APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Apr. 1995, p. 1194–1200 Vol. 61, No. 4
0099-2240/95/$04.00+0

Diversity among Field Populations of *Bradyrhizobium japonicum* in Poland†

CEZARY J. MĄDRZAK,[1,2] BARBARA GOLIŃSKA,[1,2] JOANNA KRÓLICZAK,[1] KRZYSZTOF PUDEŁKO,[1] DOROTA ŁAŻEWSKA,[1] BOŻENA LAMPKA,[1] AND MICHAEL J. SADOWSKY[2]*

Institute of Biochemistry, University of Agriculture, Poznań, Poland,[1] and Departments of Soil Science and Microbiology, University of Minnesota, St. Paul, Minnesota 55108[2]

Received 14 November 1994/Accepted 19 January 1995

Genetic structure in field populations of *Bradyrhizobium japonicum* isolated in Poland was determined by using several complementary techniques. Of the 10 field sites examined, only 4 contained populations of indigenous *B. japonicum* strains. The Polish bradyrhizobia were divided into at least two major groups on the basis of protein profiles on polyacrylamide gels, serological reaction with polyclonal antisera, repetitive extragenic palindromic PCR fingerprints of genomic DNA, and Southern hybridization analyses with *nif* and *nod* gene probes. Serological analyses indicated that 87.5% of the Polish *B. japonicum* isolates tested were in serogroups 123 and 129, while seven (12.5%) of the isolates tested belonged to their own unique serogroup. These seven strains also could be grouped together on the basis of repetitive extragenic palindromic PCR fingerprints, protein profiles, and Southern hybridization analyses. Cluster analyses indicated that the seven serologically undefined isolates were genetically dissimilar from the majority of the Polish *B. japonicum* strains. Moreover, immuno-cross-adsorption studies indicated that although the Polish *B. japonicum* strains reacted with polyclonal antisera prepared against strain USDA123, the majority failed to react with serogroup 123- and 129-specific antisera, suggesting that Polish bradyrhizobia comprise a unique group of root nodule bacteria which have only a few antigens in common with strains USDA123 and USDA129. Nodulation studies indicated that members of the serologically distinct group were very competitive for nodulation of *Glycine max* cv. Nawiko. None of the Polish serogroup 123 or 129 isolates were restricted for nodulation by USDA123- and USDA129-restricting soybean plant introduction genotypes. Taken together, our results indicate that while genetically diverse *B. japonicum* strains were isolated from some Polish soils, the majority of field sites contained no soybean-nodulating bacteria. In addition, despite the lack of long-term soybean production in Poland, field populations of unique *B. japonicum* strains are present in some Polish soils and these strains are very competitive for nodulation of currently used Polish soybean varieties.

Bradyrhizobium japonicum strains form nitrogen-fixing root nodule symbioses with soybean (*Glycine max* (L.) Merr.) plants. Results from several studies in the United States and abroad have indicated that there is considerable physiological (15, 21, 23, 24), symbiotic (19), serological (5, 7, 27, 32, 40), and genetic (10, 14, 19, 34, 37) diversity among strains and serogroups of *B. japonicum*. Soybean nodulation in production fields often occurs by competitive, yet ineffective bradyrhizobia. It has been recognized that one of the major problems in the application of N_2 fixation technology is the establishment of introduced *Bradyrhizobium* strains in the nodules of soybean plants grown in soils which contain indigenous bradyrhizobial populations (11–13, 38, 41). In the Midwestern United States, strains in serocluster 123 are the dominant competitors for soybean nodulation (3, 22, 29, 32). Immunoadsorption studies (5, 9, 32) have shown that serocluster 123 consists of at least three distinct serogroups, 123, 127, and 129, and strains within the serocluster differ in competitiveness (4). It has been estimated that even 10 competitive bradyrhizobia per g of soil can act as an effective barrier for the introduction of new *B. japonicum* strains (36).

Most studies done to examine the diversity and population structure of *B. japonicum* have used isolates obtained from production fields having relatively large and established populations of indigenous soybean-nodulating bacteria. Little information, however, is available concerning the persistence and population dynamics of bradyrhizobia recently added to fields having no or very low numbers of indigenous *B. japonicum*. Moreover, in studies in which soybean inoculants were added to soils having low numbers of indigenous *B. japonicum* strains, the investigators were concerned mainly with assessing soybean yield response and the competitiveness of the introduced strains (2, 8, 13, 33, 36, 41).

Soybeans are becoming an important newly emergent crop in Poland and will be used for human consumption (as an oil crop which replaces or augments rapeseed oil) and for feeding livestock. There are four fully registered domestic soybean cultivars for use in Poland: Aldana, Progres, Polan, and Nawiko. Estimates obtained from soybean breeders indicate that 3,000 to 5,000 hectares of soybean plants will be planted in the not-too-distant future. Despite the use of soybeans in Poland, there is, however, no information available concerning *B. japonicum* in Polish soils.

In this study, we investigated diversity among field populations of *B. japonicum* isolated in Poland. Our studies were done to assess the genetic, physiological, serological, and symbiotic diversity of nodulating strains and to obtain phenotypic characteristics that can be used for strain identification and recovery. In addition, since soybeans have been introduced into Poland within only the last 10 to 15 years, our studies were also done to obtain information concerning the spread and persistence of newly introduced *B. japonicum* in Polish soybean production fields. Our results indicated that while genet-

* Corresponding author.

† Manuscript number 21,666 from the University of Minnesota Agricultural Experiment Station.

