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Genetic Diversity of an Italian *Rhizobium meliloti* Population from Different *Medicago sativa* Varieties

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We investigated the genetic diversity of 96 *Rhizobium meliloti* strains isolated from nodules of four *Medicago sativa* varieties from distinct geographic areas and planted in two different northern Italian soils. The 96 isolates, which were phenotypically indistinguishable, were analyzed for DNA polymorphism with the following three methods: (i) a randomly amplified polymorphic DNA (RAPD) method, (ii) a restriction fragment length polymorphism (RFLP) analysis of the 16S-23S ribosomal operon spacer region, and (iii) an RFLP analysis of a 25-kb region of the p*Sym* plasmid containing *nod* genes. Although the bacteria which were studied constituted a unique genetic population, a considerable level of genetic diversity was found. The new analysis of molecular variance (AMOVA) method was used to estimate the variance among the RAPD patterns. The results indicated that there was significant genetic diversity among strains nodulating different varieties. The AMOVA method was confirmed to be a useful tool for investigating the genetic variation in an intraspecific population. Moreover, the data obtained with the two RFLP methods were consistent with the RAPD results. The genetic diversity of the population was found to reside on the whole bacterial genome, as suggested by the RAPD analysis results, and seemed to be distributed on both the chromosome and plasmid p*Sym*.

The symbiotic nitrogen-fixing soil bacteria belonging to the genera *Rhizobium* and *Bradyrhizobium* are members of genetically diverse and physiologically heterogeneous groups of bacteria that interact with the roots of legumes to form nodules where atmospheric nitrogen is fixed (2). The population genetics of these genera has been studied, and almost all of the data reported previously indicate that there is a high level of genetic diversity in these bacteria. This genetic diversity has been associated with a strong linkage disequilibrium (8, 9, 11, 12, 31, 47) or no linkage disequilibrium (3, 8, 12, 27, 40, 41), which provides evidence of genetic exchange in the population considered (46). In general, the results of studies of the genetic structures of natural soil populations support the hypothesis that recombination may play a role in generating new genotypes, since these populations are very different in the genetic structure from bacterial populations recovered from outbreaks of infectious diseases, which are typically clonal (10, 46). The difference is easily appreciated when the genotypic structures of the populations are represented on dendrograms, which have very deep branches for the clonal, nonrecombining populations and starlike structures for the freely recombining populations. In symbiotic rhizobacteria like *Rhizobium* species, the evolution of the population structure may be influenced by environmental conditions like biological barriers to gene exchange or geographical isolation and also by the type of soil or the genotype of the host plant (8). It is known that legumes can be preferentially nodulated by one of two rhizobial strains even if the preferred strain is outnumbered by the other strain (28, 44). However, little is known about the relationships between the genetic polymorphism within a *Rhizobium* population and the preferences for nodulating particular genotypes of compatible host plants (28). One additional element that can also play a critical role in the evolution of *Rhizobium* populations is the occurrence of large plasmids that can have an evolutionary history different from the evolutionary history of the strains that they live in (39, 47). Moreover, plasmids can undergo rearrangements and recombination at high frequencies, which increases genetic variability (8, 35, 47).

Finally, analysis of the genetic structure of *Rhizobium* populations also has practical importance since the results can be used to assess the fate of released strains (36), engineered or not, and their impact on resident microbial communities (43).

Most studies of the population genetics of *Rhizobium* and *Bradyrhizobium* species have been carried out by using serological or multilocus enzyme electrophoresis techniques (3, 8, 9, 11, 12, 28, 40–42). More recently, techniques based on DNA analysis have been used with or instead of multilocus enzyme electrophoresis (4, 7, 9, 12, 24–27, 45, 47). In particular, the techniques that are frequently used are (i) analysis of repetitive sequences, including repetitive extragenic palindromic (REP) and enterobacterial repetitive intergeneric consensus (ERIC) sequences, amplified with PCR (7, 23, 41), (ii) analysis of insertion elements (4, 25), and (iii) restriction fragment length polymorphism (RFLP) analysis of ribosomal genes (11, 12), of p*Sym* sequences (8, 9, 47), and of other chromosomal genes (8, 47). A recent modification of the ribosomal DNA fingerprinting technique, which has not been applied to *Rhizobium* strains yet, consists of analyzing the 16S-23S ribosomal operon spacer region, which is known to exhibit significant variations in length and sequence both at the genus level and at the species level (1, 18, 29). The development of randomly amplified polymorphic DNA (RAPD) markers provided a new tool for investigating genetic polymorphisms in many different organisms, including bacteria (5, 14, 21), and recently this method has been used for *Rhizobium* identification and *Bradyrhizobium* genetic analyses (24, 45). In this study we used the RAPD technique, together with an RFLP analysis of p*Sym* genes and restriction digestion of the ribosomal DNA (rDNA) intergenic region, to examine the genetic polymorphism of a population of *Rhizobium meliloti* strains recovered from nodules of four varieties of *Medicago sativa* from distinct geographic areas that

