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Comparison of Soybean-Nodulating Bradyrhizobia Community Structures Along North Latitude Between Japan and USA

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1. Introduction

Soybean (*Glycine max* [L.] Merr.) establishes a symbiotic relationship by infection with soybean-nodulating bacteria and subsequent root nodule formation. Soybean acquires atmospheric nitrogen as ammonia through the symbiotic nitrogen fixation by soybean-nodulating rhizobia in the root nodules. The inoculation of soybean with bradyrhizobia that has high ability of nitrogen fixation is considered to be effective in increasing soybean production. However, the efficiency of the inoculum may be poor if the inoculum can not compete with indigenous soybean-nodulating rhizobia in the soil or can not establish an efficient symbiosis with the host plants because of the increased densities of indigenous rhizobia. To solve this problem, it is very important to understand the ecology of indigenous soybean-nodulating rhizobia in terms of their genetic diversity, community structure, geographical distribution, compatibility with the host soybean, and the ecosystems including environmental factors associated with the localization and dominance of the rhizobial strains in the soil. 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. It is likely that the community of soybean-nodulating rhizobia vary from place to place because various wild soybeans are distributed and various soybean cultivars are cultivated in the northern to southern regions of the world. In Japan, Sawada et al. [1] isolated 85 indigenous soybean bradyrhizobia from soybean root nodules sampled from 46 soybean fields and the isolates were classified based on their serotype using rabbit antisera prepared against *Bradyrhizobium* USDA strains as antigens. Minamisawa et al. [2] also characterized 213 Japanese indigenous soybean bradyrhizobia isolated from six fields by analysis of their fingerprints with *RSα*, *RSβ*, *nif*DK and *hup*SL, and revealed diversity and

endemism in their population structure. They suggested that bradyrhizobia might diversify in individual fields depending on the associated host plants and local soil conditions. Soybean-nodulating bradyrhizobia show physiological and genetic diversity and the bradyrhizobial community structures are constructed under the various environmental conditions. The major soybean-nodulating rhizobia that have been identified are *Bradyrhizobium japonicum*, *Bradyrhizobium elkanii*, and *Sinorhizobium/Ensifer fredii* [3-6]. Furthermore, additional species of soybean-nodulating rhizobia have been extensively discussed in the literature owing to the complexity of their taxonomical classification [7-11]. Soybean-nodulating bacteria are found over a wide region of the world, and their genetic diversity reflects geographical and climatic differences as well as host diversity.

In the host soybean, the genes related to nodulation, the *Rj* genes, are known as nodulation regulatory genes, and the *Rj* genotypes of rj_1, Rj_2, Rj_3, Rj_4 and non-*Rj* which lack these genetical phenotypes have been confirmed to exist in nature [12-17]. Specific rhizobial strains are 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 [18-23]. The ability of a soybean plant to host bradyrhizobia depends on the characteristics of *Rj* genes. Previous experimental results have also demonstrated that the community structure of soybean-nodulating bradyrhizobia depends on the host soybean *Rj* genotype and on the soybean cultivar, and it varies with cultivation temperature even in an identical soil sample [24, 25]. Since soybean cultivars harboring *Rj* genes are involved in the inhibition of effective nodulation by certain serogroups of rhizobia as well as in the preferential selection of appropriate rhizobia for nodulation, in the analysis of indigenous soybean-nodulating bacteria, it is important to use several kinds of *Rj*-genotypes of soybean cultivars for the isolation of rhizobia.

In our research group, Saeki et al. [26] investigated the genetic diversity and geographical distribution of indigenous soybean-nodulating rhizobia isolates from five sites in Japan (Hokkaido, Fukushima, Kyoto, Miyazaki, and Okinawa) by analyzing their restriction fragment length polymorphisms of polymerase chain reaction amplicons (PCR-RFLP) of the 16S-23S rRNA gene internal transcribed spacer (ITS) region, with 11 *Bradyrhizobium* strains that have USDA numbers as reference strains [27]. We reported that a geographical distribution of indigenous bradyrhizobia varied from northern to southern Japan. Furthermore, Saeki et al. [28] reported that the distribution of soybean-nodulating rhizobial niche in Japan was strongly correlated with latitude. The representative clusters of the isolated bradyrhizobia shifted from those of *B. japonicum* strains USDA 123, 110, and 6^T to *B. elkanii* strain USDA 76^T, moving from northern to southern Japan [29, 30].

The United States of America (USA) is the world's largest producer of soybeans. North latitudes between Japan and USA are similar and soybean cultivars are grown at latitudes similar to those of the soybean production areas of both countries. Understanding the geographical distribution of soybean-nodulating rhizobia in the USA therefore, would provide important knowledge about bradyrhizobial ecology and insights into appropriate inoculation techniques for soybean-nodulating rhizobia with high nitrogen fixation ability. We investi-
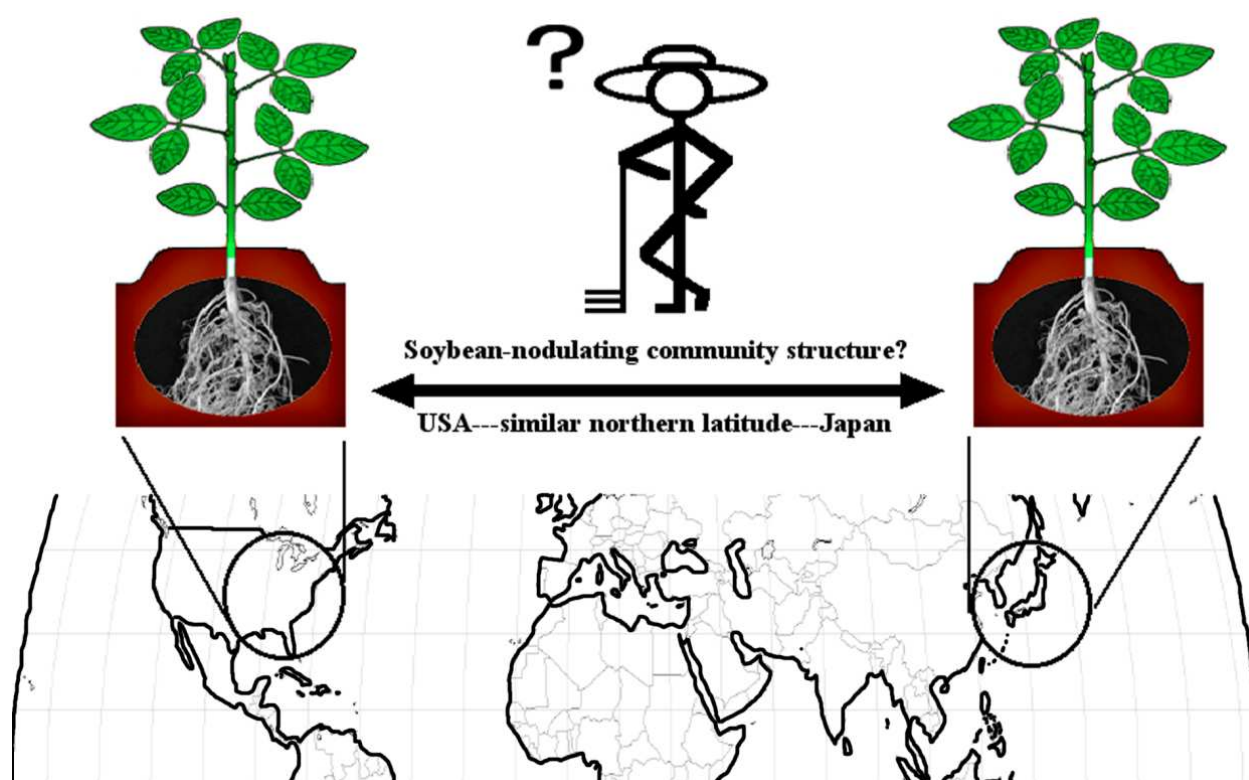
Figure 1. Schematic representation of the theme of this chapter. Are soybean-nodulating bradyrhizobial community structures similar between the same northern latitude in USA and Japan?

gated the relationship between the genetic diversity of indigenous soybean-nodulating bradyrhizobia and their geographical distribution in the USA using nine communities of isolates from eight states [31]. We analyzed their genetic diversity and community structure by means of RFLP of PCR amplicons to target the 16S-23S rRNA gene ITS region, with 11 USDA *Bradyrhizobium* strains as reference strains. We also performed diversity analysis, multidimensional scaling analysis based on the mathematical dissimilarity index, and polar ordination analysis to describe the structure and geographical distribution of the soybean-nodulating bradyrhizobial community. The major clusters were *B. japonicum* belonging to the cluster Bj123 in the northern USA, and *B. elkanii* in the middle to southern regions. Dominance of bradyrhizobia in a community was generally larger for the cluster belonging to *B. elkanii* than for the cluster belonging to *B. japonicum*. The indigenous American soybean-nodulating bradyrhizobial community structure was also strongly correlated with latitude as well as that of Japan. Our results indicated that this community varies geographically. Adhikari et al. [32] revealed the genetic diversity of soybean-nodulating bradyrhizobia in relation to climate depending on altitude and soil properties, such as soil pH, in Nepal. Furthermore, our research group demonstrated that soybean-nodulating rhizobial communities differed greatly in nearby fields depending on whether the soil was acidic or basic, and it was found that *S. fredii* strains were dominant in the alkaline soils of Vietnam and Okinawa, Japan [23, 33]. These results suggest that a relationship exists between the geographic distribution of indigenous soybean-nodulating rhizobia, soil temperature (and its variations due to latitude and altitude), and soil pH.

Therefore, knowledge of rhizobial ecology and biology in relation to numerous environmental factors and the environmental gradients is needed.

This chapter discusses the analysis of soybean-nodulating bradyrhizobial communities isolated from *Rj*-genotypes of soybean cultivars in relation to geographical differences including latitudinal gradients between Japan and USA (Fig.1). Analysis of RFLP of PCR amplicons of the 16S-23S rRNA gene ITS region and mathematical analysis of the PCR-RFLP results are demonstrated as possible approaches to the study of community diversity and ecosystem of soybean-nodulating rhizobia in relation to the rhizobial endemism in Japan and USA.

