LATITUDINAL CHARACTERISTIC NODULE COMPOSITION OF *Bradyrhizobium* spp. AFFECTED BY THEIR TEMPERATURE-DEPENDENT PROLIFERATION IN SOIL AND INFECTION

（ダイズ根粒菌の温度依存的な土壌中の生息と感染に影響を受ける緯度特異的な根粒内組成）

MD HAFIZUR RAHMAN HAFIZ

2021

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THESIS BY

MD HAFIZUR RAHMAN HAFIZ

(D16A2104Z)

In Partial Fulfillment of the Requirement for the Award of Degree of
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At
THE COURSE OF BIOENVIRONMENTAL SCIENCE
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TOTTORI UNIVERSITY

2021

Declaration by author

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. The content of my thesis is the result of work I have carried out since the commencement of my PhD studies.

This is a true copy of the thesis, including final revision.

Date: September 2021
Md Hafizur Rahman Hafiz

Signature…………………………

Approval Sheet

The thesis enclosed herewith, **“LATITUDINAL CHARACTERISTIC NODULE COMPOSITION OF *Bradyrhizobium* spp. AFFECTED BY THEIR TEMPERATURE-DEPENDENT PROLIFERATION IN SOIL AND INFECTION”** prepared and submitted by MD HAFIZUR RAHMAN HAFIZ in partial fulfillment of the requirement for the award of Doctor of Philosophy, is hereby approved as style and contents

By

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Dedicated to my beloved son Jawaad labeeb Mahi
and daughter Juwayrirrah Laibah Manha

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Md Hafizur Rahman Hafiz
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List of abbreviations and their full forms

Abbreviations	**Full forms**
Acc. No	Accession Number
ANOVA	Analysis of variance
ATP	Adenosine tri-phosphate
B. elkanii	*Bradyrhizobium elkanii*
B. japonicum	*Bradyrhizobium japonicum*
BeL7	*Bradyrhizobium elkanii* stain L7
Bj11	*Bradyrhizobium japonicum* strain 11
BjS10J	*Bradyrhizobium japonicum* strain S10J
BLAST	Basic local alignment search tool
BNF	Biological nitrogen fixation
Cfa	Humid sub-tropical climate
cm	Centimeter
DDBJ	DNA databank of Japan
Dfb	Temperate continental climate
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribose nucleotide triphosphate
fu	Fukagawa location
FU	Fukagawa soil
g	Gram
ha	Hectare
Hh	Fukagawa soil-Fukagawa location
Hk	Fukagawa soil-Kyushu location
Hm	Fukagawa soil- Matsue location
Id. (%)	Identity percent
ITS	Internal transcribed spacer
Kg	Kilogram
Kh	Kyushu soil-Fukagawa location
Kk	Kyushu soil-Kyushu location

List of abbreviations and their fullforms (Cont'd)

Abbreviations	**Fullforms**
Km	Kyushu soil-Matsue location
LCOs	lipochitooligosaccharides
ma	Matsue location
MA	Matsue soil
MEGA	Molecular Evolutionary Genetics Analysis
Mg	Magnesium
Mh	Matsue soil-Fukagawa location
µL	Micro liter
mi	Miyazaki location
MI	Miyazaki soil
Mk	Matsue soil-Miyazaki location
Mm	Matsue soil-Matsue location
mm	Millimeter
MT	Metric ton
N	Nitrogen
NCBI	National Center for Biotechnology Information
nodC	N-acetylglucosaminyltransferase
NodD	Transcriptional regulator of common nod genes
OD_{660}	Optical density at 600nm wavelength
PCR	Polymerase chain reaction
rDNA	Ribosomal DNA
Rj	A bacteria's symbiotic partner, the soybean host, has nodulation regulatory genes (Rj)
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
SNF	Symbiotic nitrogen fixation
USDA	United state department of agriculture
YMA	Yeast mannitol agar

CHAPTER 1

General Introduction

1.1. Legume

The ripe seeds of the plant belonging to the Leguminosae (*Fabaceae)* family commonly known as “legumes” or “pulses”. This legume family comprises of over 18,000 species, can categorised into around 650 genera, occupying a major part of the terrestrial biomes (Polhill and Raven, 1981). The legumes are an important source of proteins for much of the world’s population[*]. They are the crucial supply of protein for the “third world”. Among the legumes soybeans and peanuts are oil seeds and in the industrialized countries they are used as an important source of raw proteins. There are around 60 domesticated legume species belong to the Leguminosae family cultivated around the world (Fuleky, 2009). The main cultivated legumes with their common name as well as Latin name, distribution and their major use are listed in Table1 (Fuleky, 2009).

Table1. Main cultivated legumes with their common name as well as Latin name, distribution and their major use in the world.

Common name	Latin name	Distribution	Consumption
Soybean	*Glycine max L.*	USA, Brasil, China, Argentina, Japan	Human consumption, mainly processed products (soy meal, concentrate, isolate, soy milk, fermented products) Animal feed.
White lupin	*Lupinus albus L.*	Europe, America	Animal feed for poultry, pigs and fish.
Yellow lupin	*Lupinus luteus L.*	Europe, America	Animal feed for poultry, pigs and fish.
Sweet lupin	*Lupinus angustifolius L.*	Europe, America	Animal feed for poultry, pigs and fish.

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[*] Semi-ripe peas and beans are considered as vegetables

Common name	**Latin name**	**Distribution**	**Consumption**
Chickpea	*Cicer arietinum L.*	Mediterranean countries, South Asia, Eastern and Southern Africa	Green leaves are eaten as vegetable, green seeds in raw, roasted, and boiled form. The dry seeds are cooked or canned. Dry leaves are animal feed.
Mung bean	*Vigna radiata L.*	South Asia, China, India	Used as green vegetable or sprouting shoots.
Pigeon pea	*Cajanus cajan L.*	India	Human consumption and animal feed.
Cluster bean	*Cyamopsis tetregonoloba*	India	Young bean are used as vegetable, the dry beans are used for producing guar gum, it is used in paper and textile industry and for cosmetics and pharmaceuticals.
Jack bean	*Canavalia ensiformis L.*	India, Far East, North and East Africa	For human consumption used as vegetable, as dry bean.
Common bean (also called as French, garden, haricot, kidney, pinto, navy (baked bean) black, pink, black eye, cranberry, great northern or dry bean)	*Phaseolus vulgaris L.*	India, Brazil, France, Russia, German, UK, Ukraine	Human consumption green in pods (canning, freezing) or dry seeds.
Faba bean (field bean)	*Vicia faba L.*	Central Asia, Medeterranean countries, South America, Near east, Europe	Human consumption, and canning, freezing, animal feed.
Lentil	*Lens culinaris*	Turkey, Europe (France, Spain), Asia, Canada, USA	Human consumption.
Cowpea	*Vigna unguiculata L.*	Medeterranean area, Africa, Asia	Human consumption, it is eaten as dhal made from soaked, dehulled seeds.
Pea	*Pisum sativum L.*	Europe, North America,	Human consumption, combining crop for animal feed.