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TABLE 1. Sampling procedure[a]

Strains	Soil	Variety	Box	Sampling cut
A1 through A6	A	Edfo	1	1st
A7 through A12	A	Diablo Verde	2	1st
A13 through A18	A	Lodi	3	1st
A19 through A24	A	Edfo	4	4th
A25 through A30	A	Diablo Verde	5	4th
A31 through A36	A	Lodi	6	4th
A37 through A42	A	Edfo	7	1st winter
A43 through A48	A	Diablo Verde	8	1st winter
A49 through A54	A	Lodi	9	1st winter
A55 through A60	A	Edfo	10	2nd winter
A61 through A66	A	Diablo Verde	11	2nd winter
A67 through A72	A	Lodi	12	2nd winter
C1 through C12	C	L1	13	1st
C13 through C24	C	L1	14	3rd

[a] See text.

were planted in two different northern Italian soils, with the aim of determining some of the factors that play a role in shaping the genetic structure of the population. In order to fully exploit RAPD results for studies of the genetic structures of populations, it is necessary to utilize a method of data analysis that permits identification of variations within a population and also permits correlation of genetic variations with environmental effects. These requirements seem to be fulfilled by the new analysis of molecular variance (AMOVA) method (13), which was first developed for RFLP data and has also been successfully used for RAPD data obtained from an analysis of buffalo grass (20).

MATERIALS AND METHODS

Sampling procedure. This study represented the beginning of a breeding program conducted at the Istituto Sperimentale Colture Foraggere, Lodi, Italy, for improving the symbiotic ability of *M. sativa*. Soil A used in this study was a mollic Hapludalf, coarse loamy, mixed, mesic soil from the South Milan region (northern Po Valley, Lombardy, Italy) containing 11.7% clay, 32.4% silt, 55.9% sand, 1.38% organic matter, and 0.8‰ N; the pH was 6.8. Soil C was a calcixerollic-vertic Xerochrept, fine, mixed, mesic soil from the Modena region (southern Po Valley, Emilia, Italy) containing 37.6% clay, 53.7% silt, 8.7% sand, 4.61% organic matter, and 2.9‰ N; the pH was 7.3. The locations where the two soils were found were about 50 km apart and were separated by the Po River. It was estimated that the *Rhizobium* populations present in the two soils contained 10^4 cells per g of dry soil (as determined by the most-probable-number with plant infection technique). The *M. sativa* varieties used in soil A were varieties Diablo Verde (bred at W-L Research, Bakersfield, Calif.), Edfo (ecotype; provided by A. Rammah, Agriculture Research Center, Giza, Egypt), and Lodi (bred at the Istituto Sperimentale Colture Foraggere), and the *M. sativa* variety used in soil C was L1 (an experimental synthetic variety bred at the Istituto Sperimentale Colture Foraggere). Nodule samples were obtained from soil A during the first cut (27 May to 3 June) and the fourth cut (7 to 18 August) and during the winter (7 December and 13 February). Nodule samples were obtained from soil C during the first cut (11 June) and the third cut (29 July). Plants were grown at the Istituto Sperimentale Colture Foraggere in iron boxes (width, 12 cm; length, 53 cm; height, 90 cm) in a greenhouse. A total of 510 plants for each genotype and soil were transplanted in March at the early cotyledon stage in 17 boxes (30 plants per box); the growth system was characterized by an absence of N fertilization and a water supply that was not limiting. The bacterial strains examined in this study came from a single box for each sample (Table 1) and from two or three plants in each box. To obtain samples, plants were cut, the soil was removed from the roots, and the nodules were collected. The nodules were surface sterilized by immersing them in a 1‰ $HgCl_2$ solution for 3 min and then rinsed with sterile distilled water. The sterilized nodules were crushed individually in 70 μl of water and plated onto yeast extract-mannitol agar (6). After growth at 30°C, single pure colonies were isolated and stored at −80°C. For subsequent analyses single colonies were isolated from the frozen stock solutions. The samples used in this work are described in Table 1.