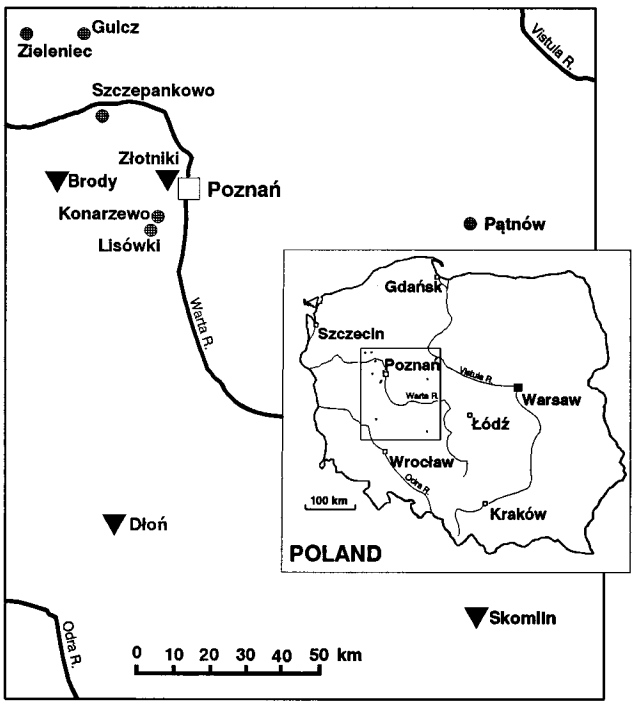

FIG. 1. Locations of field sites in Poland. The inset shows the relationship of field sites near Poznań to the rest of the country. Symbols: ▼, locations where *B. japonicum* isolates were obtained; ●, locations having soils free of *B. japonicum* strains.

ically diverse *B. japonicum* could be isolated from some Polish soils, most of the field sites studied contained no soybean-nodulating bacteria. In addition, we show that despite the lack of long-term soybean production in Poland, field populations of unique *B. japonicum* strains are present in some Polish soils and that these strains are very competitive for nodulation of the currently used soybean varieties.

MATERIALS AND METHODS

Isolation of *B. japonicum* strains from field-grown soybeans in Poland. Isolates of *B. japonicum* were obtained from nodules of field-grown plants collected in two locations in Poland, Dłoń and Skomlin, or from greenhouse plants grown in soils from the Złotniki and Brody field sites. Soils from the Złotniki, Brody, Dłoń, and Skomlin field sites were previously cultivated for soybean production. The Brody, Złotniki, and Dłoń soils are characterized as coarse, loamy, typic Hapludalfs. No information is available about the classification of soil from the Skomlin field site. Nodules were obtained from three soybean cultivars, Proges, Polan, and Nawiko. The locations of the field sites used in this study are shown in Fig. 1.

B. japonicum strains from Poland were isolated from surface-sterilized nodules (39) on AG medium (29) containing cycloheximide at 50 μg/ml. Isolates were streak purified twice on the same medium and maintained as frozen glycerol stocks at −70°C. Over 80 isolates were collected, of which 70 were used for further characterization. Twenty-one, 19, 18, and 12 isolates were obtained from the Złotniki, Brody, Dłoń, and Skomlin soils, respectively (Table 1). Reference *B. japonicum* strains with USDA designations were obtained from the culture collection of the U.S. Department of Agriculture, Beltsville, Md.

Analysis of antibiotic resistance. All isolates were tested for intrinsic resistance to antibiotics essentially as described by Josey et al. (16). The following antibiotics were tested (at the concentrations in parentheses): streptomycin (20 μg/ml), spectinomycin (10 μg/ml), kanamycin (20 μg/ml), tetracycline (30 μg/ml), nalidixic acid (30 μg/ml), erythromycin (30 μg/ml), and rifampin (1 μg/ml).

SDS-PAGE protein profiles. Total cytoplasmic proteins were extracted from bacterial cells and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 15% polyacrylamide gels containing 0.1% SDS as previously described (29). Protein profiles were converted to a two-dimensional binary matrix and analyzed as described by Judd et al. (17).

Immunologic analyses. Antibodies were prepared against surface somatic antigens of *B. japonicum* strains as described by Schmidt et al. (31) and stored at −20°C until used. The serum immunoglobulin G fraction was isolated as previously described (35). Fluorescent antibodies (FA) for detection of *B. japonicum* USDA4, USDA38, USDA110, USDA122, USDA123, USDA127, USDA129, USDA135, USDA138, and Webster 48; *B. elkanii* USDA31, USDA46, USDA76, USDA94, and USDA130; and Polish isolates 501 and 631 were prepared as previously described (31). Immunofluorescence analyses were done as previously described (27, 31), and the FA were made strain specific by immuno-cross-adsorption as described by Robert and Schmidt (25).

TABLE 1. Origins of *B. japonicum* isolates obtained from soybean production fields in Poland

Location	Soybean cultivar	Isolate designations
Złotniki	Progres	1, 6, 16, 26, 31, 151, 161, 176, 181, 191
	Polan	206, 207, 221, 226, 236, 246, 351, 356, 366, 386, 396
Brody	Nawiko	426, 431, 436
	Progres	456, 466, 501, 506, 516, 531, 536, 541, 601, 606, 611, 631, 636, 641, 701, 716
Dłoń	Nawiko	608, 610, 612, 613, 614, 615, 616, 617, 618, 619, 620, 621, 622, 623, 624, 625, 626, 627
Skomlin	Nawiko	717, 718, 719, 720, 721, 722, 723, 724, 725, 726, 727, 728

REP-PCR analysis of Polish *B. japonicum* strains. Genomic DNAs from Polish strains were isolated as previously described (29). The PCR was performed with repetitive extragenic palindromic (REP) primers (6, 17), and the REP-PCR fingerprints were converted into a two-dimensional binary matrix and analyzed as described by Judd et al. (17).