1.2. Soybean with the importance and production in Japan

Soybean (*Glycine max* [L.] Merr.) originated in central and northern China in the period 1100–700 B.C., moving before the first millennium A.D. to India, Nepal, Burma, Thailand, Indochina, Korea, Japan, Malaysia, Indonesia, and the Philippines (Hymowitz and Singh, 1987). It is a seed crop and can easily be cultivated in nitrogen poor soil. For these reasons it has been cultivated in Japan for a long time as an important crop and it is still widely cultivated throughout the country, following rice, wheat, white and sweet potatoes, and naked barley among the ordinary crops (Konno, 1983). Despite a rapid increase in demand of soybeans in Japan, production is not so increased because of the huge import of cheap soybeans from the USA, Brazil, and China (Knnno, 1983). Globally Japan ranked 19th and 7th, respectively in terms of annual production and import of soybean are present in Table 2 (USDA, 2020).

The rapid increase of meat consumption in the modern day yielded a high demand for protein-rich feedstuff. Around the world soybean meals and other oil seed meals supplied this demand. Soybean is the most important legume in the world; it is cultivated more than 121 million hectares with the production of over 361 million ton (USDA, 2020). The main producers of soybean are, Brazil, the USA, China, and Argentina. (USDA, 2020). The cultivation of soybean in Europe is rather low, other legumes such as pea, bean, lentil, cowpea, and lupins playing a more important role in food and feed market (Fuleky, 2009).

Table 2. World production and import of Soybean by country rank order in 2020.

Rank order	**Country**	**Production (1000 MT)**	**Rank order**	**Country**	**Production (1000 MT)**	**Rank order**	**Country**	**Import (1000 MT)**
1	Brazil	133,000	11	European Union	2,700	1	China	100,000
2	United States	112,549	12	Uruguay	2,200	2	European Union	15,150
3	Argentina	48,000	13	South Africa	1,500	3	Mexico	6,200
4	China	19,600	14	Nigeria	700	4	Argentina	4,500
5	India	10,500	15	Serbia	635	5	Egypt	4,150
6	Paraguay	10,250	16	Indonesia	475	6	Thailand	3,890
7	Canada	6,350	17	Zambia	285	7	Japan	3,410
8	Russia	4,300	18	Mexico	255	8	Turkey	3,000
9	Ukraine	3,100	19	Japan	235	9	Taiwan	2,900
10	Bolivia	2,900	20	North Korea	225	10	Bangladesh	2,800

(Source USDA, 2020)

Soybeans have high nutritive value and a good source of protein and fat. In Japan about 70% of soybean is used for oil crushing and the rest part is used for making ‘Tofu’[¶], ‘Miso’[¶], ‘Natto’[¶], other food stuffs and feed (Knnno, 1970). Improvement on research in cultivation technique and breeding methods are essential to ensure the production increase. It has been reported that inoculation of bradyrhizobia with soybean evidence increased crop yield. For example., In Argentina, Hungria et al. (2006) achieved 6000 kg ha^{-1} soybean inoculated with strain E109 without N-fertilizer, in Brazil, soybean yielded

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[¶] ‘Tofu’, ‘Miso’ and ‘Natto’[¶] are soybean curd, soybean paste and fermented soybean respectively, which are necessities in the Japanese diet.

5890 kg ha^{-1} without N^{-}fertilizer (Zotarelli, 2000), Pakistan (Afzal et al., 2010) soybean increased 38% yield with the inoculation of *B. japonicum* TAL 337 and India (Chebotar et al., 2001) reported that inoculation of *B. japonicum* A1017 increased soybean yield than uninoculated plant. Thus, inoculation of soybean with nodulating rhizobia could be a possible way to increase the soybean yield.

1.3. Rhizobia and Nitrogen Fixation

Nnitrogen fixation is the conversion of nitrogen (N_2) in the atmosphere into ammonia (NH_3) (Postgate, 1998). But this atmospheric nitrogen does not easily react with other chemicals to form new compound, thus fixation of nitrogen free up the nitrogen atoms from their diatomic form (N_2) to be useable for other purpose. Nitrogen is essential for all forms of life because nitrogen is required to biosynthesize of basic building blocks of plants, animals, and other life forms. It is the component of nucleotides for DNA and RNA and amino acids for proteins (Burén and Rubio, 2017). Therefore, nitrogen fixation is essential for agriculture and for the manufacture of fertilizer.

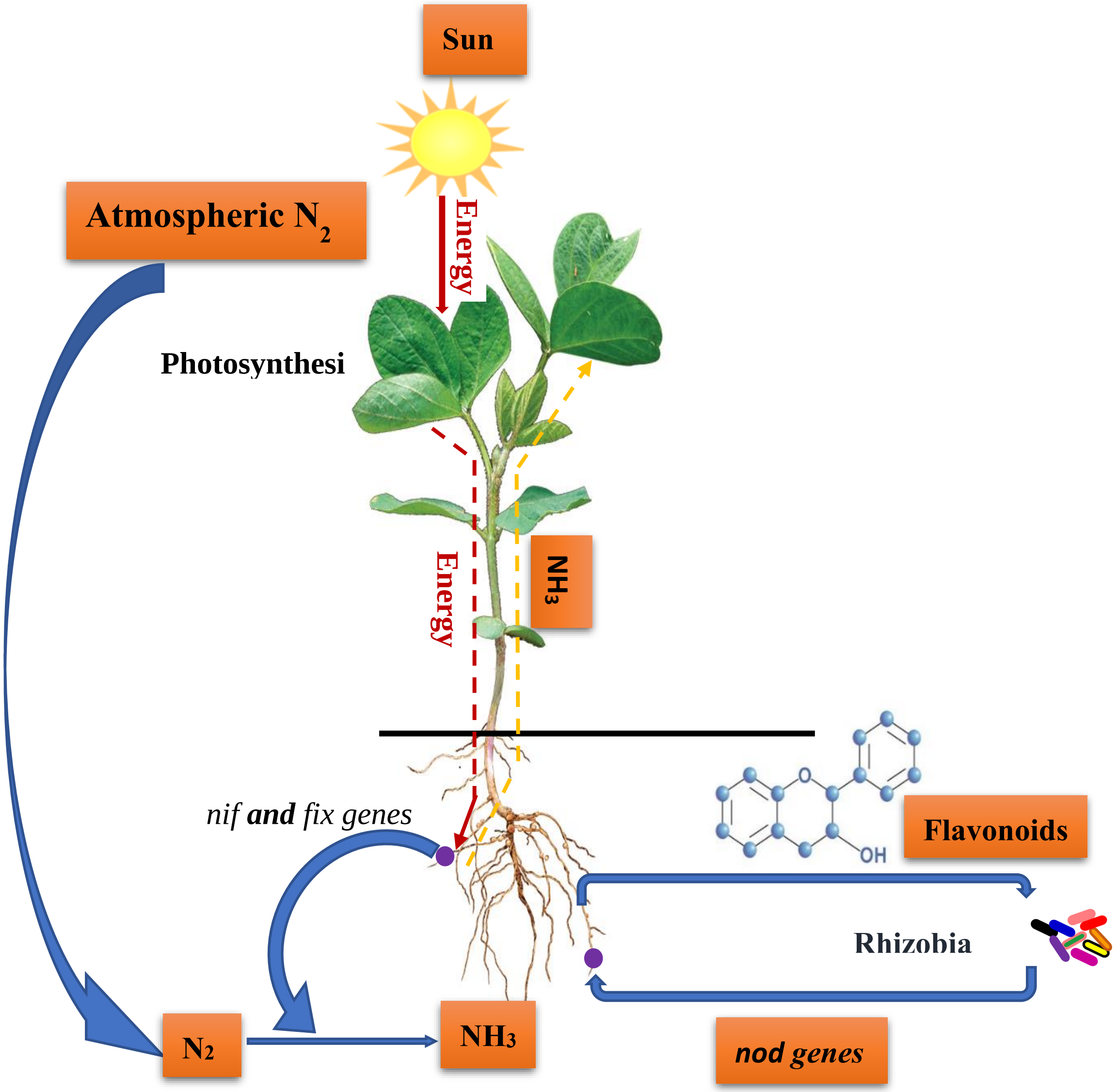
The natural fixation of nitrogen refers to Biological nitrogen fixation (BNF). An enzyme called *nitrogenase* is essential for BNF, although the process involves several complex biochemical reactions, it may be summarized in a relatively simple way by the following equation (Simpson and Burris, 1984).