2. Classification of indigenous soybean-nodulating bradyrhizobia

Methods that are being developed and available for characterizing bradyrhizobial communities, include denaturing gradient gel electrophoresis (DGGE) analysis [34], terminal RFLP (T-RFLP) analysis [35], and automated ribosomal intergenic spacer analysis (ARISA) [36] using environmental DNA, and sequence polymorphisms targeting 16S rRNA gene (rDNA), the 16S–23S rDNA ITS region and other genomic and RNA sequences such as house-keeping genes and symbiotic functional genes [37-42]. In this section on experimental procedures, a relatively simple and reliable method for the study of indigenous soybean-nodulating bradyrhizobia isolated from nodules as described in detail previously [29] is demonstrated as one approach to the study of bradyrhizobial ecology.

2.1. Soil samples

Fresh soils for laboratory soybean cultivation were collected from some fields. We have analyzed soil samples from sixteen fields in Japan collected from 2004 to 2010 [30], and soil samples from nine experimental fields and farm fields in eight American states (US soils) in August 2010 [31] for isolation of soybean-nodulating bradyrhizobia (Table 1). These samples were weakly acidic-neutral soils collected from different regions along north latitude in these countries. At least, three soil samples were obtained from each field, to a depth of 10 cm, after removal of the surface litter, and the samples were homogenized to produce a single composite sample. Table 1 summarized the location, soil pH, and electric conductivity (EC) at these sites.

2.2. Isolation of indigenous soybean-nodulating bradyrhizobia

Since indigenous soybean-nodulating bacteria should be isolated from cultivars with different *Rj* genotypes, the soybean cultivars Akishirome, Bragg, or Orihime for non-*Rj* genotype, Bonminori, CNS, Hardee, or IAC-2 for Rj_2Rj_3 genotype, and Akisengoku, Fukuyutaka, or Hill for Rj_4 genotype were cultivated in culture pots for 4 weeks in our laboratory. Soybean cultivars were planted in 1 L culture pots. The pots were filled with vermiculite and a 40% (v/v) N-free nutrient solution [21] and then autoclaved at 121°C for 20 min. The soybean seeds were sterilized by soaking for 30 s in 70% ethanol and 3 min in a dilute sodium hypochlorite solution (0.25% available chlorine), and then rinsed with sterile distilled water. A soil sample (2-3 g) was placed into the vermiculite at a depth of 2–3 cm, and the soybean seeds were sown into

the soil. The plants were grown for 4 weeks in a growth chamber (day, 28°C for 16 h; night, 23°C for 8 h) with a weekly supply of sterile distilled water. After harvesting, the roots were washed thoroughly with tap water. The nodules were randomly collected and surface sterilized for 3 min in 70% ethanol and 30 min in a diluted sodium hypochlorite solution, and then rinsed with sterile distilled water. Each nodule was homogenized in sterile distilled water, and was streaked onto a yeast extract–mannitol agar (YMA) [43] plate and incubated for 5–7 days in the dark at 28°C. To determine the genus of the isolates, a single colony was streaked onto YMA plates containing 0.002% (w/v) bromothymol blue (BTB) to determine whether the genus of the isolate was *Bradyrhizobium* or *Sinorhizobium/Ensifer*, based on change of BTB color [44], and incubated as described above. After incubation, each isolate was maintained on YMA slant medium at 4°C for later analysis. As a negative control, soybean plants grown without soil were confirmed to form no nodules, eliminating the possibility of contamination with soybean-nodulating bacteria. Total number of soybean-nodulating bradyrhizobia isolated from each *Rj*-genotype soybean, for a sample soil, was considered as a soybean-nodulating rhizobial community in the soil sample.

Japan	USA	Latitude	Longitude	⊿Latitude	pH(H$_2$O)	EC(dS m^{-1})
	Michigan	43.05N	82.53W	18.67	7.7	0.15
Hokkaido		42.89N	143.07E	18.51	5.2	0.17
	Ohio	40.78N	81.93W	16.40	6.3	0.10
Akita A		40.01N	139.98E	15.63	6.1	0.06
Akita B		40.00N	139.96E	15.62	5.9	0.05
	Kentucky	38.93N	86.47W	14.55	6.1	0.10
Fukushima		37.71N	140.39E	13.33	5.0	0.05
	North Carolina	35.79N	78.69W	11.41	5.2	0.06
Yamanashi		35.68N	138.49E	11.30	6.1	0.07
Kyoto		35.29N	135.26E	10.91	5.1	0.15
Shizuoka A		34.70N	137.93E	10.32	5.3	0.18
Shizuoka B		34.90N	138.27E	10.52	5.8	0.46
Fukuoka		33.61N	130.46E	9.23	5.6	0.02
Kochi		33.55N	133.68E	9.17	4.9	0.34
	Alabama1	32.59N	85.49W	8.21	5.8	0.07
	Alabama2	32.59N	85.48W	8.21	5.2	0.04
Miyazaki		31.83N	131.42E	7.45	5.7	0.06
	Georgia	31.48N	83.52W	7.10	5.7	0.03
	Florida	30.68N	85.31W	6.30	5.6	0.02
	Louisiana	30.22N	91.10W	5.84	5.5	0.05
Tokunoshima		27.74N	128.97E	3.36	7.3	0.06
Okinawa A		26.25N	127.76E	1.87	4.7	0.06
Okinawa B		26.25N	127.76E	1.87	5.7	0.04
Miyakojima		24.77N	125.33E	0.39	7.5	0.05
Ishigaki		24.38N	124.19E	0.00	6.1	0.03

Table 1. Soil sample and the location of the sampling site, soil pH and EC in Japan and USA.

2.3. PCR-RFLP analysis of the 16S-23S rRNA gene ITS region

For DNA extraction, we cultured each isolate in 1.5 mL of HEPES-MES (HM) medium [45] supplemented with 0.1% L-arabinose [46] for 5 days at 28°C. Total DNA for the PCR template was extracted from the HM culture of the isolate as described by Hiraishi et al. [47]. Bacteria cells cultured in the HM medium were collected by centrifugation and washed with sterile distilled water. The cell pellet was suspended in 200 μL sterile distilled water. Then 40 μL of the suspension was mixed 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 at 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. In the phylogenetic analysis, reference strains were used to classify the isolates, namely, 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 rRNA gene ITS region [27]. Total DNAs of the reference strains were extracted by means of the same procedure as that used for the isolates. In our study, PCR was carried out with *Ex Taq* DNA polymerase (TaKaRa Bio Inc., Otsu, Shiga, Japan). For ITS amplification, an ITS primer set (BraITS-F: 5′-GACTGGGGGTGAAGTCGTAAC-3′, BraITS-R: 5′-ACGTCCTT-CATCGCCTC-3′) designed for amplification of the 16S-23S rRNA gene ITS region of bradyrhizobia [26] was used for the PCR reaction. 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 amplicon was performed with the restriction enzymes *Hae*III, *Hha*I, *Msp*I, and *Xsp*I (TaKaRa Bio Inc.). The restriction fragments were separated by a capillary electrophoresis apparatus (QIAxcel, Qiagen, UK) and submerged gel electrophoresis with ethidium bromide for visualization.

2.4. Cluster analysis

To construct a dendrogram based on the result of PCR-RFLP analysis 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 reproducible fragments longer than 50 bp were used for the cluster analysis, but some irreproducible fragments were excluded. The genetic distance between pairs of isolates (*D*) was calculated using the following equation (1):

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

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, respectively [48, 49]. The *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 the PHYLIP software (J. Felsenstein, University of Washington, Seattle, WA, USA; http://evolution.genetics.washington.edu/phylip.html).

Community structures of soybean-nodulating bradyrhizobia from each soil samples were summarized into Table 2, as bradyrhizobial community structures from Japanese soils, and into Table 3, as those from US soils, respectively. Dendrograms constructed from these data are demonstrated in our reports [26, 30, 31, 33]. As reference strains, we used *B. japonicum* USDA strains 4, 6^T, 38, 110, 115, 123, 124, and 135 and *B. elkanii* USDA strains 46, 76^T, and 94. 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 clusters and/or 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.

Site \\ Cluster	Bj6	Bj38	Bj115	Bj110	Bj123	BjH	BjF	BjO	Be61	Be76	Be94	BeO
Hokkaido					58 (19,19,20)	2 (1,1,0)						
Akita A				38 (13,12,13)	22 (7,8,7)							
Akita B				46 (16,17,13)	14 (4,3,7)							
Fukushima		20 (7,1,12)	3 (1,0,2)	22 (10,10,2)	12 (2,8,2)		2 (0,1,1)			1 (0,0,1)		
Yamanashi				58 (20,18,20)						2 (0,2,0)		
Kyoto	14 (8,3,3)	8 (5,0,3)	6 (2,1,3)	29 (5,13,11)							3 (0,3,0)	
Shizuoka A	1 (0,0,1)			54 (17,20,17)							5 (3,0,2)	
Shizuoka B				60 (20,20,20)								
Fukuoka	15 (3,3,9)			33 (15,9,9)						12 (2,8,2)		
Kochi	24 (10,13,1)	1 (0,0,1)		35 (10,7,18)								
Miyazaki	40 (16,7,17)	7 (4,0,3)									13 (0,13,0)	
Tokunoshima	28 (3,16,9)			30 (15,4,11)						2 (2,0,0)		
Okinawa A	25 (7,6,12)							1 (0,1,0)		25 (11,8,6)	1 (1,0,0)	8 (1,5,2)
Okinawa B	16 (4,2,10)									41 (15,18,8)	2 (0,0,2)	1 (1,0,0)
Miyakojima	8 (3,3,2)									49 (16,16,17)	1 (0,1,0)	2 (1,0,1)
Ishigaki									1 (0,0,1)	59 (20,20,19)		
No. isolate	171	36	9	405	106	2	2	1	1	191	25	11
Percentage(%)	17.8	3.8	0.9	42.2	11.0	0.2	0.2	0.1	0.1	19.9	2.6	1.1

Upper number in each column indicates a whole number of isolates and lower indicates a number of isolates from non-*Rj*, *Rj$_2$Rj$_3$*, and *Rj$_4$*, respectively.

Table 2. Cluster and the number of soybean-nodulating bradyrhizobia from Japan based on the report from Saeki et al. [30].

