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Geographical distribution and genetic diversity of *Bradyrhizobium* spp. isolated from Korean soybean root nodules

Ye-eun Kim†, Hanseob Shin†, Youri Yang and Hor-Gil Hur*

Abstract

This study investigated the distribution and genetic diversity of the indigenous *Bradyrhizobium* spp. in the Korean agricultural field. A total of 254 *Bradyrhizobium* strains were isolated from 97 soybean samples (9 cultivars) collected in 14 regions. *B. elkanii* dominated in the southern regions, while *B. diazoefficiens* dominated in most central and northern regions. Through non-parametric multidimensional scaling (NMDS) analysis, we confirmed the possibility that environmental factors such as annual average temperature and soybean cultivars might affect the distribution of *Bradyrhizobium* spp. in some regions. The DNA fingerprint using repetitive DNA sequences showed the genetic diversity among the *Bradyrhizobium* strains isolated from the different regions. Clustering the strains based on the genetic diversity indicated that *Bradyrhizobium* spp. grouped into different clusters depending on geographic location. This study suggests that the Korean indigenous *Bradyrhizobium* spp. distribute differently according to the geographical feature, and the high genetic diversity of each strain attribute to the geographic location.

Keywords: *Bradyrhzibium* spp., Genetic diversity, Nitrogen-fixing bacteria, Geographical distribution, Rep-PCR, Soybean-nodulating bacteria

Introduction

Soybean [*Glycine max* (L.) Merr.] has been used as one of the major grains for food production, animal fodder and biofuel in South Korea. Although soybean production was able to fill up 98% of domestic demand in 1975, the self-sufficiency rate has gradually decreased since then, reaching only 8.7% in 2015. Therefore, soybean consumption in Korea is covered by soybeans mostly imported from the United States [1]. To increase the self-sufficiency rate of soybean, a solution for improving productivity of soybeans should be addressed.

The biological symbiosis between legumes and *rhizobia* is well known as the effective nitrogen-fixing process [2]. Especially, *Bradyrhizobium* is major soybean-nodulating bacteria that have high efficiency in soybean growth and productivity [3]. Thus, the elite species such as *B. elkanii*, *B. japonicum* and *B. diazoefficiens* have long been utilized as biological fertilizers in many other countries because they have the eco-friendly and economic advantage for replacing the chemical fertilizer [4, 5]. Retaining sufficient number of the elite soybean-nodulating bacteria in soybean root and rhizosphere is necessary to improve the efficiency of the green fertilizer. However, the inoculation of nitrogen-fixing strains was mostly unsuccessful, due to less competence against indigenous strains to occupy a superior ecological niche [6]. To prevent the decline of inoculation efficiency, it is crucial to comprehend the geographical distribution and genetic diversity of indigenous *Bradyrhizobium* spp. in terms of the influence of environmental factors attributed to the regional location [7].

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According to the previous studies in Japan and the United States, the distribution and genetic diversity of indigenous *Bradyrhizobium* has differed based on geographical location [7, 8]. Moreover, *Bradyrhizobia* community structures of Japan and the USA revealed that the ecological niche of *Bradyrhizobia* was affected by north latitude and consequential soil temperature [9]. In addition, a study in Japan reported that the host soybean cultivars and local soil conditions may also affect the diversity of the *Bradyrhizobia* community [10]. In addition, *Bradyrhizobia* species and symbiotic gene diversity were related to climate and soil conditions in different regions in China [11].

Although the phylogenetic information of *Bradyrhizobium* spp. in Korea has been reported partially using 16r-RNA and ITS region analysis method [12], in-depth study for geographical distribution and genetic diversity of indigenous *Bradyrhizobium* spp. using the repetitive sequence-based polymerase chain reaction (rep-PCR) DNA fingerprinting method have been rarely reported. The rep-PCR technique detects the interspersed repetitive DNA sequence to enable differentiation between each other closely related strains [13]. Because of the high discriminatory ability and reproducibility of DNA patterns, rep-PCR has been massively applied as the method of estimation for the genetic diversity of soybean-nodulating *rhizobia* [14].