$$N_2 + 8\ H^+ + 8\ e^- + 16\ MgATP \quad \rightarrow 2\ NH_3 + H^{2\ +}\ 16\ MgADP + 16\ Pi$$

The best-known group of symbiotic nitrogen-fixing bacteria are the rhizobia. Rhizobia is a generic name for a certain Gram-negative group of Alphaproteobacteria and Betaproteobacteria that can form nodules on the root, or in some cases on the stems, of their hosts and fix nitrogen in symbiosis with legumes as their host plants (Garrity et al., 2005; Sprent, 2008).

The legume-rhizobia symbiosis is an interaction between rhizobia and legumes in which both plants and bacteria are benefited. In this symbiotic relationship, rhizobia are hosted and supplied with carbon sources by legumes and in return legumes receive ammonia provided by rhizobia (Figure 1).

Legume-*Rhizobium* symbiosis or symbiotic nitrogen fixation (SNF) starts with a molecular signal between the two partners. In this process the legume secretes a mixture of phenolic molecules, predominantly flavonoids and isoflavonoids, into the rhizosphere. These signals are taken up by rhizobia, bind the transcriptional regulator *NodD*, and activate a suite of bacterial nodulation genes (Long, 1996). Various genes that are responsible for SNF are listed in Table 3. These nodulation genes produce the lipochitooligosaccharides (LCOs) called Nod factors. This Nod factors are the key symbiotic indicators and are essential for the specific host-*Rhizobium* interaction and thereby infection process start, and nodule organogenesis occurred (Oldroyd and Downie, 2008). Production of LCOs play a crucial role in determining whether the Nod factors can be perceived by a specific host (Long, 1996). Genes are listed in Table 3, are very important for bacterial invasion and induction of nodules in the early stages of nodulation by inducing host responses such as root hair deformation and cortical cell division (Moulin et al., 2004).



Source: Modification from Lindström and Mousavi, 2020.

Figure 1. Summarized model for symbiotic nitrogen fixation in legumes by rhizobia.

Table 3. A list of the most common rhizobial *nod*, *nif* and *fix* genes (after Laranjo et al., 2014).

Genes	**Function of gene product**
Nodulation genes	
nodA	Acyltransferase
nodB	Chitooligosaccharide deacetylase
nodC	N-acetylglucosaminyltransferase
nodD	Transcriptional regulator of common nod genes
nodIJ	Nod factor transport
nodPQ	Synthesis of Nod factor substituents
nodX	Synthesis of Nod factor substituents
nofEF	Synthesis of Nod factor substituents
Other *nod* genes	Several functions in synthesis of Nod factors
nol genes	Several functions in synthesis of Nod factor substituents and secretion
noe	genes Synthesis of Nod factos substituents
Nitrogen fixation genes	
nifH	Dinitrogenase reductase (Fe protein)
nifD	a subunits of dinitrogenase (MoFe protein)
nifK b	subunits of dinitrogenase (MoFe protein)
nifA	Transcriptional regulator of the other nif genes
nifBEN	Biosynthesis of the Fe-Mo cofactor
fixABCX	Electron transport chain to nitrogenase
fixNOPQ	Cytochrome oxidase
fixLJ	Transcriptional regulators
fixK	Transcriptional regulator
fixGHIS	Copper uptake and metabolism
fdxN	Ferredoxin

Colonization of legume roots by rhizobia results in the deformation and curling of root hairs, and several genes, e.g. early nodulation (ENOD) genes and Msrip1, are expressed in the epidermis of legumes (Franssen et al., 1995; McAdam et al., 2018). Several recent reviews deal with the complex biological interactions leading to infection and nodulation by rhizobia, such as how the legume controls the massive infection caused by rhizobia (Berrabah et al., 2018; Buhian and Bensmihen, 2018; Poole et al., 2018). Two main types of nodules are formed in legume species like indeterminate or determinate (Hirsch, 1992). The type nodule is determined by the host plant and in rare cases legumes may form both nodule types (Andrews and Andrews, 2017).

1.4. Soybean-Nodulating Rhizobia

The genus Rhizobium was the first described group symbiotic bacteria, and that is why it has been used frequently for the nitrogen-fixing bacteria in legumes. (Lloret and Martinez-Romero, 2005) Today, this group of bacteria are scattered in 18 genera in the families *Rhizobiaceae (Rhizobium, Ensifer (syn. Sinorhizobium), Allorhizobium, Pararhizobium, Neorhizobium, Shinella), Phyllobacteriaceae (Mesorhizobium, Aminobacter, Phyllobacterium), Brucellaceae (Ochrobactrum), Methylobacteriaceae (Methylobacterium, Microvirga), Bradyrhizobiaceae (Bradyrhizobium), Xanthobacteraceae (Azorhizobium), Hyphomicrobiaceae (Devosia) and Burkholderiaceae (Paraburkholderia, Cupriavidus).* The genus Rhizobium, accommodating 112 species, is the largest genus of rhizobia (Mousavi, 2016; de Lajudie et al., 2019). Soybean-nodulating rhizobia are genetically diverse and are classified into different genera and species and are characterized as slow to fast growing habit. The slow-growing soybean-nodulating rhizobia are distributed in five species of *Bradyrhizobium*, such as *B. japonicum* (Jordan, 1982), *B. elkanii* (Kyukendall et al., 1992), *B. liaoningense* (Xu et al., 1995), *B. yuanmingense* (Yao et al., 2002), and *B. canariense* (Vinuesa et al., 2005). Two fast-growing species of *Sinorhizobium* (*Ensifer*), namely *S. fredii* (Chen et al., 1988) and *S. xinjiangense* (Peng et al., 2002), are also reported to be soybean- nodulating rhizobia. *Mesorhizobium tianshanense* (Chen et al., 1995), which has varying growth rates, has also been reported as a soybean rhizobial species.