Cluster \ Site	Bj6	Bj38	Bj110	Bj123	Bj124	Be46	Be76	Be94	BeOH
Michigan	1 (0, 0, 1)		3 (0, 0, 3)	65 (23, 23, 19)					
Ohio			9 (5, 1, 3)	28 (5, 2, 21)				4 (3, 1, 0)	31 (11, 20, 0)
Kentucky	12 (4, 0, 8)		26 (12, 2, 12)				33 (8, 22, 3)	1 (0, 0, 1)	
North Carolina	29 (9, 0, 20)	1 (0, 0, 1)				1 (0, 0, 1)	13 (4, 7, 2)	20 (8, 12, 0)	8 (3, 5, 0)
Alabama1	8 (2, 1, 5)		1 (1, 0, 0)			6 (2, 2, 2)	56 (19, 21, 16)	1 (0, 0, 1)	
Alabama2	9 (6, 0, 3)	1 (0, 0, 1)			1 (0, 0, 1)	16 (5, 5, 6)	19 (5, 7, 7)	23 (8, 9, 6)	3 (0, 3, 0)
Georgia	2 (0, 0, 2)		9 (4, 0, 5)			4 (2, 2, 0)	26 (6, 9, 11)	27 (12, 10, 5)	4 (0, 3, 1)
Florida					1 (1, 0, 0)	4 (0, 0, 4)	55 (18, 20, 17)	11 (5, 3, 3)	1 (0, 1, 0)
Louisiana			4 (3, 0, 1)			36 (12, 12, 12)	14 (3, 4, 7)	18 (6, 8, 4)	
No. isolate	61	2	52	93	2	67	216	105	47
Percentage (%)	9.5	0.3	8.1	14.4	0.3	10.4	33.5	16.3	7.3

Upper number in each column indicates a whole number of isolates and lower indicates a number of isolates from non-Rj, Rj_2Rj_3, and Rj_4, respectively.

Table 3. Cluster and the number of soybean-nodulating bradyrhizobia from USA based on the report from Shiro et al. [31].

2.5. Confirmation of nodule formation of isolate

Several representative isolates in each operational taxonomic unit (OTU) of the dendrogram were confirmed for their nodulation capability on host soybean by inoculation test. Each isolate was cultured in yeast extract-mannitol broth (YMB) culture [43] for 6 days at 28°C, and the cultures were then diluted with sterile distilled water to approximately 10^6 cells mL^{-1}. The soybean seeds were sown into 500 mL prepared culture pots without soil, as described above, and inoculated with a 1 mL aliquot of each isolate per seed, with two or three replicates. We assessed nodule formation after 3 weeks in a growth chamber under the conditions described above.

3. Mathematical ecology analysis of soybean-nodulating bradyrhizobial communities

In previous section, we investigated the polymorphisms of 16S-23S rRNA gene ITS region of soybean-nodulating isolates and analyzed the community structures to elucidate bradyrhizobial ecology along latitude between Japan and USA. Several soybeans that are different in Rj-genotypes were cultivated for four weeks on the soil for isolation of soybean-

nodulating bradyrhizobia. Polymorphisms of the isolates were detected by RFLP of 16S-23S rRNA gene ITS regions with *Hae*III, *Hha*I, *Msp*I, and *Xsp*I, with *B. japonicum* USDA 4, 6[T], 38, 110, 115, 123, 124, 135, *B. elkanii* USDA 46, 76[T], 94, as reference strains. As a result, 12 clusters from Japan and 9 clusters from USA were obtained (Tables 2 and 3). Isolates in this study were confirmed to belong to the genus *Bradyrhizobium* on the basis of their ability to form nodules on soybean roots, alkaline production on YMA medium containing BTB, and the PCR-RFLP analysis results. Among the clusters, Bj123, Bj110, Bj6, and Be76, from northern to southern regions, were isolated most frequently, followed by Be94, Bj115 from Japanese soils. In US soils, Bj123 was also dominant in the northern regions, whereas Be46, Be76, and Be94 were dominant in the central to southern regions, and Bj6 and Bj110 were moderately dominant in the central regions. In this section, mathematical approaches to the analysis of soybean-nodulating bradyrhizobial community diversity are demonstrated using the community structures of indigenous bradyrhizobia isolated from Japanese soils and US soils, presented in Tables 2 and 3.

3.1. Diversity indices analysis among field sites

To estimate differences among the diversities of the soybean-nodulating bradyrhizobial communities at different field sites, we used the Shannon-Wiener diversity index [50, 51]. Shannon-Wiener diversity index (H') was calculated for each field site with equation (2):

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

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 species, *B. japonicum* (Bj) or *B. elkanii* (Be), or a particular cluster at each field site, respectively. Thereafter, we calculated the alpha diversity (H'_α), beta diversity (H'_β), and gamma diversity (H'_γ) to estimate the differences in the bradyrhizobial communities between pairs of soil samples [52, 53]. The H'_α index represents a weighted average of the diversity indices of comparing two bradyrhizobial communities, the H'_β index represents the differences between the bradyrhizobial communities from two soil samples (i.e., differences between sites), and the H'_γ index represents the diversity of the total isolate communities from the two soil samples (Figure 2).

The relationship among these indices is expressed as the equation (3):

$$H'_\beta = H'_\gamma - H'_\alpha. \tag{3}$$

Thereafter, we estimated the differences among the compositions of the bradyrhizobial communities by comparing the ratio of beta to gamma diversity as the equation (4), taking into consideration the difference in gamma diversity in each pairwise comparison of bradyrhizobial communities.
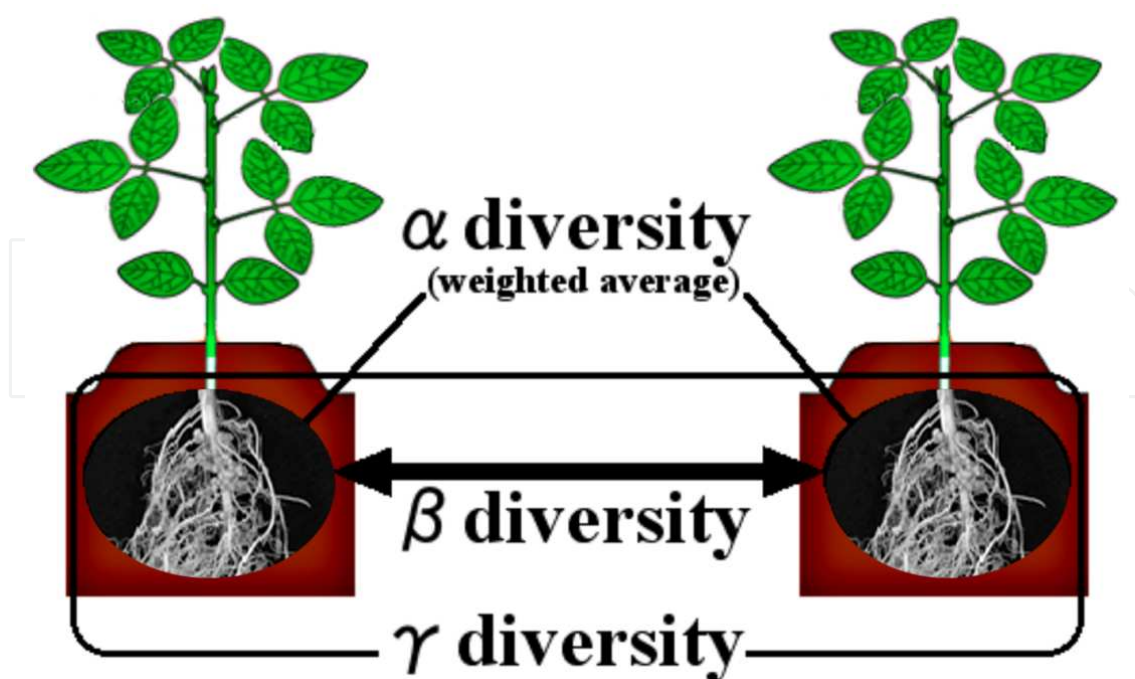
Figure 2. Schematic representation of alpha, beta, and gamma diversities of soybean-nodulating rhizobial communities.

$$H'_\beta \,/\, H'_\gamma. \tag{4}$$

3.2. Result of diversity indices analysis

At the northern sites, most isolates were classified into cluster Bj123, and the proportion of isolates in cluster Bj123 decreased southward. On the other hand, the frequency of isolation of *B. elkanii* increased southward, and the proportion of *B. elkanii* isolates was highest at the southern sites among the soil sample sites. As described above, Bj123, Bj110, Bj6, and Be76 clusters were isolated most frequently, from northern to southern regions in Japan. In US soils, Bj123 was also dominant in the northern regions, whereas Bj6 and Bj110 clusters were moderately dominant in the central regions, and Be46, Be76, and Be94 clusters were major in the central to southern regions in USA. In In comparing the northern and southern sites of both countries, beta diversities ($H'_\beta \,/\, H'_\gamma$) were higher than other comparisons (Tables 4 and 5, Figs. 3-6). These results mean that difference between bradyrhizobial community structures become larger in the case of comparison between northern and southern sites, and smaller in the case of similar latitudes. However, since varieties of beta diversities ($H'_\beta \,/\, H'_\gamma$) were larger in community structures of Japanese soils than those of US soils, tendency of beta diversity ($H'_\beta \,/\, H'_\gamma$), in comparison of bradyrhizobial community structures, is obscure as compared to those of US soil samples. This reason is due to the fact that some soil samples indicated high proportion of Bj110 cluster in Japanese bradyrhizobial community structures.

Bj - Be / Cluster	Hokkaido	Akita A	Akita B	Fukushima	Yamanashi	Kyoto	Shizuoka A	Shizuoka B	Fukuoka	Kochi	Miyazaki	Tokuno shima	Okinawa A	Okinawa B	Miyako jima	Ishigaki
Hokkaido		0.00	0.00	0.12	0.14	0.15	0.17	0.00	0.23	0.00	0.24	0.14	0.43	0.56	0.71	1.00
Akita A	0.43		0.00	0.12	0.14	0.15	0.17	0.00	0.23	0.00	0.24	0.14	0.43	0.56	0.71	1.00
Akita B	0.54	0.02		0.12	0.14	0.15	0.17	0.00	0.23	0.00	0.24	0.14	0.43	0.56	0.71	1.00
Fukushima	0.36	0.15	0.16		0.01	0.03	0.06	0.12	0.15	0.12	0.16	0.01	0.36	0.50	0.65	0.94
Yamanashi	0.83	0.28	0.22	0.20		0.01	0.03	0.14	0.10	0.14	0.11	0.00	0.32	0.45	0.61	0.89
Kyoto	0.48	0.24	0.23	0.14	0.24		0.01	0.15	0.07	0.15	0.08	0.01	0.29	0.42	0.57	0.86
Shizuoka A	0.73	0.25	0.21	0.26	0.15	0.17		0.17	0.04	0.17	0.04	0.03	0.23	0.36	0.51	0.79
Shizuoka B	0.90	0.31	0.25	0.30	0.14	0.25	0.16		0.23	0.00	0.24	0.14	0.43	0.56	0.71	1.00
Fukuoka	0.55	0.26	0.24	0.23	0.21	0.13	0.21	0.27		0.23	0.00	0.10	0.11	0.22	0.35	0.63
Kochi	0.59	0.28	0.26	0.24	0.29	0.08	0.22	0.31	0.09		0.24	0.14	0.43	0.56	0.71	1.00
Miyazaki	0.58	0.48	0.50	0.34	0.58	0.21	0.48	0.62	0.31	0.28		0.11	0.10	0.20	0.34	0.61
Tokunoshima	0.59	0.29	0.28	0.26	0.29	0.03	0.25	0.35	0.05	0.03	0.27		0.32	0.45	0.61	0.89
Okinawa A	0.52	0.44	0.45	0.34	0.50	0.27	0.46	0.55	0.21	0.30	0.23	0.25		0.02	0.10	0.35
Okinawa B	0.60	0.49	0.51	0.37	0.57	0.32	0.52	0.64	0.24	0.38	0.31	0.32	0.06		0.03	0.26
Miyakojima	0.65	0.52	0.54	0.39	0.62	0.36	0.57	0.69	0.28	0.44	0.40	0.39	0.10	0.02		0.20
Ishigaki	0.86	0.65	0.69	0.47	0.84	0.49	0.75	0.94	0.44	0.62	0.60	0.58	0.27	0.23	0.17	

Data in the upper triangle were based on the species (Bj and Be) data, and that in lower triangle were based on the cluster data.

