strains USDA 185, USDA 228, USDA 433, and MN9. These strains hybridized weakly to pFR2508 and did not show the hyperreiterated DNA hybridization pattern which is characteristic of many serocluster 123 strains (data not shown). For these strains, genetic relatedness could only be determined by using REP and ERIC PCR analysis.

In summary, our results indicate that the REP and ERIC PCR technique provides an effective means of differentiating between and classifying genetically related serocluster 123 strains. The REP and ERIC PCR technique has proven to be especially useful in distinguishing between genetically and phenotypically near-identical *B. japonicum* strains in serogroups 123, 127, and 129. Consequently, REP and ERIC PCR is a very useful tool to examine intraserogroup competition for nodulation among serocluster 123 isolates. To maximize genomic differences detected by REP and ERIC PCR product patterns, the REP and the ERIC PCR data sets were combined and analyzed in a single dendrogram. We are exploring whether REP and ERIC primers can be interchanged or combined with outwardly directed primers from other *B. japonicum* repeated sequences (such as HRS1 [12] or RSα [7]) to more accurately fingerprint the *B. japonicum* genome. By using this approach, we hope to detect genetic differences not found by using REP and ERIC primers alone and more accurately determine the phylogenetic relatedness of *B. japonicum* serocluster 123 strains.

ACKNOWLEDGMENTS

This study was supported in part by U.S. Department of Agriculture competitive research grant 91-37305-6831, by a grant from the University of Minnesota Agriculture Experiment Station (to M.J.S.), and by U.S. Department of Energy grant DE-FG02-90ER20021 (to F.J.d.B.).

We thank Kurt Steipnitz (MSU-DOE Plant Research Laboratory) for help in preparing photographs.

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