In the present study, we provide the first assessment of the geographical distribution and genetic diversity of the indigenous *Bradyrhizobia* community isolated from Korean soybean root nodules. We collected 97 soybean plants from 14 regions of Korea with covering at least one site for each 8 local Province except Jeju island, and obtained a total of 279 isolates. Among them, a total of 254 *Bradyrhizobium* spp. were identified. The influence of environmental factors and host plant cultivars on the distribution of *Bradyrhizobium* spp. was assessed by Principal component analysis (PCA) and non-metric multi-dimensional scaling (NMDS) analysis. Then, the genetic diversity of predominant *Bradyrhizobium* strains was compared by using the rep-PCR based DNA fingerprinting technique. Thus, this study for investigating the ecological survey of soybean-nodulating *Bradyrhizobia* in Korea may provide the basic step of developing sustainable agricultural technique with reducing agricultural energy input into the soil environment in the era of carbon neutrality.

Materials and methods

Collecting soybean plants and information of sampling sites

A total of 97 soybean plants were collected for 2 days from 14 regions (23 upland fields and 5 paddy fields) of South Korea, September, 2019. Three soybean plants placed 50 Meter away each other at each sampling site were randomly sampled. The GPS address, field type and variety of soybean plants at the sampling sites were described in Additional file 1: Table S1. Approximately 400 g of soil was sampled and prepared for soil analyses. The climate parameters including annual average temperature, and annual average precipitation were collected from the 2019 annual climatological report published by Korea Meteorological Administration (KMA). The geographic information such as latitude, longitude and altitude of the sampling sites was collected by Google Earth satellite image mapping service (https://www.google.co.kr/intl/ko/earth/).

Identification of *Bradyrhizobium* spp. isolated from soybean nodules

One nodule from each soybean plant was sampled and sterilized the surface with soaking in 0.1% $HgCl_2$ for 30 s, followed by rinsing briefly in distilled water and in 70% ethanol for 30 s, and in distilled water 7–10 times [15]. Then, the nodules were squeezed into 1 mL of distilled water using a disinfected tweezer. An aliquot (100 µL) of the bacteria suspension was put into 900 µL of sterile distilled water for serial dilution and subsequently diluted to 10^{-4}. The diluted suspensions were spread on AG media [16], followed by incubated at 28 °C for 7–10 d. Three colonies were picked from each plate and were purely cultured using the streak plate method [17]. 16S-rRNA PCR was carried out using Accupower® PCR premix (Bioneer Co., Ltd., Daejeon, Korea) and universal bacterial primer 27F (5′-AGAGTTTGATCMTGG CTCAG-3′) and 1492R (5′-GYTACCTTGTTACGACTT -3′). The PCR amplification was performed by Eppendorf Mastercycler Gradient (Eppendorf Co., Ltd., Hamburg, Germany) with the following standard temperature condition: initial denaturation at 96 °C for 4 min; 30 cycles of denaturation at 94 °C for 30 s, annealing at 57 °C 30 s and extension at 72 °C for 1 min; final extension at 72 °C for 10 min [18]. Aliquots of 3 µL of PCR product were analyzed on 1.5% (w/v) agarose gel in TAE buffer with ethidium bromide (0.5 µg/mL) using Mupid-exU Electrophoresis system (Takara Bio Inc., Shiga, Japan). The gel was analyzed by UV Transilluminator (Wealtec Crop., Meadowvale Way Sparks, NV, USA). PCR products were purified and sequenced by Macrogen Institute (Macrogen Co., Ltd., Seoul, Korea). About 1300 bp of 16S rRNA gene sequence was assembled with the BioEdit program. The sequences were ensured by comparing with corresponded taxonomy from the National Center for Biotechnology Information (NCBI) GenBank database and identified by a 16S-based ID tool of the EZBioCloud server (https://www.ezbiocloud.net/) [19]. Based on the

16S rRNA sequencing results, the information of the strains was described in Additional file 2: Table S2.