Table 4. The ratio of beta to gamma diversity (H'_β / H'_γ) among soybean-nodulating bradyrhizobial communities in Japan.
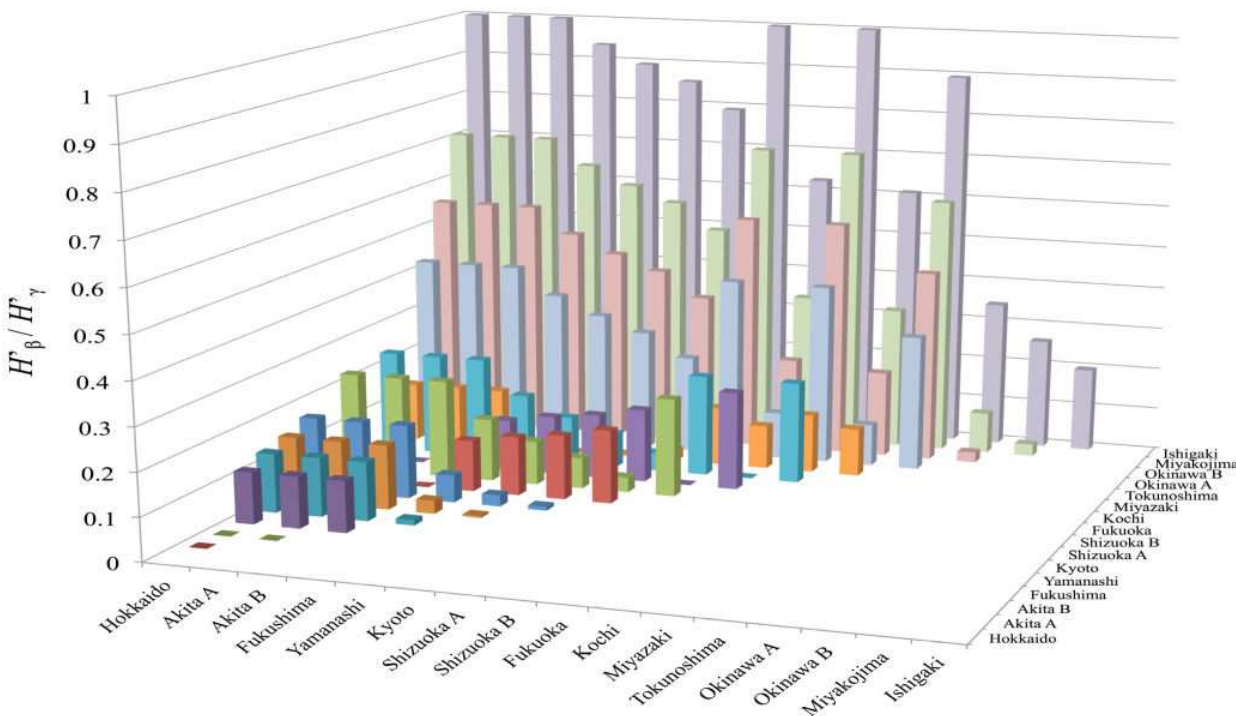

Figure 3. The ratio of beta to gamma diversity (H'_β / H'_γ) among pairs of soil sample sites based on the species (Bj and Be) data in Japan.
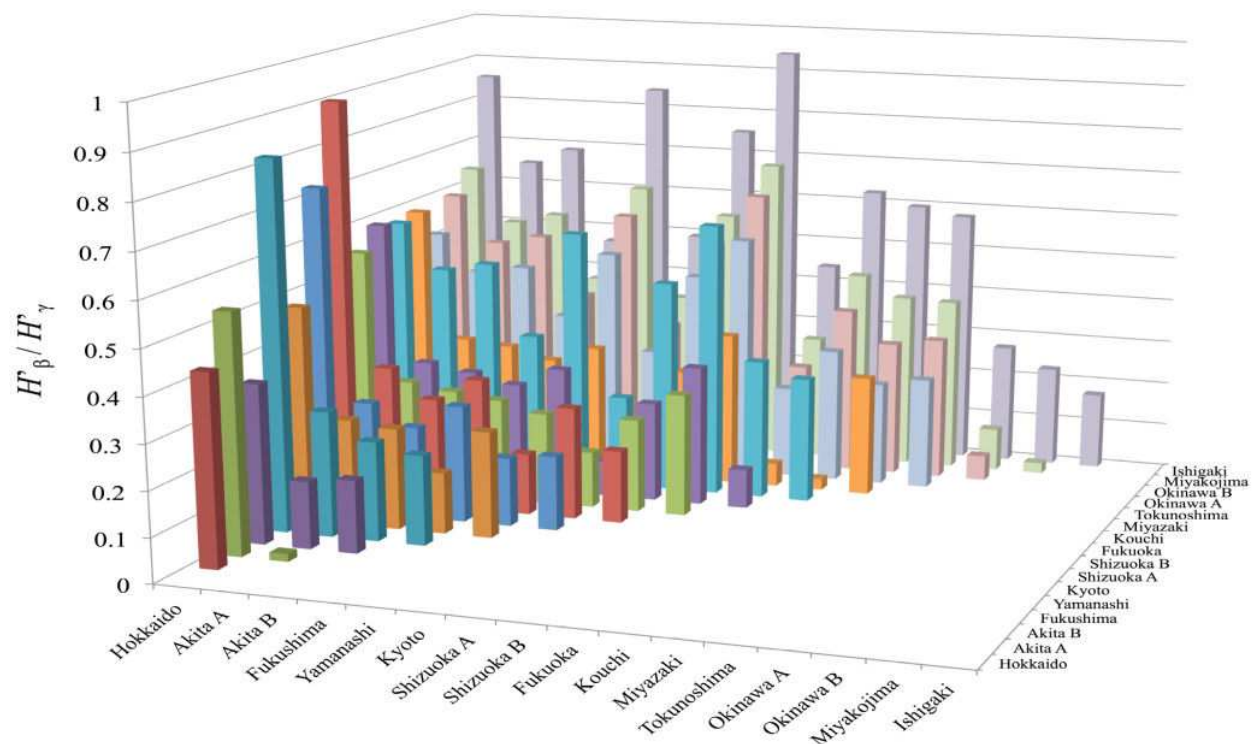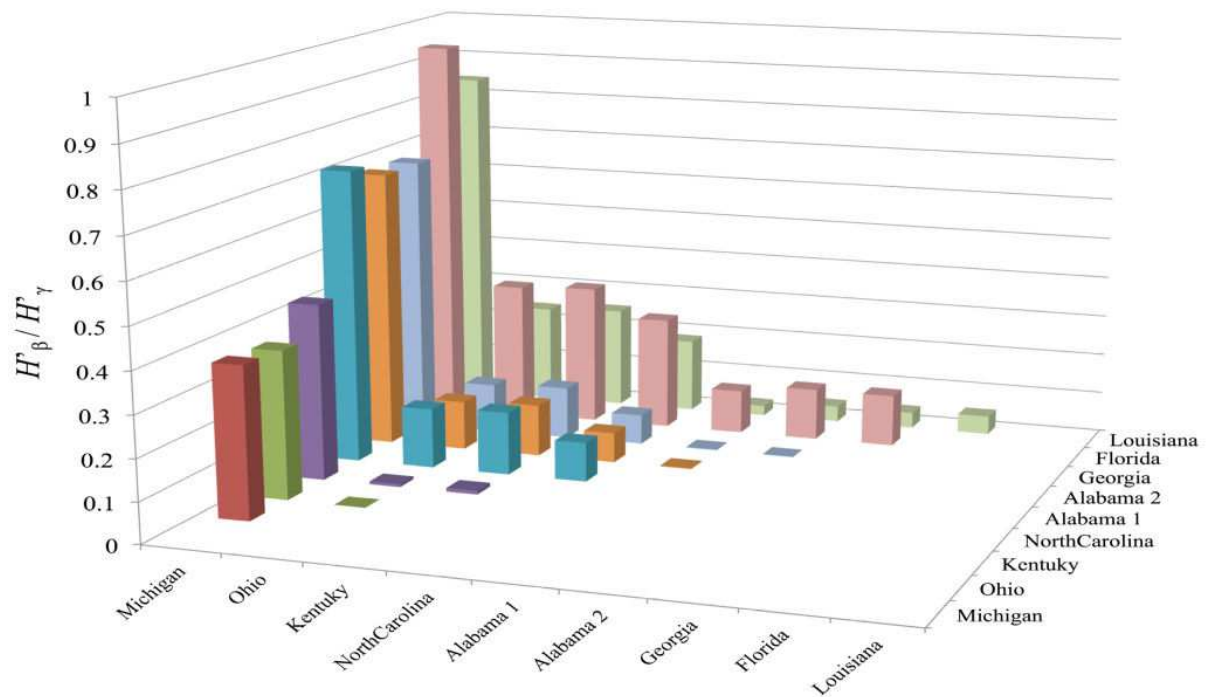
Figure 4. The ratio of beta to gamma diversity (H'_β/ H'_γ) among pairs of soil sample sites based on the cluster data in Japan.

