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Compatibility of adzuki bean (*Vigna angularis*) and *Bradyrhizobium* USDA strains, and geographical distribution and community structure on indigenous adzuki bean-nodulating bradyrhizobia in Japan

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Abstract: We investigated the compatibility between adzuki bean and *Bradyrhizobium* USDA strains and the relation between the genetic diversity of indigenous adzuki bean-nodulating bradyrhizobia and their geographical distribution in Japan. Six *Bradyrhizobium* USDA strains were used in the inoculation test, and *B. elkanii* USDA 94 showed higher symbiotic efficiency than other strains. Two adzuki bean cultivars were used to isolate indigenous adzuki bean-nodulating bradyrhizobia. Their genetic diversity and community structure were analyzed by restriction fragment length polymorphisms of PCR amplicons to target the 16S-23S rRNA gene internal transcribed spacer region, using 11 USDA *Bradyrhizobium* strains as reference strains. Furthermore, we performed diversity analysis, non-metric multidimensional scaling analysis based on the Chao index, and polar ordination analysis to explain the relation between community structure and geographical distribution of the adzuki bean-nodulating bradyrhizobia. The major clusters were Bj123 in Hokkaido and Kyoto, Bd110 in Fukushima and Shimane A, Be76 in Shimane B and Fukuoka, Bj6 in Miyazaki, and Bj6 and BeOK in Okinawa. Japan's indigenous adzuki bean-nodulating bradyrhizobial community was strongly and significantly correlated with latitude, longitude, annual mean temperature, and electrical conductivity. This result suggests that the adzuki bean-nodulating bradyrhizobial community is influenced by multiple environmental factors.

Keywords: *Bradyrhizobium*; adzuki bean; compatibility; genetic diversity; environmental factors

Adzuki bean (*Vigna angularis*) is widely cultivated in East Asian countries such as Japan, China, Korea, Taiwan and Australia (Lumpkin and McClary 1994; Tomooka et al. 2002; Redden et al. 2012). In Japan, adzuki bean is an important crop and an ingredient in traditional confectionery. Additionally, they

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are a highly valuable crop for food and medicinal use because they contain rich starch, a balanced variety of amino acids, and polyphenols such as proanthocyanidins, which exhibit potential radical scavenging activities (Ariga & Hamano 1990; Kitano-Okada et al. 2012; Kitano-Okada et al. 2019; Wang et al. 2022). Adzuki bean is a legume that forms root nodules after infection with rhizobia, which perform symbiotic nitrogen fixation by taking up atmospheric nitrogen through the root nodules.

The genus *Vigna* includes mung bean (*V. radiata*), cowpea (*V. unguiculata*), and black gram (*V. mungo*), which are widely cultivated in many tropical and subtropical countries for food and feedstuff (Fery 2002). The rhizobia of these *Vigna* species were isolated from root nodules and the analysis of genetic diversity and symbiotic function was conducted, and the major adzuki bean-nodulating rhizobia found were *Bradyrhizobium japonicum*, *B. elkanii*, *B. yuanmingense*, *B. liaoningense*, *B. vignae*, and *Ensifer/Sinorhizobium fredii* (Krasova-Wade et al. 2003; Yokoyama et al. 2006; Zhang et al. 2008; Appunu et al. 2009; Han et al. 2009; Odori et al. 2020; Songwattana et al. 2021). These rhizobia are common to soybean-nodulating rhizobia (Jordan 1982; Scholla & Elkan 1984; Chen et al. 1988; Kuykendall et al. 1992; Xu et al. 1995; Yao et al. 2002; Young 2003).

The mung bean, cowpea, and black gram have been reported to improve biological nitrogen fixation (BNF) and productivity, such as plant growth and seed yield, by inoculation with *Bradyrhizobium* strains and co-inoculation of arbuscular mycorrhizal fungi (Christopher et al. 2018; Ayalew & Yoseph 2020; Pereira et al. 2020; Gough et al. 2021; de Oliveira et al. 2022). In adzuki bean, inoculation with *Bradyrhizobium* strains has been reported to increase shoot dry weight and shoot total N content due to improved BNF (Delić et al. 2010). However, the efficiency of inoculum with high nitrogen fixation ability is poor in fields because the inoculum cannot compete with indigenous rhizobia in the soil. To solve this problem, it is very important to understand the ecology of indigenous rhizobia in terms of genetic diversity, geographical distribution, compatibility with the host legume plants, and environmental factors associated with localisation and dominance in the soil.

Soybean (*Glycine max*. L. Merr.), as well as adzuki bean, is known to perform symbiotic nitrogen fixation with *Bradyrhizobium* and *Ensifer*/*Sinorhizobium* (Jordan 1982; Scholla & Elkan 1984; Chen et al. 1988; Kuykendall et al. 1992; Young 2003). Saeki et al. (2013) investigated the genetic diversity and geographical distribution of indigenous soybean-nodulating rhizobia isolates from 16 sites in Japan by PCR restriction fragment length polymorphism (PCR-RFLP) analysis of the 16S-23S rRNA gene internal transcribed spacer (ITS) region and revealed that the community structures and their geographical distribution of indigenous soybean-nodulationg rhizobia varied from the northern to southern regions in Japan according to latitude and related climate. Furthermore, the representative clusters of isolated indigenous soybean-nodulating rhizobia were in the order of *B. japonicum* strains USDA 123, 110 [species name changed to *B. diazoefficiens* USDA 110^T (Delamuta et al. 2013)], and 6^T and *B. elkanii* strain USDA 76^T, moving from northern to southern regions in Japan. However, it is unknown if the different host legume plants would show the same tendency as in this report.

Thus, in this study, we confirmed the compatibility of adzuki bean and *Bradyrhizobium* USDA strains, and isolated indigenous adzuki bean-nodulating bradyrhizobia from eight Japanese field site soils. The isolates were investigated for genetic diversity, community structure, and geographical distribution based on PCR-RFLP analysis of the 16S-23S rRNA gene ITS region. Furthermore, the environmental factors that characterise the adzuki bean-nodulating bradyrhizobial community structure and geographical distribution were estimated using mathematical ecology analyses.

MATERIAL AND METHODS

Inoculation test. To estimate the nodulation compatibility between adzuki bean and *Bradyrhizobium* USDA strains, we performed inoculation tests of bradyrhizobia to adzuki bean. Tanbadainagon was used as adzuki bean cultivar and *B. japonicum* USDA 6^T and 123, and *B. diazoefficiens* USDA 110^T, and *B. elkanii* USDA 46, 76^T, and 94 were used as test strains. These strains were cultured in yeast extract-mannitol broth (YMB) medium (Vincent 1970) at 28 ℃ for 6 days. The cultures were diluted to 10^6 cells/mL with sterile distilled water. 1 L culture pots were used to cultivate adzuki bean. The culture pots were filled with vermiculite containing N-free nutrient solution (Saeki et al. 2000) at 40% (v/v) water content and were then autoclaved at 121 ℃ for 20 min. Adzuki bean seeds were sterilized by soaking them in 70% ethanol for 30 s and in a dilute sodium hypochlorite solution (0.25% available chlorine) for 3 min, followed by

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washing with sterile distilled water. The adzuki bean seeds were sown and inoculated with 1 mL of diluted bacterial culture per seed. There were four plants per pot, with no replications. The adzuki bean was grown for 4 weeks in a growth chamber (day, 28 ℃ for 16 h; night, 25 ℃ for 8 h) with a weekly supply of sterile distilled water. A control pot (non-inoculation) as a negative control for nodulation of rhizobia was also prepared and cultivated under similar conditions. After 4 weeks, shoot length, shoot dry weight, SPAD, nodule number, and nodule dry weight were assessed. The shoots and nodules were dried at 80 ℃ for 48 h using a drying machine.