Genetic diversity of *Bradyrhizobium* strains at strain level using rep-PCR

The genomic DNA of the isolated strains was extracted using Exgene™ Clinic SV DNA extraction kit (GeneAll Biotechnology Co., Ltd, Seoul, Korea) [20]. The concentration of genomic DNA was calculated using a Nabi UV–Vis Nano spectrophotometer (Microdigital Co., Seong-nam, Korea). For rep-PCR amplification reactions, REP1R-I (5′-IIIICGICGICATCIGGC-3′) and REP2-I (5′-ICGICTTATCIGGCCTAC-3′) primer sets were used. The PCR was carried out using Eppendorf Mastercycler Gradient (Eppendorf Co., Ltd.,, Hamburg, Germany) under the following condition: initial denaturation at 95 °C for 7 min; 30 cycles of denaturation at 94 °C for 1 min, annealing at 44 °C for1 min and extension at 65 °C for 8 min; final extension at 65 °C for 15 min [21]. The PCR products were analyzed by gel electrophoresis using 1X TAE buffer and 1.5% Seakem LE agarose gels (FMC Bioproducts, Philadelphia, Pennsylvania, USA) for 16 h 45 min at 100 A and 70 V. The results were visualized by Typhoon 9410 scanner (Amersham Biosciences Co., Ltd., Amersham, UK). The results of DNA fingerprinting were normalized and analyzed using the BioNumerics V8.0 software package (Applied Maths, Ghent, Belgium). Genetic similarities of DNA fingerprint profiles between isolates were calculated using the Dice similarity coefficient and unweighted pair-group method with arithmetic means (UPGMA) clustering technique.

Results and discussion

Geographical distribution of *Bradyrhizobium* spp. isolated from Korean soils

Among 279 of strains isolated from soybean nodules, 254 isolates were identified as *Bradyrhizobium* with allocating into 6 species of *B. diazoefficiens*, *B. elkanii*, *B. japonicum*, *B. ottawaense*, *B. guangxiense*, and *B. lianoningense* (Additional file 1: Table S1). As shown in Fig. 1A, *B. diazoefficiens* (48.1%) was the most dominant species, followed by *B. elkanii* (18.2%), *B. japonicum* (13.8%) and *B. ottawaense* (8.6%). Figure 1B shows the regional distribution patterns of *Bradyrhizobium* isolates in South Korea. *B. diazoeffciens* occupied most part of the nation with except for JN_MA, JB_JU, CB_JC, GW_PC, and CN_TA. *B. elkanii* was dominant species in JN_MA and JB_JU at 94.4% and 88.9%, respectively. *B. ottawaense* was also found to be major *Bradyrhizobium* species in CB_JC (66.7%), and KW_PC (38.9%). *B. japonicum* was the most common strain in CN_TA with 61.1%. Overall, *B. diazoeffciens,* to which the most commonly used nitrogen fixing biofertilizer type strain USDA110 belongs, was found in the all sampling sites as a mostly dominant colonizer. Interestingly, *B. elkanii* species was found in the warm western part of the Korean peninsula, and occupied southwestern part as a major *Bradyrhizobium* species. However, *B. ottawaense* was found in the mountainous sites with relatively low temperature. The geographical distribution of indigenous *Bradyrhizobium* spp in Japan and the United States have shown similar results that *B. elkanii* was mainly dominant in the southern regions, while *B. japonicum* and *B. diazoefficiens* were dominant in the northern regions.