Bj - Be Cluster	Michigan	Ohio	Kentuky	North Carolina	Alabama 1	Alabama 2	Georgia	Florida	Louisiana
Michigan		0.37	0.36	0.43	0.72	0.68	0.68	0.95	0.84
Ohio	0.25		0.00	0.01	0.15	0.12	0.12	0.34	0.24
Kentuky	0.47	0.32		0.01	0.15	0.13	0.13	0.35	0.25
North Carolina	0.44	0.27	0.20		0.10	0.07	0.07	0.28	0.18
Alabama 1	0.56	0.41	0.14	0.18		0.00	0.00	0.11	0.02
Alabama 2	0.42	0.29	0.20	0.07	0.15		0.00	0.13	0.04
Georgia	0.43	0.25	0.14	0.11	0.16	0.06		0.13	0.04
Florida	0.57	0.38	0.22	0.21	0.10	0.12	0.10		0.05
Louisiana	0.48	0.33	0.25	0.21	0.23	0.06	0.11	0.19	

Data in the upper triangle were based on the species (Bj and Be) data, and that in the lower triangle were based on the cluster data.

Table 5. The ratio of beta to gamma diversity (H'_β/ H'_γ) among soybean-nodulating bradyrhizobial communities in USA.

Figure 5. The ratio of beta to gamma diversity (H'_β / H'_γ) among pairs of soil sample sites based on the species (Bj and Be) data in USA.
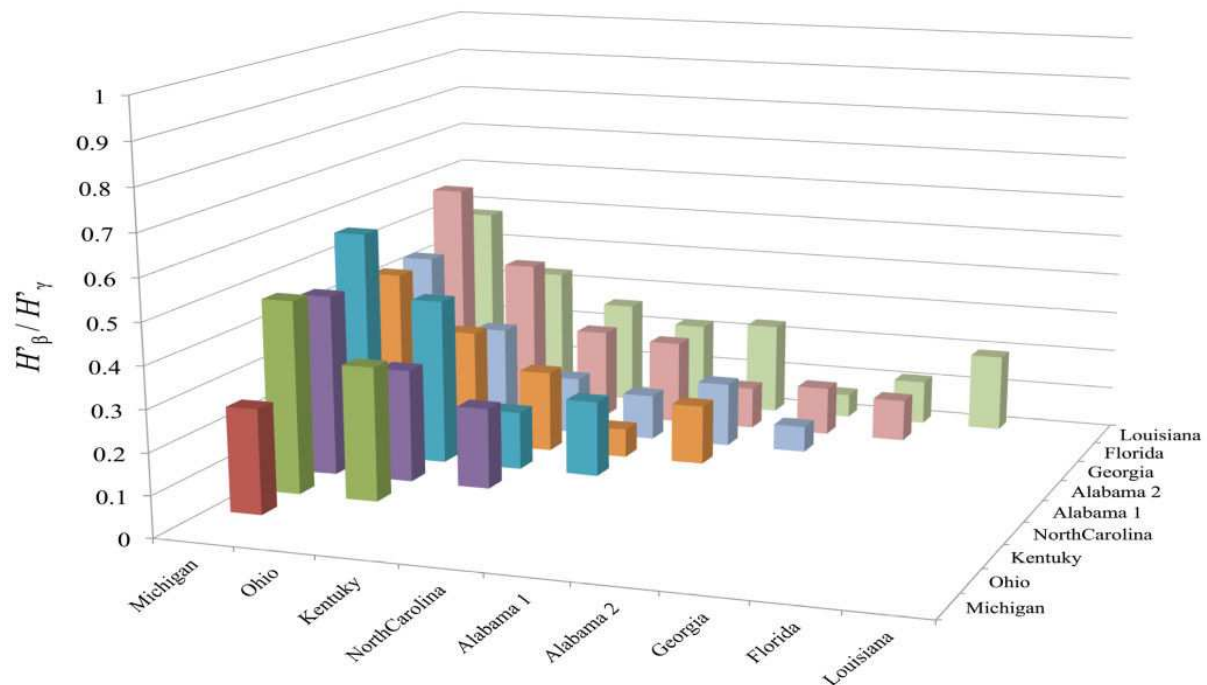

Figure 6. The ratio of beta to gamma diversity (H'_β / H'_γ) among pairs of soil sample sites based on the cluster data in USA.

In the comparison between Japan and USA, bradyrhizobial community structures based on the species Bj and Be at similar geographical latitude indicated lower beta diversities and higher beta diversity at different geographical latitudes even in the comparison of different countries, Japan and USA (Table 6). On the other hand, in the comparison of beta diversities of community structures based on the cluster compositions, varieties of beta diversities (H'_β / H'_γ) were also larger due to high proportion of Bj110 cluster in some Japanese bradyrhizobial community structures, though tendency of results were similar to the result of H'_β / H'_γ based on the species data (Table 7).

Bj-Be	Michigan	Ohio	Kentuky	North Carolina	Alabama 1	Alabama 2	Georgia	Florida	Louisiana
Hokkaido	0.00	0.35	0.34	0.41	0.70	0.66	0.66	0.94	0.83
Akita A	0.00	0.35	0.34	0.41	0.70	0.66	0.66	0.94	0.83
Akita B	0.00	0.35	0.34	0.41	0.70	0.66	0.66	0.94	0.83
Fukushima	0.13	0.29	0.28	0.35	0.65	0.61	0.61	0.89	0.78
Yamanashi	0.15	0.25	0.24	0.31	0.61	0.57	0.57	0.85	0.73
Kyoto	0.16	0.22	0.21	0.28	0.57	0.53	0.53	0.81	0.70
Shizuoka A	0.19	0.17	0.16	0.23	0.52	0.48	0.48	0.75	0.64
Shizuoka B	0.00	0.35	0.34	0.41	0.70	0.66	0.66	0.94	0.83
Fukuoka	0.25	0.07	0.06	0.12	0.37	0.33	0.33	0.59	0.49
Kochi	0.00	0.35	0.34	0.41	0.70	0.66	0.66	0.94	0.83
Miyazaki	0.00	0.06	0.06	0.10	0.35	0.31	0.31	0.58	0.47
Tokunoshima	0.15	0.25	0.24	0.31	0.61	0.57	0.57	0.85	0.73
Okinawa A	0.45	0.00	0.01	0.00	0.11	0.08	0.08	0.31	0.20
Okinawa B	0.58	0.05	0.05	0.02	0.03	0.02	0.02	0.21	0.11
Miyakojima	0.73	0.13	0.14	0.09	0.00	0.00	0.00	0.12	0.03
Ishigaki	1.00	0.36	0.37	0.31	0.17	0.19	0.19	0.10	0.14

Table 6. The ratio of beta to gamma diversity (H'_β/ H'_γ) among soybean-nodulating bradyrhizobial communities in the comparison between Japan and USA based on the species (Bj and Be) data.

3.3. Multidimensional scaling analysis

To describe the characteristics of the bradyrhizobial communities and the differences among field sample sites, we performed a multidimensional scaling (MDS) analysis based on the Bray-Curtis similarity measure. The Bray-Curtis similarity measure [54] has a robust monotonic relationship with ecological distance and a robust linear relationship with ecological distance up to large values of the distance. Thus, the Bray-Curtis similarity measure (*BC*) is one of the indices that best reflect the properties between communities [55]. The Bray-Curtis similarity measure was calculated using the following equation (5):

$$BC_{AB} = \left[\sum |n_A - n_B| \right] / \left[\sum (n_A + n_B) \right], \tag{5}$$

where BC_{AB} is the dissimilarity between communities A and B, and n_A and n_B represent the total number of strains in *B. japonicum* (Bj) and *B. elkanii* (Be), or the number of strains in a particular cluster for communities A and B. The MDS analysis based on the Bray-Curtis similarity measure was conducted using the command "cmdscale" in the R software program version 2.15.1 (http://www.r-project.org/). Results of MDS analysis were indicated as two-dimensional (2-D) MDS analysis and three-dimensional (3-D) MDS analysis based on the species (Bj and Be) as independent variables or the clusters as independent variables.

Cluster	Michigan	Ohio	Kentucky	North Carolina	Alabama 1	Alabama 2	Georgia	Florida	Louisiana
Hokkaido	0.13	0.29	0.51	0.45	0.59	0.43	0.45	0.59	0.49
Akita A	0.34	0.22	0.29	0.39	0.48	0.37	0.32	0.49	0.39
Akita B	0.45	0.26	0.28	0.41	0.50	0.39	0.33	0.51	0.40
Fukushima	0.32	0.22	0.25	0.31	0.37	0.30	0.27	0.38	0.31
Yamanashi	0.75	0.41	0.27	0.44	0.55	0.41	0.34	0.56	0.43
Kyoto	0.44	0.29	0.17	0.22	0.34	0.25	0.23	0.38	0.30
Shizuoka A	0.66	0.36	0.25	0.38	0.51	0.36	0.28	0.51	0.38
Shizuoka B	0.81	0.44	0.33	0.48	0.61	0.45	0.39	0.62	0.47
Fukuoka	0.49	0.33	0.04	0.22	0.26	0.24	0.20	0.34	0.29
Kochi	0.54	0.35	0.18	0.27	0.41	0.31	0.30	0.47	0.38
Miyazaki	0.55	0.38	0.31	0.11	0.38	0.20	0.27	0.41	0.34
Tokunoshima	0.53	0.35	0.14	0.23	0.36	0.28	0.27	0.44	0.35
Okinawa A	0.50	0.37	0.15	0.13	0.15	0.17	0.21	0.23	0.29
Okinawa B	0.57	0.40	0.13	0.15	0.07	0.16	0.18	0.16	0.29
Miyakojima	0.61	0.42	0.15	0.21	0.06	0.19	0.19	0.14	0.31
Ishigaki	0.80	0.51	0.27	0.35	0.16	0.29	0.27	0.17	0.38

Table 7. The ratio of beta to gamma diversity (H'_β / H'_γ) among soybean-nodulating bradyrhizobial communities in the comparison between Japan and USA based on the cluster data.

3.4. Result of MDS analysis

As a result of MDS analysis of Bj and Be data set, the width of third dimension range was small in the 3-D MDS result, and it was considered that 2-D MDS result was able to explain their relationships among community structures (Figs. 7 and 8). As shown in Figure 7, communities mainly consisted of Bj were arranged at left part, and those mainly consisted of Be were arranged at right part of the MDS result. This result suggests that similar community compositions by Bj and Be exist in Japan and USA. Difference between Japan and USA is that frequency of isolation of Be of USA is higher than that of Japan. Thus, many bradyrhizobial communities of USA were positioned at right part of the 2-D MDS result.