Soil samples. The soil samples for the isolation of adzuki bean-nodulating bradyrhizobia were obtained from eight experimental fields and farm fields in seven Japanese prefectures: Hokkaido, Fukushima, Kyoto, Shimane, Fukuoka, Miyazaki, and Okinawa (Table 1). Only soil samples from Shimane were collected from two field sites (Shimane A and Shimane B). Six soil samples of Hokkaido, Fukushima, Kyoto, Fukuoka, Miyazaki, and Okinawa are the same soils used in previous studies (Saeki et al. 2006; Saeki et al. 2013; Suzuki et al. 2008). These soils have a history of adzuki bean or soybean cultivation but no history of inoculation of bradyrhizobia. The sampling location, soil group, annual mean temperature, annual mean precipitation, soil pH, and electrical conductivity (EC) are listed in Table 1. The data of annual mean temperature and annual mean precipitation were collected from the Japan Metrological Agency.

Isolation of the indigenous adzuki bean-nodulating bradyrhizobia. To isolate indigenous adzuki bean-nodulating bradyrhizobia, we used two Japanese adzuki bean cultivars of Tanbadainagon and Kitahotaru, and planted each adzuki bean cultivar in 1 L culture pots (n = 3 plants per cultivar). The preparation of culture pots and sterilization of seed were as described above. A soil sample (2 to 3 g) was placed in the vermiculite at a depth of 2 to 3 cm, the adzuki bean seeds were sown in the soil, and then the pot weight was measured. The plants were grown for 6 weeks in a growth chamber (day, 28 ℃ for 16 h; night, 25 ℃ for 8 h), and sterile distilled water was supplied twice a week until it reached the initial pot weight.

After cultivation for 6 weeks, 16 to 24 nodules were randomly collected from all of the nodules harvested from adzuki bean root and sterilized by soaking them in 70% ethanol for 3 min and in a diluted sodium hypochlorite solution (0.25% available chlorine) for 30 min and then washed with sterile distilled water. Each nodule was homogenized in sterile distilled water, streaked onto yeast extract-mannitol agar (YMA) (Vincent, 1970) plate medium, and incubated for 5 to 7 days in the dark at 28 ℃. To determine the genus of

Table 1. Information of soil samples used in this study

Sample soil	Soil group	Sampling site	Latitude, longitude	Δ Latitude (°N)	Δ Longitude (°E)	AMT (℃)	AMP (mm)	pH (H_2O)	EC (mS/m)	Reference
Hokkaido	Andosols	Memuro, Hokkaido	42.89 °N, 143.07 °E	16.64	15.31	6.3	971.6	5.2	17.0	Saeki et al. 2006
Fukushima	Andosols	Arai, Fukushima	37.71 °N, 140.39 °E	11.46	12.63	13.4	1 207.0	5.0	5.0	Saeki et al. 2006
Kyoto	Andosols	Ayabe, Kyoto	35.29 °N, 135.26 °E	9.04	7.50	14.6	1 623.8	5.1	15.0	Saeki et al. 2006
Shimane A	Gray lowland soils	Matsue, Shimane	35.49 °N, 133.07 °E	9.24	5.31	15.2	1 791.9	6.6	8.0	This study
Shimane B	Gray lowland soils	Masuda, Shimane	34.68 °N, 131.93 °E	8.43	4.17	15.9	1 570.5	5.8	10.0	This study
Fukuoka	Gray lowland soils	Kasuya, Fukuoka	33.61 °N, 130.46 °E	7.36	2.70	17.3	1 686.9	5.6	2.0	Saeki et al. 2013
Miyazaki	Andosols	Kibanadai, Miyazaki	31.83 °N, 131.42 °E	5.58	3.66	17.7	2 625.5	5.7	6.0	Saeki et al. 2006
Okinawa	Red yellow soils	Nishihara, Okinawa	26.25 °N, 127.76 °E	0.00	0.00	23.3	2 161.0	5.7	4.0	Suzuki et al. 2008

AMT – annual mean temperature; AMP – annual mean precipitation

The data of AMP and AMT are 30-year averages from 1991 to 2020 and were obtained from the web site of Japan Meteorological Agency (https://www.data.jma.go.jp/obd/stats/etrn/index.php)

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the isolates, a single colony was streaked onto YMA plate medium containing 0.002% (w/v) bromothymol blue (Keyser et al. 1982) and incubated as described above. After incubation, each isolate was maintained on YMA slant medium at 4 ℃ until further analysis. Forty to 48 isolates per soil sample were used to represent the adzuki bean-nodulating bradyrhizobial community, and we obtained a total of 357 isolates from the eight fields and used them in the diversity analysis and non-metric multidimensional scaling (NMDS) analysis described below.

Representative isolates in each operational taxonomic unit (OUT) of the dendrogram were confirmed for their nodulation capability on host adzuki bean by an inoculation test. Each isolate was cultured in YMB medium for 6 days at 28 ℃, and the cultures were then diluted with sterile distilled water to 10^6 cells/mL. The Tanbadainagon and Kitahotaru each adzuki bean seeds were sown into 1 L culture pots without soil, as described above, and inoculated with 1 mL of diluted bacterial culture per seed. There were two to three plants per pot, with no replications. We assessed nodule formation after 4 weeks in a growth chamber under the conditions described above.

PCR-RFLP analysis of 16S-23S rRNA gene internal transcribed spacer region. For DNA extraction, each isolate was cultured in 1.5 mL HEPES-MES (HM) medium (Cole & Elkan 1973) supplemented with 0.1% L-arabinose (Sameshima et al. 2003) for 5 days at 28 ℃. Total DNA for the PCR template was extracted from the isolates with BL extraction buffer as described previously (Minami et al. 2009) based on the method reported by Hiraishi et al. (1995).

As reference strains, *B. japonicum* USDA 4, 6^T, 38, 115, 123, 124, and 135, and *B. diazoefficiens* USDA 110^T, and *B. elkanii* USDA 46, 76^T, and 94 were used (Saeki et al. 2004). Total DNA of reference strains for the PCR template was also extracted in the same manner as the isolates. PCR amplification of the ITS region was carried out using TaKaRa *Ex Taq*® Hot Start Version (TaKaRa Bio, Shiga, Japan) and the ITS primer set (BraITS-F: 5'-GACTGGGGTGAAGTCGTAAC-3' and BraITS-R1: 5'-ACGTCCTTCATCGCCTC-3') (Saeki et al. 2006). The PCR cycle consisted of a pre-run at 94 ℃ for 5 min, denaturation at 94 ℃ for 30 s, annealing at 55 ℃ for 30 s, and extension at 72 ℃ for 1 min. This temperature control sequence was repeated for a total of 30 cycles, followed by a final post-run at 72 ℃ for 10 min. The restriction fragment length polymorphism (RFLP) analysis of the 16S-23S rRNA gene ITS region was performed using restriction enzymes *Hae*III, *Hha*I, *Msp*I, and *Xsp*I (TaKaRa Bio). A 5 µL aliquot of the PCR product was digested with restriction enzyme at 37 ℃ for 16 h in a 20 µL reaction mixture. The restricted fragments were separated by 3% or 4% agarose gel electrophoresis and visualized with ethidium bromide.