RAPD analysis. Amplification reactions were performed directly with single colonies as described previously (17) by using primers 1247 (5′-AAGAGC CCGT) and RF2 (5′-CGGCCCCTGT). Each 25-μl amplification reaction mixture contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 3 mM $MgCl_2$, 0.001% (wt/vol) gelatin, each deoxynucleoside triphosphate at a concentration of 200 μM, 500 ng of primer, 0.625 U of AmpliTaq DNA polymerase (Perkin-Elmer, Norwalk, Conn.), and 2 μl of a lysed cell suspension. After incubation for 60 s at 90°C and for 90 s at 95°C, the reaction mixtures were subjected to 45 cycles in a Perkin-Elmer model 9600 thermocycler by using the following temperature profile: 95°C for 30 s, 36°C for 1 min, and 72°C for 2 min. Then the samples were incubated at 72°C for 10 min. A 6-μl aliquot of each reaction mixture was analyzed by electrophoresis on a MetaPhor (FMC BioProducts, Rockland, Maine) agarose (2%, wt/vol) gel. The amplification patterns were analyzed with a scanner densitometer (model GDS2000; Ultra-Violet Products, Ltd., Cambridge, United Kingdom).

AMOVA of the amplification products. We used the vector of presence and absence of RAPD markers (1 indicated that a band was present on a gel, and 0 indicated that a band was not present) for each strain to compute the genetic distance for each pair of strains. The measurement used was the Euclidean metric measurement (E) of Excoffier et al. (13) as defined by Huff et al. (20), as follows: $E = \varepsilon^2_{xy} = n(1 - 2n_{xy}/2n)$, where $2n_{xy}$ is the number of markers shared by two individuals and n is the total number of polymorphic sites. We used the AMOVA procedure (13) to estimate the variance components of RAPD patterns, and to partition the variation among soils, among varieties within a soil, among single boxes within a soil, and among single boxes within a variety. All analyses were performed with the WINAMOVA program provided by L. Excoffier (University of Geneva).

RFLP analysis of the intergenic region between 16S and 23S rDNAs. The intergenic region between 16S and 23S rDNAs was amplified by PCR by using two primers, one derived from the 3′ end of the 16S rDNA (primer FGPS1490; 5′-TGCGGCTGGATCACCTCCTT) (30) and the other derived from the 5′ end of the 23S rDNA (primer FGPL132′; 5′-CCGGGTTTCCCCATTCGG) (32). PCR amplification was performed by using a Perkin-Elmer model 9600 thermocycler. Each reaction mixture contained 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100, 1 mM $MgCl_2$, each deoxynucleoside triphosphate at a concentration of 20 μM, each primer at a concentration of 50 pM, 2.5 U of *Taq* DNA polymerase (Promega, Madison, Wis.), 2 μl of lysed cell suspension (17), and enough sterile distilled water to bring the volume to 100 μl. The PCR temperature profile used was 95°C for 3 min and then 35 cycles consisting of 95°C for 1 min, 55°C for 1 min, and 72°C for 2 min, with a final extension step at 72°C for 2 min. A 25-μl aliquot of each PCR mixture was treated with 5 U of restriction enzyme (Boehringer, Mannheim, Germany) in a total volume of 30 μl at 37°C for 3 h. The reaction products were analyzed by electrophoresis on a MetaPhor (FMC BioProducts) agarose (3%, wt/vol) gel.

