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# Characterization of Soybean-Nodulating Rhizobial Communities and Diversity

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## 1. Introduction

Soybean establishes a symbiotic relationship with soybean-nodulating bacteria during the development of its nitrogen-fixing organ, the root nodule. The major soybean-nodulating rhizobia that have been identified are *Bradyrhizobium japonicum*, *Bradyrhizobium elkanii*, and *Sinorhizobium/Ensifer fredii* (Jordan, 1982; Scholla & Elkan, 1984; Kuykendall et al., 1992; Young, 2003). Furthermore, additional species of soybean-nodulating rhizobia have been extensively discussed in the literature owing to the complexity of their taxonomical classification (Xu et al., 1995; Tan et al., 1997; Peng et al., 2002; Hungria et al., 2006; Vinuesa et al., 2008). Soybean-nodulating bacteria are found over wide regions of the world, and their genetic diversity may reflect geographical and climatic differences as well as host diversity. Some strain of *Bradyrhizobium japonicum* effectively fixes atmospheric nitrogen and is expected to be an effective bacterial inoculant. Inoculation of soybean seeds with an effective inoculant can increase soybean yield, but field-indigenous rhizobia frequently compete with the inoculant (Yamakawa et al., 2003). Therefore, analysis of the genetic diversity and field distribution of indigenous soybean-nodulating rhizobia is important to improve our understanding of rhizobial ecology as well as inoculation methodology under various environmental conditions (Fig. 1). Many studies have investigated rhizobial ecology. In case of research for indigenous soybean-nodulating rhizobia in Japan, Sawada et al. (1989) isolated 85 indigenous soybean bradyrhizobia from soybean root nodules sampled from 46 soybean fields in Japan and classified the isolates according to their serotype by using rabbit antisera prepared against USDA strains of *Bradyrhizobium* as serotype antigens. Minamisawa et al. (1999) also characterized Japanese indigenous soybean bradyrhizobia isolated from six fields by analysis of their fingerprints with the hybridization probes RSα, RSβ, *nifDK*, and *hupSL*, and revealed the diversity and endemism of their population structures. Furthermore, phylogenetic comparison of common nod gene was also conducted between isolates in Thailand and Japan (Yokoyama et al., 1996). Their results suggested that bradyrhizobia might diversify in individual fields depending on the associated host plants and local soil conditions. The soybean-nodulating rhizobial community might change depending on the host cultivar and cultivation temperature even in the same field as well as in different fields with geographical, soil texture, soil pH, salinity, and other differences (Howieson & Ballard, 2004). Previously, our research group demonstrated that soybean-nodulating rhizobial communities differed greatly in nearby fields depending on whether

the soil was acidic or basic (Suzuki et al., 2008). Therefore, knowledge of rhizobial ecology and biology in relation to numerous environmental gradients is needed.

Some soybean cultivars possess nodulation regulatory genes known as *Rj* genes, and the genotypes non-*Rj*, $rj_1$, $Rj_2$, $Rj_3$, and $Rj_4$ have been confirmed to exist in nature (Devine & Kuykendall, 1996). A non-nodulating soybean line was identified from a cross between the soybean cultivars Lincoln and Richard, and incompatible nodulation was found to be controlled by a single recessive gene; this genotype was named the $rj_1$ genotype (Williams & Lynch, 1954). Subsequently, several dominant *Rj* genotypes ($Rj_2$, $Rj_3$, and $Rj_4$) were discovered that restrict effective nodule formation in certain *Bradyrhizobium* strains (Caldwell, 1966; Caldwell et al., 1966; Vest, 1970; Vest & Caldwell, 1972). The *Rj* genes play a role in controlling the plant's compatibility with specific rhizobial strains, and indigenous soybean-nodulating rhizobia may display a preference for a certain *Rj* genotype (Ishizuka et al., 1991; Saeki et al., 2000). Therefore, characterization of soybean-nodulating rhizobial communities requires that compatibility and preferences between soybean *Rj* genotypes and soybean-infecting rhizobia be taken into consideration.

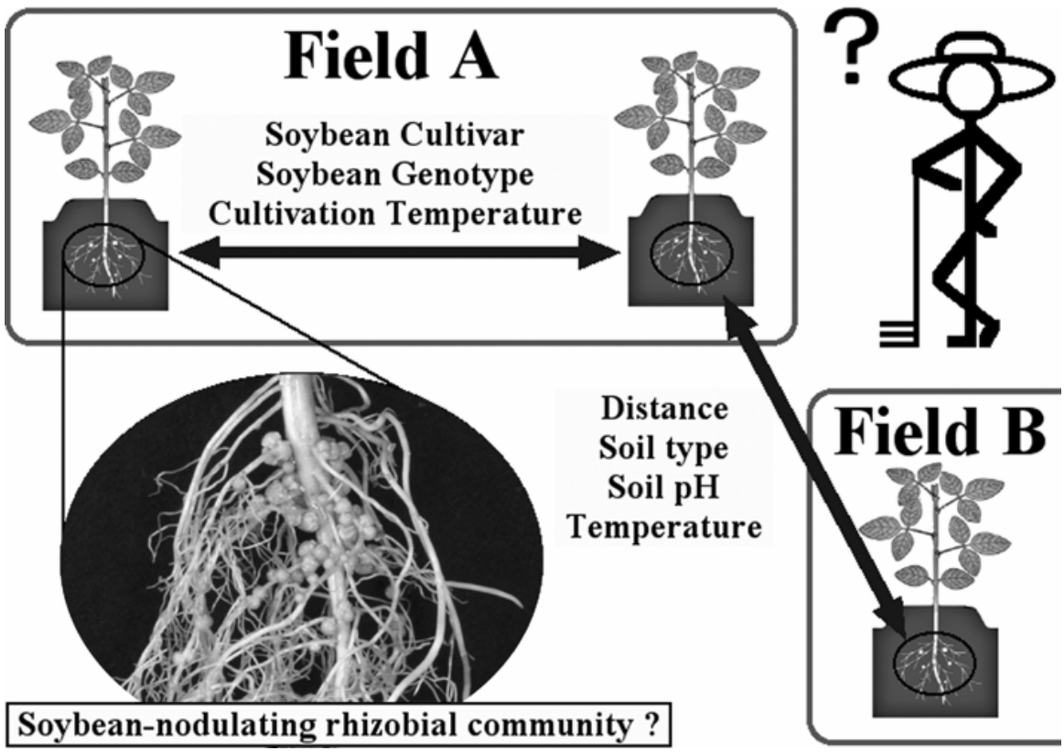


Fig. 1. Schematic representation of the theme of this chapter.

This chapter discusses the analysis of soybean-nodulating bradyrhizobial communities in relation to geographical differences, including latitudinal gradients, and the genotypes of the host soybean cultivars. Analysis of restriction fragment length polymorphisms of polymerase chain reaction (PCR-RFLP) products of the 16S–23S rDNA internal transcribed spacer (ITS) region and mathematical analysis of the PCR-RFLP results are demonstrated as possible approaches to the study of community diversity of soybean-nodulating rhizobia in relation to the *Rj* genotypes of the host plants and rhizobial endemism in Japan.

## 2. Basic methodology for analysis of indigenous soybean-nodulating rhizobia

Many methods using environmental DNA, including denaturing gradient gel electrophoresis (DGGE), terminal RFLP (T-RFLP) analysis, and automated ribosomal intergenic spacer analysis (ARISA), targeting 16S rDNA, the 16S–23S rDNA ITS region, and other genomic and RNA sequences, are developing and available for characterizing bacterial communities. In this section on experimental procedures, a relatively simple and reliable method for the study of indigenous soybean-nodulating bradyrhizobia by isolation procedure from nodules is demonstrated as one approach to the study of bradyrhizobial ecology.

### 2.1 Isolation of indigenous soybean-nodulating rhizobia

Fresh soils for laboratory soybean cultivation were collected from some fields. Since indigenous soybean-nodulating bacteria should be isolated from cultivars with different *Rj* genotypes, the soybean cultivars Akishirome, Bragg, and Orihime (non-*Rj* genotype), Bonminori, CNS, Hardee, and IAC-2 ($Rj_2Rj_3$ genotype), D-51 ($Rj_3$ genotype), and Akisengoku, Fukuyutaka, and Hill ($Rj_4$ genotype) were cultivated in culture pots for 4–5 weeks in our laboratory. The culture pots (volume 1 L) were filled with vermiculite and a N-free nutrient solution (Saeki et al., 2000), at 40% (v/v) water content, and autoclaved at 121 °C for 20 min. A soil sample (2–3 g) was then placed in the vermiculite at 2–3 cm depth. Surface-sterilized soybean seeds (i.e., treated with 70% ethanol for 30 s and sodium hypochlorite solution with 0.25% available chlorine for 3 min) were sown in the soil. After the plants had been cultivated in a greenhouse (an incubation chamber could also be used) for 4–5 weeks, with sterile distilled water supplied weekly, nodules were randomly detached from the roots and sterilized in a 70% ethanol and sodium hypochlorite solution (0.25% available chlorine). Each nodule was washed with sterile distilled water and homogenized in 50–100 μL of sterile 0.9% NaCl or sterile distilled water. The homogenate was streaked onto a yeast extract–mannitol agar (YMA; Vincent, 1970) plate and incubated at 28 °C for 4–6 days. The single colonies were re-streaked on YMA plates containing 0.002% (w/v) bromothymol blue to determine whether the genus of the isolate was *Bradyrhizobium* or *Sinorhizobium*/*Ensifer* (Keyser et al., 1982). This is a simple method for determining the genus of soybean-nodulating rhizobia. After the 4–6 days of incubation, YMA slants were made from single-colony isolates and stored at 4 °C. As a negative control, it was confirmed that soybean plants grown without soil, eliminating the possibility of contamination with soybean-nodulating bacteria, formed no nodules.