Cluster analysis. The fragment sizes on the electrophoresed gel were measured with a 50 bp reference ladder marker (TaKaRa Bio) and the fragment sizes from the sequences of the reference strains. All reproducible fragments longer than 50 bp were used for cluster analysis and some irreproducible fragments were excluded. The genetic distance between pairs of isolates (D) was calculated using the following equation:

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

where: N_{AB} – the number of RFLP bands shared by strains A and B; N_A and N_B – the number of RFLP bands in strains A and B, respectively (Nei & Li 1979; Sakai et al. 1998).

Cluster analysis was performed using the unweighted pair group with arithmetic average (UPGMA) method. The dendrograms were constructed using the PHYLIP software program (version 3.695).

Sequence analysis of 16S rRNA gene, ITS region and *rpo*B gene. The sequence analysis of 16S rRNA gene, 16S-23S rRNA gene ITS region and *rpo*B gene of the representative strains that selected based on the results of the PCR-RFLP analysis and cluster analysis, were performed. Amplification of these genes were conducted using TaKaRa Ex Premier DNA Polymerase (TaKaRa Bio), the primer set for 16S rRNA gene (16S-F: 5'-AGAGTTTGATCCTGGCTCAG-3' and 16S-R2: 5'-CGGCTACCTTGTTACGACTT-3') (Weisberg et al. 1991), the primer set for *rpo*B gene (*rpo*B83F: 5'-CCTSATCGAGGTTCACAGAAGGC-3' and *rpo*B1540R: 5'-AGCTGCGAGGAACCGAAG-3') (Martens et al. 2008), and the primer set for ITS region (BraITS-F and BraITS-R1) (Saeki et al. 2006). The PCR cycle consisted of a pre-run at 94 ℃ for 3 min, denaturation at 98 ℃ for 10 s, annealing at 58 ℃ for 15 s, and extension at 68 ℃ for 40 s. This temperature control sequence was repeated for a total of 30 cycles, followed by a final post-run at 68 ℃ for 2 min. The PCR amplified products were cleaned-up according to the protocol of Exo-CIP

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Rapid PCR Cleanup Kit (New England BioLabs, USA). The sequence analysis of cleaned-up samples was conducted using the contracting service of the company (Eurofins Genomics K.K., Tokyo, Japan). The samples were prepared according to the protocol provided by this company and the samples were sent to the company. The forward primer of each primer set was used as the sequence primer.

To search the homology of sequences, Basic Local Alignment Search Tool (BLAST) in National Center for Biotechnology Information (NCBI) was used. The multiple alignment of the obtained sequences was performed using ClustalW. Phylogeny was determined by the Neighbor-Joining method (Saitou & Nei 1987). Genetic distances were calculated using Kimura 2-parameter model (Kimura 1980). These analyses were performed according to a previous study (Mason et al. 2017) and using the Molecular Evolutionary Genetics Analysis software (version 10.2.6) (Kumar et al. 2018). Phylogenetic trees were bootstrapped with 1 000 replications of each nucleotide sequence to evaluate the reliability of the tree topology. All the nucleotide sequences obtained in this study were deposited in DNA Data Bank of Japan (DDBJ) under accession numbers LC702982 to LC702999; LC703000 to LC703017; LC703018 to LC703035 at http://www.ddbj.nig.ac.jp/.

Diversity analysis for bradyrhizobial communities. To estimate the diversity of the bradyrhizobial communities in Japan isolated from the host adzuki beans, we used the Shannon-Wiener diversity index (MacArthur 1965; Pielou 1969; Saeki et al. 2008) for alpha diversity and Chao index for beta diversity as a measure of dissimilarity (Chao et al. 2005). The Shannon-Wiener index (H') was calculated using the following equation:

$$H' = -\sum Pi \ln Pi \quad (2)$$

where: Pi – the dominance of the isolates expressed by ni/N; N – the total number of tested isolates (from n = 16 to 24); ni – the total number of tested isolates belonging to a particular dendrogram cluster.

The Chao dissimilarity measure (d_{Ch}) was calculated using the following equation:

$$d_{ChAB} = 1 - U_A U_B/(U_A + U_B - U_A U_B) \quad (3)$$

$$U_A = C_A/N_A + (N_B - 1)/N_B \times a1/(2a2) \times S_A/N_A \quad (4)$$

where: d_{chAB} – the dissimilarity between communities A and B; C_A – the total number of individuals in the cluster of community A that are shared with community B; N_A – the total number of individuals in community A; N_B – the total number of individuals in community B; $a1, a2$ – the number of clusters in community A that have only one or two individuals in community B; S_A – the total number of individuals in the species present in community A that occur with only one individual in community B; U_B – similar to the U_A equation.

The Chao index is one of the indices that best reflects the properties between communities when considering the probability of rare species in communities (Chao et al. 2005; Chao et al. 2006).

The Chao dissimilarity index was calculated using function "vegdist" of the "vegan" package (version 2.5-7) in the R software program (version 4.0.3) (The R Project for Statistical Computing: http://www.r-project.org/).

Permutation multivariate analysis of variance, non-metric multidimensional scaling analysis and polar ordination analysis. To determine the environmental factors such as Δlatitude (°N), Δlongitude (°E), annual mean temperature, annual mean precipitation, pH, and EC that are related to indigenous bradyrhizobial community structure, permutation multivariate analysis of variance (PERMANOVA) (Anderson 2001) based on the Chao index as a measure of dissimilarity (permutations of 9 000) was performed using function "adonis" of the "vegan" package (version 2.5-7) in the R software program (version 4.0.3).

To characterize the relationships among the bradyrhizobial community, we performed non-metric multidimensional scaling (NMDS) analysis (Somerfield & Clarke 1995) based on the Chao index as a measure of dissimilarity. The three-dimensional NMDS analysis and the cluster analysis based on the Chao dissimilarity measure were conducted using the function "metaMDS" of the "vegan" package (version 2.5-7) in the R software program (version 4.0.3).

To determine the relative distances among the bradyrhizobial communities based on three-dimensional NMDS plots of bradyrhizobial communities and each environmental factor, the polar differences were calculated from the Euclidean distances between the bradyrhizobial communities using a trigonometric figure and were plotted between the polar axis (Whittaker 1967; Kobayashi 1995; Saeki et al. 2008). The bradyrhizobial communities isolated from the Okinawa site (26.25 °N) and the Hokkaido

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site (42.89 °N) were applied as plot samples (100% difference) due to the greatest difference among the latitudes of the sample soil sites. The distances between each pole plot and other plots on the three-dimentional NMDS plot were calculated with coordinates of *x*-, *y*-, and *z*-axis as Euclidean distance (*Ed*) using the following equation:

$$Ed_{AB} = (|X_A - X_B|^2 + |Y_A - Y_B|^2 + |Z_A - Z_B|^2)^{1/2} \quad (5)$$

where: Ed_{AB} – the linear distance between communities A and B on the three-dimensional NMDS plots; X_A, X_B, Y_A, Y_B, Z_A, Z_B – the coordinates on the *x*-, *y*-, and *z*-axis of communities A and B, respectively.