RFLP analysis of the *nod* region. Total DNAs of *R. meliloti* strains were purified as described by Giovannetti et al. (16). Restriction digestions with *Eco*RI (Boehringer) were carried out by treating 6 μg of DNA with 6 U of enzyme at 37°C for 4 h. Fragments were separated by electrophoresis on a 0.6% (wt/vol) agarose gel. The probe, which consisted of plasmid p*PP369* (33) (see Fig. 3) and

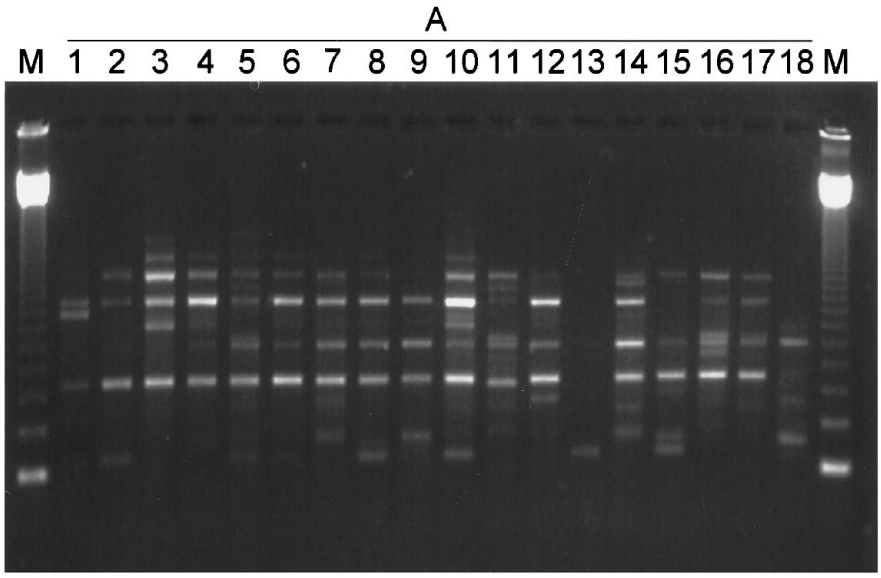

FIG. 1. Electrophoretic patterns generated after an RAPD analysis of 18 *R. meliloti* strains with primer 1247. Lanes M, 123-bp molecular weight marker ladder.

was prepared by using standard protocols (38), was labelled, and the hybridization signals were detected with a digoxigenin labelling and detection kit (Boehringer) by using the chemiluminescence method and the instructions of the supplier. Southern blotting was carried out on nylon membranes (Hybond N; Amersham, Amersham Place, Little Chalfont, Buckinghamshire, United Kingdom) as described by Fani et al. (15).

RESULTS

RAPD analysis. Lysed cell suspensions of 96 *R. meliloti* strains isolated from nodules of four *M. sativa* varieties (varieties Edfo, Diablo Verde, Lodi, and L1) obtained from distinct geographic areas and planted in two northern Italian soils (soils A and C) were amplified by the RAPD technique by using two 10-mer primers, primers 1247 and RF2, which had G+C contents of 60 and 80%, respectively. The products of the amplification reactions were analyzed by agarose gel electrophoresis. The reproducibility of the results were verified in independent experiments. An example of the amplification patterns obtained is shown in Fig. 1. Altogether, 27 and 28 bands of different sizes were obtained with primers 1247 and RF2, respectively, and the 55 RAPD markers identified ranged from 180 to 2,100 bp long. The amplification patterns obtained with primer 1247 exhibited a higher level of polymorphism than the patterns obtained with primer RF2.

Distance matrix. The patterns obtained were compared with each other, and a Euclidean distance matrix was constructed. For this analysis, we split the total population into subpopulations of bacteria. On the basis of the matrix (data not shown) the following three salient points concerning the data set were identified: (i) there was a considerable variation within the population, as each bacterial strain was a distinct genetic unit; (ii) despite the internal variation, there was considerable divergence between the two soils (strains from different soils were more divergent than strains from the same soil); and (iii) within-population variation was greater in soil C than in soil A. However, it was not possible to ascribe the genetic differences between soils A and C to the soils or the varieties, because only one variety was planted in soil C and this variety was different from the varieties planted in soil A.