### 2.2 Inoculation test

Inoculation tests to determine effective nodule formation were conducted, not only to confirm compatibility of each isolate with the different host soybean cultivars but also to confirm its compatibility with each *Rj* genotype. Culture pots were filled with vermiculite and the N-free nutrient solution described above and sterilized by autoclaving. Soybean seeds were sterilized with a 70% ethanol and diluted sodium hypochlorite solution (0.25% available chlorine). We used soybean cultivars CNS ($Rj_2Rj_3$) and Hill ($Rj_4$) to confirm the compatibility of each isolate with the different *Rj* genotypes for nodulation. The seeds were sown in pots filled with sterilized vermiculite. Each rhizobial isolate was cultured in a yeast extract–mannitol broth (YMB, Vincent, 1970). The culture was then diluted with sterile 0.9% NaCl or sterile distilled water to $10^6$ cells $mL^{-1}$, and a 1-mL aliquot of the diluted bacterial

culture was used to inoculate each of the sown seeds. After 21 days of culture in a greenhouse or an incubation chamber, during which sterile distilled water was supplied weekly, the roots of the soybean plants were checked for effective or ineffective nodule formation. Each combination of *Rj* genotype and isolate was scored as positive (+) if nodule formation was effective and as negative (–) if nodule formation was ineffective or absent. Two or three replicates of each *Rj* genotype–isolate combination were tested. The number of effective nodules per plant or an acetylene reduction assay for measuring nitrogenase activity can be used to characterize the isolates, depending on the purpose of the study.

### 2.3 PCR-RFLP analysis

Total DNA for the PCR template was extracted from an HM culture (Sameshima et al., 2003) of the isolate as described by Hiraishi et al. (1995). Bacteria cells cultured in the HM medium were collected by centrifugation and washed with sterile distilled water. The pellet was suspended in 200 µL sterile distilled water. Then 40 µL of the sample was treated with 50 µL of BL buffer (40 mM Tris-HCl, 1% Tween 20, 0.5% Nonidet P-40, 1 mM EDTA, pH 8.0) and 10 µL of proteinase K (1 mg $mL^{-1}$) and incubated at 60 °C for 30 min. Thereafter, the digested sample was incubated in boiling water or 95 °C for 5 min. The sample was centrifuged at 15,000 × *g* for 10 min to remove undisrupted cells and large debris, and the supernatant was collected with a pipette. The total DNA lysate was adjusted as necessary to obtain an $A_{260}$ value of 3.0, and then 1 µL of the lysate was used directly as the template DNA in a 50-µL PCR reaction mixture. In our study, PCR was carried out with *Ex Taq* DNA polymerase (TaKaRa Bio Incorporated, Otsu, Japan). For ITS amplification, an ITS primer set (BraITS-F: 5′-GACTGGGGTGAAGTCGTAAC-3′, BraITS-R: 5′-ACGTCCTTCATCGCCTC-3′) designed for amplification of the 16S–23S rDNA ITS region of bradyrhizobia (Saeki et al., 2006) was used for the PCR reaction (Fig. 2). The PCR cycle consisted of a pre-run at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 1 min, with a final extension at 72 °C for 10 min. The RFLP analysis of the 16S–23S rDNA ITS region was performed with the restriction enzymes *Hae*III, *Hha*I, *Msp*I, and *Xsp*I. Five microliters of the PCR product was digested with the restriction enzymes at 37 °C for more than 3 h in a 20-µL reaction mixture. The restriction fragments were separated by submerged gel electrophoresis and visualized with ethidium bromide. For amplifications of 16S rDNA, the following primer sets were used (Weisburg et al., 1991): 16SrDNA-F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 16SrDNA-R (5′-AAGGAGGTGATCCAGCC-3′) or 16SrDNA-R2 (5′-CGGCTACCTTGTTACGACTT-3′). For amplifications of 23S-5S rDNA, the following primer set was used (van Berkum et al., 2003; Saeki et al., 2007): 23S-F1 (5′-GRYGGATGCCTTGGC-3′) and 5S-R (5′-GGCAGCGACCTACTCTC-3′). Furthermore, for amplifications of the 16S–23S rDNA ITS region from *Sinorhizobium/Ensifer*, the primer set ITS1512F (5′-GTCGTAACAAGGTAGCCGT-3′) and ITSLS23R (5′-TGCCAAGGCATCCACC-3′), based on Hiraishi et al. (1997) with slight modification for *Sinorhizobium/Ensifer* (Saeki et al., 2005), was used.

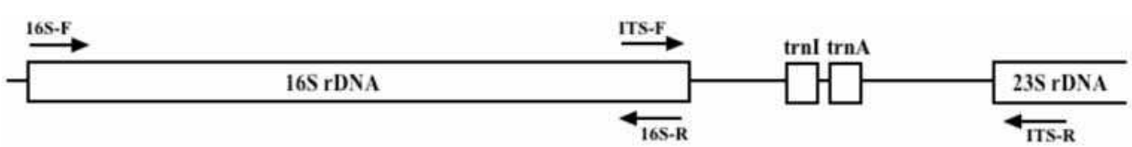


Fig. 2. Schematic representation of the 16S rDNA and 16S–23S rDNA internal transcribed spacer region of *Bradyrhizobium* strains.

### 2.4 Cluster analysis

To construct a dendrogram based on the PCR-RFLP analysis results for soybean-nodulating isolates, the fragment sizes on the electrophoresed gel were calculated by using appropriate fragment size markers and the fragment sizes deduced from the sequences of the reference strains. All detectable and reproducible fragments were used for the cluster analysis, but some irreproducible fragments were excluded. The genetic distance between pairs of isolates (*D*) was calculated with the following equation:

$$D = 1 - [2N_{AB}/(N_A + N_B)], \quad (1)$$

where $N_{AB}$ is the number of RFLP bands shared by strains A and B, and $N_A$ and $N_B$ are the numbers of restriction fragments of each of the two strains (Nei & Li, 1979; Sakai et al., 1998). *D* values were calculated for all pairs of isolates, the cluster analysis was conducted by the unweighted pair group method using the arithmetic average (UPGMA), and a dendrogram was constructed with Phylip software (J. Felsenstein, University of Washington, Seattle, WA, USA).

Although the topology can differ between dendrograms based on reference strain sequence data and those based on RFLP patterns, for analysis of soybean-nodulating bradyrhizobial communities, the dendrogram obtained as described is acceptable, because it is important to classify the numerous isolates into reliable groups based on reference strains. Furthermore, since the topology of a dendrogram can also differ depending on the electrophoretic resolution, fragment detection sensitivity, and analysis accuracy, it is important to classify isolates into appropriate RFLP patterns and clusters by using appropriate reference strains and restriction enzymes. Cluster analysis provides both qualitative and quantitative information for analyses of soybean-nodulating rhizobial communities. If there are multiple copies of rRNA gene operons such as in *Sinorhizobium/Ensifer* soybean-nodulating rhizobia (Kündig et al., 1995), ITS sequences might differ among the copies. In this situation, it is necessary to take the electrophoresed fragment intensity into consideration in a cluster analysis (Saeki et al., 2009).

In the phylogenetic analysis, reference strains were used to classify the isolates, namely, four *Bradyrhizobium* USDA strains (*B. japonicum* USDA 6$^T$, 110, and 124, and *B. elkanii* USDA 76$^T$) were used in the RFLP analysis of the 16S rDNA, and eleven *Bradyrhizobium* USDA strains (*B. japonicum* USDA 4, 6$^T$, 38, 110, 115, 123, 124, and 135, and *B. elkanii* USDA 46, 76$^T$, and 94) were used in the RFLP analysis of the 16S–23S rDNA ITS region. Their selection is described below (Saeki et al., 2004).

### 2.5 Reference strains

To characterize indigenous bradyrhizobia in detail, a reasonable method for classification and grouping using reliable reference strains should be applied. The serotype strains *B. japonicum* USDA 4, 6$^T$, 38, 62, 110, 115, 122, 123, 124, 125, 127, 129, and 135, and *B. elkanii* USDA 31, 46, 76$^T$, 94, and 130 are generally used for classification of bradyrhizobia (van Berkum & Fuhrmann, 2000). Recently, the sequence of the ITS region between the 16S and 23S rRNA genes are applied for the classification of bradyrhizobia (van Berkum & Fuhrmann, 2000; Willems et al., 2001; Willems et al., 2003). We have also used the PCR-RFLP analysis of the 16S–23S rDNA ITS region to group isolates of soybean-nodulating rhizobia for characterizing soybean-nodulating rhizobial communities (Saeki et al., 2004, 2006). In our previous study, five restriction enzymes, *Hae*III, *Hha*I, *Msp*I, *Xsp*I, and *Hin*dIII, were used to

classify *Bradyrhizobium* USDA serotype strains into four 16S rDNA types (*B. japonicum* USDA 6$^T$, 110, and 124, and *B. elkanii* USDA 76$^T$) and 11 ITS types (*B. japonicum* USDA 4, 6$^T$, 38, 110, 115, 123, 124, and 135, and *B. elkanii* USDA 46, 76$^T$, and 94) for use as reference strains (Saeki et al., 2004). Thus, the restriction enzymes *Hae*III, *Hha*I, *Msp*I, and *Xsp*I can be reasonably used with these 11 reference strains for grouping bradyrhizobia (Fig. 3).