Southern hybridization analysis of Polish *B. japonicum* strains with *nod* and *nif* gene probes. Genomic DNAs from selected *B. japonicum* isolates were digested with *Eco*RI, and the resulting fragments were separated on 0.8% agarose gels by using Tris-EDTA-borate buffer (30). The DNA was blotted onto Nytran membranes (Schleicher & Schuell, Inc., Keene, N.H.) and hybridized to the *nif* or *nod* gene probe as previously described (28, 29). The *nod* gene probe used was pRjUT10 (26), and the *nif* probe was pCHK12 (29). The probes were labeled with [^{32}P]dCTP by using the Multiprime DNA Labeling System (Amersham) in accordance with the manufacturer's protocol. The autoradiograms were analyzed after conversion of the hybridization patterns into a two-dimensional binary matrix (17).

Nodulation performance of Polish *B. japonicum* strains on soybean cv. Kasota and plant introduction (PI) genotypes. Soybean [*Glycine max* cv. Kasota, PI 377578, and PI 417566 (3, 4, 18)] seeds were surface sterilized (39) and planted in modified Leonard jars containing a 3:1 mixture of vermiculite and perlite as previously described (28, 39). Seeds were individually inoculated with 1 ml (about 10^8 cells) of late-log-phase, AG-grown cultures of 32 Polish *B. japonicum* isolates and with strains USDA122, USDA123, USDA129, and USDA110. Three Leonard jars, each containing three plants, were inoculated with each strain. Uninoculated plants served as controls. Plants were grown in a plant growth chamber at 20°C with an 18-h photoperiod and watered with N-free Keyser's plant nutrient solution (18) as needed. Plants were harvested after 5 weeks, and root nodules were counted. Statistical significance was determined by using the analysis of variance and Duncan's new multiple-range procedures of SAS.

Competition-for-nodulation studies. On the basis of REP-PCR profiles and serological characteristics, five Polish *B. japonicum* isolates were selected for competition studies. Polish soybean cv. Nawiko was used as the host plant. Surface-sterilized soybean seeds were planted in modified Leonard jars as described above. Seeds were inoculated with equal numbers (about 10^8 cells of each strain per ml) of AG-grown cultures of two Polish *B. japonicum* strains. Four combinations of Polish strains were inoculated in triplicate. *B. japonicum* USDA110, USDA123, and USDA129 were used as controls. Plants were grown in a growth chamber as described above. After 5 weeks of growth, plants were harvested and 36 randomly selected nodules were collected from each replicate. Twelve nodules per replicate were collected from the control plants. Nodules were placed in a microtiter plate and crushed in 100 μl of distilled water. Nodule occupants were determined by immuno-spot blot analysis as previously described (1, 4).

RESULTS

Isolation of Polish *B. japonicum* strains. We examined 10 field sites in Poland with the goal of isolating and characteriz-

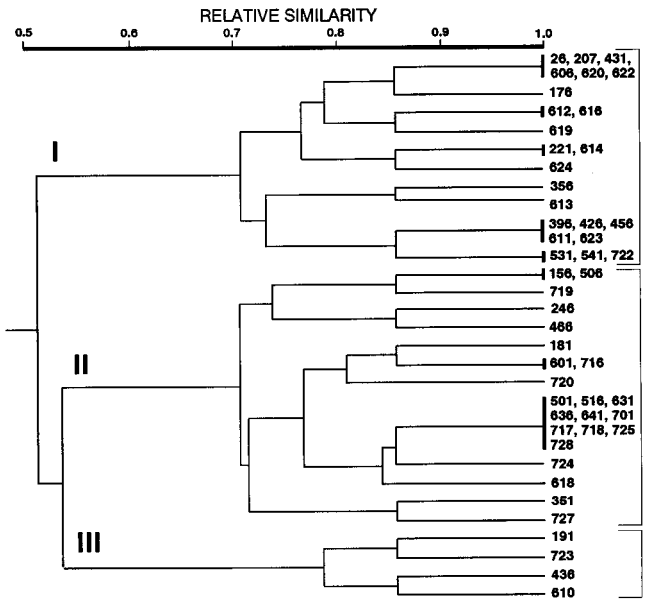

FIG. 2. Dendrogram of intrinsic resistance to antibiotics among Polish *B. japonicum* isolates.

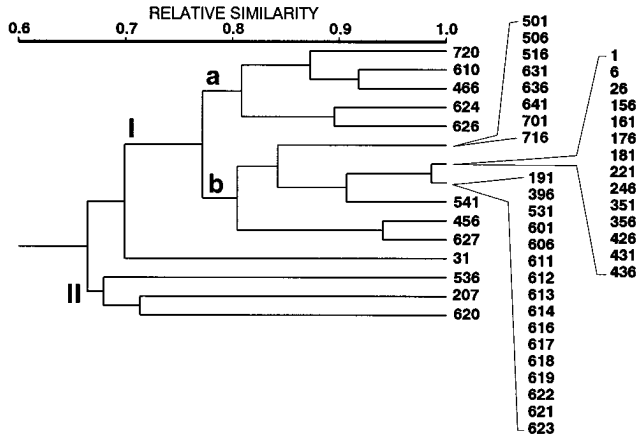

FIG. 3. Dendrogram of Polish *B. japonicum* strains determined from protein profile data. Strains having identical profiles are grouped together.

ing indigenous *B. japonicum* strains. Seventy *B. japonicum* strains were isolated from four locations (Brody, Złotniki, Dłoń, and Skomlin) (Table 1 and Fig. 1). The strains were isolated from three soybean varieties which are registered for use in Poland: Polan, Nawiko, and Progres. All four locations had been used to grow soybeans, and information obtained from farmers indicated that these fields had previously received soybean inoculants. However, the origin of strains used to prepare commercial inocula in Poland is unknown. Greenhouse plants grown in soils from field sites where soybeans have never been grown (Gulcz, Zieleniec, Szczepankowo, Lisówki, Konarzewo, and Pątnów) showed no nodulation. Based on this limited study in a small portion of Poland, our results suggest that Polish soils are most likely free of indigenous *B. japonicum* strains.