AMOVA partition. From the Euclidean distance matrix we extracted AMOVA data which confirmed the high level of significance ($P < 0.001$) of the genetic differences (19.22%) between the soil A and soil C collections (Table 2, experiment f); as described above, it was not possible to correlate the genetic differences with soil or variety. Most of the total molecular variance was attributable to divergence among strains; the values for divergence among strains (data not shown) could be calculated by determining the complement to 100% for each variance value reported in Table 2. A within-soil analysis was performed only with soil A data, because only one variety was planted in soil C. Within soil A highly significant genetic differences (22.17%) (Table 2, experiment a) were found when the strains derived from each of 12 boxes were analyzed (Table

TABLE 2. AMOVA of 96 *R. meliloti* strains isolated from nodules by using 55 RAPD markers[a]

Expt	Variance component	Variance	% Total	P^b	ϕ Statistic
a	Among boxes[c]	1.179	22.17	<0.001	0.222
b	Among varieties[c]	0.062	1.17	0.05	0.012
c	Among single variety Edfo boxes	1.910	29.06	0.05	0.291
d	Among single variety Diablo Verde boxes	1.180	22.86	0.01	0.229
e	Among single variety Lodi boxes	0.950	20.14	0.03	0.201
f	Soil A vs soil C	1.362	19.22	<0.001	0.192

[a] The total data set contained strains from two soils (soils A and C) and three varieties (varieties Edfo, Diablo Verde, and Lodi) for soil A. An AMOVA was also performed for the 24 strains (6 strains in single boxes) within each variety.
[b] Probability of having a more extreme variance component and ϕ statistic (13).
[c] Only soil A boxes and varieties were considered.

1); in contrast, only 1.17% of the genetic diversity was attributable to differences among varieties (Table 2, experiment b). When the within-variety soil A data were analyzed (Table 2, experiments c through e), 29.06, 22.86, and 20.14% of the genetic differences were attributable to varieties Edfo, Diablo Verde, and Lodi, respectively. None of the other comparisons, in particular comparisons of data for variety L1 in soil C and comparisons among different cuts, revealed significant differences (data not shown).

The relationships among the 96 strains studied were represented by a dendrogram (Fig. 2) constructed by using the Euclidian distance matrix and the neighbor-joining method (37). This unrooted dendrogram looks more like a bush than a tree, as described previously (46) for a bacterial population in which recombination occurs frequently. Moreover, the soil C variety L1 strains appeared to be separated from the remainder of the population by a large distance.

In an attempt to ascertain whether the two different primers used produced results (in terms of levels of genetic diversity) that were consistent with each other and with the results obtained when the RAPD results were considered together, we performed the same analyses (AMOVA and dendrogram analysis) independently with the data sets obtained with each primer. Although the levels of polymorphism of the RAPD data obtained with the two primers were different, the results of this analysis (data not shown) were in agreement with the results reported above; in particular, the dendrograms had the same starlike structure, with clusters of strains often conserved among the three dendrograms.

IGS analysis. To investigate further the genetic differences among the 96 *R. meliloti* strains, we analyzed the 16S-23S ribosomal operon spacer region. We amplified by PCR the intergenic sequence (IGS) from the same 96 strains used in the RAPD analysis. The length of the IGS amplified region, which included circa 200 bp of the 16S and 23S genes, appeared to be the same (about 1,350 bp) for all of the strains except strain A19, which had a fragment that was about 100 bp longer (and a faint band at about 800 bp). The amplification products were digested with endonuclease *Hae*III, and the restriction fragments were analyzed. The patterns obtained showed that there were about five or six fragments which ranged from 74 to 574 bp long. Three restriction fragments (297, 228, and 165 bp) were found in all of the strains and may be considered species specific. Only three restriction patterns were recognizable; pattern 1 was observed more commonly in strains obtained from soil A, whereas pattern 2 was observed more frequently in strains obtained from soil C (16.7% of the strains obtained from soil C). Pattern 3 was typical of strain A19 and contained at least two bands that were not present in the two other patterns. Further restriction analyses performed with endonuclease *Hae*II and with a mixture of endonucleases *Hae*II and *Alu*I (data not shown) led to perfectly comparable results which showed the same degree of polymorphism in the 96 strains considered.

RFLP analysis. The RFLP analysis was performed with 16 strains, 10 strains from soil A and 6 strains from soil C, that were chosen from all regions of the dendrogram. The total DNA of each strain was digested with *Eco*RI and hybridized to a probe (Fig. 3) containing the *nod*/*nol* operons of *R. meliloti*. The results obtained (Fig. 4) showed that seven strains, five of which were from soil C, gave six restriction patterns different from the pattern of the probe, while nine strains gave a pattern identical to the probe pattern. These variations in the restriction patterns were mapped (Fig. 3), and the restriction patterns obtained were compared. The variations seemed to be localized within two intergenic regions between *nodQ* and *nodJ* and between *nolN* and *nodC*. Since the strains used were able to nodulate plants, we assume that the mutations were localized where known nodulation genes are absent.