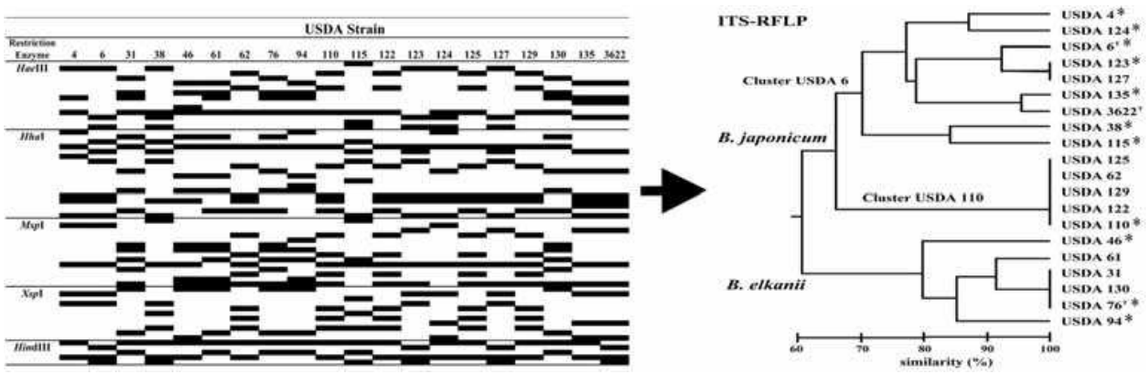


Fig. 3. Construction of a dendrogram from the results of PCR-RFLP analysis of the 16S–23S rDNA ITS region of *Bradyrhizobium* USDA serotype strains (modified from Saeki et al., 2004). *Selected as reference strains for the PCR-RFLP analysis.

## 3. Analysis of indigenous bradyrhizobial communities

In this section, the results of analyses of indigenous bradyrhizobial communities isolated from Japanese soil are presented, and mathematical approaches to the analysis of soybean-nodulating bradyrhizobial community diversity are demonstrated.

### 3.1 Grouping of indigenous bradyrhizobia in Japan

The *Rj* genes are the nodulation regulatory genes in soybean, and the genotypes non-*Rj*, *rj*$_1$ *Rj*$_2$, *Rj*$_3$, and *Rj*$_4$ have been confirmed to exist in nature (Devine & Kuykendall, 1996). Specific rhizobial strains can be incompatible with soybean cultivars harboring a particular *Rj* gene. In addition, indigenous soybean-nodulating rhizobia may show a preference for particular genotypes among the compatible genotypes, even among soybean plants cultivated in soil samples from the same field (Ishizuka et al., 1991; Saeki et al., 2000, 2005). It is therefore important to use soybean cultivars with different *Rj* genotypes for both isolating and analyzing indigenous soybean-nodulating rhizobia.

In this section, the results of phylogenetic analysis of indigenous soybean-nodulating rhizobia based on PCR-RFLP analysis of the 16S–23S rDNA ITS region using the *Bradyrhizobium* reference strains *B. japonicum* (USDA 4, 6$^T$, 38, 110, 115, 123, 124, and 135) and *B. elkanii* (USDA 46, 76$^T$, and 94) are presented, and the diversity and endemism of soybean-nodulating rhizobia in Japan are discussed.

Weakly acidic soils for soybean cultivation were collected from experimental fields in five regions of Japan (Saeki et al., 2006): Memuro, Hokkaido (Hokkaido site); Arai, Fukushima (Fukushima site); Ayabe, Kyoto (Kyoto site); Gakuen-Kibanadai-Nishi, Miyazaki (Miyazaki site); and Nishihara, Okinawa (Okinawa site). Four soils (from the Hokkaido, Fukushima, Kyoto, and Miyazaki sites) were Andosols, and that from the Okinawa site was Acrisol. A total of 300 isolates were derived from combinations of the three *Rj* genotypes of the

soybean cultivars, Akishirome (non-*Rj*), CNS (*Rj*$_2$*Rj*$_3$), and Fukuyutaka (*Rj*$_4$), and the five field sites. A dendrogram based on the electrophoretic patterns obtained with four restriction enzymes, *Hae*III, *Hha*I, *Msp*I, and *Xsp*I, revealed 22 RFLP patterns. A similarity of 86%, which is the maximum similarity required to distinguish USDA 38 and 115, the most closely related RFLP patterns among the reference strains used in this study, was applied as the criterion for differentiating clusters for constructing the dendrogram. As a result, a dendrogram with 11 clusters was obtained (Fig. 4).

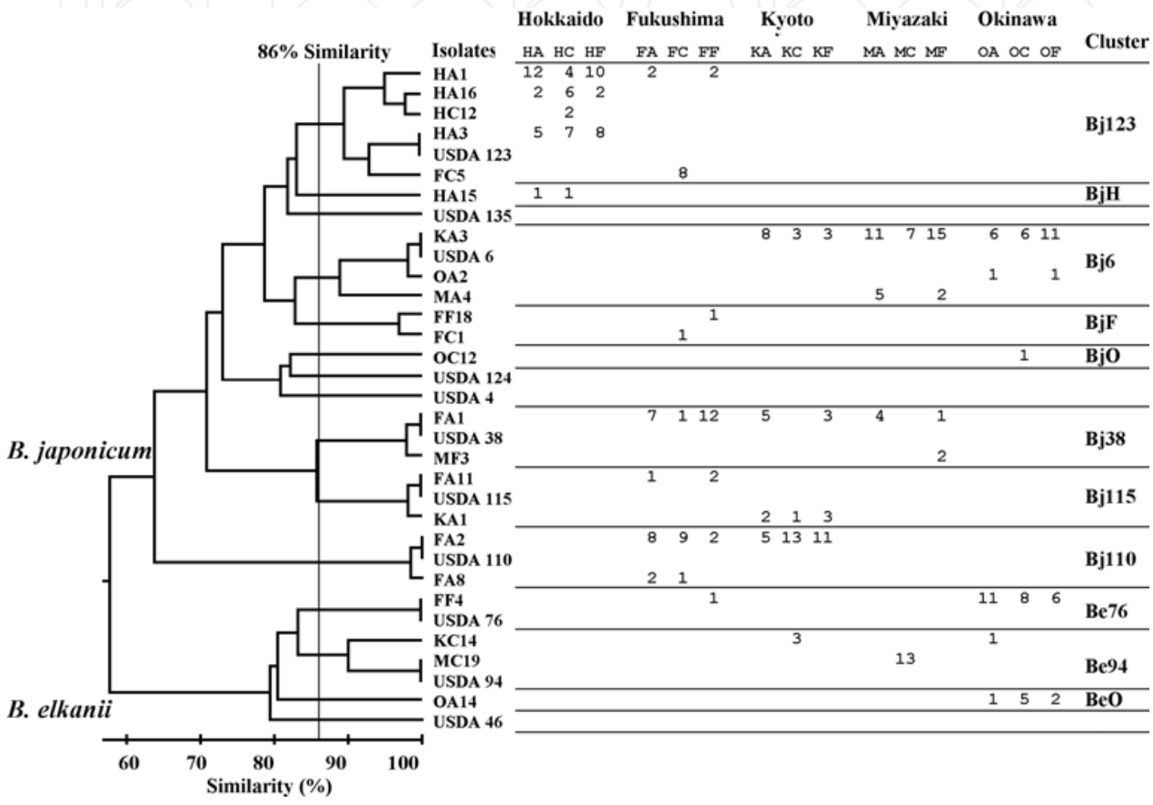


Fig. 4. Dendrogram based of the PCR-RFLP pattern obtained with four restriction enzymes of 16S–23S rDNA ITS region of indigenous bradyrhizobia in Japan (Saeki et al., 2006). The first letter of each abbreviation indicates the field site (H, Memuro, Hokkaido; F, Arai, Fukushima; K, Ayabe, Kyoto; M, Kibanadai, Miyazaki; O, Nishihara, Okinawa) and the second letter indicates the host soybean cultivar (A, Akishirome [non-*Rj*]; C, CNS [*Rj*$_2$*Rj*$_3$]; F, Fukuyutaka [*Rj*$_4$]). The numbers of each isolate obtained from each site–cultivar combination is shown.

Isolates in this study were designated as belonging to the genus *Bradyrhizobium* on the basis of their ability to form nodules on soybean roots, alkaline production on YMA medium, and the PCR-RFLP analysis results. Among the 11 clusters, Bj6, Bj123, Bj110, Bj38, and Be76 were isolated most frequently, followed by Be94, Bj115, and the other clusters. The RFLP patterns of these seven major clusters were similar or identical to *B. japonicum* USDA 6$^T$, 38, 110, 115, and 123, and *B. elkanii* USDA 76$^T$ and 94. The distribution and abundance of the major clusters of indigenous bradyrhizobia at each field site suggest that the dominant clusters from north to south were Bj123, Bj110, Bj6, and Be76 (Fig. 5).

### 3.2 Diversity and equitability indices at each field site

To estimate the diversity and equitability of the soybean-nodulating bradyrhizobial communities at each field site, Shannon's diversity index ($H'$) and equitability ($E_H$) were calculated for each field site. Shannon's diversity index was calculated with Equation (2) (MacArthur, 1965; Pielou, 1969), and equitability with Equation (3) (Pielou, 1969):

$$H' = -\sum Pi \ln Pi , \tag{2}$$

$$E_H = H' / \ln S. \tag{3}$$

The $Pi$ is the dominance of the isolate, expressed as ($ni$/$N$), where $N$ and $ni$ are the total number of isolates tested and the number of isolates belonging to a particular RFLP pattern or cluster, respectively. $S$ is the total number of RFLP patterns or clusters, indicating the taxonomic group, at each field site.