NMDS analysis also revealed that the distribution of *Bradyrhizobium* spp. was thought to be affected by the annual average temperature (Fig. 2). Additionally, PERMANOVA test revealed that the community of *Bradyrhuzobium* spp. of soil samples was significantly different by temperature level ($R = 0.1806$, $p = 0.0399$). Otherwise, the annual temperature is thought as a potential environmental factor to classify the geographical regions by annual temperature as high temperature (13–14 °C), moderate (11.5–13 °C) and low (10–11.5 °C) (Fig. 2), supporting the different composition of *Bradyrhizobium* spp. as shown in Fig. 1B. Yuichi Saeki and Sokichi Shiro reported that the indigenous *Bradyrhizobium* community of Japan and the United State correlated with latitude, and the community could be influenced by the soil temperature associated with the latitude of the particular geographical location or the diversity of acclimatized host plants to the climate [9]. Suzuki et al. describes that *B. elkanii* is dominant at high temperature, whereas *B. japonicum*, is dominant at low temperature under the inoculation experiment condition [22]. In Japan, Kyoto (in which the latitude is similar to that in Jeollado provinces and similar temperature to that in Uiryeong, Gyeongsangnamdo) showed the high distribution of the various strains of *B. japonicum* and the *B. diazoefficiens* USDA110^T. However, *B. elkanii* USDA76^T occupied the largest proportion of isolates in Okinawa located in the southernmost of Japan with higher temperature than Kyoto [7]. In the USA, *B. japonicum* was predominant in Ohio region (of which the annual average temperature similar to that of Gangwondo provinces). Furthermore, *B. elkanii* USDA76 was predominant in Kentucky where annual average temperature and latitude are similar to those in Muan, Jeollanamdo [8]. Depending on the study of Ethiopia, the clusters group included in *B. ottawaense* were dominant in Borcha that have similar temperature and latitude with the northern region of Korea [23]. Those studies support the pattern of distribution of Korean indigenous *Bradyrhizobium* spp. with similar tendency to those of other countries affected by temperature, geographical, and physiochemical