DISCUSSION

We analyzed the genetic diversity of 96 *R. meliloti* strains that were isolated from nodules of four different varieties of *M. sativa* obtained from distinct geographic areas and planted in two different Italian soils. Our analysis was performed by using RAPD and RFLP techniques. We used the AMOVA method to analyze the RAPD variation within the *R. meliloti* population isolated from nodules. A high level of genetic diversity was found within this population, as each microorganism had a unique fingerprint. Genetic differences among varieties within soil A (Table 2, experiment b) and among boxes within the same variety were also detected (Table 2, experiments c through e). The genetic difference detected among boxes (Table 2, experiment a) was highly significant ($P < 0.001$), indicating that after populations are separated and placed into individual boxes, they tend to differentiate. On the other hand, we did not detect significant differences among cuts, indicating that the population was stable throughout the seasons. The genetic difference between the two soils (Table 2, experiment f) was highly significant, but it is possible that the genetic differences between the soils resulted from the variety planted in soil C, which was unique and different from the varieties planted in soil A. The chemical and physical differences between the two soils may be at least partially responsible for the genetic differences among the strains. In fact, soil pH has been described as a critical factor in establishing genetic differences among *Rhizobium leguminosarum* populations (19, 41); moreover, the clay and organic matter contents, together with pH, were found to have a significant effect on the rate of plasmid transfer in soil among *Rhizobium fredii* strains (34), which influenced the level of genetic diversity of the population. Moreover, data derived from the AMOVA of the RAPD results were useful in the construction of a dendrogram representing the genetic relatedness of the strains. Although our dendrogram is not meant to imply phylogenetic relationships, nevertheless it has considerable value for interpreting data; it allows us to define the population considered as a population that is not clonal and probably freely recombines, and that there are major differences between the strains isolated from the two different soil types.

We decided to use two other analytical methods in order to compare their results and validate the RAPD results. The IGS between 16S and 23S rRNA genes could be an effective marker for detecting genetic differences at the intrageneric level and also at the intraspecific level. These differences are in part due to variations in the number and type of tRNA genes found in these spacers (22). We found a high level of homogeneity in the sizes of the amplification bands (95 1,350-bp bands and only 1 1,450-bp band) in the population of *R. meliloti* examined. We suppose that the difference in strain A19 (1,450-bp band) may be explained by an additional tRNA gene in the IGS of this strain. However, the high level of size homogeneity is consistent with results of Jensen et al. (22) which were obtained in an extensive study of about 300 bacterial strains belonging to different genera, which indicated that the degree of variability in both the size and the number of amplified IGS bands did not seem to be high enough to discriminate between strains belonging to the same species. However, restriction digestion of the amplified IGS allowed us to distinguish three different patterns. These data, which revealed a low level of polymorphism, confirmed the overall results of the RAPD