As shown in Figs. 4 and 5, the flora of indigenous soybean rhizobia changed gradually from north to south, and a distinctive flora was detected at each field site. At the Hokkaido site most isolates were classified into cluster Bj123, showing low equitability of clusters, and the proportion of isolates in cluster Bj123 decreased southward. In Fukushima and Kyoto sites,

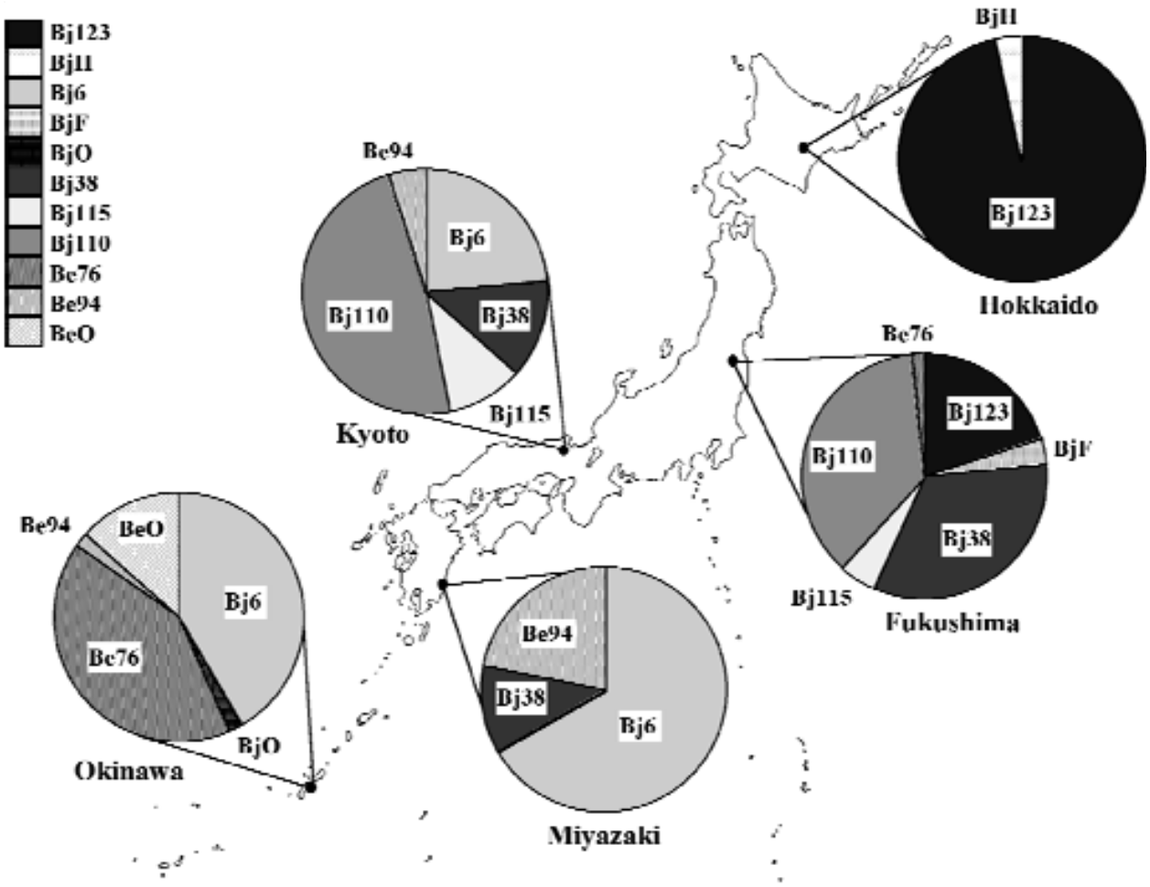


Fig. 5. Distribution of clusters of indigenous bradyrhizobia in Japan (Saeki et al., 2006). The sites are at the following latitudes: Okinawa, 26° 15'N; Miyazaki, 31° 49'N; Kyoto, 35° 17'N; Fukushima, 37° 42'N; and Hokkaido, 42° 53'N.

| | No. of RFLP patterm | | No. of Cluster | | Index* | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | RFLP pattern | | Cluster | |
| Field site | Bj | Be | Bj | Be | Diversity ($H'$) | Equitability ($E_H$) | Diversity ($H'$) | Equitability ($E_H$) |
| Hokkaido | 5 | - | 2 | - | 1.25 | 0.78 | 0.15 | 0.21 |
| Fukushima | 8 | 1 | 5 | 1 | 1.68 | 0.77 | 1.39 | 0.77 |
| Kyoto | 4 | 1 | 4 | 1 | 1.34 | 0.83 | 1.34 | 0.83 |
| Miyazaki | 4 | 1 | 2 | 1 | 1.23 | 0.77 | 0.85 | 0.78 |
| Okinawa | 3 | 3 | 2 | 3 | 1.25 | 0.7 | 1.13 | 0.71 |

Table 1. Diversity index and equitability of RFLP patterns and clusters at each field site (Saeki et al., 2006).
*The indices were calculated from the RFLP patterns or clusters, which were used as the operational taxonomic unit (OTU), and the number of isolates.

both the diversity index and cluster equitability were high, owing to higher variation in *B. japonicum* (Table 1). On the other hand, the frequency of isolation of *B. elkanii* increased southward, and the proportion of *B. elkanii* isolates was highest at the Okinawa site among the five field sites, although the diversity index and equitability at that site were not different from those in central Japan. Four minor clusters, BjH, BjF, BjO, and BeO, did not belong to the clusters of the reference strains used in this study. Even though the isolates in these minor clusters might belong to other, independent clusters, the relative proportions of the clusters overall would not change drastically because of their low frequency of isolation.
These results suggest that the genetic diversity of indigenous *B. japonicum* is higher in central Japan, where variations of RFLP patterns and clusters for *B. japonicum* were higher, whereas the genetic diversity of *B. elkanii* is higher at the Okinawa site. Furthermore, the dominant clusters of indigenous bradyrhizobia in Japan from north to south were Bj123, Bj110, Bj6, and Be76 in this study, suggesting that environmental factors such as temperature or vegetation type may influence the localization of indigenous soybean-nodulating bacterial communities.

### 3.3 Polar ordination of community diversity and latitude

These PCR-RFLP data may also provide important information for mathematical analysis of the geographical distributions of indigenous soybean-nodulating bradyrhizobia. The RFLP pattern diversity results were analyzed mathematically by comparing percentage differences between pairs of soybean-nodulating indigenous bradyrhizobial communities and by using a polar ordination analysis (Whittaker, 1967) to examine the geographical distributions of soybean-nodulating bradyrhizobia in Japan.
To characterize the relationship between the diversities of bradyrhizobial communities based on the RFLP patterns of bradyrhizobia from a soybean cultivar with a particular *Rj* genotype and grown on a soil sample at a specific latitude (°N), the percentage differences (*PD*) between the bradyrhizobial communities were used to construct a trigonometric diagram. Then, polar differences were calculated from the trigonometric diagram and plotted against the latitudinal difference between sites (Fig. 6; Kobayashi, 1995; Whittaker, 1967).
The percentage difference (*PD*) was calculated as follows:

$$PD = 50\sum |Pi_{\mathrm{A}} - Pi_{\mathrm{B}}|, \tag{4}$$

where $Pi_{\mathrm{A}}$ and $Pi_{\mathrm{B}}$ are the dominance of the isolates, expressed as ($n_i/N$), in communities A and B, respectively. Simultaneous equations were constructed from the trigonometric figure

by using the Pythagorean theorem (Fig. 6, left). Parameter $x$ represents the polar difference (%) from the 0% pole and is calculated as follows (Whittaker, 1967):

$$x = (l^2 + d_1^2 - d_2^2) / 2 \cdot l, \quad (5)$$

where $d_1$ and $d_2$ are the percentage differences between a particular bradyrhizobial community and the communities from cultivar CNS at Okinawa and cultivar Akishirome at Hokkaido, respectively. Parameter $l$ represents a 100% polar difference between the poles (communities A and B). Then, the polar differences calculated from the percentage differences were plotted on the $y$-axis, and differences in latitude between the field sites were plotted on the $x$-axis (Fig. 6, right). Because among combinations of the three hosts, Akishirome, CNS, and Fukuyutaka, the differences were greatest between Akishirome and CNS (Table 2), bradyrhizobial communities isolated from CNS at the Okinawa site (26° 15′N, the southernmost site) and from Akishirome at the Hokkaido site (42° 53′N, the northernmost site) were used as the poles (i.e., communities A and B).

The results of the polar ordination analysis showed that indigenous soybean-nodulating bradyrhizobial community composition was correlated with latitude (Fig. 6). This result suggests that the community of indigenous bradyrhizobia at a particular geographical location might be affected by soil temperature or the diversity of the associated host plants acclimatized to that region's climate. Keyser et al. (1984) examined the distribution of indigenous soybean-nodulating rhizobia in the United States and found that serogroup 123 was dominant in northern regions and *B. elkanii* in southern regions. Other studies support these results, with some exceptions in the case of alkaline soils, in which *Bradyrhizobium* serogroup 135 or *Sinorhizobium/Ensifer fredii* predominate (Damirgi et al., 1967; Ham et al., 1971; Kowalski et al., 1974; Gross et al., 1979; Suzuki et al., 2008). Further studies are still needed, however, to examine the many environmental factors potentially affecting indigenous bradyrhizobia.