1.5. Geographical Distribution of Soybean-Nodulating Rhizobia

Several researches have been studied world-wide to find out the distribution of soybean-nodulating rhizobia. It has been reported that the distribution of soybean-nodulating rhizobia at the species level is largely depends on temperature of a particular environment in different soil. Among the slow growers, *B. japonicum* and *B. elkanii* have been found in various climates across the world (Adhikari et al., 2012; Ando and Yokoyama 1999; Chen et al., 2000; Li et al., 2011; Man et al., 2008; Risal et al., 2010; Saeki et al., 2006; Suzuki et al., 2008; van Berkum and Fuhrmann 2000; Vinuesa et al., 2008; Wasike et al., 2009; Yang and Zhou, 2008). *B. japonicum* was dominant in the soils of cooler environments as reported in the USA (Shiro et al., 2013), Japan (Saeki et al., 2006; Suzuki et al., 2008) and Nepal, (Adhikari et al., 2012; Vinuesa et al., 2008 and Risal et al., 2010). Adhikari et al., (2012) also reported that *B. elkanii*, *B. yuanmingense*, and *B. liaoningense* dominated in the subtropical region respectively, at acidic, moderately acidic, and slightly alkaline soils. The relatively extra-slow-growing species like *B. liaoningense*, has been isolated from alkaline soils of temperate to subtropical climates in south and south-east Asia (Xu et al., 1995; Saeki et al., 2005; Appunu et al., 2008; Vinuesa et al., 2008; Yang and Zhou, 2008; Han et al., 2009; Li et al., 2011). The occurrence of *B. yuanmingense* in India (Appunu et al., 2008), Kenya (Wasike et al., 2009) and Nepal (Risal et al., 2010) suggests its preference for warm climates, although *B. yuanmingense* is reported in the alkaline-saline soils of temperate China (Li et al., 2011). Yang and Zhou (2008) first time reported that *B. canariense* have found in the soybean nodules in north and northeast China. *B. canariense* is a distinguished as an extra acid tolerant than *B. japonicum* (Vinuesa et al., 2005). Chen et al. (1988) reported that *S. fredii* and *S. xinjiangense* are acid-producing were the fast-growing rhizobia which have been isolated from the saline-alkaline soils in China, Vietnam and Japan (Appunu et al., 2009; Han et al., 2009; Li et al., 2011; Man et al., 2008; Peng et al., 2002; Saeki et al., 2005; Suzuki et al., 2008). These results suggest that temperature and soil pH determine the species-specific distribution of soybean-nodulating rhizobia in soils.

However, it is uncertain which factors responsible for the temperature dependent distribution of soybean-nodulating rhizobia. To clarify this uncertainty several competitive inoculation experiments has been done at different temperature. Kluson et al. (1986) studied the competition between the strains of *B. japonicum* and *B. elkanii* and reported that *B. japonicum* dominated in the noduled at low temperature while at higher temperature *B. elkanii* found to be dominant. In a competitive conditions Suzuki et al. (2014) examined the nodule occupancy as well as relative population of *B. japonicum* and *B. elkanii* strains in rhizosphere of soybean cultivated using sterilized vermiculite and reported that infection of *B. japonicum* lead the nodule dominancy at lower temperature though the population of both *B. japonicum* and *B. elkanii* were similar in the rhizosphere. They also observed that the population of *B. elkanii* relatively larger than *B. japonicum* at higher temperature led to the dominance of *B. elkanii* in the nodules. Similarly, Yokoyama (2005) investigated the soybean nodule formation at different temperatures under competitive condition using a mixture of *Bradyrhizobium* spp. strains and reported that *B. elkanii* USDA 31 dominated in the nodules at high temperature, while *B. japonicum* USDA 110 dominated at low temperature. Shiro et al. (2016) study the *nodC* gene expression and nodule occupancy of a mixture of *Bradyrhizobia* at different temperatures. They reported that *B. japonicum* dominates in the nodule at lower temperature along with the increase of *NodC* gene expression, while *B. elkanii* did at higher temperature. All these results support the temperature-dependent distribution of soybean-nodulating rhizobia in the field and suggested that that temperature dependent infection and/or proliferation in soil might determine the nodule dominance in the soil. Yet need to clarify which factors is more involved for nodulation.

1.6. Objectives of the Study

Soybean-nodulating rhizobia can fix nitrogen in the nodule of the host plant through symbiosis. Because effective nitrogen fixation depends on the potential of an individual rhizobial strain, competition for proliferation and infection among the rhizobial strains in a soil is crucial subject. In addition, ecological behavior of the indigenous soybean-nodulating rhizobia in relation to the environmental conditions need to be considered.

It has been reported that *B. japonicum* and *B. elakanii* are the major soybean nodulating rhizobia. Previous reports suggested that these *B. japonicum* and *B. elakanii,* respectively dominates in the nodules of soybean cultivated form northern to southern latitudes (Saeki et al., 2006, Shiro et al., 2013, Adhikari et al., 2012). Previous report also suggest that latitudinal temperature influence the rhizobial preference for infection and proliferation in soils (Kluson et al., 1986, Suzuki et al., 2014; Shiro et al., 2016). Generally, it is very hard to determine the distribution of specific rhizobia in field soil. For these reasons, the species-specific distribution of rhizobia in field soils is evaluated by their distribution in nodules. But it is uncertain which factor, temperature-dependent infection or proliferation in soil contributes to the latitudinally characteristic nodule composition of the bradyrhizobia. Considering the above mentioned two factors in the field under local climatic condition, we selected three study locations, Fukagawa with temperate continental climate, and Matsue and Miyazaki with humid sub-tropical climate in Japan. Each soil sample of the sites was used for soybean cultivation at all the locations for successive two years to examine the changes in distribution of rhizobia in the nodules after transfer of the soil samples to the different climatic conditions, and to follow the changes in the second year in the new environments. If the predominance of some rhizobia in the soil determines nodule occupancy, changing climatic conditions would not affect the nodule occupancy, on the other hand, if temperature-dependent infection determines nodule occupancy, it would be changed in different climatic conditions. Therefore, the objectives of the present study are listed below.

- To elucidate the reason for the latitudinally characteristic nodule composition of *Bradyrhizobium* spp., we examined the changes in the nodule compositions of soybean which was cultivated using the soil samples under the different local climate conditions. (**Chapter 1**).
- To confirm the assumption derived from Chapter 1, we examined the temperature-related growth and infection properties of the *Bradyrhizobium* spp. strains, which were isolated from the soybean nodules cultivated in the Fukagawa and Miyazaki soils and locations (**Chapter 2**).

CHAPTER 2

Latitudinal Characteristics Nodule Composition of Soybean-Nodulating Bradyrhizobia: Temperature-Dependent Proliferation in Soil or Infection?