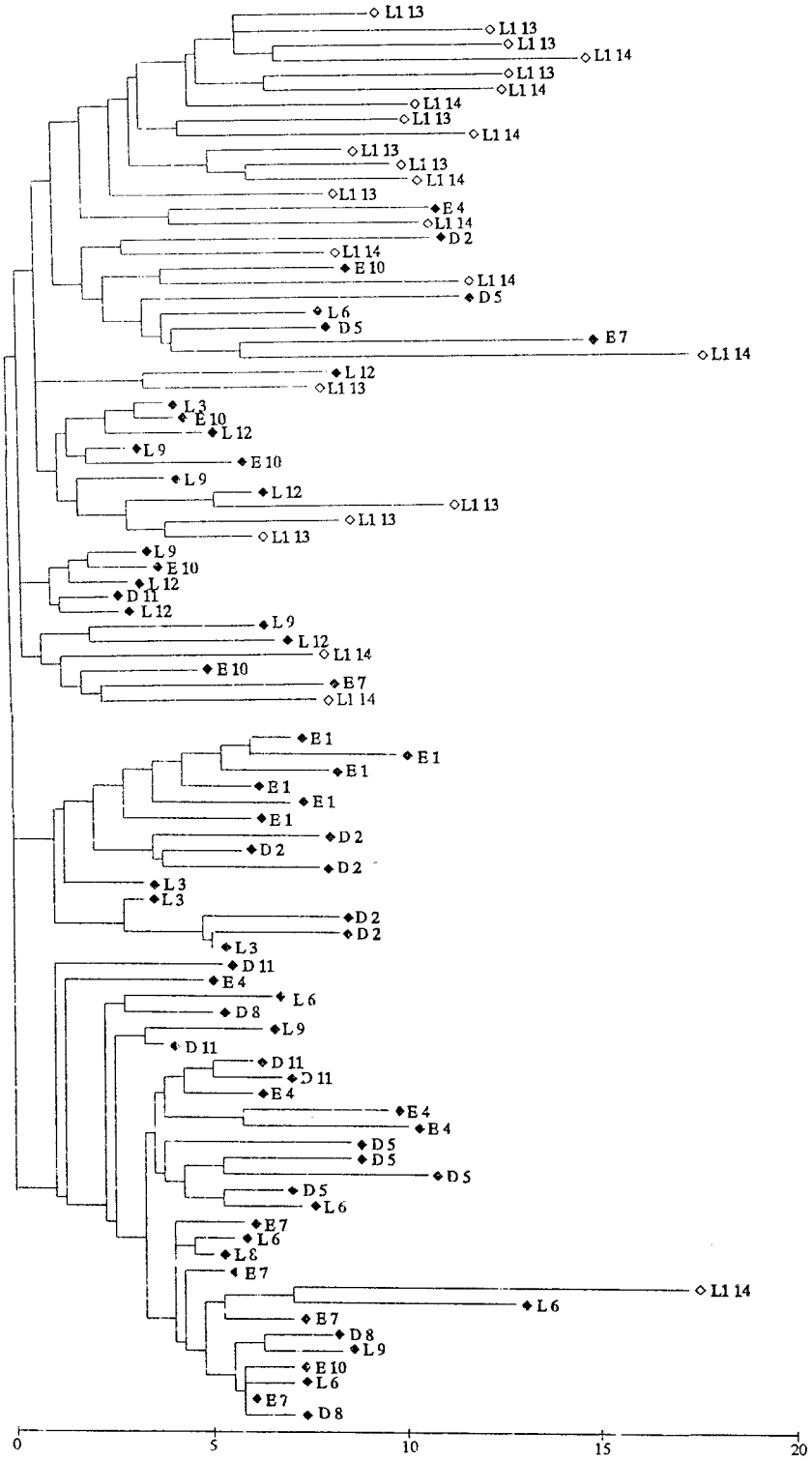

FIG. 2. Dendrogram. Symbols: ◇, soil C strain; ◆, soil A strain. E, variety Edfo; D, variety Diablo Verde; L, variety Lodi; L1, variety L1. The numbers from 1 to 14 indicate different boxes.

analysis, in which the primary genetic variability was found to be between strains isolated from the two different soils and thus distinguished two subpopulations.

A higher level of polymorphism was found on symbiotic plasmid p*Sym*. Young and Wexler (47) found that compared with the chromosomally encoded *lac* region, the plasmid-encoded *Sym* region of the same *R. leguminosarum* strains was substantially more polymorphic. We examined 16 strains and found seven different restriction patterns, five of which were from soil C organisms. Although the restriction maps drawn in