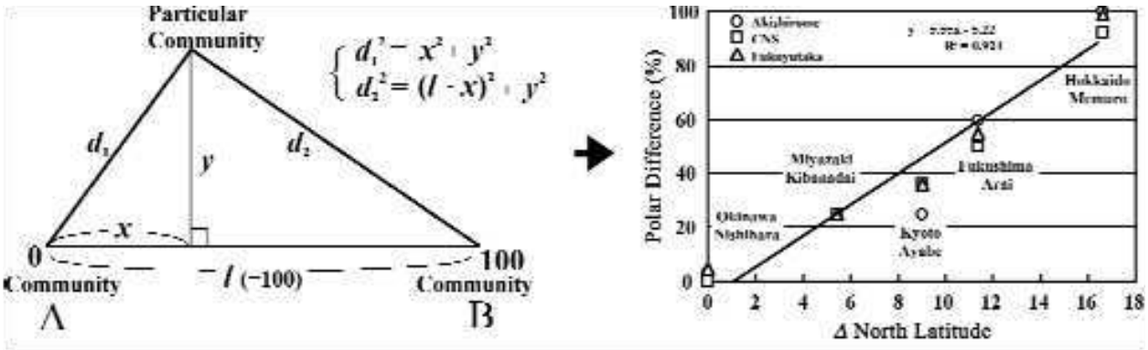


Fig. 6. Calculation of the polar difference (%) (left), and the relationship between polar differences in soybean-nodulating bradyrhizobial communities isolated from soybean cultivars with three different *Rj* genotypes (Akishirome, CNS, and Fukuyutaka) and the latitude of the field sites, with 0° used for the latitude of the Okinawa site (Saeki et al., 2008) (right).

### 3.4 Analysis of bradyrhizobial community diversity

Shannon's diversity index $H'$, Equation (2), was used to quantify the diversity of the bradyrhizobial communities (MacArthur, 1965; Pielou, 1969).

To quantify differences in the composition of bacterial communities among three host soybean cultivars with different *Rj* genotypes, Akishirome (non-*Rj*), CNS (*Rj*$_2$*Rj*$_3$), and Fukuyutaka (*Rj*$_4$), by pair-wise comparison (i.e., Akishirome–CNS, CNS–Fukuyutaka, and Fukuyutaka–Akishirome), alpha ($H'_\alpha$), beta ($H'_\beta$), and gamma ($H'_\gamma$) diversity indices were calculated (Kobayashi, 1995; Whittaker, 1972) (Fig. 7). In a comparison between two bradyrhizobial communities from two different host cultivars, alpha diversity is the weighted average of the diversity indices ($H'$) of the two bradyrhizobial communities, gamma diversity is the diversity index of the combination of the two bradyrhizobial communities, and beta diversity is the difference between the two bradyrhizobial communities (Whittaker, 1972):

$$H'_\beta = H'_\gamma - H'_\alpha. \quad (6)$$

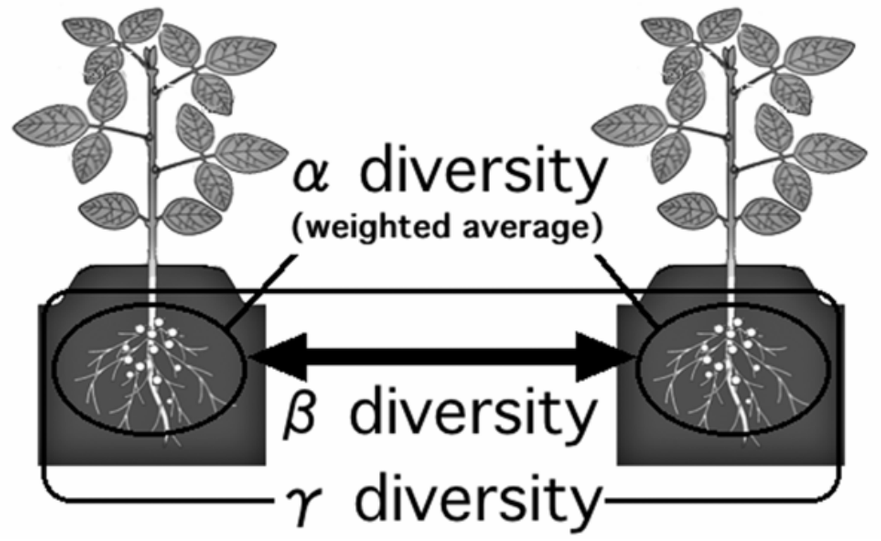


Fig. 7. Schematic representation of alpha, beta, and gamma diversities of soybean-nodulating rhizobial communities.

For this analysis, the RFLP patterns, the number of isolates belonging to each RFLP pattern, and three cultivars with three different genotypes (A, C, and F, Fig. 4) were used, and the results are shown in Table 2. To facilitate comparison of the compositional differences among the bacterial communities, ratios of beta diversity to gamma diversity ($H'_\beta$/ $H'_\gamma$) were used, in order to take into account the differences in gamma diversity in each pair-wise comparison of bacterial communities.

| | Field site and host combination | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Hokkaido | | | Fukushima | | | Kyoto | | | Miyazaki | | | Okinawa | | |
| Diversity | HA-HC | HC-HF | HF-HA | FA-FC | FC-FF | FF-FA | KA-KC | KC-KF | KF-KA | MA-MC | MC-MF | MF-MA | OA-OC | OC-OF | OF-OA |
| α diversity ($H'_\alpha$) | 1.23 | 1.19 | 0.99 | 1.26 | 1.24 | 1.32 | 1.14 | 1.09 | 1.24 | 0.82 | 0.74 | 0.91 | 1.18 | 1.15 | 1.10 |
| β diversity ($H'_\beta$) | 0.12 | 0.11 | 0.03 | 0.28 | 0.47 | 0.14 | 0.22 | 0.12 | 0.02 | 0.39 | 0.26 | 0.08 | 0.09 | 0.07 | 0.06 |
| γ diversity ($H'_\gamma$) | 1.35 | 1.30 | 1.02 | 1.54 | 1.71 | 1.46 | 1.36 | 1.21 | 1.26 | 1.21 | 0.99 | 0.99 | 1.28 | 1.22 | 1.16 |

Table 2. Alpha, beta, and gamma diversity indices of bradyrhizobial communities isolated from three soybean cultivars with different *Rj* genotypes by pair-wise comparisons (Saeki et al., 2008).

The operational taxonomic unit (OTU) used was the particular RFLP pattern obtained by PCR-RFLP analysis of the 16S–23S rDNA ITS region using *Hae*III, *Hha*I, *Msp*I, and *Xsp*I.

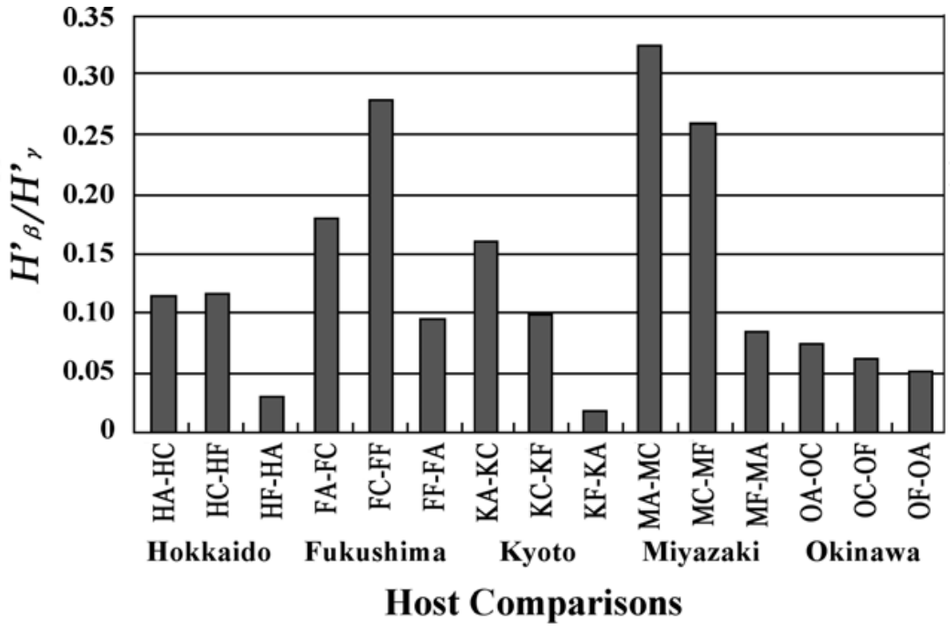


Fig. 8. Ratios of beta diversity, an indicator of pair-wise differences between bacterial communities, to gamma diversity, an indicator of the total diversity of two combined communities, in bradyrhizobia isolated from soybean cultivars with three different genotypes (Saeki et al., 2008).

Differences in the degree of beta diversity, expressed by $H'_{\beta}/ H'_{\gamma}$, between pairs of bradyrhizobial communities isolated from the three soybean genotypes and the five sites are shown in Fig. 8. $H'_{\beta}/ H'_{\gamma}$, which represents compositional differences in both RFLP patterns and the isolation ratios (*Pi*) of rhizobial communities, was lowest at every site in comparisons of Akishirome (non-*Rj*) and Fukuyutaka (*Rj*$_4$ genotype) cultivars, and twofold to ninefold higher in comparisons of CNS (*Rj*$_2$*Rj*$_3$ genotype) and cultivars with other genotypes at every site except Okinawa (Fig. 8). Thus, based on the degree of beta diversity, the composition of bradyrhizobial communities isolated from *Rj*$_2$*Rj*$_3$ genotype cultivars differ in either the relative proportions of the isolates or in the strains of soybean-nodulating bacteria represented compared with the bradyrhizobial communities associated with the other *Rj* genotypes.