The distances from each pole were converted to percentage differences (%), D_1 and D_2, from the pole communities, Hokkaido, and Okinawa sites with a 100% difference. Simultaneous equations were constructed from the trigonometric figure using the Pythagorean theorem, as described previously (Saeki et al. 2008). Parameter *Pd* represents the polar difference (%) from the 0% pole (bradyrhizobial community of Okinawa) and is calculated as follows:

$$Pd = (L^2 + D_1^2 - D_2^2)/2L \quad (6)$$

where: D_1, D_2 – the percentage differences between a particular bradyrhizobial community and the communities in Okinawa and Hokkaido, respectively (Whittaker 1967); L – the difference between the two polar sites (*i.e.*, 100%).

This analysis was conducted using the combined data from the soil sampling sites of each adzuki bean cultivars.

Linear regression analysis (Pearson correlation) for studying the relationship between the latitudes of soil sampling sites and polar ordination was conducted using command "cor.test" in the R software program (version 4.0.3).

RESULTS

Compatibility of adzuki bean and *Bradyrhizobium* USDA strains. The compatibility of adzuki bean cultivar Tanbadainagon and *Bradyrhizobium* USDA strains for nodulation and plant growth is shown in Table 2. Nodulation due to infection by USDA strains was observed in all strains except *B elkanii* USDA 76^T, and the number of nodules was highest in *B. japonicum* USDA 123 compared to the other strains. The control plants that were not inoculated with rhizobia did not show nodules. In addition, *B. elkanii* USDA 94 showed higher values for shoot length, shoot dry weight and nodule dry weight, and *B. japonicum* USDA 6^T showed higher values for SPAD than the other strains.

Genetic diversity and geographical distribution of indigenous adzuki bean-nodulating bradyrhizobia by RFLP analysis of the 16S-23S rRNA gene ITS region. Sixteen to 24 isolates were obtained from two adzuki bean cultivars and a total of 357 indigenous adzuki bean-nodulating bradyrhizobia that could be used for further analysis. The indigenous bradyrhizobia isolates from each soil sample and host adzuki bean cultivar combinations were labeled using a combination of the site abbreviations, an abbreviation for the two adzuki bean cultivars (T: Tanbadainagon and K: Kitahotaru), and the number of isolates (1–16 to 1–24) (*e.g.*, for Hokkaido, THO1-24, and KHO1-22). The YMA cultures of all isolates turned blue due to the presence of bromothymol blue, indicating that all isolates belonged

Table 2. Infection of *Bradyrhizobium* USDA strains and difference in growth rates on adzuki bean

USDA strains	Shoot length (cm/plant)	Shoot dry weight (g/plant)	SPAD	Nodule number (No./plant)	Nodule dry weight (mg/plant)
B. japonicum USDA 6^T	13.6 ± 1.36^b	0.33 ± 0.04^{ab}	30.4 ± 2.60^a	79.5 ± 13.3^b	24.5 ± 0.01^a
B. japonicum USDA 123	11.7 ± 0.96^b	0.31 ± 0.01^{ab}	13.5 ± 1.41^b	166.3 ± 17.4^a	25.3 ± 0.00^a
B. diazoefficiens USDA 110^T	15.6 ± 0.90^{ab}	0.35 ± 0.03^{ab}	28.1 ± 0.88^a	97.0 ± 6.22^b	26.7 ± 0.01^a
B. elkanii USDA 46	15.2 ± 0.35^{ab}	0.38 ± 0.04^a	28.6 ± 1.01^a	78.3 ± 8.20^b	29.0 ± 0.00^a
B. elkanii USDA 76^T	12.6 ± 0.58^b	0.24 ± 0.03^b	12.8 ± 0.70^b	2.5 ± 1.55^c	0.0 ± 0.00^b
B. elkanii USDA 94	18.8 ± 0.95^a	0.45 ± 0.02^a	26.1 ± 2.09^a	110.3 ± 11.0^b	31.6 ± 0.00^a
ANOVA	***	**	***	***	***

Values are expressed as the mean ± SE of four plants

Asterisks indicate significant differences at * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$, respectively

Different letters indicate significant differences (Tukey's HSD test) at $P < 0.05$

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to the genus *Bradyrhizobium*. Furthermore, in the inoculation test of representative isolates, they indicated nodulation capability on the host adzuki bean.

PCR products of the amplified 16S-23S rRNA gene ITS region of isolates were digested using four restriction enzymes, and the restriction fragments were separated by gel electrophoresis. The dendrogram was constructed based on the differences in fragment size and pattern, as shown in Figure 1. The results of the cluster analysis based on the PCR-RFLP analysis data is shown Figure 2. We detected 25 operational taxonomic units (OTUs) containing 11 reference strains. The maximum similarity between the OTUs and the reference strains was 92%, and it occurred between USDA 76^T and USDA 94 of OTUs. These results were then applied as the criterion for distinguishing clusters in the dendrogram, which produced 15 clusters, 11 of which included