2.1. Introduction

Soybean (*Glycine max* [L] Merr.) originated in north-eastern China and is presently cultivated around the globe under various soils and climatic conditions (Hymowitz and Harlan, 1983; Dupare et al., 2008; Khojely, et al., 2018). High concentrations of protein and oil in soybean seeds indicated its significance in daily life. Soybean is an easy-to-cultivate crop belonging to the Leguminosae family that can grow in nitrogen poor soils. Soybean-nodulating rhizobia can establish symbiosis with soybeans through effective nitrogen fixation.

Diverse soybean-nodulating rhizobia belongs to the genera, *Bradyrhizobium, Sinorhizobium* (*Ensifer*) and *Mesorhizobium* (Vinuesa et al., 2008; Tan et al., 1997), among which *Bradyrhizobium* is recognized as a slow grower, while *Sinorhizobium* (*Ensifer*) as a fast grower, and *Mesorhizobium* as variable one (Chen et al., 1995). *B. japonicum* and *B. elakanii* are the major soybean-nodulating rhizobia having the high nitrogen-fixing ability and have been used as inoculants for improving crop production. But the inoculants could not dominate in nodules due to competition with indigenous rhizobia in the field (Streeter, 1994). Therefore, it is essential to evaluate the ecological behavior of the indigenous soybean-nodulating rhizobia in relation to the environmental conditions.

Saeki et al. (2006) studied geographical distribution of soybean-nodulating rhizobia in Japan using soil samples around the country as inoculants and showed the species-specific latitudinal distribution of *B. japonicum* and *B. elkanii*, in which the former dominated in nodules when the northern soils were used, while the latter did in southern soils. Shiro et al. (2013) examined the genetic diversity of indigenous soybean-nodulating rhizobia in USA using the similar method and found the same latitudinal distribution of *B. japonicum* and *B. elkanii* from north to south. Adhikari et al. (2012) examined the nodules form different locations in Nepal, and found that *B. japonicum* dominated in temperate regions, while in subtropical locations, *B. elkanii*, *B. yuanmingense*, and *B. liaoningense* dominated in acidic, moderately acidic, and slightly alkaline soils, respectively. Li et al. (2011) also reported the pH-dependent distribution of rhizobia in Chinese soils, in which *B. japonicum* and *B. elkanii* dominated in neutral soils, while *B. yuanmingense*, *B. liaoningense*, and *Sinorhizobium* in alkaline soils. These results suggest that temperature and soil pH determine the species-specific distribution of soybean-nodulating rhizobia in soils.

To examine the possible reasons of the temperature-dependent distribution of soybean-nodulating rhizobia, competitive inoculation experiments at different temperature have been conducted. Kluson et al. (1986) reported that *B. japonicum* USDA 6 and *B. diazoefficiens* USDA 110 dominated in nodules at lower temperatures, while *B. elkanii* USDA 76 and *B. elkanii* USDA 94 did at higher temperature. Suzuki et al. (2014) examined the nodule occupancy as well as relative population of *B. japonicum* and *B. elkanii* strains in rhizosphere of soybean cultivated using sterilized vermiculite. Under competitive conditions, *B. japonicum* strains dominated in nodules at lower temperature even though the relative populations of both strains were similar in the rhizosphere, while at higher temperature, *B. elkanii* strains dominated in nodules due to their larger relative population in the rhizosphere. Shiro et al. (2016) examined gene expression of *nodC* and nodule occupancy of *Bradyrhizobia* at different temperatures. In the inoculation experiment with the mixes of three strains, the nodule occupancy of *B. elkanii* USDA 31 increased at higher temperatures, whereas that of *B. japonicum* USDA 123 increased at lower temperatures, corresponding to their temperature-dependent *nodC* gene expressions. These results support the temperature-depended distribution of soybean-nodulating rhizobia in the field and suggested that temperature influenced their preference for infection and/or proliferation in soils. Because the species-specific distribution of rhizobia in field soils is evaluated by their distribution in nodules, it is uncertain which factor, temperature-dependent infection or proliferation in soil, contributes to the temperature-dependent distribution of the rhizobia in nodules.

Considering the above mentioned two factors, we selected three study locations of different climatic conditions in Japan, and each soil sample of the sites was used for soybean cultivation at all the locations for successive two years to examine the changes in distribution of rhizobia in the nodules after transfer of the soil samples to the different climatic conditions, and to follow the changes in the second year in the new environments. If the predominance of some rhizobia in the soil determines nodule occupancy, changing climatic conditions would not affect the nodule occupancy, on the other hand, if temperature-dependent infection determines nodule occupancy, it would be changed in different climatic conditions. The aim of this study is to elucidate the possible reasons of the latitudinal characteristic distribution of soybean-nodulating rhizobia in the local climate conditions.

2.2. Materials and Methods

2.2.1. Study Locations

To examine the temperature-dependent nodule occupancy of soybean rhizobia, three study locations, Fukagawa (fu), Matsue (ma) and Miyazaki (mi), were selected in Japan. According to Koppen’s climatic classification, Fukagawa belongs to Dfb (Temperate continental climate) region, and Matsue and Miyazaki belong to Cfa (Humid sub-tropical climate) region. Soil samples were collected from the experimental fields of Takushoku University of Hokkaido College, Shimane University, and Miyazaki University, and used for soybean cultivation at all study locations. There had been no history of legumes cultivation in all soils. Basic information on the site and climatic parameters are presented in Table 1. The soil properties were reported previously (Table S1, Puri et al., 2018).

2.2.2. Soybean Cultivation

The soil samples with a total weight of about 25 Kg were collected from several sites of the experimental field and mixed together for each study location. Each soil sample was divided into three parts (ca. 7.5 Kg), and used for soybean cultivation at each study location. Each soil sample was put in three plastic pots (20 cm in diameter and 25 cm in height), which were placed on a plastic sheet or a wooden duck board in the open field. Seeds of soybean cv. Orihime (non-*Rj*) from the same lot were used at all study locations. Three healthy seedlings per pot were remained at seven days after germination, then cultivated for ca. 2–3 months depending on the conditions of the study locations in each year until harvest without fertilization, then fresh weight of the

Table 1. Geographical and climatic characteristics of the study locations in Japan.

Location	**Latitude (°N)**	**Longitude (°E)**	**Temperature (°C)** [a]	**Rainfall (mm)** [a]
Fukagawa	43.71	142.01	16–26/16–26 (14–24/17–27) [b]	432/243
Matsue	35.48	133.06	23–30/23–30 (22–29/25–32)	177/481
Miyazaki	31.82	131.41	24–32/24–31 (25–32/25–33)	240/860

[a] Average daily minimum and maximum temperatures and total rainfall during the cultivation period in 2016/2017.

[b] Figures in parenthesis indicate those during one month after sowing. (https://www.jma.go.jp).

whole plant and number of nodules were measured. After harvesting of the soybean plants in 2016, in the case of Matsue location, the pots with the soil were kept in the open filed until next cultivation season. For Miyazaki and Fukagawa locations, the triplicate soil samples were mixed and kept in a paper bag in a warehouse under the same temperature conditions as outdoors until the next cultivation in 2017. The different procedures were due to space issues at the study location.