The results of the inoculation tests to determine the compatibility of the isolates with the various *Rj* genotypes showed that even isolates belonging to the same OTU showed differences in compatibility (Table 3). At the Hokkaido site, isolates that were incompatible with the *Rj*$_2$*Rj*$_3$ CNS cultivar had RFLP patterns Bj123-HA1 and Bj123-HA3. In addition, isolates with the identical RFLP pattern showed different compatibilities with the same *Rj* genotype at the Hokkaido, Fukushima, Kyoto, and Okinawa sites. These results suggest that diversities within the 16S–23S rDNA ITS region alone cannot completely explain the observed compatibilities between *Rj* genotypes and bradyrhizobial isolates. Moreover, although *B. japonicum* USDA 122 has a nearly identical ITS sequence to that of *B. japonicum* USDA 110 and is classified within the *B. japonicum* USDA 110 cluster (Saeki et al., 2004), it is

well known that the compatibilities of these strains with *Rj* genotypes differ: USDA 110 is compatible with genotypes $Rj_2$, $Rj_3$, and $Rj_4$, whereas USDA 122 is incompatible with soybean cultivars with the $Rj_2$ genotype.

Thirteen isolates from the Miyazaki site having an RFLP pattern identical to that of *B. elkanii* USDA 94 were incompatible with the $Rj_4$ genotype, the only isolates from the five sites incompatible with that genotype. These bradyrhizobia were isolated solely from the $Rj_2Rj_3$ genotype CNS cultivar, although they were also compatible with non-*Rj* genotype soybeans for nodulation. This result suggests that bradyrhizobia incompatible with $Rj_4$ genotype cultivars tend to infect $Rj_2Rj_3$ genotype soybean cultivars. By contrast, isolates incompatible with $Rj_2Rj_3$ genotype cultivars were isolated from both non-*Rj* and $Rj_4$ genotype soybean cultivars, suggesting that bradyrhizobia incompatible with $Rj_2Rj_3$ genotype soybeans do not show a specific nodulation preference for $Rj_4$ genotype soybeans.

| | Field site | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Hokkaido | | | Fukushima | | | Kyoto | | | Miyazaki | | | Okinawa | | |
| | Compatibility with $Rj_2Rj_3$ and $Rj_4$ for effective nodule formation* | | | | | | | | | | | | | | |
| OTUs | ++ | −+ | +− | ++ | −+ | +− | ++ | −+ | +− | ++ | −+ | +− | ++ | −+ | +− |
| Bj123-HA1 | 20 | 6 | - | 4 | - | - | | | | | | | | | |
| Bj123-HA16 | 10 | - | - | | | | | | | | | | | | |
| Bj123-HC12 | 2 | - | - | | | | | | | | | | | | |
| Bj123-HA3 | 17 | 3 | - | | | | | | | | | | | | |
| Bj123-FC5 | | | | 8 | - | - | | | | | | | | | |
| BjH-HA15 | 2 | - | - | | | | | | | | | | | | |
| Bj6-KA3 | | | | | | | 13 | 1 | - | 33 | - | - | 21 | 2 | - |
| Bj6-OA2 | | | | | | | | | | | | | - | 2 | - |
| Bj6-MA4 | | | | | | | | | | 7 | - | - | | | |
| BjF-FF18 | | | | 1 | - | - | | | | | | | | | |
| BjF-FC1 | | | | 1 | - | - | | | | | | | | | |
| BjO-OC12 | | | | | | | | | | | | | 1 | - | - |
| Bj38-FA1 | | | | 19 | 1 | - | 8 | - | - | 5 | - | - | | | |
| Bj38-MF3 | | | | | | | | | | 2 | - | - | | | |
| Bj115-FA11 | | | | - | 3 | - | | | | | | | | | |
| Bj115-KA1 | | | | | | | 6 | - | - | | | | | | |
| Bj110-FA2 | | | | 18 | 1 | - | 29 | - | - | | | | | | |
| Bj110-FA8 | | | | 3 | - | - | | | | | | | | | |
| Be76-FF4 | | | | 1 | - | - | | | | | | | 25 | - | - |
| Be94-KC14 | | | | | | | 3 | - | - | | | | 1 | - | - |
| Be94-MC19 | | | | | | | | | | - | - | 13 | | | |
| BeO-OA14 | | | | | | | | | | | | | 8 | - | - |

Table 3. Isolates showing compatibility or incompatibility with soybean cultivars with $Rj_2Rj_3$ or $Rj_4$ genotypes (Saeki et al., 2008).

*Compatibility scores: ++, compatible with both $Rj_2Rj_3$ and $Rj_4$ genotype soybean cultivars; –+, incompatible with the $Rj_2Rj_3$ genotype soybean cultivar CNS; +–, incompatible with the $Rj_4$ genotype soybean cultivar Hill; –, no such isolates detected. OTU, operational taxonomic unit.

One reason for the different composition of bradyrhizobial communities isolated from $Rj_2Rj_3$ genotype cultivars, as shown in Fig. 8, might be the differences in nodulation compatibility of the various isolates with the *Rj* genotypes (Table 3). However, because the degree of beta diversity between the cultivars Fukuyutaka ($Rj_4$) and Akishirome (non-*Rj*) was lower than that between CNS ($Rj_2Rj_3$) and cultivars with the other genotypes, even at the Miyazaki site, where bradyrhizobia incompatible with the $Rj_4$ genotype were found, these nodulation differences cannot be explained by compatibility differences alone.

Although the isolates associated with each *Rj* genotype of soybean cultivars varied among the field sites, isolates from the $Rj_2Rj_3$ genotype soybean cultivar CNS tended to show different community structures compared with those of isolates from soybean cultivars of the other two genotypes at each field site in the present study. However, because the mechanism determining the preference for particular *Rj* genotypes of a given isolate is not yet clear, future studies should examine the mechanisms of nodulation compatibility and preference between *Rj* genotypes and bradyrhizobia to elucidate these complicated biological relationships.

In this section, the nodulation preferences of specific bradyrhizobia for *Rj* genotypes were estimated for only three bradyrhizobial communities of isolates from three soybean cultivars (one each of non-*Rj*, $Rj_2Rj_3$, and $Rj_4$ genotypes). These results suggest that the *Rj* gene of the cultivar might affect not only the nodulation compatibility between the *Rj* genotype of the soybeans and the bradyrhizobial strain but also bradyrhizobial nodulation preferences that depend on soil conditions. Further analyses of bradyrhizobial communities isolated from several cultivars of each *Rj* genotype must be conducted to estimate more accurately the nodulation preferences of isolates among different *Rj* genotypes.

### 3.5 Diversity among rhizobial communities within identical or different genotypes

In this section, soybean-nodulating bradyrhizobial communities are compared among cultivars or *Rj* genotypes.

$Rj_2Rj_3Rj_4$ genotype lines bred by crossing the IAC-2 ($Rj_2Rj_3$) and Hill ($Rj_4$) cultivars have been used to investigate the preference of these *Rj* genotypes for various bradyrhizobia strains (Ishizuka et al., 1993; Yamakawa et al., 1999). Because the $Rj_2$, $Rj_3$, and $Rj_4$ genes are associated with phenotypes that restrict nodulation by some bradyrhizobia, the $Rj_2Rj_3Rj_4$ lines were expected to be more suitable for inocula of bradyrhizobia with high nitrogen-fixing ability. Yamakawa et al. (2003) reported that the preference of the $Rj_2Rj_3Rj_4$ lines for serogroup *B. japonicum* USDA 110 was insufficiently high to rule out competition with other serogroups, though the $Rj_2Rj_3Rj_4$ lines were superior to other *Rj* genotypes in inoculation efficiency of the strain USDA 110. Although an effective inoculation technique that results in effective nodule formation for *Rj* genotype cultivars is needed, there is still limited information regarding compatibility, competition, and preferences of *Rj* genotype soybean cultivars for bradyrhizobia. To investigate further the nodulation tendencies of soybean-nodulating bradyrhizobia on different *Rj* genotype soybean cultivars, indigenous bradyrhizobia isolated from Fukushima soil, which had the highest diversity index among the five field sites (Table 1) were analyzed using 13 soybean cultivars, each with one of five *Rj* genotypes (non-*Rj*, $Rj_2Rj_3$, $Rj_3$, $Rj_4$, or $Rj_2Rj_3Rj_4$). The 260 isolates were analyzed by PCR-RFLP of the ITS region, and a dendrogram was constructed to classify the isolates into clusters. The soybean cultivars used were Akishirome, Bragg, and Orihime (non-*Rj*); CNS, Hardee, and IAC-2 ($Rj_2Rj_3$); D-51 ($Rj_3$); Akisengoku, Fukuyutaka, and Hill ($Rj_4$); and A-250-3, B349, and C242 ($Rj_2Rj_3Rj_4$) (Yamakawa et al., 1999). A dendrogram was constructed and the number of isolates in each OTU was determined to characterize the diversity of the bradyrhizobial communities obtained from each *Rj* genotype cultivar (Fig. 9). Shannon's diversity index (MacArthur, 1965; Pielou, 1969) was then used to estimate the diversity of the bradyrhizobial communities isolated from each host soybean cultivar, which was further characterized by using the alpha, beta, and gamma diversity indices of each cultivar pair (Table 4).