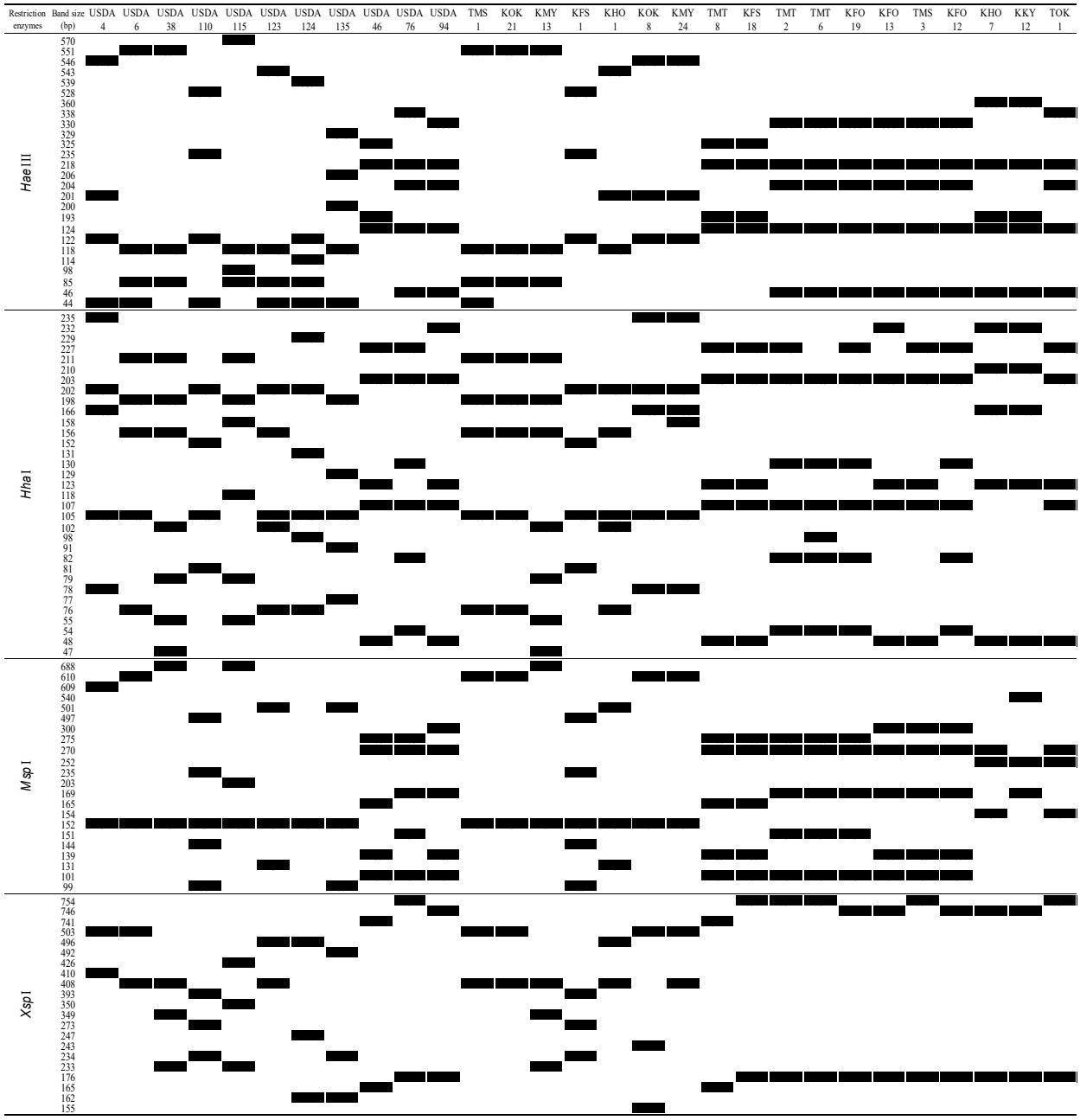

Figure 1. Schematic representation of electrophoresis pattern of PCR-RFLP of 16S-23S rRNA gene ITS region of adzuki bean-nodulating bradyrhizobia isolates

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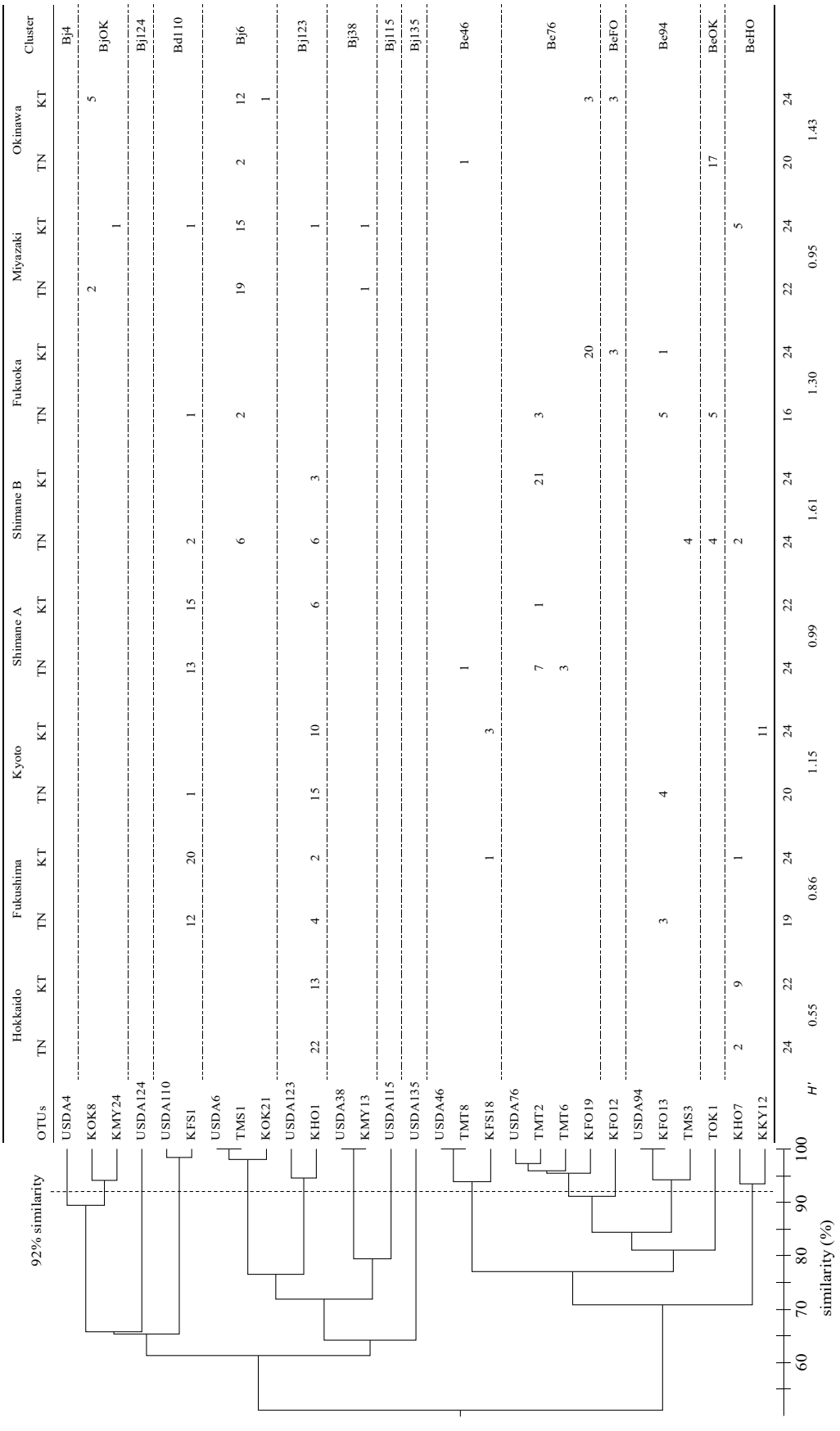

Figure 2. Dendrogram of the 16S-23S rRNA gene ITS region of indigenous adzuki bean-nodulating bradyrhizobia and *Bradyrhizobium* USDA reference strains

The letters under the sample soil site name indicate the adzuki bean cultivars, Tanbadainagon (TN) and Kitahotaru (KT). The similarity between *Bradyrhizobium elkanii* USDA 76^{T} and 94 (which was 92 %) was applied as the criterion for the differentiation of the clusters. Clusters are indicated on the right. The diversity index (H') was calculated using the following equation: $H' = -\Sigma\, Pi \ln Pi$