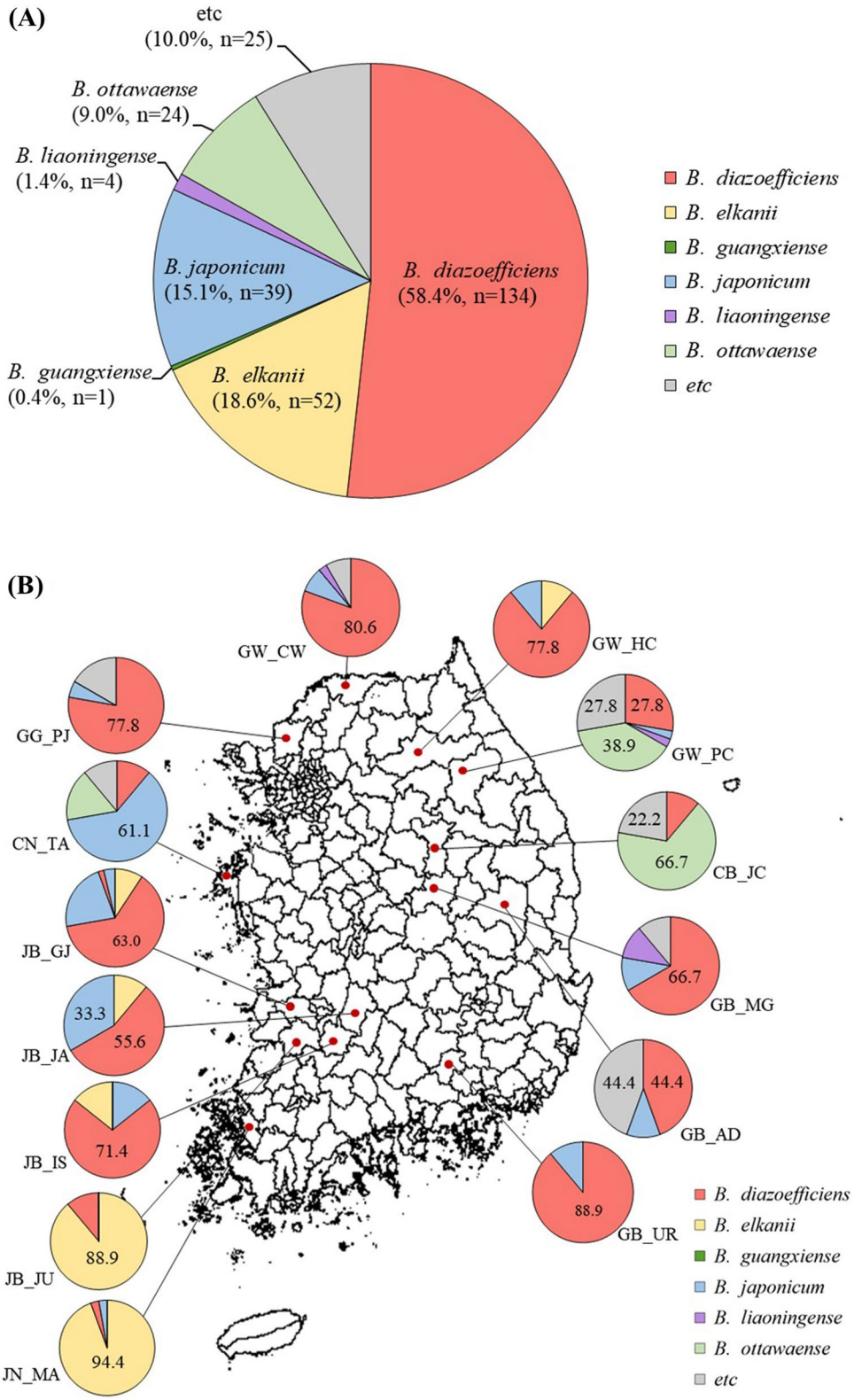

Fig. 1 The distribution of *Bradyrhizobium* spp. isolated from the Korean soybean root nodule. **a** Represent the abundance of total *Bradyrhizobium* spp. isolated in Korea and **b** represents the distribution of *Bradyrhizobium* spp. by the regions

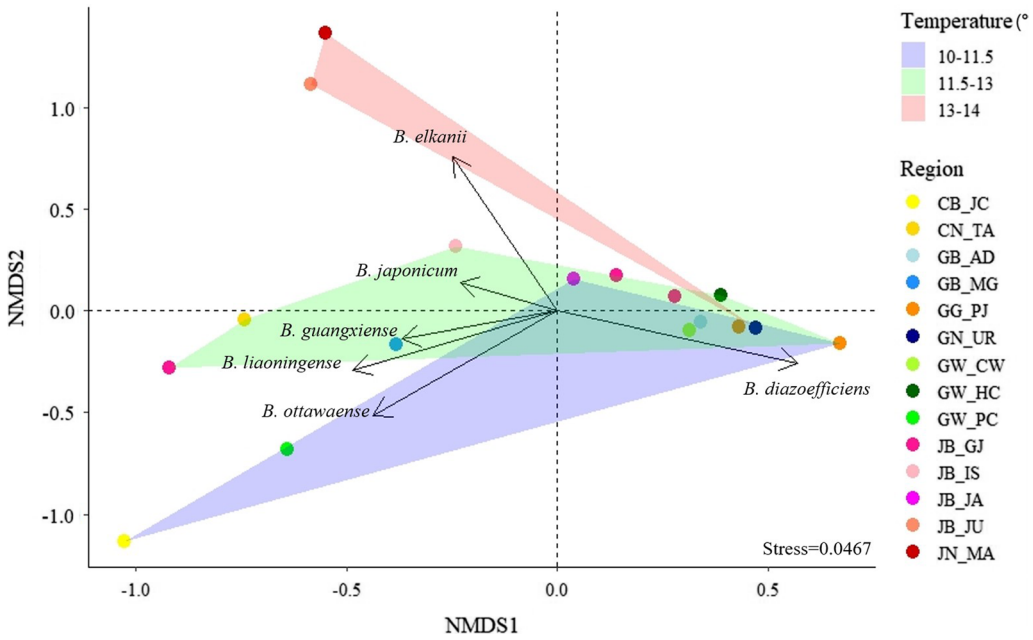

Fig. 2 The result of NMDS analysis for verifying the influence of temperature. The plot represents the influence of temperature on the regional distribution of *Bradyrhizobium* spp. based on annual average temperature. CB_JC; Chungcheongbukdo_Jecheon, CN_TA; Chungcheongnamdo_Taean, GB_AD; Gyeongsangbukdo_Andong, GB_MG; Gyeongsangbukdo_Mungyeong, GG_PJ; Gyeonggido_Paju, GN_UR; Gyeongsangnamdo_Uiryeong, GW_CW; Gangwondo_Cheorwon, GW_HC; Gangwondo_Hongcheon, GW_PC; Gangwondo_Pyeongchang, JB_GJ; Jeollabukdo_Gimje, JB_IS; Jeollabukdo_Imsil, JB_JA; Jeollabukdo_Jinan, JB_JU; Jeollabukdo_Jeongeup, JN_MA; Jeollanamdo_Muan