Intrinsic antibiotic resistance. To initially characterize Polish *B. japonicum*, the 50 randomly selected isolates were analyzed for intrinsic resistance to seven different antibiotics. Five concentrations of each antibiotic were tested, and the most discriminatory concentration was chosen for use in analyses. Results in Fig. 2 show that the 50 strains could be divided into three main antibiotic resistance groups (at a relative similarity level of greater than 0.5). Within groups I and II (which contained the same number of isolates), several strains were identical or nearly identical to each other in overall resistance to the antibiotics tested. Group III, on the other hand, contained four different strains that were related at a similarity level of 0.80. Despite these groupings, the Polish *B. japonicum* isolates were relatively heterogeneous with respect to resistance to specific antibiotics. For example, of the 50 strains analyzed, only four isolates (8%) were resistant to kanamycin and five (10%) showed resistance to streptomycin. On the other hand, 40 isolates (80%) were resistant to erythromycin, 31 (62%) were resistant to rifampin, 29 (58%) were resistant to spectinomycin, and 29 (58%) were resistant to nalidixic acid. Fewer isolates, 22 (44%), were resistant to tetracycline.

SDS-PAGE protein profiles. Total cellular proteins were extracted from 50 Polish *B. japonicum* isolates, and solubilized proteins were analyzed by SDS-PAGE. The protein profiles of the 50 strains were analyzed as a two-dimensional binary matrix (as described above), and the resulting dendrogram of similarity coefficients is shown in Fig. 3. These results show that the isolates clustered within two major groups on the basis of their protein profiles. Group I, in turn, could be divided into two subgroups, a and b, at a similarity level of 0.78. Most isolates were clustered within protein group I, subgroup b, which represented 76% of the strains.

Serological characterization of Polish *B. japonicum* isolates. To determine the serological relatedness of Polish *B. japonicum* isolates, we examined 55 isolates for serological cross-reactivity with FA prepared against 10 USDA *B. japonicum* strains and five *B. elkanii* strains. Results of this study indicated that 47 of 54 isolates (87%) showed immunoreaction with FA prepared against strain USDA123 and 28 isolates (51.8%) reacted with FA prepared against strain USDA129 (Table 2). Among these cross-reactive strains, 28 reacted with FA prepared against both strains USDA129 and USDA123. The Polish *B. japonicum* isolates did not react with any of the other antisera that we examined, suggesting that strains representing only a limited number of serogroups were present in Polish soils.

To determine whether the serologically reactive Polish *B. japonicum* strains showed serological identity to strains USDA123 and USDA129 (which define serogroups 123 and 129, respectively), we used cross-adsorbed FA that were specific for strains USDA123 and USDA129. While four isolates (191, 396, 531, and 536) reacted with strain USDA123-specific antibodies, none of the Polish bradyrhizobia had a serological reaction with strain 129-specific antibodies. These results indicate that while a majority of the Polish bradyrhizobia have some antigens in common with strains in serogroups 123 and 129, they are serologically distinct from the typical U.S. strains which constitute serocluster 123.

Seven of the Polish isolates (501, 506, 541, 631, 636, 641, and 701) failed to react with any of the antisera tested. These strains were isolated on soybean cv. Progres at the Brody field site. Polyclonal antibodies raised against two of these strains, 501 and 631, cross-reacted with all seven isolates (Table 2) but failed to react with any of the other Polish bradyrhizobia (or USDA type strains). Since these seven strains had nearly identical protein profiles and were all members of Protein profile group Ib, our results indicate that these strains represent a distinct group of bradyrhizobia.

REP-PCR analysis of bacterial genomes. Total genomic

TABLE 2. Serological reactions of selected *B. japonicum* isolates from Poland

Serological group	FA specificity	Isolates reacting with FA[a]
1	USDA123, USDA129[b]	1, 6, 16, 26, 31, 151, 156, 176, 181, 191, 206, 236, 246, 396, 431, 436, 466, 516, 531, 601, 611, 612, 614, 616, 620, 623, 624, 625
2	USDA123[c]	221, 226, 351, 356, 366, 386, 426, 456, 536, 606, 612, 617, 618, 619, 621, 622, 626, 627, 716
3	123[d]	191, 396, 531, 536
4	501 or 631[e]	501, 506, 541, 631, 636, 641, 701

[a] Reactions were scored on a scale of 0 to 4; only those greater than 2 are reported.
[b] Strains reacting with polyclonal antisera prepared against *B. japonicum* USDA123 and USDA129.
[c] Strains reacting only with antiserum prepared against *B. japonicum* USDA123.
[d] Strains reacting with cross-adsorbed polyclonal antiserum specific for *B. japonicum* strains in serogroup 123.
[e] Strains reacting only with antiserum prepared against Polish *B. japonicum* 501 or 631.

DNAs from 35 isolates of Polish *B. japonicum* were used as templates for PCR with REP primers. To maximize genetic diversity, the strains were selected on the basis of the heterogeneity of their antibiotic resistance profiles. The PCR products from a representative group of strains were separated on 1.5% agarose gels and stained with ethidium bromide. All strains were run on one agarose gel to make comparisons between adjacent lanes consistent. The primers yielded multiple DNA products ranging in size from 0.14 to 2.3 kb. To classify the strains by using this technique, we converted the results from the stained agarose gels into a two-dimensional binary matrix, analyzed the results with a commercially available biostatistics program, and generated a dendrogram based on pairwise comparisons of PCR products. The dendrogram in Fig. 4 shows that the Polish *B. japonicum* isolates studied could be divided into two major groups based on their REP-PCR profiles. Group I strains, which represent 80% of the isolates were, in turn, divided into two major subgroups, A and B, at a similarity level of 0.8. There were, however, a few group I strains which had relatively unique REP-PCR profiles and consisted of single isolates which diverged at a similarity level of 0.68. Since the group II strains (which represented 20% of the isolates tested) diverged from the group I strains at a similarity level of 0.4, our results indicate that these two major groups of Polish isolates differ significantly in genomic organization. Results in Figure 4, however, indicate that the group II strains were nearly identical to each other and only diverged at a similarity level of about 0.95. These group II strains (501, 506, 541, 631, 636, 641, and 701) are the same ones which reacted with antisera prepared against strains 501 and 631 (Table 2) and had identical protein profiles (Fig. 3). Taken together, these results further strengthen our contention that the seven strains represent a unique group of Polish bradyrhizobia.