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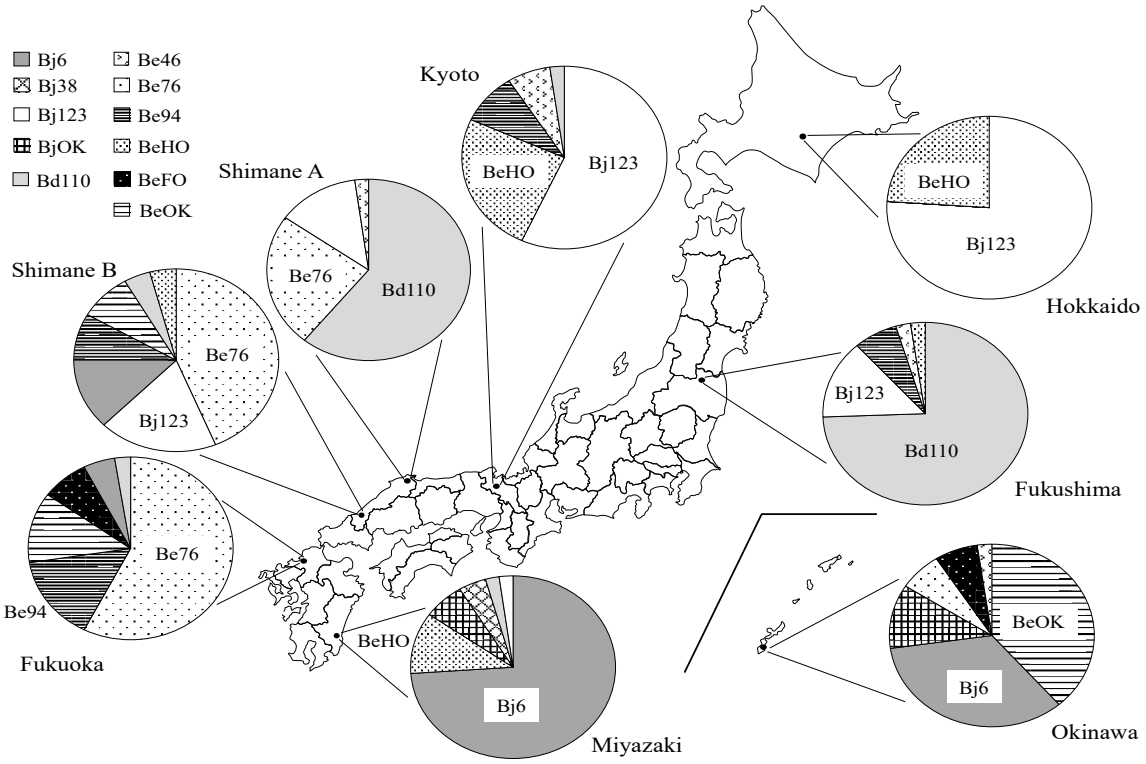

Figure 3. Distribution of clusters and the population ratio of indigenous adzuki-bean nodulating bradyrhizobia in Japan. Map data is obtained from website (https://power-point-design.com/ppt-design/japan-map-available-for-powerpoint/)

four of the reference strains. All indigenous bradyrhizobia were classified into 11 clusters: Bj6, Bj38, Bj123, BjOK, Bd110, Be46, Be76, Be94, BeHO, BeFO, and BeOK. Four of the clusters included only a single reference strain and no indigenous bradyrhizobia. Bj6, Bj38, and Bj123 showed RFLP patterns identical or similar to those of *B. japonicum* USDA 6^T, 38, and 123, respectively. Bd110 showed RFLP patterns identical or similar to those of *B. diazoefficiens* USDA 110^T. Be46, Be76, and Be94 showed RFLP patterns identical or similar to those of *B. elkanii* USDA 46, 76^T, and 94, respectively. BjOK, BeHO, BeFO, and BeOK showed patterns that were independent of those of the reference strains.

The geographical distribution of adzuki bean-nodulating bradyrhizobial isolates that belonged to each cluster is shown Figure 3. Most of the isolates from the Hokkaido site were classified into cluster Bj123, and BeHO was a minor cluster. The Fukushima site revealed Bd110 to be the dominant cluster, and Bj123 and Be94 were minor clusters. The Kyoto site revealed Bj123 to be the dominant cluster, and BeHO and Be94 were minor clusters. The Shimane A site revealed Bd110 to be the dominant cluster, and Be76 and Be46 were minor clusters. The Shimane B site revealed Be76 to be the dominant cluster, and Bj123 and Bj6 were minor clusters. The Fukuoka site revealed Be76 to be the dominant cluster, and Be94 and BeOK were minor clusters. The Miyazaki site revealed Bj6 to be the dominant cluster, and BeHO and BjOK were minor clusters. The Okinawa site revealed BeOK and Bj6 to be the dominant clusters, and BjOK and Be76 were minor clusters. The occupancy rates of *B. japonicum*, *B. diazoefficiens*, and *B. elkanii* in Japan soil were 40.7%, 18.6%, and 40.7%, respectively.

Genetic diversity of indigenous adzuki bean-nodulating bradyrhizobia by sequence analysis of 16S rRNA gene, ITS region and *rpo*B gene. The phylogenetic tree based on neighbor-joining for 16S rRNA gene, 16S-23S rRNA gene ITS region and *rpo*B gene of 18 representative strains and 11 *Bradyrhizobium* USDA reference strains is shown Figure 4. In the sequence analysis of 16S rRNA gene, 7 representative strains belonging to cluster of *B. japonicum* and *B. diazoefficiens* were included in group including *B. japonicum* and *B. diazoefficiens* USDA strains, and 11 representative strains belonging to cluster of *B. elkanii* were included in group including *B. elkanii* USDA strains (Figure 4A). In

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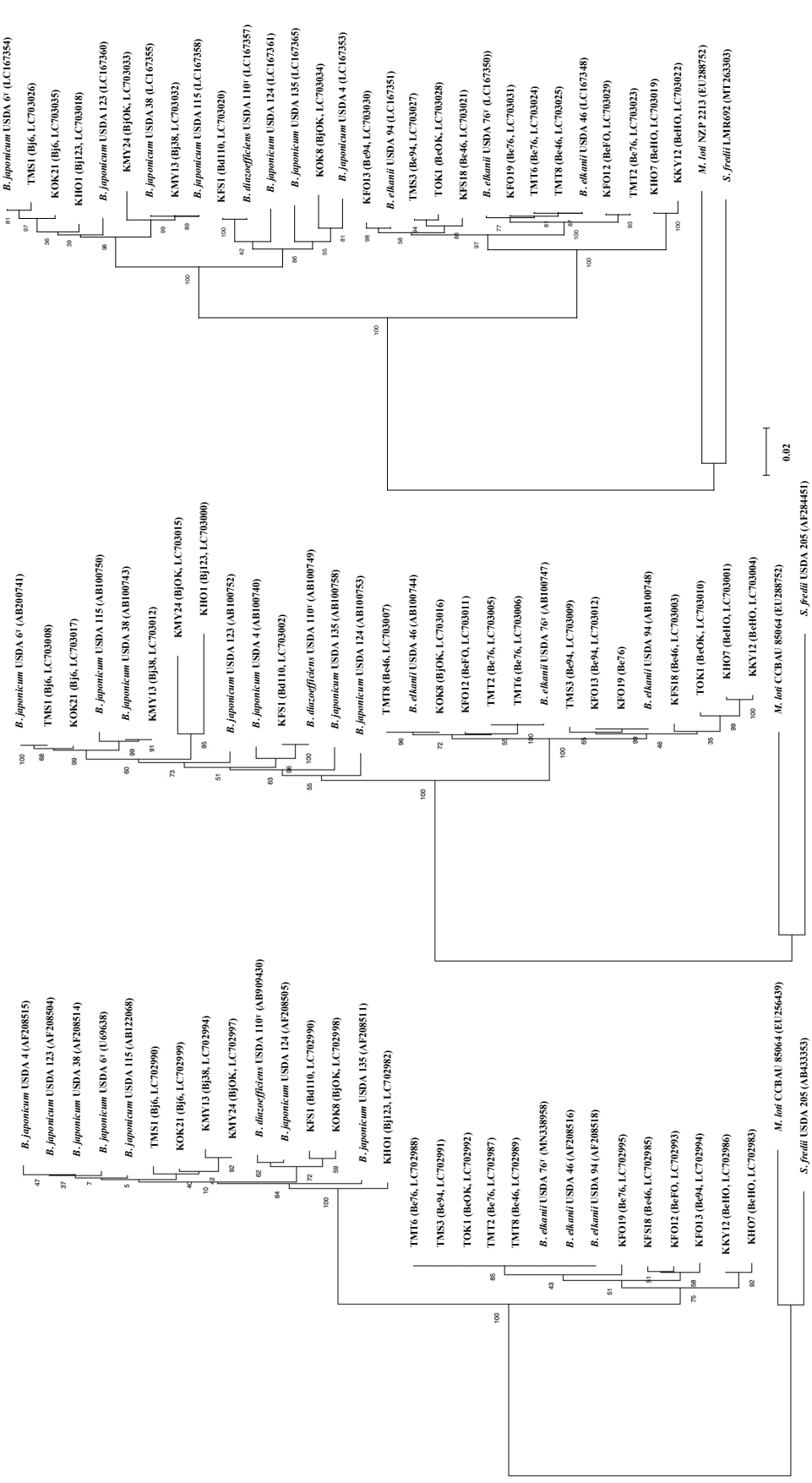