In the case of characterization of community structures by MDS analysis based on Bj and Be as variables, 2-D MDS was reasonable to explain their relationships among communi-

ties because of only two variables, Bj and Be. However, in the case of characterization of community structure by MDS based on the cluster sets, 3-D MDS was more suitable to explain their relationships among communities (Figs. 9 and 10). This means that much number of clusters as variables are necessary for characterization of relationships among bradyrhizobial community structures. As described above, cluster Bj123, Bj110, Bj6, and Be76, from northern to southern regions in Japan, were isolated with high frequency, followed by Be94, Bj115 from Japanese soils. In US soils, Bj123 was also dominant in the northern regions, and Be46, Be76, and Be94 were dominant in the central to southern regions, and Bj6 and Bj110 were moderately dominant in the central regions. In the 3-D MDS result, similar coordinates were shown among bradyrhizobial communities isolated from soil samples, if the latitudes of the sample sites were near, without affecting the distance between Japan and USA. To inspect this result, a relationship between bradyrhizobial community structures and latitudes was investigated by a polar ordination of 3D-MDS coordinates of the community structures in the next analysis.
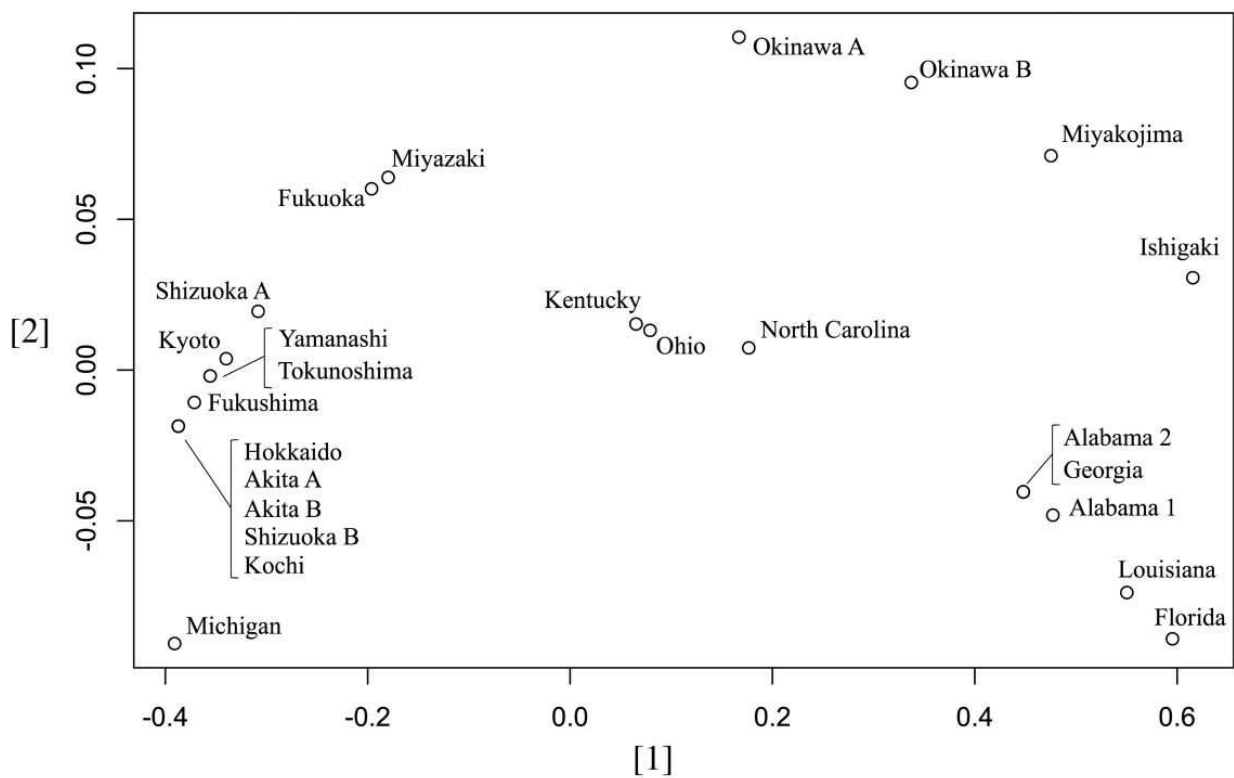
Figure 7. Result of 2D-MDS analysis based on Bj and Be and the number of isolates as independent variables.
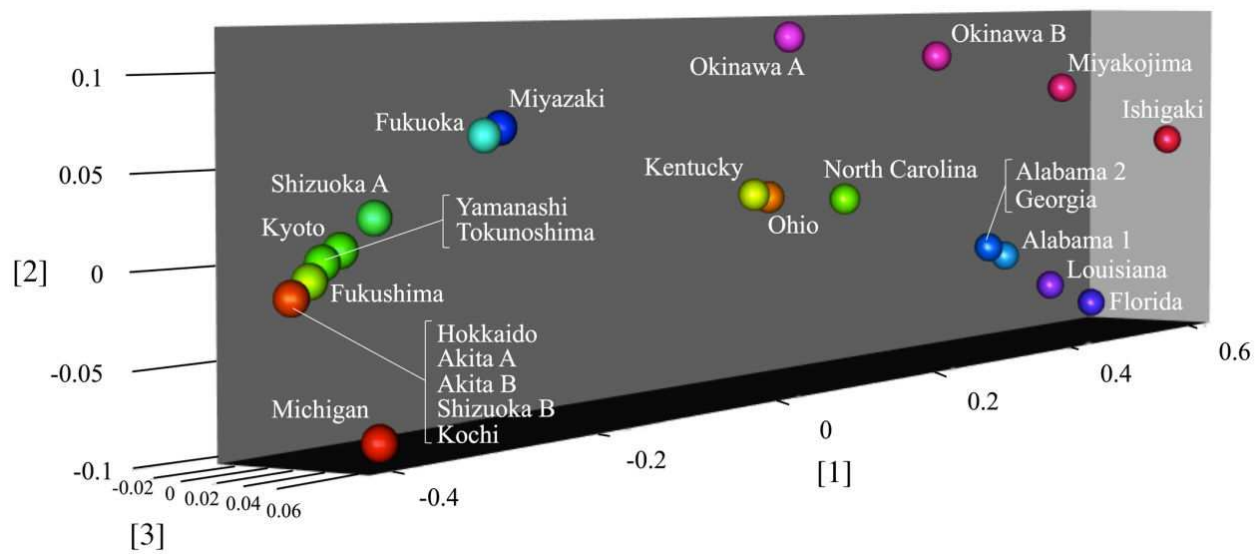
Figure 8. Result of 3D-MDS analysis based on Bj and Be and the number of isolates as independent variables.
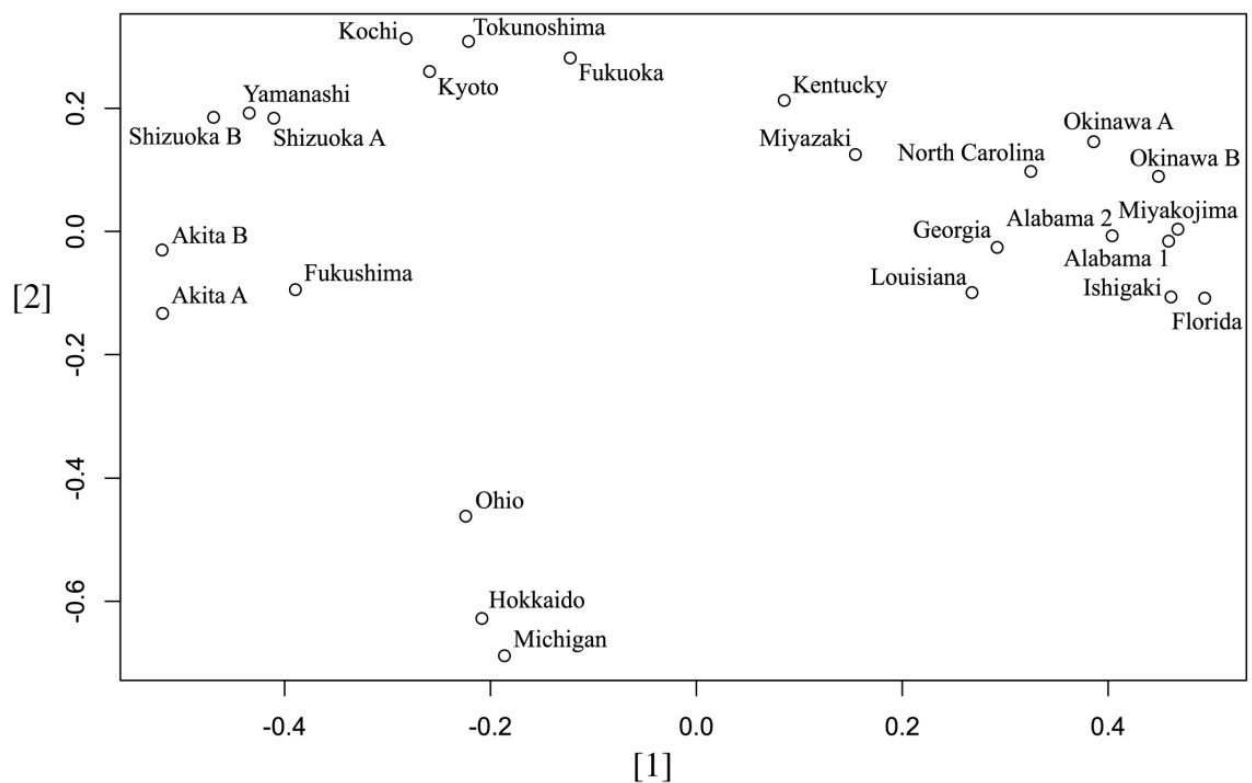
Figure 9. Result of 2D-MDS analysis based on the clusters and the number of isolates as independent variables.
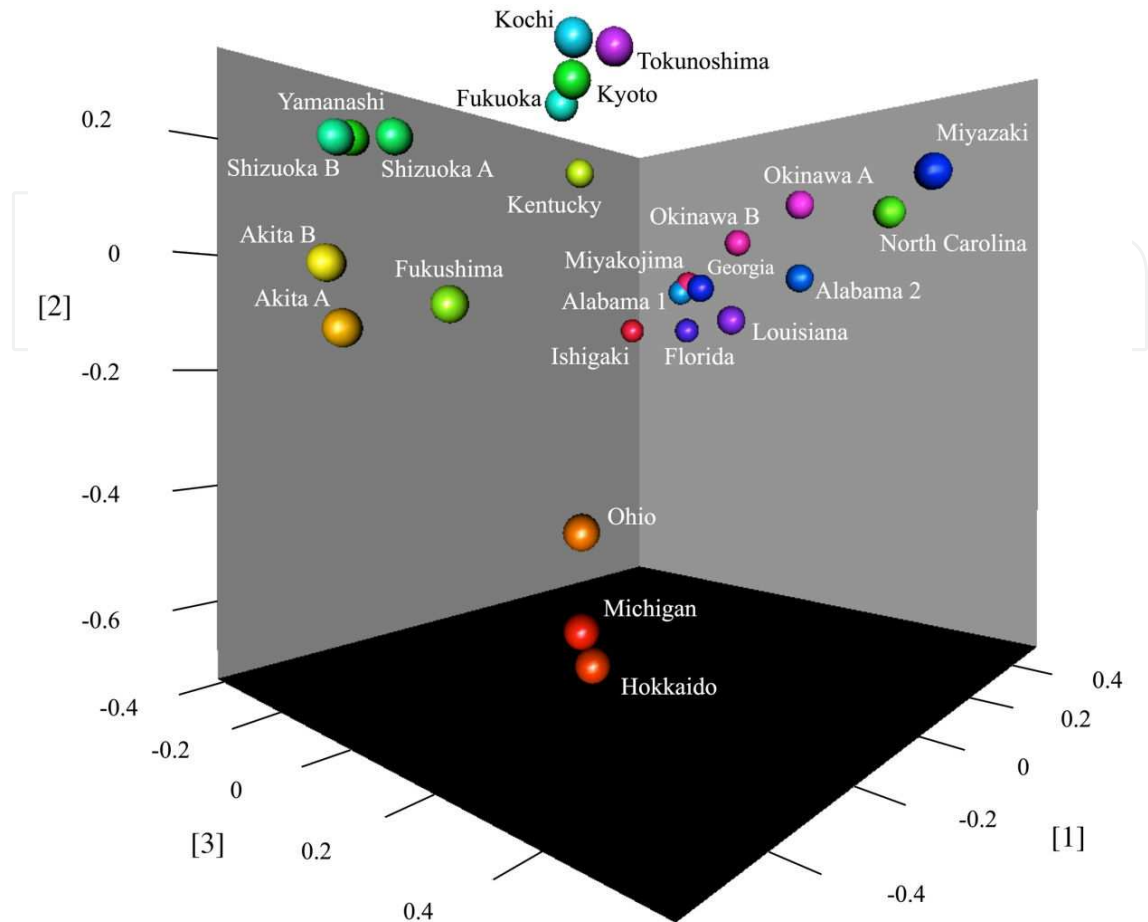
Figure 10. Result of 3D-MDS analysis based on the clusters and the number of isolates as independent variables.