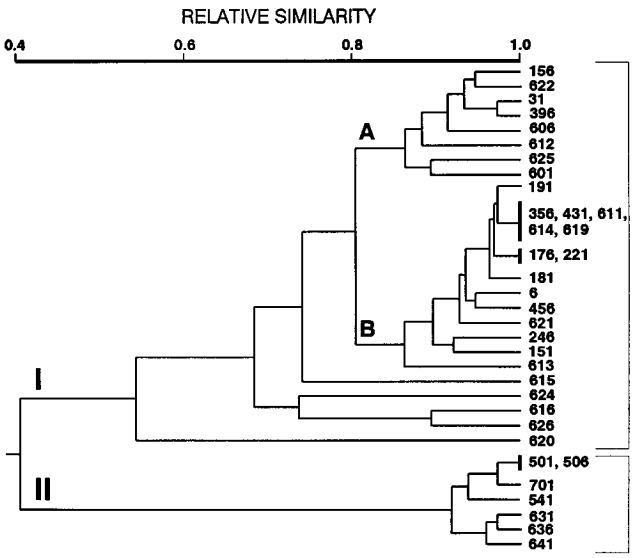

FIG. 4. Dendrogram of Polish *B. japonicum* strains derived from REP-PCR fingerprints generated by using REP primers.

Southern hybridization analyses using symbiotically relevant gene probes. To further examine the genomic organization of the Polish bradyrhizobia, we hybridized *nod* and *nif* gene probes to *Eco*RI-digested genomic DNAs from 34 selected isolates. To maximize the regions of the genome examined, Southern hybridizations were separately done with cosmid clone pRjUT10, which detects *B. japonicum* nodulation genes *nodD1*, *nodD2*, *nodY*, *nodA*, *nodB*, *nodC*, *nodSUIJ*, *nolMNO*, *nodZ*, and *nolA* and nitrogen fixation genes *fixR*, *nifA*, and *fixA*, and plasmid pCHK12, which detects *nifH* and *nifD* hybridizing sequences. After autoradiography, the resulting hybridizing fragments from each autoradiogram were combined into a single two-dimensional binary matrix and analyzed with NTSYS. The dendrogram generated by using pairwise comparisons of hybridizing bands is shown in Fig. 5. Our results show that the Polish *B. japonicum* isolates were divided into two major groups based on their *nif* plus *nod* gene hybridization profiles. Group I strains accounted for 79.4% of the isolates. These 27 isolates were further subdivided into two subgroups, a and b, which diverge from each other at a similarity value of 0.78. The hybridization group I isolates were the same as those revealed by serological and REP-PCR analyses. Results of hybridization analyses shown in Fig. 5 also indicate that the group II isolates (accounting for 20.6% of the strains), which diverge from the group I strains at a similarity value of 0.5, represent a unique group of bradyrhizobia. These seven group II isolates (501, 506, 541, 631, 636, 641, and 701) are the same ones which failed to react with any of the antisera tested (Table 2) and had identical protein and REP-PCR profiles (Fig. 3 and 4). As was revealed by using the other methods of analysis, the hybridization group II strains were relatively similar to one another and formed a close group which clustered at a similarity value of 0.93.

Nodulation performance of Polish *B. japonicum* isolates. We have previously reported that soybean genotypes PI 377578 and PI 417566 restrict nodulation by most serocluster 123 strains (3, 4, 18, 29). To determine if the Polish isolates, which reacted with antisera prepared against USDA123 and USDA129, were similarly restricted for nodulation by the PI genotypes, we inoculated *G. max* cv. Kasota, PI 417566, and PI 377578 with cultures of 50 Polish *B. japonicum* strains. Results

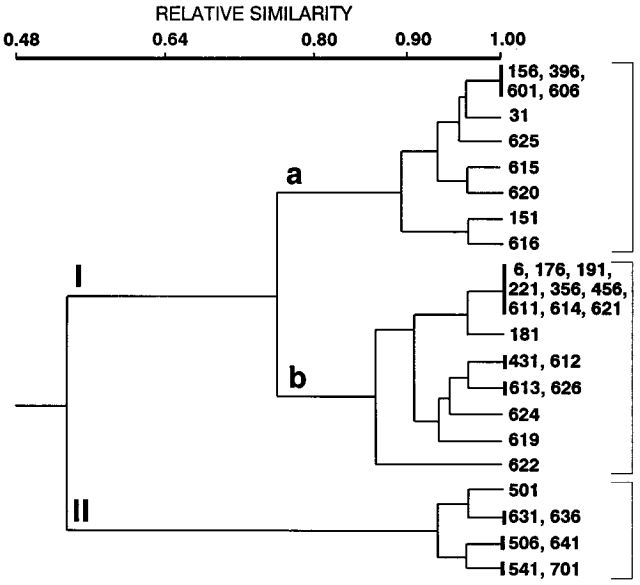

FIG. 5. Dendrogram of Polish *B. japonicum* strains derived from combined *nif* and *nod* gene hybridization profiles.

of these studies indicated that none of the soybean genotypes restricted nodulation by the Polish *B. japonicum* strains (data not shown). Moreover, the four strains which had previously been shown to be members of serogroup 123 (191, 396, 531, and 536) were not restricted for nodulation by PI 377578. The Polish isolates produced mean nodule numbers of 9.5 to 24.5, 6.0 to 42.0, and 11.0 to 32.5 on soybean genotypes Kasota, PI 377578, and PI 417566, respectively. Consequently, the nodulation data indicate that despite having serological relatedness with strains in serogroups 123 and 129, the Polish *B. japonicum* strains do not share symbiotic properties with a majority of the previously characterized strains in serocluster 123.