2.2.3. Nodule Sampling and Isolation of Rhizobia

After harvesting from flowering to early fruiting period, the roots were washed carefully with tap water and the whole plant fresh weight was measured after removal of surface water with tissue towel, and the number of nodules in each plant was counted, then the nodules were preserved at low temperature in a vial containing desiccating silica gel until isolation of rhizobia (Figure S1).

For isolation of rhizobia, ten nodules were randomly selected from one plant for each replication and kept in sterilized distilled water overnight. When the number of nodules was less than 10, two plants were used. After surface sterilization with 95% ethanol for 30 s followed by 3% sodium hypochlorite solution for 30 s, and rinsed in sterilized distilled water at least seven times, each nodule was crushed in an Eppendorf tube with 1 mL of sterilized distilled water, then a drop of suspension was streaked onto yeast mannitol agar (YMA) medium (Vincent, 1970) (Table S2), and incubated at 25 °C for 5–12 days. Randomly selected two colonies per nodule were purified, and a total of 540 isolates (3 replications, 3 soils from 3 study locations, 10 nodules per plant, and 2 isolates per nodule) were further analyzed molecularly (Appendix 1 and 2).

2.2.4. Phylogenetic Analysis of the Rhizobia Based on Genes of 16S rRNA and 16S–23S rRNA Internal Transcribed Spacer (ITS) Region

A small amount of colony was directly subjected as template for the PCR amplification of the partial 16S rRNA gene using the universal primers fD1 and rP2 (Weisburg et al., 1991). The components of the PCR mixtures and the PCR running conditions and primer used are summarized in Tables S3, S4 and S5, respectively. PCR products were purified and subjected to PCR cycle sequencing, according to the procedures described previously Adhikari et al. (2012). Taxonomic position of the isolates was determined based on the database (https://www.ncbi.nlm.nih.gov/) using BLAST (Altschul et al., 1997) search. Multiple sequence alignments were constructed using ClustalW 2.1 (Larkin et al., 2007). Alignments were manually edited and phylogenetic trees with the related reference strains were constructed using ClustalW 2.1 with the neighbor-joining method and the tree was visualized by MEGA 7 (Kumar et al., 2016).

Among the isolates with the same phylogeny in 16S rRNA gene in each Soil-Location-Year combination, two representatives were randomly selected for analysis of the ITS region. PCR amplification of ITS region was done using the universal ITS primers, 1512F and 23R (Hiraishi et al., 1997). The procedures were the same as described above.

2.2.5. Nucleotide Sequence Accession Numbers

The sequence data generated in this study were deposited in the DDBJ Nucleotide Submission System under the accession numbers LC582850 to LC582907 for 16S rRNA gene, and LC579845 to LC579902 for 16S–23S rRNA ITS region.

2.2.6. Statistical Analysis

Statistical analysis of the soybean cultivation data was carried out using the MSTAT-C 6.1.4 (Freed, 2007) software package. The data were subjected to Duncan's multiple range test after one-way ANOVA.

2.3. Results

2.3.1. Fresh Plant Weight and Number of Nodules of Soybean

At each study location, the fresh plant weight and the nodule numbers were not significantly different among soils in both years with a few exceptions (Figure 1). When all data in each study location were analyzed, the fresh plant weight showed the tendency increasing from northern (FU) to southern (MI) sites, whereas the nodule number showed the opposite tendency significantly decreasing from northern to southern sites (Figure 2).

2.3.2. Phylogenetical Characterizations of the Rhizobia

Based on the 16S rRNA gene analysis, the isolated rhizobia were most closely related to one of the three groups, *Bradyrhizobium japonicum* Bj11 (KY000638.1), *Bradyrhizobium japonicum* S10J (MF664374.1), and *Bradyrhizobium elkanii* L7 (KY412842.1) (Figure S2). Similarities (%) of the sequences between the isolates and the corresponding type strains were 98–100%, 96–100%, and 97–100% for *B. japonicum* Bj11, *B. japonicum* S10J, and *B. elkanii* L7, respectively. The phylogenetic tree of the ITS region of the selected isolates indicated that the rhizobial strains were further