3.5. Polar ordination of community diversity and latitude

The 3-D MDS results were analyzed mathematically by comparing percentage differences between pairs of soybean-nodulating indigenous bradyrhizobial communities and by using a polar ordination analysis [56, 53] to examine the geographical distributions of soybean-nodulating bradyrhizobia between Japan and USA (Fig. 11). To determine the relative distances between the diversities based on the 3-D MDS plots of the communities in the 3-D Euclidean space as a function of latitude (°N), we calculated the Euclidean distances between the bradyrhizobial communities and poles. The MDS plot of Michigan was set as the northern pole, and that of Ishigaki was set as the southern pole, according to their latitudes of sample sites (Table 1). The distances between the MDS plots were calculated using the coordinates on the x-, y-, and z-axes as the Euclidean distance (*Ed*) using the equation (6):

$$Ed_{AB} = [(X_A - X_B)^2 + (Y_A - Y_B)^2 + (Z_A - Z_B)^2]^{1/2}, \tag{6}$$

where Ed_{AB} is the linear distance between communities A and B in the MDS plot and X_A and X_B, Y_A and Y_B, and Z_A and Z_B represent the x (axis 1 in 3-D MDS), y (axis 2 in 3-D MDS), and z (axis 3 in 3-D MDS) coordinates of communities A and B, respectively. The distances from each pole were converted into percent differences, D_1 and D_2, from the two polar communities (i.e., the Michigan and Ishigaki sites, which were considered to have a 100% difference). Simultaneous equations were constructed from the trigonometric figure using the Pythagorean theorem as described previously [28, 29].
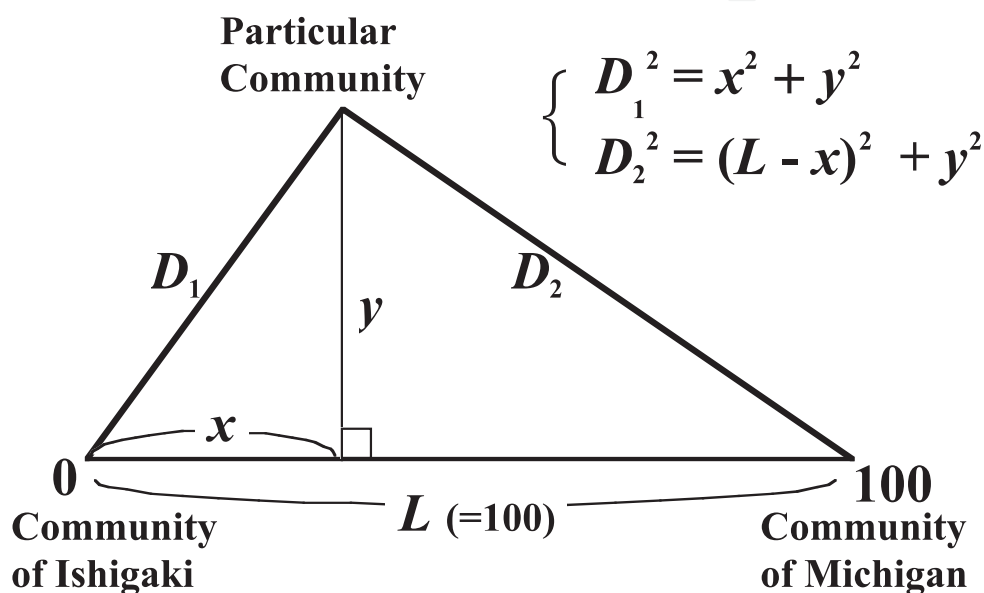
Figure 11. Schematic representation of polar ordination analysis.

Parameter x represents the polar difference (%) from the 0% pole (Ishigaki site) and is calculated as the following equation (7):

$$x = (L^2 + D_1^2 - D_2^2) / 2L, \tag{7}$$

where D_1 and D_2 are the percentage differences between a particular bradyrhizobial community and the communities at Ishigaki and at Michigan, respectively. Parameter L represents the 100% polar difference between the poles. Then, polar differences were calculated from the trigonometric diagram and plotted against the latitudinal difference between sites. The relationship of the polar ordination and the latitudes of the field sampling sites were estimated. This analysis was conducted for united data of sample soil site, based on the species (Bj and Be) and the clusters, for estimation of community structure distribution.
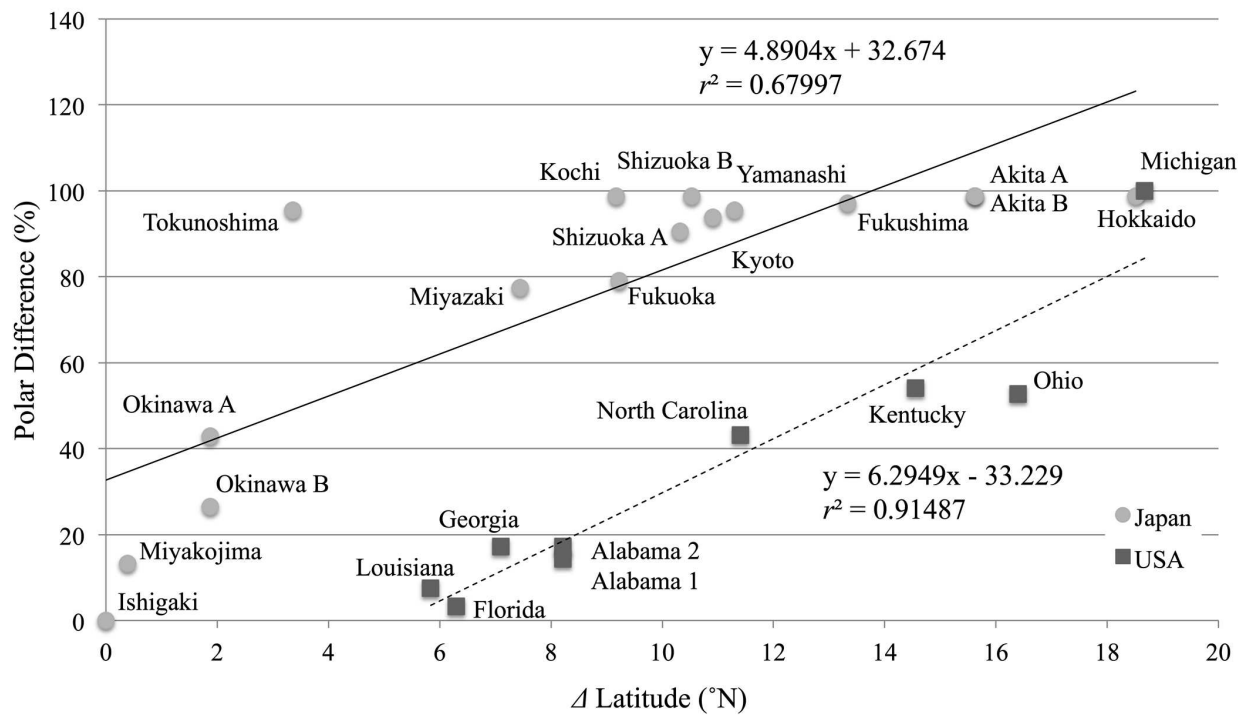
Figure 12. Relationship between latitude and polar difference based on 3D-MDS from Bj-Be data set.
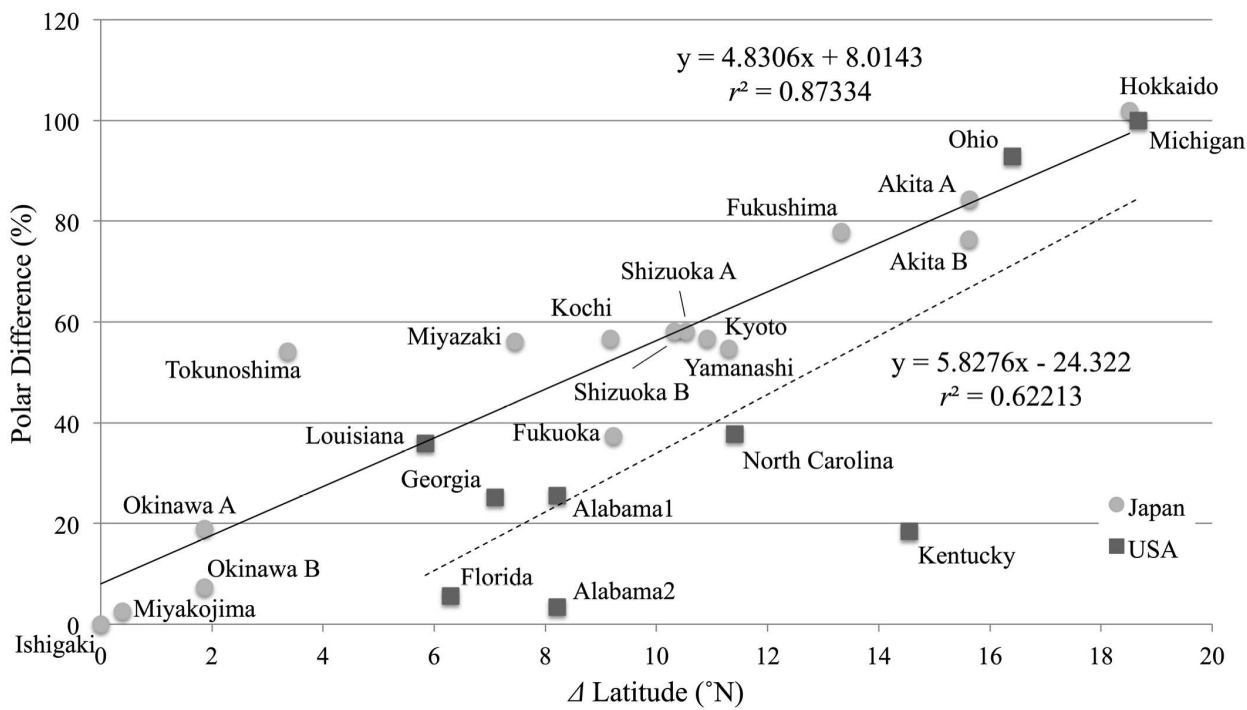