Competition for nodulation between selected Polish *B. japonicum* isolates. To determine if the serologically and genetically distinct Polish *B. japonicum* strains from the Brody field site were competitive for soybean nodulation, we performed paired, equal-concentration competition assays on cv. Nawiko with one of the seven strains, 541, and four randomly selected serogroup 123 strains isolated from other field sites in Poland. Results of these studies are shown in Table 3. In all cases, isolate 541 out competed the challenge strains for nodulation of soybean cv. Nawiko. Strain 541 singly occupied 50 to 76% of the soybean nodules, whereas the challenge strains singly occupied 0 to 29% of the nodules. Nodules doubly occupied by both strains ranged from 15 to 38%. Strain 541 was the most competitive against strain 620, and this combination of strains produced the least doubly infected nodules. Results in Table 3 indicate that the Polish *B. japonicum* strains differ in competitive ability and that a serologically distinctive strain, 541, has the ability to compete successfully against some serocluster 123 strains for nodulation of soybean cv. Nawiko.

TABLE 3. Nodulation competitiveness of Polish *B. japonicum* strains against Polish *B. japonicum* 541 on *G. max* cv. Nawiko

Competitor strain	No. of nodules analyzed	% of nodules occupied by:		
		Competitor strain	Strain 541	Both[a]
6	44	0	66	34
396	107	29	50	21
619	104	9	53	38
620	107	9	76	15

[a] Occupation of nodules by both inoculant strains was determined by using strain-specific antisera.

DISCUSSION

In this study, we investigated the diversity of *B. japonicum* strains in Polish soils. Since soybean is a relatively new crop in Poland, the distribution of *B. japonicum* in Polish soils may provide us with interesting information concerning the survival and symbiotic performance of bacteria introduced into areas previously free of indigenous populations of soybean bacteria.

Results of our greenhouse studies indicated that soybean plants did not nodulate when grown in soils collected from several field sites (Lisówki, Konarzewo, Pątnów, Szczepankowo, Zieleniec, and Gulcz) where there was no previous cultivation of this crop. Consequently, our isolation-nodulation studies indicated that most of the Polish soils which we examined contained no strains capable of nodulating *G. max*. The lack of indigenous *B. japonicum* strains has been confirmed by results from several cultivation and breeding studies which indicate that soybean plants in several soils throughout Poland fail to form nodules (24a). Our results also indicate, however, that *B. japonicum* strains survive and persist once they are introduced into Polish soils. Efficient nodulation was observed when surface-sterilized soybean seeds were sown in soils from sites (Brody, Złotniki, Dłoń, and Skomlin) which had previously been inoculated and cultivated for soybean production.

While we have been unable to determine the exact origin of strains used by inoculum producers in Poland, our results suggest that at least some of the *B. japonicum* strains maintained in the collection at the Institute of Cultivation, Fertilization and Soil Science in Puławy, Poland, were obtained from North America. In fact, one of the most frequently used Polish soybean inoculum strains, designated 94P (Institute of Cultivation, Fertilization and Soil Science collection number), was imported over 30 years ago from The University of Wisconsin-Madison (35a). Moreover, greater than 87% of the *B. japonicum* isolates obtained from the Brody, Złotniki, Dłoń, and Skomlin sites were in *B. japonicum* serocluster 123, which contains the dominant indigenous competitors for soybean nodulation in midwestern U.S. soils.

While the range of soybean genotypes cultivated in Poland is rather limited and despite the limited occurrence of *B. japonicum* in Polish field soils, we nevertheless observed significant diversity among the Polish bradyrhizobia which we isolated. Our analysis of the degree of relative similarity between these isolates, irrespective of almost every method used for this purpose, indicated that the isolates could be divided into two major groups with relative similarity values of about 0.4 to 0.9. On the basis of genetic criteria obtained by using REP-PCR and DNA hybridization probes, however, the group I isolates, which represented over 87% of the strains examined, formed a fairly tight cluster, with the majority of strains being greater than 75% similar. Without exception, the group I isolates consisted of strains belonging to *B. japonicum* serocluster 123. This result suggests that the strains have a common ancestor, perhaps originally from the United States.

The other major group of strains (group II) consisted of seven closely related, but not identical, *B. japonicum* isolates (501, 506, 541, 631, 636, 641, and 701) which differed significantly from most of the other strains examined. These seven isolates did not react with any of the 15 FA tested (Table 2),

were obtained from the Brody field site (but were not the only type of *B. japonicum* found there), had closely related REP-PCR fingerprints (Fig. 4), and had very similar *nif* and *nod* hybridization profiles (Fig. 5). Although these seven strains were not genetically identical, as shown by using several characterization techniques, they were very closely related and had similarity values of greater than 0.9. Moreover, all seven strains reacted with the same two antisera, indicating that they were also very similar at the serological level.

Since the seven group II isolates occupied about 50% of the nodules on cv. Progres plants at the Brody field site, we investigated their nodulation competitiveness relative to that of other (group I) Polish *B. japonicum* strains. To do this, we did equal-concentration, paired competition assays on soybean cv. Nawiko between group I isolates 6, 396, 619, and 620 and one typical group II isolate, 541. Results of these studies (Table 3) indicated that the group II strain (541) was relatively competitive for nodulation of cv. Nawiko and singly occupied 50 to 76% of the nodules. While we are aware that only limited conclusions can be drawn from analyses done with such a small number of strains, our results do suggest that it is likely that the "indigenous" seven strains are well adapted and competitive for soybean nodulation. Moreover, the high degree of genetic similarity suggests that all of the strains are very closely related and may, in fact, be derived from a common ancestral inoculant strain.