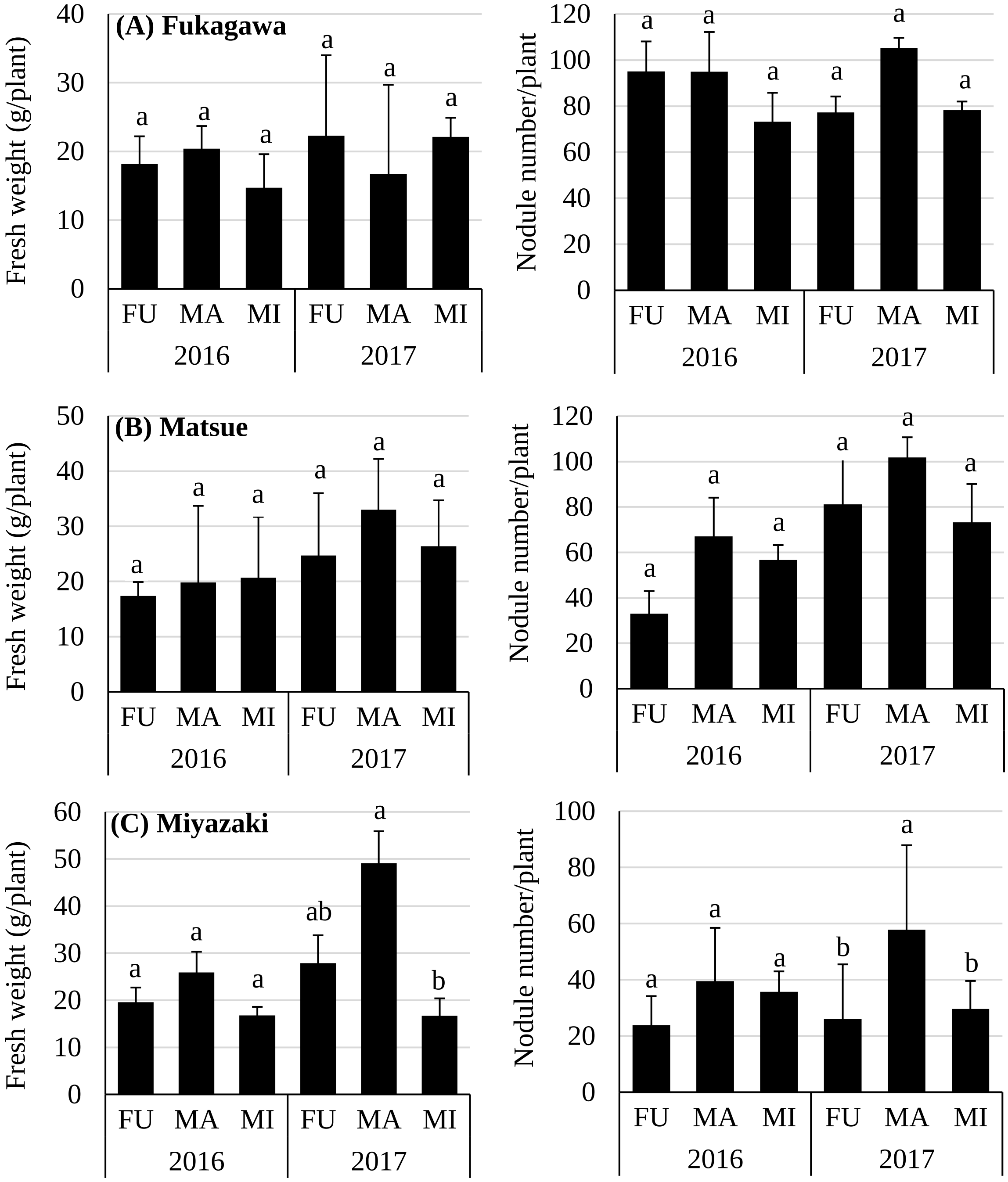


Figure 1. Fresh weight and number of nodules of soybean cultivated at Fukagawa (**A**), Matsue (**B**), and Miyazaki (**C**) locations using the soil samples (FU, MA and MI) collected from the corresponding study locations. The bars represent standard deviation (n = 3) and different letters indicate significant differences at $p < 0.05$ by Duncan's test.

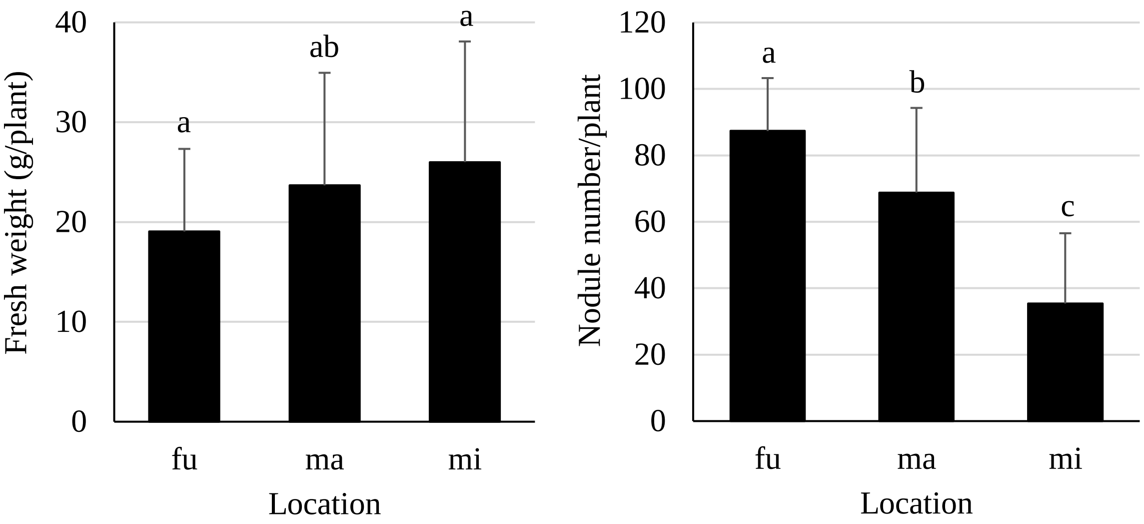


Figure 2. Fresh weight and number of nodules of soybean cultivated at Fukagawa (fu), Matsue (ma), and Miyazaki (mi) locations. The bars represent standard deviation (n = 18) and different letters indicate significant differences at $p < 0.05$ by Duncan's test.

grouped into the subgroups (Figure 3). The most similar sequences in the data base are listed in Table 2.

B. japonicum Bj11 was grouped into Bj11-1 and Bj11-2 based on the ITS sequence, and the two groups were characterized by their physiological properties, that is, it took more than one week for Bj11-1 to form visible colonies on the YMA agar plate, compared to 5–6 days for Bj11-2. *B. japonicum* S10J was grouped from BjS10J-1 to BjS10J-4 based on the ITS sequence. The ITS sequences of BjS10J-1 had more similarity to those of *B. japonicum* Bj11 than the other groups of *B. japonicum* S10J. BjS10J-2 and BjS10J-3 were characterized by their origin, that is, BjS10J-2 and BjS10J-3 were isolated from soybeans cultivated in Miyazaki and Matsue soils, respectively. *B. elkanii* L7 was isolated from soybeans cultivated in all soils and study locations, and their ITS sequences were not distinguished among them.

2.3.3. Relative Composition of the Strains in Relation to Soil and Climate in 2016 and 2017

In Fukagawa soil, the soybean rhizobia consisted of Bj11-1, Bj11-2, and BeL7 in all study locations (Table 3 and Figure 4). Bj11-1 dominated (80–87%) in all study locations in 2016. In 2017, Bj11-1 maintained in Fukagawa soil at 80%, however, the compositions decreased in Matsue and Miyazaki locations at 53% and 60%, respectively, along with the increase of BeL7 to 40% and 30%, respectively. Bj11-2 was present minorly at 7–17% in all study locations and in both years.