characteristics. The characteristics of geographical distribution of soil samples was also explained using principal component analysis (PCA) using temperature, precipitation, total nitrogen, total phosphate, total organic carbon, carbon to nitrogen ratio, cation exchange capacity, soil texture (Additional file 3: Fig. S1) of soil samples, showing the separation of JN and JB regions from other regions. Eigenvalues of environmental factors obtained from PCA analysis were included in Additional file 4: Table S3.

The abundance of *Bradyrhizobium* spp. from soybean cultivars is shown in Fig. 3. *B. diazoefficiens* was

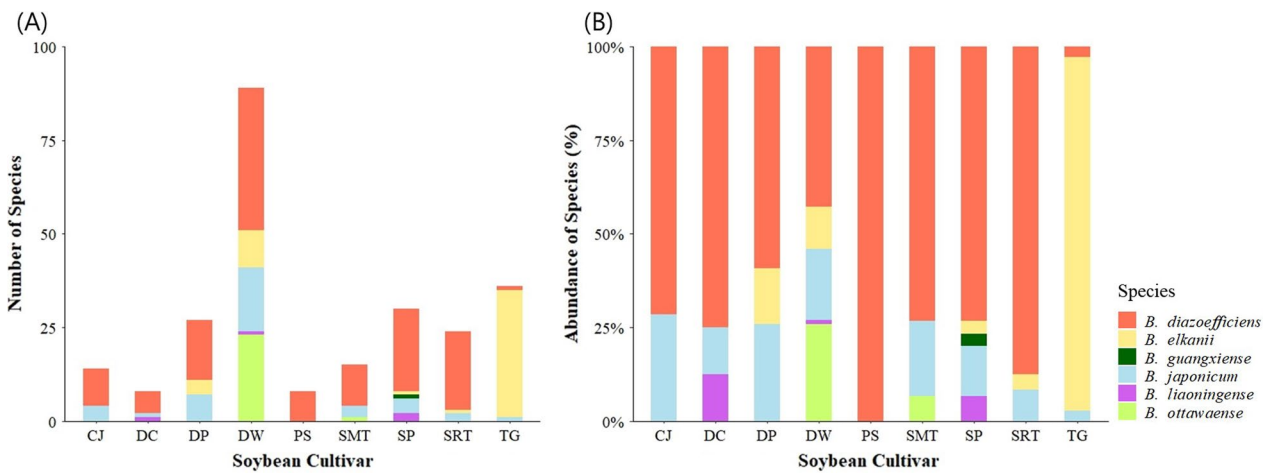

Fig. 3 Number and relative abundance of *Bradyrhizobium* spp. by soybean cultivars. The left absolute bar plot shows number of *Bradyrhizobium* species by soybean cultivars and the right bar plot indicates the relative abundance. CJ; Chungja, DC; Daechan, DP; Daepung, DW; Daewon, PS; Pungsan, SMT; Seomoktae, SP; Sunpung, SRT; Seoritae, TG; Taekwang soybean cultivar

predominated in most of the soybean cultivars. Exceptionally, *B. elkanii* was particularly dominant in the Taekwang soybean cultivar collected from Jeollanamdo province. Devine et al. suggested that certain *Bradyrhizobia* strains produce the substance named rhizobitoxine, and it could be induced foliar chlorosis into the host plant. The result of the study has been described that *B. elkanii* USDA 76 T has the highest chlorosis score among 25 *Bradyrhizobium* strains [24]. The foliar chlorosis affected to postproduction of soybeans by inhibiting the production of flowers and fruits as well as disturbing the initial growth [25]. In this study, most of the *B. elkanii* identified were classified as strain USDA 76^T. In Korea, the distribution of *Bradyrhizbium* spp. is spatially various, and environmental conditions such as temperature associated with climate and soybean cultivars are thought to affect the distribution structure.