Figure 13. Relationship between latitude and polar difference based on 3D-MDS from the cluster data set.

3.6. Result of polar ordination analysis

As shown in Figs. 12 and 13, the flora of indigenous soybean rhizobia changed gradually from north to south, and a distinctive flora was detected at each field site. The results of the polar ordination analysis showed that indigenous soybean-nodulating bradyrhizobial community structure was correlated with latitude. This result suggests that the community of indigenous bradyrhizobia at a particular geographical location might be affected by soil temperature associated with latitude or the diversity of the associated host plants acclimatized to that region's climate. In our report, the higher dominance of localized *B. elkanii* strains in the soil was detected in USA [31]. In USA, the regression equations were shifted to lower than those of Japan. This is due to higher proportions of *B. elkanii* isolates in USA than those of Japan, though Bj123 was dominant in northern regions in both countries. The reason of dominance of *B. elkanii* in USA can be discussed based on other previous reports. Keyser et al. [57] examined the distribution of indigenous soybean-nodulating rhizobia in USA and found that serogroup of *B. japonicum* USDA123 was dominant in northern regions and *B. elkanii* in southern regions. Minamisawa et al. [58] investigated the preference of nodulation of soybean cultivars, a wild soybean progenitor (*Glycine soja*), and siratro (*Macroptilium atropurpureum*) by *B. japonicum* and *B. elkanii* strains. The result was that *B. japonicum* and *B. elkanii* preferentially nodulated *G. max* and *M. atropurpureum*, respectively, whereas both bradyrhizobial species formed nodules on *G. soja* with similar efficiency. Marr et al. [59] reported that *Amphicarpaea bracteata* performed nodule formation with *B. japonicum* and *B. elkanii* and performed nitrogen fixation with *B. elkanii*, though *A. bracteata* will not be the original host for *B. elkanii*. Furthermore, the microevolution and origins of *Bradyrhizobium* populations in eastern North America associated with soybean and native legumes (*A. bracteata* and *Desmodium canadense*) was investigated using genetic characterization by multilocus sequence typing of six core (housekeeping) gene sequences and two symbiotic gene sequences, and the results suggested that soybean-nodulating bacteria associated with native legumes represent a novel source of ecologically adapted bacteria for soybean inoculation [60]. Siratro is a major pasture legume that is cultivated in the tropics and subtropics, including parts of Australia, South and Central America, and some Pacific islands [61]. Additionally, *A. bracteata* is an annual legume that distributes widely in eastern North America [62, 63]. The presence of these legumes that have compatibility for nodulation with *B. elkanii* might be a reason to contribute strongly to the high dominance of *B. elkanii* in eastern North America.

In previous study, *B. elkanii* isolates were detected with high frequencies from southern regions of Japan [26, 33]. However, in those soil samples, isolation rates of *B. elkanii* were relatively low, and dominant isolates from fine-particle soils belonged to the Bj110 cluster (Table 2). In the United States, indigenous soybean-bradyrhizobia belonging to Bj123 cluster are dominant in northern regions, and Be clusters are dominant in central and southern regions [54, 31]. The Bj110 cluster is detected in central regions, but in association with few bradyrhizobia. These results suggest that soil chemical and/or physical properties determined by soil texture (e.g., silt versus clay) might affect indigenization and/or nodulation of soybean-nodulating bradyrhizobia. Fine-particle soils developed under paddy field conditions such as Gray Lowland soils might be suitable for indigenization of strains such as USDA110 strain under temperate climate regions. It has also been reported that soybean cultivation management practices affect the

genetic variance of soybean-nodulating bacteria [64, 65]. Concerning the structure of soybean-nodulating bacterial communities, it is therefore necessary to consider soil types, cultivation conditions (e.g., sowing period), soil temperature, and soil management practices.

As a summary of these results, it is indicated that the composition of indigenous soybean-nodulating bradyrhizobial community was correlated with latitude in temperate regions. And it is suggested that the community structure of indigenous bradyrhizobia at a particular geographical location will be affected by soil temperature and/or the diversity of the associated host plants acclimatized to that region's climate, with some exceptions in the case of fine-particle soils as discussed above, and alkaline-salinity soils, in which *Bradyrhizobium* serogroup 135 or *Sinorhizobium/Ensifer fredii* dominate [23, 33, 66-69].

4. Conclusion and future prospects

In this chapter, the RFLP analysis of the 16S–23S rRNA gene ITS region and mathematical analysis of the PCR-RFLP results were demonstrated as possible approaches to the study of community diversity and ecosystem of soybean-nodulating bradyrhizobia in relation to the rhizobial endemism in Japan and USA. As a result, generally, *B. japonicum* and *B. elkanii* generally indicated dominant existence from north to middle regions and from middle to south regions in both countries, respectively. Cluster Bj123 was dominant in northern regions, and cluster Be76 was dominant in southern regions in both countries. The bradyrhizobial community in USA was consisted from mainly Bj123, Bj110, Bj6, Be46, Be76, and Be94 clusters and diversity of Be cluster was higher than in Japan, and the bradyrhizobial community in Japan was consisted from mainly Bj123, Bj110, Bj6 and Be76 clusters and diversity of Bj clusters was higher than in USA. High coefficient of correlation was detected between community structures and north latitude. These results suggested that ecological niche of soybean-nodulating bradyrhizobial community will be detected along latitude, as a function of latitude and soil temperature.

The geographical distribution of bradyrhizobia along latitude reflects soil taxonomy such as zonal soils, the distribution of which on earth are affected by climate changes as a function of latitude. In contrast, the cluster of *B. japonicum* USDA 110 was dominant on fine-particle soils. The distribution of these strains reflects in part the distribution of soils such as intrazonal soils and is affected by water conditions and the oxidation-reduction potential in the soil. One of the reasons for the high occupancy of the Bj110 cluster in fine-particle soils might be strain capability for denitrification of bradyrhizobia. Though the end products of denitrification depend on the strain capability, *B. japonicum* strain USDA110 possesses a full set of functional denitrifying genes and reduces NO_3^- to N_2 [70]. Furthermore, this strain evinces the denitrifying capability to reduce N_2O surrounding the soybean root system [71]. Recently, Itakura et al. [72] demonstrated the mitigation of N_2O emission from soils by inoculation of soils with *B. japonicum* US-DA110 under field conditions. Therefore, utilization of useful bradyrhizobia that evince high N-fixing and full denitrifying capabilities is important not only for increasing yields but also for environmental conservation in agriculture and concerning the global warming.

Additionally, there are many reports on genetic diversity of soybean-nodulating rhizobia in subtropical-tropical regions. Appunu et al. [73] reported the genetic diversity of bradyrhizobia isolated from soybeans in India, and Jaiswal et al. [42] also reported the genetic diversity of soybean-nodulating rhizobia in India. They indicated the difference of soybean-nodulating rhizbial ecosystem from temperate regions and their broad host range. Yokoyama et al. [74] and Ando and Yokoyama [75] reported on *Bradyrhizobium* spp., which are different from *B. japonicum* and *B. elkanii*, based on genetic diversity of soybean-nodulating rhizobia in Thailand. Abaidoo et al. [76] reported heterogeneity of *Bradyrhizobium* spp. isolated from the new soybean cultivars in Africa as compared to bradyrhizobia from North American soybeans. These results suggest that diversity of soybean-nodulating rhizobia in subtropical-tropical regions and their ecosystems will be different from those in temperate regions. Further research on diversity and ecology of soybean-nodulating rhizobia in subtropical-tropical regions must be conducted for numerous environmental factors, containing soil types, climate conditions and soil managements to elucidate their ecology and to utilize their ecological traits for agriculture.

Because direct characterization of bradyrhizobial community structure in soil has so far been difficult, the characterization of rhizobial community structure has been limited with information coming only from analysis of soybean-nodulating rhizobial communities. It must be developed that the direct methods for the characterization of indigenous bradyrhizobial populations and community diversity in soils. Methods of characterizing indigenous rhizobial community structure from environmental DNA and use of media selective for bradyrhizobia from soil samples must therefore be developed to advance our understanding of indigenous rhizobial ecology and for construction of reliable models of soybean-nodulating rhizobial community structure.

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