Figure 4. Neighbor-joining phylogenetic tree of representative and *Bradyrhizobium* USDA reference strains based on sequence analysis of (A) 16S rRNA gene (935 aligned bp), (B) 16S-23S rRNA gene ITS region (990 aligned bp) and (C) *rpo*B gene (895 aligned bp) sequences

Bootstrap values are indicated a percentage of 1 000 replications at the branching points. The scale bar indicates substitutions per site. The characters in parenthesis beside to representative strains indicate cluster obtained by PCR-RFLP analysis of ITS region and the accession number registered in DDBJ

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Table 3. Chao dissimilarity indices for each soil sample site pair

	Hokkaido	Fukushima	Kyoto	Shimane A	Shimane B	Fukuoka	Miyazaki	Okinawa
Hokkaido	–	–	–	–	–	–	–	–
Fukushima	0.837	–	–	–	–	–	–	–
Kyoto	0.182	0.000	–	–	–	–	–	–
Shimane A	0.875	0.278	0.308	–	–	–	–	–
Shimane B	0.771	0.629	0.635	0.343	–	–	–	–
Fukuoka	1.000	0.825	0.919	0.400	0.257	–	–	–
Miyazaki	0.870	0.795	0.837	0.957	0.424	0.915	–	–
Okinawa	1.000	0.983	0.981	0.920	0.446	0.270	0.591	–

the sequence analysis of ITS region, 6 representative strains belonging to cluster of *B. japonicum* and *B. diazoefficiens* except for KOK8 were included in group including *B. japonicum* and *B. diazoefficiens* USDA strains, and 11 representative strains belonging to cluster of *B. elkanii* were included in group including *B. elkanii* USDA strains (Figure 4B). In the sequence analysis of *rpo*B gene, the results were similar to those in the sequence analysis of 16S rRNA gene (Figure 4C). The phylogenetic trees of 16S rRNA gene and *rpoB* gene of the 18 representative strains clearly separated the groups of *B. japonicum* and *B. diazoefficiens* strains from the group of *B. elkanii* strains (Figure 4A and Figure 4C).

Diversity analysis for the indigenous bradyrhizobial community, Permutation multivariate analysis of variance, non-metric multidimensional scaling analysis and polar ordination analysis. The differences in indigenous bradyrhizobial communities among the eight sample sites were estimated using beta diversity based on the Chao dissimilarity measure. The Shannon-Wiener diversity index (H') values are indicated at the bottom of Figure 2, and the Chao dissimilarity indices of each soil sample site pair is shown in Table 3. In the Japanese indigenous bradyrhizobial communities, Shimane B had the highest H' value (1.61), whereas Hokkaido had the lowest diversity index (0.55). The values of Chao dissimilarity indices were largest for the comparison of Hokkaido to Fukuoka and Okinawa sites. Beyond that, the comparison of Fukushima to Okinawa, Kyoto to Fukuoka and Okinawa, Shimane A to Miyazaki and Okinawa, Fukuoka to Miyazaki indicate high values of Chao dissimilarity indices.

The PERMANOVA showed that there was a significant difference between the indigenous adzuki bean-nodulating bradyrhizobial community and Δlatitude (P = 0.014), Δlongitude (P = 0.014), annual mean temperature (P = 0.005) and EC (P = 0.027). On the other hand, no significant difference was observed between the indigenous adzuki bean-nodulating bradyrhizobial community and annual mean precipitation (P = 0.111), and pH (P = 0.283). Furthermore, Figure 5 shows the relationships between the polar ordination analysis of the coordinates of the three-dimensional NMDS analysis and the Δlatitude, Δlongitude, annual mean temperature, and EC of the sample sites. The relationship between the Δlatitude, Δlongitude, temperature difference of annual mean temperature, and EC and polar differences of the adzuki bean-nodulating bradyrizhobial communities indicates a significant correlation, r = 0.8379 (P < 0.01), r = 0.8823 (P < 0.01), r = 0.8685 (P < 0.01), and r = 0.8391 (P < 0.01), respectively (Figure 5).

DISCUSSION

In this study, we confirmed the compatibility of adzuki bean and *Bradyrhizobium* USDA strains and investigated the genetic diversity and geographical distribution of indigenous adzuki bean-nodulating bradyrhizobia in Japan. The results of the inoculation test of *Bradyrhizobium* USDA strains on adzuki bean showed that normal nodulation was observed in all USDA strains except USDA 76^T, with USDA 123 indicating the highest value of nodule number (Table 2). On the other hand, the shoot length was significantly higher in USDA 94, which had the second highest value of nodule number, compared with USDA 6^T, USDA 123, and USDA 76^T (Table 2). An excessive increase in nodule number has been reported to inhibit soybean growth (Akao & Kouchi 1992). In this study, USDA 123, which had a high nodule number, showed reduced growth of adzuki bean, suggesting that it is necessary to select *Bradyrhizobium* strains that can ensure an appropriate number of nodules. In addition,