Differences in bacterial community composition were compared among genotypes by pair-wise comparison of $H'_{\beta}$/ $H'_{\gamma}$ ratios, with significant differences estimated with the Tukey–Kramer test ($P < 0.05$) (Fig. 10).

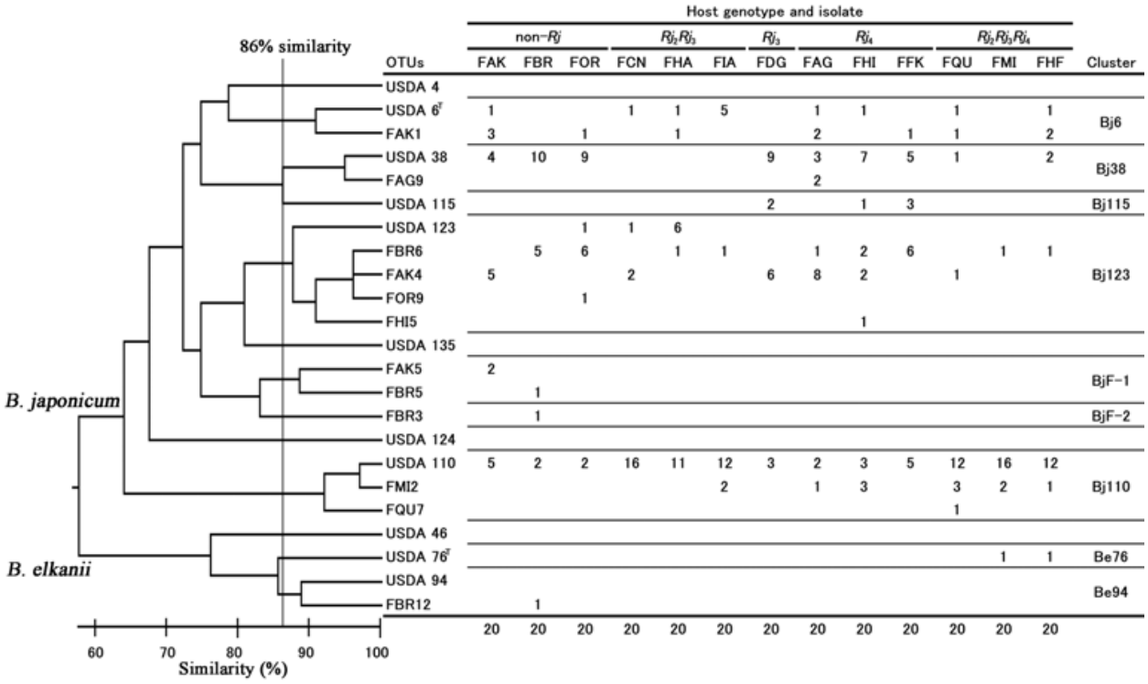


Fig. 9. Dendrogram based on RFLP patterns of the 16S–23S rDNA internal transcribed spacer region (Minami et al., 2009). The similarity between *B. japonicum* USDA 38 and USDA 115 (86%) was used as the criterion to differentiate clusters. Cluster designations are shown on the right. The numbers indicate the number of isolates in each OTU for all soil–host soybean combinations. Isolated bradyrhizobia were numbered for each host soybean cultivar as follows: Akishirome, FAK1-20; Bragg, FBR1-20; Orihime, FOR1-20; CNS, FCN1-20; Hardee, FHA1-20; IAC-2, FIA1-20; D-51, FDG1-20; Akisengoku, FAG1-20; Fukuyutaka, FFK1-20; Hill, FHI1-20; A-250-3, FQU1-20; B349, FMI1-20; and C242, FHF1-20.

| | Pair-wise comparison among bradyrhizobial communities from *Rj*-genotype soybeans | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | non-*Rj* | *Rj*$_2$*Rj*$_3$ | *Rj*$_4$ | *Rj*$_2$*Rj*$_3$*Rj*$_4$ | non-*Rj* | non-*Rj* | non-*Rj* | non-*Rj* | *Rj*$_2$*Rj*$_3$ | *Rj*$_2$*Rj*$_3$ | *Rj*$_2$*Rj*$_3$ | *Rj*$_3$ | *Rj*$_3$ | *Rj*$_4$ |
| Diversity | | | | | *Rj*$_2$*Rj*$_3$ | *Rj*$_3$ | *Rj*$_4$ | *Rj*$_2$*Rj*$_3$*Rj*$_4$ | *Rj*$_3$ | *Rj*$_4$ | *Rj*$_2$*Rj*$_3$*Rj*$_4$ | *Rj*$_4$ | *Rj*$_2$*Rj*$_3$*Rj*$_4$ | *Rj*$_2$*Rj*$_3$*Rj*$_4$ |
| α diversity ($H'_{\alpha}$) | 1.35 | 0.76 | 1.38 | 0.73 | 1.06 | 1.29 | 1.36 | 1.04 | 1.00 | 1.07 | 0.75 | 1.31 | 0.98 | 1.06 |
| β diversity ($H'_{\beta}$) | 0.12 | 0.06 | 0.05 | 0.07 | 0.31 | 0.11 | 0.09 | 0.32 | 0.35 | 0.22 | 0.08 | 0.06 | 0.35 | 0.25 |
| γ diversity ($H'_{\gamma}$) | 1.47 | 0.82 | 1.43 | 0.80 | 1.36 | 1.40 | 1.46 | 1.36 | 1.35 | 1.29 | 0.83 | 1.37 | 1.33 | 1.30 |

Table 4. Alpha, beta, and gamma diversity indices calculated by pair-wise comparison of bradyrhizobial communities isolated from different *Rj* genotype soybean cultivars (Minami et al., 2009).

The OTU used was the particular cluster obtained by PCR-RFLP analysis of the 16S–23S rDNA ITS region using *Hae*III, *Hha*I, *Msp*I, and *Xsp*I.

The major soybean-nodulating rhizobia are *B. japonicum*, *B. elkanii*, and *Sinorhizobium*/*Ensifer fredii*. As soybean-nodulating sinorhizobia will inhabit alkaline soils exclusively (Saeki et al.,

2005; Suzuki et al., 2008), and because the soils sampled in this study were all acidic, it is reasonable to assume that all isolates in the present study belonged to genus *Bradyrhizobium*, and that most were *B. japonicum*, as suggested by the soybean root nodulation, alkaline production on YMA medium, and the PCR-RFLP analysis results.

Bradyrhizobial community diversities, determined by pair-wise comparison, among soybean cultivars of each *Rj* genotype are shown in Table 4 and Fig. 10. Pairs with the same *Rj* genotype showed no significant differences in bradyrhizobial communities (Fig. 10), suggesting that different soybean cultivars with the same *Rj* gene can be expected to have a similar communities of nodulating indigenous bradyrhizobia. In comparisons between different *Rj* genotypes, soybean cultivars with non-*Rj*, $Rj_3$, and $Rj_4$ genotypes also had similar bradyrhizobial community compositions. In contrast, in general agreement with the findings of our previous study (Saeki et al., 2008), significant differences were generally detected in pair-wise comparisons between $Rj_2$ and the other *Rj* genotypes. Bj110 cluster isolates accounted for more than 68% of isolates in the rhizobial communities of soybean cultivars with $Rj_2Rj_3$ or $Rj_2Rj_3Rj_4$ genotypes, whereas the proportions of Bj38 and Bj123 cluster isolates in the bradyrhizobial communities of these cultivars were lower than those in the communities of cultivars with other genotypes (Table 5).

The present study results suggest that $Rj_2$ genotype soybeans might prefer nodulation by bradyrhizobia in the Bj110 cluster, as reflected by the high nodule occupancy in cultivars of that genotype with isolates in that cluster and by the relatively low nodulation occupancy by bradyrhizobial isolates in the Bj38 and Bj123 clusters. These results, however, are inconsistent with those of Yamakawa et al. (2003), who reported that the preference of $Rj_2Rj_3Rj_4$ for serogroup 110 was not sufficient to rule out competition with other serogroups, even though $Rj_2Rj_3Rj_4$ cultivars were superior to other *Rj* genotypes for inoculation with *B. japonicum* USDA 110. It might be possible to observe the soybean preferences for bradyrhizobia found in the present study only in fields with indigenous populations of bradyrhizobia belonging to the Bj38, Bj110, and Bj123 clusters. The field studied by

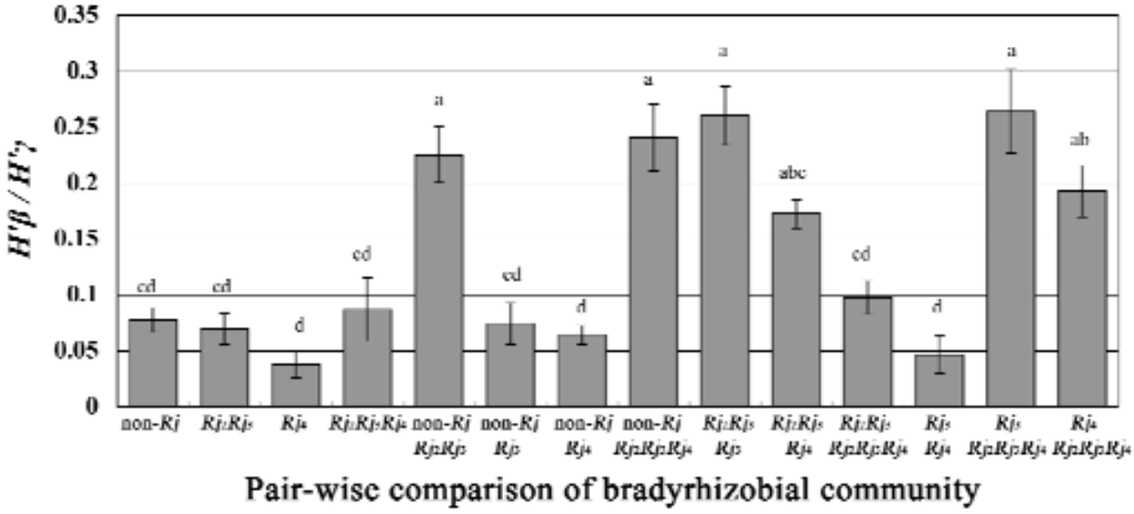


Fig. 10. Pair-wise comparisons of bradyrhizobial compositional differences expressed as the ratio $H'_\beta / H'_\gamma$ (Minami et al., 2009) (mean ± SE, $n$ = 3 or 9). Bars with different superscripts are significantly different (Tukey–Kramer test) at $P < 0.05$.