Genetic diversity of *Bradyrhizobium* spp. in strain level

Dendrograms of each dominant *Bradyrhizobium* spp. were represented the genetic similarity of respective DNA fragments extracted from each strain. A total of 42 *B. elkanii* strains were grouped as 4 major clusters based on 70% similarity (Additional file 5: Fig. S2). Cluster A includes the most strains from Jeongeup, Jeollabukdo and cluster B was grouped with the strains obtained from Muan, and Jeollanamdo. Both clusters C and D included strains from Gimje, and Jeollabukdo. The strains were regionally distinguished with the similarity of more than 80%. A total of 129 *B. diazoefficiens* strains were distinguished into 4 clusters based on 50% similarity (Additional file 6: Fig. S3). Cluster A and D were grouped strains from northern regions, while cluster C was mainly comprised of strains from southern regions. Although cluster B included strains obtained from both the north and south regions, they were divided into two subgroups, which were distinguished by latitudinal variation depending on 55.6% similarity. A total of 31 *B. japonicum* strains were grouped as 3 clusters based on 50% similarity (Additional file 7: Fig. S4). Most strains isolated from southern regions were classified in cluster C. However, the strains isolated from central and northern regions were clustered in cluster A. A total of 20 strains identified as *B. ottawaense* were grouped as 3 clusters based on 70% similarity (Additional file 8: Fig. S5). Strains obtained from both northern regions and central regions were clustered in cluster A, and the regions were classified with a similarity of more than 86.4%. Overall, the strains were genetically clustered by geographical location, showing specific strains are thought to have indigenous specificity to regions. In particular, the strains *B. diazoefficiens* and *B. japonicum* from southern regions were classified as the genetically different groups from central and northern regions, even though they have over 98.5% similarity of 16S rRNA-based DNA sequence. Exceptionally, the strains isolated from Gimje, Jeollabukdo have represented the high similarity with both strains isolated from central and northern regions and isolated from the southern regions.

Because of horizontal gene transfer of symbiotic chromosomal genes, rep-PCR has been used as an enhanced genomic fingerprinting method to identify the higher taxonomic level of *Bradyrhizobium* [26]. Some previous studies suggested that the genetic diversity of *Bradyrhizobium* strains could be influenced by physicochemical soil characteristics such as the proportion of clay in the soil, pH and salt condition [26–28]. Elboutahiri et al. verified the high level of genetic changeability under the high salinity and drought conditions in their study through the increasing profile of rep-PCR results using *Sinorhizobium* species. They proposed that exposure to stressful environments enabled the evaluation of the genome by exchange and acquisition through horizontal gene transfer [29]. In addition, the previous study of Japan described that the genotype of soybean cultivars changes the genetic composition of the Bradyrhizobial community [30].

In conclusion, a high level of diversity at the strain level was confirmed between the strains from different regions. This result may suggest that the *Bradyrhizobium* strains, symbionts of soybean, were genetically different depending on geographical location and associated environmental differences. It could be explained by the horizontal gene transfer between *Bradyrhizobium* spp. that occurred for adjusting to environmental condition [31]; however, further studies should be required to prove the direct cause of genetic diversity. Taken together, this study surveyed the distribution and genetic difference of the indigenous *Bradyrhizobium* community in South Korea for the sustainable agriculture techniques with easing global warming problem.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13765-022-00708-8.

Additional file 1: Table S1. The information of sampling sites.

Additional file 2: Table S2. The 16S rRNA BLAST information of isolated Bradyrhizobium strains.