The Polish *B. japonicum* strains tested were also unique with respect to the ability to nodulate two soybean plant introduction genotypes. Results of previous studies (3, 4, 29) indicated that soybean genotypes PI 377578 and PI 417566 have the ability to restrict nodulation by serogroup 123-127 and 129 strains, respectively. Results of the current study indicated that none of the Polish *B. japonicum* strains, a large number of which are members of serogroup 123, were restricted for nodulation by the PI genotypes. Consequently, the relationship between serological classification and nodulation restriction may be more complicated than originally assumed. It should be noted, however, that the Polish *B. japonicum* strains are not serologically identical but only antigenically related to North American serocluster 123 isolates, as judged by the lack of reaction with some serogroup-specific antisera. This may be related to their ability to form symbioses with the nodulation-restricting soybean PI genotypes. Given enough time and distance, however, it is not unexpected that the Polish *B. japonicum* isolates, which act as clonal populations (20), have genetically diverged from an original ancestor introduced in a soybean inoculant.

ACKNOWLEDGMENTS

This study was supported, in part, by grant HRN-5600-G-00-2074-00 from the U.S. Agency for International Development (to M.J.S. and C.J.M.) and by the University of Minnesota Agricultural Experiment Station (M.J.S.).

REFERENCES

1. **Ayanaba, A., K. D. Weiland, and R. M. Zablotowicz.** 1986. Evaluation of diverse antisera, conjugates, and support media for detecting *Bradyrhizobium japonicum* by indirect enzyme-linked immunosorbent assay. Appl. Environ. Microbiol. **52:**1132–1138.
2. **Boonkerd, N., D. F. Weber, and D. F. Bezdicek.** 1978. Influence of *Rhizobium japonicum* strains and inoculation methods on soybean grown in rhizobia-populated soils. Agron. J. **70:**547–549.
3. **Cregan, P. B., and H. H. Keyser.** 1986. Host restriction of nodulation by *Bradyrhizobium japonicum* strain USDA123 in soybean. Crop Sci. **26:**911–916.
4. **Cregan, P. B., H. H. Keyser, and M. J. Sadowsky.** 1989. Host plant effects on nodulation and competitiveness of the *Bradyrhizobium japonicum* serotype strains constituting serocluster 123. Appl. Environ. Microbiol. **55:**2532–2536.
5. **Date, R. A., and A. M. Decker.** 1971. Minimal antigenic constitution of 28 strains of *Rhizobium japonicum*. Can. J. Microbiol. **11:**1–8.
6. **de Bruijn, F. J.** 1992. Use of repetitive (repetitive extragenic palindromic and enterobacterial repetitive intergeneric consensus) sequences and the polymerase chain reaction to fingerprint the genomes of *Rhizobium meliloti* isolates and other soil bacteria. Appl. Environ. Microbiol. **58:**2180–2187.
7. **Dudman, W. F.** 1971. Antigenic analysis of *Rhizobium japonicum* by immunodiffusion. Appl. Environ. Microbiol. **21:**973–985.
8. **Dunigan, E. P., P. K. Bollich, R. L. Hutchinson, P. M. Hicks, F. C. Zaunbrecher, S. G. Scott, and R. P. Mowers.** 1984. Introduction and survival of an inoculant strain of *Rhizobium japonicum* in soil. Agron. J. **76:**464–466.
9. **Gibson, A. H., W. F. Dudman, R. W. Weaver, J. C. Horton, and I. C. Anderson.** 1971. Variation within serogroup 123 of *Rhizobium japonicum*. Plant Soil (special volume), p. 33–37.
10. **Hadley, R. G., A. R. Eaglesham, and A. A. Szalay.** 1983. Conservation of DNA regions adjacent to nifKDH homologous sequence in diverse slow-growing *Rhizobium* strains. J. Mol. Appl. Genet. **2:**225–236.
11. **Ham, G. E.** 1976. Competition among strains of rhizobia, p. 144–150. *In* L. D. Hill (ed.), World soybean research. Interstate Printers & Publishers, Danville, Ill.
12. **Ham, G. E.** 1980. Interactions of *Glycine max* and *Rhizobium japonicum*, p. 289–296. *In* R. J. Summerfield and A. H. Bunting (ed.), Advances in legume science. Royal Botanical Gardens, Kew, England.
13. **Ham, G. E., V. B. Caldwell, and H. W. Johnson.** 1971. Evaluation of *Rhizobium japonicum* inoculants in soils containing naturalized populations of rhizobia. Agron. J. **63:**301–303.
14. **Haugland, R., and D. P. S. Verma.** 1981. Interspecific plasmid and genomic DNA homologies and localization of nif genes in effective and ineffective strains of *Rhizobium japonicum*. J. Mol. Appl. Genet. **1:**205–217.
15. **Huber, T. A., A. K. Agarwal, and D. L. Keister.** 1984. Extracellular polysaccharide composition, ex planta nitrogenase activity, and DNA homology in *Rhizobium japonicum*. J. Bacteriol. **158:**1168–1171.
16. **Josey, D. P., J. L. Beynon, A. W. B. Johnston, and J. E. Beringer.** 1979. Strain identification in *Rhizobium* using intrinsic antibiotic resistance. J. Appl. Bacteriol. **46:**343–350.
17. **Judd, A. K., M. Schneider, M. J. Sadowsky, and F. J. de Bruijn.** 1993. Use of repetitive sequences and the polymerase chain reaction technique to classify genetically related *Bradyrhizobium japonicum* serocluster 123 strains. Appl. Environ. Microbiol. **59:**1702–1708.
18. **Keyser, H. H., and P. B. Cregan.** 1987. Nodulation and competition for nodulation of selected genotypes among *Bradyrhizobium japonicum* serogroup 123 isolates. Appl. Environ. Microbiol. **53:**2631–2635.
19. **Keyser, H. H., P. van Berkum, and D. F. Weber.** 1982. A comparative study of the physiology of symbioses formed by *Rhizobium japonicum* with *Glycine max*, *Vigna unguiculata*, and *Macroptilium atropurpureum*. Plant Physiol. **70:**1626–1630.
20. **Martinez-Romero, E.** 1994. Recent developments in *Rhizobium* taxonomy. Plant Soil **161:**11–20.
21. **Meyer, M. C., and S. G. Pueppke.** 1980. Differentiation of *Rhizobium japonicum* strain derivatives by antibiotic sensitivity patterns, lectin binding, and utilization of biochemicals. Can. J. Microbiol. **26:**606–612.
22. **Moawad, H. A., W. R. Ellis, and E. L. Schmidt.** 1984. Rhizosphere response as a factor in competition among three serogroups of indigenous *Rhizobium japonicum* for nodulation of field-grown soybeans. Appl. Environ. Microbiol. **47:**607–612.
23. **Noel, K. D., and W. J. Brill.** 1980. Diversity and dynamics of indigenous *Rhizobium japonicum* populations. Appl. Environ. Microbiol. **40:**931–938.
24. **Parke, D., and L. N. Ornston.** 1984. Nutritional diversity of *Rhizobiaceae* revealed by auxanography. J. Gen. Microbiol. **130:**1743–1750.