| Cluster | number of isolates | Rate of cluster (%) | Nodule occupancy (%) of clusters on each *Rj*-genotype* | | | | | Compatibility (%)*** with | |
|---|---|---|---|---|---|---|---|---|---|
| | | | non-*Rj* | $Rj_2Rj_3$ | $Rj_3$** | $Rj_4$ | $Rj_2Rj_3Rj_4$ | $Rj_2Rj_3$ | $Rj_4$ |
| Bj6 | 23 | 8.8 | 8.3 ± 6.0 | 13.3 ± 6.0 | - | 8.3 ± 3.3 | 8.3 ± 4.4 | 95.7 | 100 |
| Bj38 | 52 | 20.0 | 38.3 ± 9.3 | - | 45 | 28.3 ± 3.3 | 5.0 ± 2.9 | 63.5 | 100 |
| Bj115 | 6 | 2.3 | - | - | 10 | 6.7 ± 7.6 | - | 87.3 | 100 |
| Bj123 | 58 | 22.3 | 30.0 ± 5.0 | 18.3 ± 8.8 | 30 | 33.3 ± 6.0 | 5.0 ± 0.0 | 60.3 | 100 |
| BjF-1 | 3 | 1.2 | 5.0 ± 5.0 | - | - | - | - | 33.3 | 100 |
| BjF-2 | 1 | 0.4 | 1.7 ± 2.9 | - | - | - | - | 0.0 | 100 |
| Bj110 | 114 | 43.8 | 15.0 ± 5.0 | 68.3 ± 7.3 | 15 | 23.3 ± 4.4 | 78.3 ± 7.3 | 99.1 | 100 |
| Be76 | 2 | 0.8 | - | - | - | - | 3.3 ± 2.9 | 100 | 100 |
| Be94 | 1 | 0.4 | 1.7 ± 2.9 | - | - | - | - | 0.0 | 100 |

Table 5. Number of isolates and percentage of total isolates of soybean-nodulating bradyrhizobia in each cluster, nodule occupancy of each cluster by genotype, and compatibility of each cluster with soybean genotypes $Rj_2Rj_3$ and $Rj_4$ (Minami et al., 2009).
*Nodule occupancy is the mean ± standard error of isolates belonging to each cluster in each cultivar with the identical *Rj* genotype. ** Soybean cultivar D-51 was the only cultivar studied with the $Rj_3$ genotype; thus, the percentages of all isolates in each cluster in that cultivar are shown. *** Percentage of isolates that were compatible for effective nodule formation with the $Rj_2Rj_3$ genotype cultivar CNS and the $Rj_4$ genotype Hill. −, no isolate detected.

Yamakawa et al. (2003) using Kyushu University field located in Southern region of Japan, might not have contained any bradyrhizobia belonging to the Bj38 and Bj123 clusters, which are localized to northern to central Japan (Saeki et al., 2006).
One possible reason for the observed soybean preferences for particular bradyrhizobia may be differences in nodulation compatibility of indigenous bradyrhizobia with the $Rj_2$ genotype. The results of the inoculation tests showed that 63.5% and 60.3% isolates of the Bj38 and Bj123 clusters, respectively, were compatible with CNS ($Rj_2Rj_3$) (Table 5). If this difference in bradyrhizobial composition was derived from nodulation compatibility, the theoretical occupancy rate (%) of the Bj38 cluster isolated from the $Rj_2Rj_3$ and $Rj_2Rj_3Rj_4$ genotypes should be 18–24%, and the rates of the Bj123 cluster should be 18–20%, based on calculations of the nodule occupancy of the non-*Rj* and $Rj_4$ genotypes and their compatibility (Table 5). Occupancy rates of the Bj38 and Bj123 clusters in cultivars of the $Rj_2Rj_3$ genotype were 0% and 18.3%, respectively, and those in cultivars of the $Rj_2Rj_3Rj_4$ genotype were both 5.0% (Table 5). In particular, Bj38 cluster bradyrhizobia were rarely isolated from $Rj_2Rj_3$ or $Rj_2Rj_3Rj_4$ genotype cultivars. These results indicate that the $Rj_2$ gene might not only control incompatibility with some bradyrhizobia but also have some influence on nodulation preference. However, only the Bj110 cluster showed high occupancy in communities from $Rj_2Rj_3$ and $Rj_2Rj_3Rj_4$ genotypes, so the nodulation preference of the $Rj_2$ genotype might not be explained by nodulation compatibility alone.
The ineffective nodulation gene $Rj_2$ is located in a disease-resistance gene cluster in the soybean cultivar Williams 82 (Kanazin et al., 1996; Graham et al., 2002; Yang et al., 2010). Thus, the different preference response of the $Rj_2$ genotype to various bradyrhizobia might be related to the disease resistance response, which thus might be involved in regulating the composition of the nodulating bradyrhizobial community. The results of this study showed that the compositions of bradyrhizobial communities nodulating the $Rj_2Rj_3$ genotype cultivar CNS at sites in Hokkaido, Fukushima, Kyoto, and Miyazaki were different from those of other *Rj* genotypes. Even at the Miyazaki site, which contained bradyrhizobia incompatible with the $Rj_4$ genotype, beta diversities between CNS and the other genotype cultivars were higher than in the other pair-wise comparisons. In contrast, there were almost no differences in beta diversities among cultivars at the Okinawa site compared with the

other sites. These results indicate that the *Rj*$_2$ genotype might have different preferences for bradyrhizobia of the Bj38, Bj110, and Bj123 clusters and strains that are incompatible with *Rj*$_4$ genotype soybeans. Thus, differences in beta diversity might not have been detected at the Okinawa site because isolates belonging to the Bj38 and Bj123 clusterts were not previously isolated from the Okinawa site (Saeki et al., 2006, 2008). However, the mechanism and the genetics of the described nodulation preferences are as yet not understood, and further analysis is needed to clarify the molecular mechanisms underlying bradyrhizobial compatibility and the preferences of certain host genotypes for nodulation.

In the study described in this section, differences in the composition of soybean-nodulating bradyrhizobial communities among the soybean genotypes non-*Rj*, *Rj*$_2$*Rj*$_3$, *Rj*$_3$, *Rj*$_4$, and *Rj*$_2$*Rj*$_3$*Rj*$_4$ were investigated. In the sampled soils, soybean cultivars that did not have the *Rj*$_2$ gene did not show differences in bradyrhizobial composition among genotypes, whereas cultivars with the *Rj*$_2$ gene did show different preferences for nodulation by some bradyrhizobia than cultivars with the other genotypes. As preference and compatibility are important traits in the development of effective inoculation techniques, further analysis of these phenomena is necessary.

## 4. Future research and overall goal

This chapter demonstrated experimental approaches to characterization of soybean-nodulating rhizobial communities in relation to host genotype and geographical distribution. The study results highlight the diversity and geographical distribution of indigenous soybean-nodulating bradyrhizobia in Japan and suggest that bradyrhizobial communities occupy different niches from north to south determined by environmental factors such as temperature and host-plant diversity. These results were, however, based on limited numbers of isolates from nodules. For further advances in rhizobial ecology, a direct method should be developed to characterize the rhizobial ecology of indigenous rhizobia in relation to interactions with host soybean cultivars. In particular, rhizobial communities in the soil should be compared with soybean-nodulating rhizobial communities to clarify ecological interactions among host plant, rhizobia, and environmental factors, such as temperature (Saeki et al., 2010).

The abundances and the occupation of soybean-nodulating bacteria in the soil under various environmental factors and competition between inoculants and indigenous soybean-nodulating bacteria must be studied to determine the relationships between environmental factors and competition and compatibility among *Rj* genotypes, and to improve inoculation efficiency for the yield increase. In addition, establishment of agricultural techniques to increase the occupancy of nodules by effective inoculants of soybean-nodulating rhizobia with $N_2O$ reductase activity are also important for decreasing $N_2O$ emissions from soybean fields (Sameshima-Saito et al., 2006; Inaba et al., 2009). Further studies to investigate differences in the bradyrhizobia nodulating various soybean cultivars in different fields with various soil types should be conducted to clarify the genetic diversities and the ecological relationships among bradyrhizobia, host soybean plants, and environmental factors.

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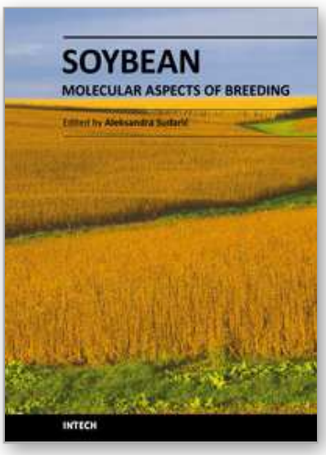


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