Additional file 3: Fig. S1. The PCA plot indicates the influence of environmental factors on the sampling site. The graph represent the effect of environmental factor on the sampling regions. Temperature; annual average temperature, Precipitation; annual average precipitation, TN; total nitrogen, TP; total phosphate, TOC; total organic carbon, C.N; carbon to nitrogen ratio, CEC; cation exchange capacity, Texture; soil texture. CB_JC; Chungcheongbukdo_Jecheon, CN_TA; Chungcheongnamdo_Taean, GB_AD; Gyeongsangbukdo_Andong, GB_MG; Gyeongsangbukdo_Mungyeong, GG_PJ; Gyeonggido_Paju, GN_UR;

Gyeongsangnamdo_Uiryeong, GW_CW; Gangwondo_Cheorwon, GW_HC; Gangwondo_Hongcheon, GW_PC; Gangwondo_Pyeongchang, JB_GJ; Jeollabukdo_Gimje, JB_IS; Jeollabukdo_Imsil, JB_JA; Jeollabukdo_Jinan, JB_JU; Jeollabukdo_Jeongeup, JN_MA; Jeollanamdo_Muan.

Additional file 4: Table S3. Eigenvalues of environmental factors from principal component analysis (PCA).

Additional file 5: Fig. S2. Dendrogram of indigenous *B. elkanii* in Korea. The dendrogram indicates the genetic similarity among repetitive DNA fragment fingerprint patterns with *Bradyrhizobium elkanii* strains. The similarity is calculated by UPGMA, the dice co-efficient method. Each color indicates the region of the sampling site. JN_MA; Jeollanamdo_Muan, JB_JU; Jeollabukdo_Jeongeup, JB_IS; Jeollabukdo_Imsil, JB_GJ; Jeollabukdo_Gimje.

Additional file 6: Fig. S3. Dendrogram of indigenous *B. diazoefficiens* in Korea. The dendrogram indicates the genetic similarity among repetitive DNA fragment fingerprint patterns with *Bradyrhizobium diazoefficiens* strains. The similarity is calculated by UPGMA, the dice co-efficient method. Each color indicates the region of the sampling site. JN_MA; Jeollanamdo_Muan, JB_GJ; Jeollabukdo_Gimje, JB_IS; Jeollabukdo_Imsil, JB_JA; Jeollabukdo_Jinan, JB_JU; Jeollabukdo_Jeongeup, GG_PJ; Gyeonggido_Paju, GW_CW; Gangwondo_Cheorwon, GW_HC; Gangwondo_Hongcheon, GW_PC; Gangwondo_Pyeongchang, GB_AD; Gyeongsangbukdo_Andong, GB_MG; Gyeongsangbukdo_Mungyeong, GN_UR; Gyeongsangnamdo_Uiryeong.

Additional file 7: Fig. S4. Dendrogram of indigenous *B. japonicum* in Korea. The dendrogram indicates the genetic similarity among repetitive DNA fragment fingerprint patterns with *Bradyrhizobium japonicum* strains. The similarity is calculated by UPGMA, the dice co-efficient method. Each color indicates the region of the sampling site. JN_MA; Jeollanamdo_Muan, JB_GJ; Jeollabukdo_Gimje, JB_IS; Jeollabukdo_Imsil, JB_JA; Jeollabukdo_Jinan, GW_CW; Gangwondo_Cheorwon, CN_TA; Chungcheongnamdo_Taean, GB_MG; Gyeongsangbukdo_Mungyeong.

Additional file 8: Fig. S5. Dendrogram of indigenous *B. ottawaense* in Korea. The dendrogram indicates the genetic similarity among repetitive DNA fragment fingerprint patterns with *Bradyrhizobium ottawaense* strains. The similarity is calculated by UPGMA, the dice co-efficient method. Each color indicates the region of the sampling site. GW_PC; Gangwondo_Pyeongchang, GW_CW; Gangwondo_Cheorwon, CN_TA; Chungcheongnamdo_Taean, CB_JC; Chungcheongbukdo_Jecheon.

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Author contributions
YE performed experiments, analyzed the data and wrote the draft. HS offered the guidelines edited and improved the original draft. YR offered guidelines for experiments. H-G supervised the process of this research. All authors read and approved the final manuscript.

Declarations

Competing interests
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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