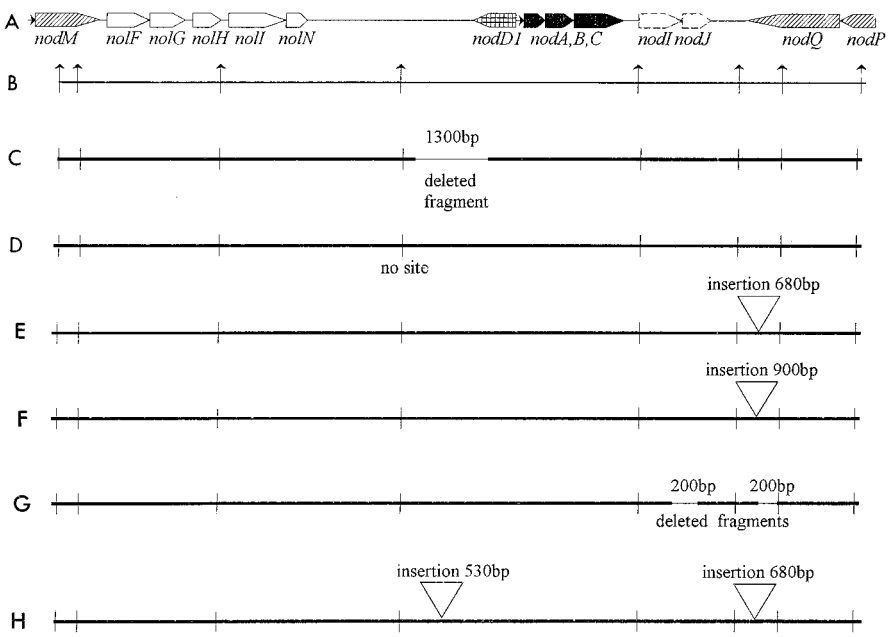

FIG. 3. Restriction and genetic maps of 25 kb of the *nod* region. Line A, genetic map of *nod* operons; line B, *Eco*RI restriction map of the probe. The other lines show the restriction maps of variants of the same region found in the following seven strains: A13 and C9 (line C), A30 (line D), C4 (line E), C11 (line F), C15 (line G), and C20 (line H). Strains A10, A23, A42, A61, A54, A56, A64, A72, and C16 had the same restriction map as the probe. The locations of insertions and deletions within the restriction fragment are not indicated.

the present study are only preliminary, it is interesting that the polymorphism found in the *nod* region is insertion and deletion polymorphism, which confirms previous observations (35) concerning the variability of these sequences. Whether the rearrangements affect the nodulation behavior of the strains it is not known, but it should be interesting to investigate the competitiveness and the host genotype preferences of some of the allelic variants.

The genetic diversity of the population was found to reside on the whole bacterial genome, as suggested by the results of the RAPD analysis, but seems to be distributed either on the chromosome (IGS) or on symbiotic plasmid p*Sym*. Of course, the RAPD amplified sequences, the IGS, and the p*Sym* region containing *nod* genes might not be representative of the chromosomal and plasmid sequences in general, but our data confirm the suggestion (35, 47) that plasmid sequences might be more variable than chromosomal sequences.

Since the strains analyzed were obtained only from nodules, this study is limited to a fraction of the soil population, a fraction that is not representative of the whole population (4).

Overall, the data obtained with the three methods are in agreement, demonstrating that there is general genetic diversity in the total population. The AMOVA method was confirmed to be a very powerful tool for investigating the genetic variation within intraspecific populations. The physical separation and the genotype of the host plant were the principal factors which determined the distribution of the genetic diversity within the natural population recovered from nodules.

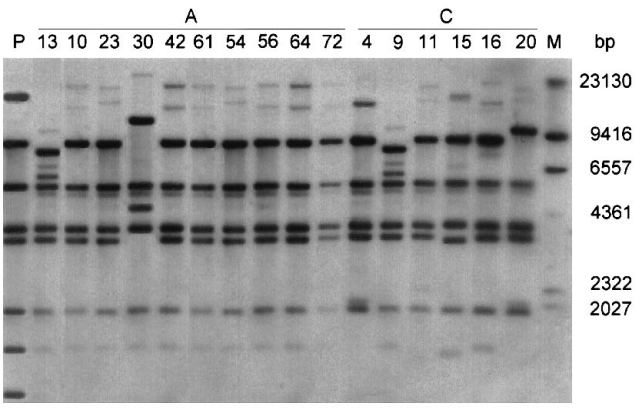

FIG. 4. Hybridization of total DNAs of 16 *R. meliloti* strains with the *nod* gene probe. DNAs were digested with *Eco*RI. The probe used was a 25-kb *Eco*RI DNA fragment of plasmid p*PP369* (see Fig. 3). Lane P, p*PP369*. Lanes 13, 10, 23, 30, 42, 61, 54, 56, 64, and 72 contained soil A strains A13, A10, A23, A30, A42, A61, A54, A56, A64, and A72, respectively. Lanes 4, 9, 11, 15, 16, and 20 contained soil C strains C4, C9, C11, C15, C16, and C20, respectively. Lane M, digoxigenin-labelled molecular weight marker ladder.

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