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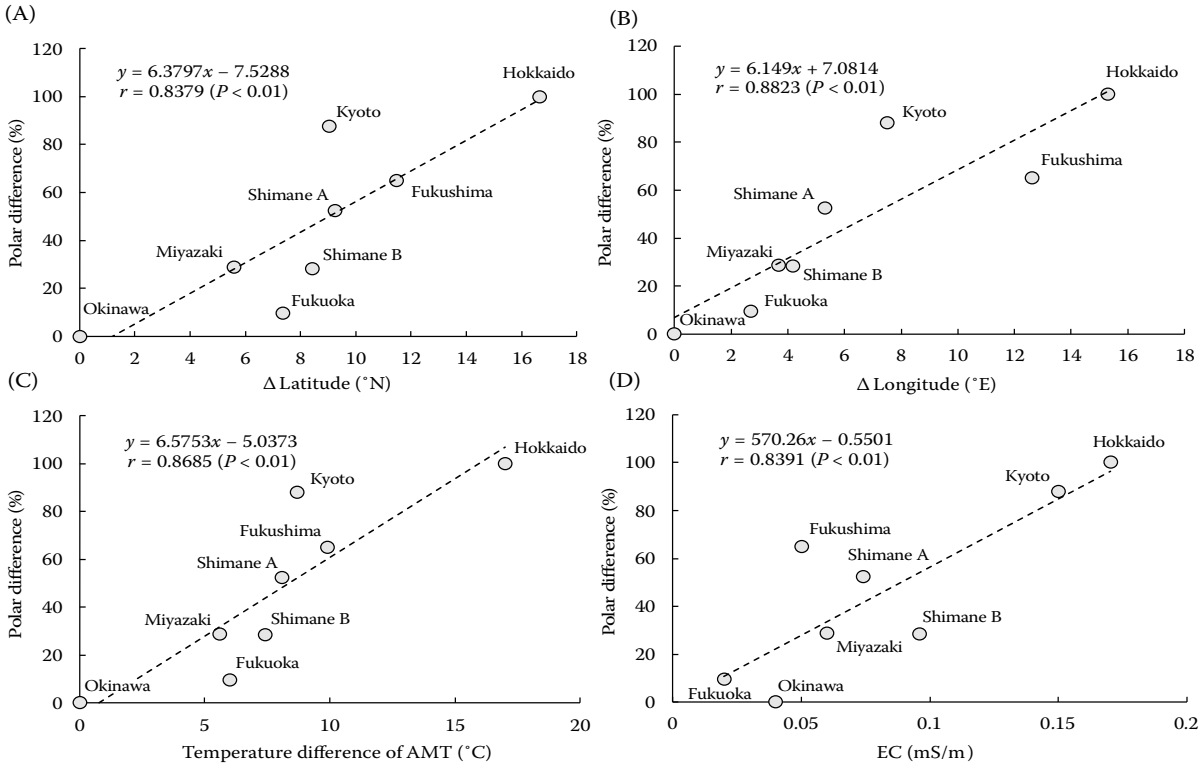

Figure 5. Relationship between adzuki bean-nodulating bradyrhizobial community of adzuki bean and (A) Δlatitude, (B) Δlongitude, (C) temperature difference of AMT and (D) electric conductivity of the soil sample site

The values of Δlatitude, Δlongitude, and temperature difference represent the difference from Okinawa site and each site

the number of nodules of USDA 76^T is significantly lower than that of other USDA strains, suggesting that it has a low compatibility with adzuki bean. This USDA 76^T also has low compatibility for soybean, and *B. elkanii* USDA 31, which shows the same RFLP patterns of 16S-23S rRNA gene ITS region, has been reported to have a higher compatibility for soybean (Saeki et al. 2004; Shiro et al. 2016). Therefore, USDA 31 might have a higher compatibility than USDA 76^T in adzuki bean. Although the growth of adzuki bean differed depending on the inoculated USDA strains, overall, strains such as USDA 46, 94, and 110^T may contribute to the improvement of adzuki bean productivity.

The major clusters from the sample soils were Bj123 in Hokkaido and Kyoto, and Bd110 in Fukushima and Shimane A, and Be76 in Shimane B and Fukuoka, Bj6 in Miyazaki and Okinawa (Figure 3). In some reports on the geographical distribution of indigenous soybean-nodulating bradyrhizobia in Japan, Bj123 has been detected in Hokkaido and Tohoku regions such as Akita and Fukushima, but not in western Kyushu, in Japan (Saeki et al. 2006; Saeki et al. 2013). In the results of this analysis, Bj123 was detected not only in Hokkaido and Fukushima, but also in Kyoto, Shimane, and Miyazaki (Figure 2 and Figure 3). The results of the inoculation test showed that USDA 123 formed more nodules than the other strains (Table 1). It has been reported that the number of nodules found when soybean was inoculated with USDA 123 tended to be less than that with USDA 110^T (Shiro et al. 2016). These results suggest that the compatibility with USDA 123 for nodulation is possibly different between adzuki bean and soybean. The number of detections for the Bj, Bd, and Be clusters was 143 (40.7%), 65 (18.6%), and 143 (40.7%), respectively, with Bj and Be clusters having the same number of detections and distributed widely in Japan (Figure 2 and Figure 3). In a report on the geographical distribution of soybean-nodulating bradyrhizobia in Japan, the number of detections for Bj, Bd, and Be clusters was 327 (34.1%), 405 (42.2%), and 227 (23.7%), respectively, with Bj and Bd clusters being detected more frequently than Be clusters, and Be clusters were rarely detected in Hokkaido and Tohoku regions (Saeki et al. 2013). In this study, in-

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digenous adzuki bean-nodulating bradyrhizobia were isolated from the soils used in this study, and it is suggested that adzuki bean is more likely to be infected with bradyrhizobia of Be cluster than soybean. Furthermore, based on the results of the inoculation test, *B. elkanii* was considered to be highly compatible with adzuki bean.

In the diversity analysis, the soil sampling site with the highest diversity index (*H'*) was Shimane B (1.61), and Hokkaido had the lowest diversity index (0.55) (Figure 2). The value of beta diversity tends to be larger than that in the southern regions, except for the comparison between Hokkaido to Kyoto and Fukuoka to Okinawa (Table 3). The results of geographical distribution analysis using polar ordination showed that indigenous adzuki bean-nodulating bradyrhizobial community structure in Japan indicated a significant positive correlation with the northern latitude of the sample sites (r = 0.8379, $P < 0.01$) (Figure 5A). This result suggests that this community may change depending on the functions associated with latitude, such as climate. The relationship between indigenous soybean-nodulating bradyrhizobial community structures in Japan and America and their geographical distribution using polar ordination analysis has been reported to be strongly and positively correlated with latitude (Saeki et al. 2008; Saeki et al. 2013; Shiro et al. 2013). These results suggest that different host legumes show similar tendencies when the symbiotic partner rhizobia species are the same. Furthermore, the same significant correlation was shown for several environmental factors, such as Δlongitude, annual mean temperature, and electric conductivity (Figure 5B, Figure 5C, and Figure 5D). Especially, significant correlation was obtained for annual mean temperature with related latitude, suggesting a close relationship between changes in indigenous adzuki bean-nodulating bradyrhizobial community structure and temperature. Shiro et al. (2012) and Shiro et al. (2016) reported that changes in indigenous soybean-nodulating bradyrhizobial community structure with increasing cultivation temperature are caused by temperature affecting the expression of bradyrhizobial nodulation gene. Although the relationship has been revealed for latitude-related factor such as temperature, the relationship between indigenous bradyrhizobial community structure and these environmental factors of Δlongitude and electrical conductivity has not been discussed in previous reports, and it is necessary to investigate how these environmental factors respond to the physiological responses of bradyrhizobia in the future.

Furthermore, based on the knowledge obtained in this study, we expect that the development of a study on competition between inoculum and indigenous bradyrhizobia, and environmental adaptability of bradyrhizobia, can contribute to establish of inoculation techniques for effective adzuki bean-nodulating bradyrhizobia toward the productivity improvement adzuki bean.

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