24a. **Pudełko, J., and J. Nawracała.** Personal communication.

25. **Robert, F. M., and E. L. Schmidt.** 1983. Population changes and persistence of *Rhizobium phaseoli* in soil and rhizospheres. Appl. Environ. Microbiol. **45:**550–556.
26. **Russell, P., M. G. Schell, K. K. Nelson, L. J. Halverson, K. M. Sirotkin, and G. Stacey.** 1985. Isolation and characterization of the DNA region encoding nodulation functions in *Bradyrhizobium japonicum*. J. Bacteriol. **164:**1301–1308.
27. **Sadowsky, M. J., B. B. Bohlool, and H. H. Keyser.** 1987. Serological relatedness of *Rhizobium fredii* to other rhizobia and to the bradyrhizobia. Appl. Environ. Microbiol. **53:**1785–1789.
28. **Sadowsky, M. J., P. B. Cregan, M. Göttfert, A. Sharma, D. Gerhold, F. Rodriguez-Quinones, H. H. Keyser, H. Hennecke, and G. Stacey.** 1991. The *Bradyrhizobium japonicum* nolA gene and its involvement in the genotype-specific nodulation of soybeans. Proc. Natl. Acad. Sci. USA **88:**637–641.
29. **Sadowsky, M. J., R. E. Tully, P. B. Cregan, and H. H. Keyser.** 1987. Genetic diversity in *Bradyrhizobium japonicum* serogroup 123 and its relation to genotype-specific nodulation of soybean. Appl. Environ. Microbiol. **53:**2624–2630.
30. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
31. **Schmidt, E. L., R. O. Bankole, and B. B. Bohlool.** 1968. Fluorescent-antibody approach to study of rhizobia in soil. J. Bacteriol. **95:**1987–1992.

32. **Schmidt, E. L., M. J. Zidwick, and H. M. Abebe.** 1986. *Bradyrhizobium japonicum* serocluster 123 and diversity among member isolates. Appl. Environ. Microbiol. **51:**1212–1215.
33. **Singleton, P. W., and J. W. Tavares.** 1986. Inoculation response of legumes in relation to the number and effectiveness of indigenous *Rhizobium* populations. Appl. Environ. Microbiol. **51:**1013–1018.
34. **Stanley, J., G. G. Brown, and D. P. S. Verma.** 1985. Slow-growing *Rhizobium japonicum* comprises two highly divergent symbiotic types. J. Bacteriol. **163:** 148–154.
35. **Steinbuch, M., and R. Audran.** 1969. The isolation of IgG from mammalian sera with the aid of caprylic acid. Arch. Biochem. Biophys. **134:**279–284.
35a. **Strzelec, A.** Personal communication.
36. **Thies, J. E., P. W. Singleton, and B. B. Bohlool.** 1991. Influence of the size of indigenous rhizobial populations on establishment and symbiotic performance of introduced rhizobia on field-grown legumes. Appl. Environ. Microbiol. **57:**19–28.
37. **van Berkum, P., S. I. Kotob, H. A. Basit, S. Salem, E. M. Gewaily, and J. S. Angle.** 1993. Genotypic diversity among strains of *Bradyrhizobium japonicum* belonging to serogroup 110. Appl. Environ. Microbiol. **59:**3130–3133.
38. **Vest, G., D. F. Weber, and C. Sloger.** 1973. Nodulation and nitrogen fixation, p. 353–390. *In* B. E. Caldwell (ed.), Soybeans: improvement, production, and uses. American Society of Agronomy, Madison, Wis.
39. **Vincent, J. M.** 1970. A manual for the practical study of root nodule bacteria. IBP handbook 15. Blackwell Scientific Publications, Oxford, England.
40. **Vincent, J. M.** 1982. The basic serology of rhizobia, p. 13–26. *In* J. M. Vincent (ed.), Nitrogen fixation in legumes. Academic Press, Inc., New York.
41. **Weaver, R. W., and L. R. Frederick.** 1974. Effect of inoculum rate on competitive nodulation of *Glycine max* (L.) Merrill. II. Field studies. Agron. J. **66:**233–236.