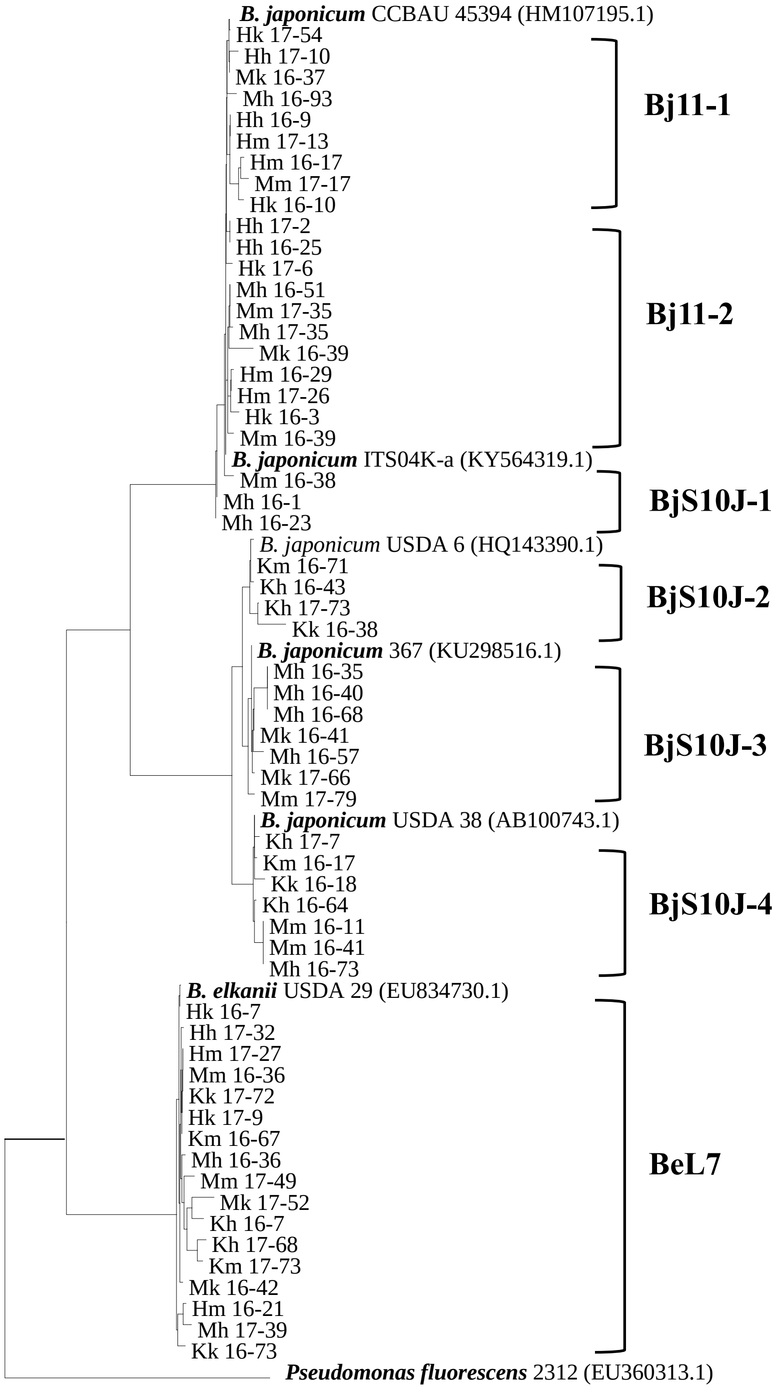


Figure 3. Phylogenetic tree of the 16S–23S rRNA ITS gene regions of the soybean rhizobial strains isolated in this study with reference strains. The isolates were designated by the soil [Fukagawa (H), Matsue (M) and Miyazaki (K)], the study location (h, m, and k), year of the cultivation, and the strain number. The scale bar indicates the number of substitutions per site.

Table 2. Group of soybean rhizobial strains isolated in this study based on phylogeny of 16S–23S rRNA genes ITS region.

Closest 16S rDNA	**ITS group**	**Closest ITS** [a]	**Acc. No.** [b]	**Id. (%)**	**Remarks**
B. japonicum Bj11	Bj11-1	Bj CCBAU 45394	HM107195	98–99	Slow grower Primarily Fukagawa soil
	Bj11-2	Bj CCBAU 45394	HM107195	99–100	Fast grower Fukagawa & Matsue soils
B. japonicum S10J	BjS10J-1	Bj ITS04K-a	KY564319	99–100	Matsue soil
	BjS10J-2	Bj USDA 6	HQ143390	98–99	Miyazaki soil
	BjS10J-3	Bj 367	KU298516	96–100	Matsue soil
	BjS10J-4	Bj USDA 38	AB100743	99–100	Primarily Miyazaki soil
B. elkanii L7	BeL7	Be USDA 29	EU834730	97–100	Ubiquitous in all soils

[a] Bj; *Bradyrhizobium japonicum*, Be; *Bradyrhizobium elkanii*.

[b] Gene accession number in database.

In Matsue soil, Bj11, BjS10J and BeL7 were isolated in all study locations (Table 3 and Figure 4). The composition and behavior were similar between Matsue and Miyazaki locations, with Bj11 decreasing from 20% and 24% in 2016 to 10% and 0% in 2017, respectively, while BeL7 increased from 70% and 73% in 2016 to 87% and 97% in 2017, respectively. BjS10J was present minorly at 3–10% in both years. In Fukagawa location, Bj11 was present at 24% in 2016, and maintained at 23% in 2017, although the major group shifted from Bj11-1 to Bj11-2. The dominant group BeL7 increased from 53% in 2016 to 77% in 2017 as the other study locations, while BjS10J, which was 23% in 2016, disappeared in 2017.

In Miyazaki soil, the rhizobia consisted of BjS10J-2, BjS10J-4, and BeL7 (Table 3 and Figure 4). In Miyazaki location, BeL7 was dominant at 77% in 2016, and completely eliminated BjS10J in 2017. In Fukagawa and Matsue locations, BjS10J-2, which was dominant at 73% and 53% in 2016, decreased to 13% and 0% in 2017, respectively, while BeL7 increased from 13% and 33% in 2016 to 80% and 100% in 2017, respectively. BjS10J-4 also decreased from 13% in 2016 to 0–7% in 2017, respectively.

When Fukagawa soil was moved to Matsue and Miyazaki locations, the dominant rhizobia changed from Bj11 to BeL7 in the second year. For Matsue and Miyazaki soils, BeL7 decreased in Fukagawa and Matsue locations in the first year and recovered to the original level in the second year.

2.4. Discussion

Although the fresh weight and number of nodules were not significantly different among soils at all study locations (Figure 1), the fresh weight increased from northern to southern study locations, while the number of nodules showed the opposite tendency depending on the study location (Figure 2). The cultivation temperature might be involved in the change in the parameters (Table 1), and similar tendency of the temperature-dependent growth of soybean have been reported (Stoyanova, 1996; Zhang and Smith, 1994; Montañez et al., 1995).

In case of the number of nodules, previous reports showed opposite temperature-dependent tendency from ours (Stoyanova, 1996; Zhang and Smith, 1994; Montañez et al., 1995). Reduction of nodules at higher temperature might be due to strain-specific properties. Shiro et al. (2016) reported that the nodule numbers were different by about 10 times depending on the strains, and the temperature-dependent expression of *nodC* gene was also strain-specific but it was not related to the nodule numbers of the corresponding strains. Hungria and Vargas (2000) showed an example of adverse effects of high temperature on soil population of bradyrhizobia and nodule number of soybean. Strain-dependent tolerance against high temperature in soil was also reported (Suzuki et al., 2014). Because the bradyrhizobial community structure was changed at the different study locations, the microbial transition might be a reason for the reduction of nodules at the southern study location**.** Increase of soybean nodule with the higher nitrogen concentration have also been reported by Xia et al., 2017 and Abdel-Wahab and Abd-Alla, 1996. In Miyazaki soil, we observed that number of nodules comparatively lower than Fukagawa and Matsue soil. The soil nitrogen status of Miyazaki was higher than other soils (Table

Table 3. Relative composition (%) of rhizobial strains isolated in this study based on phylogeny of 16S–23S rRNA ITS gene regions.

Soil/ Location	**Year**	**Bj11**		**BjS10J**				**BeL7**
		1	**2**	**1**	**2**	**3**	**4**	
FU/fu	2016	87	13	-	-	-	-	-
	2017	80	17	-	-	-	-	3
FU/ma	2016	80	17	-	-	-	-	3
	2017	53	7	-	-	-	-	40
FU/mi	2016	83	7	-	-	-	-	10
	2017	60	10	-	-	-	-	30
MA/fu	2016	17	7	7	-	13	3	53
	2017	-	23	-	-	-	-	77
MA/ma	2016	-	20	3	-	-	7	70
	2017	3	7	-	-	3	-	87
MA/mi	2016	7	17	-	-	3	-	73
	2017	-	-	-	-	3	-	97
MI/fu	2016	-	-	-	73	-	13	13
	2017	-	-	-	13	-	7	80
MI/ma	2016	-	-	-	53	-	13	33
	2017	-	-	-	-	-	-	100
MI/mi	2016	-	-	-	7	-	17	77
	2017	-	-	-	-	-	-	100

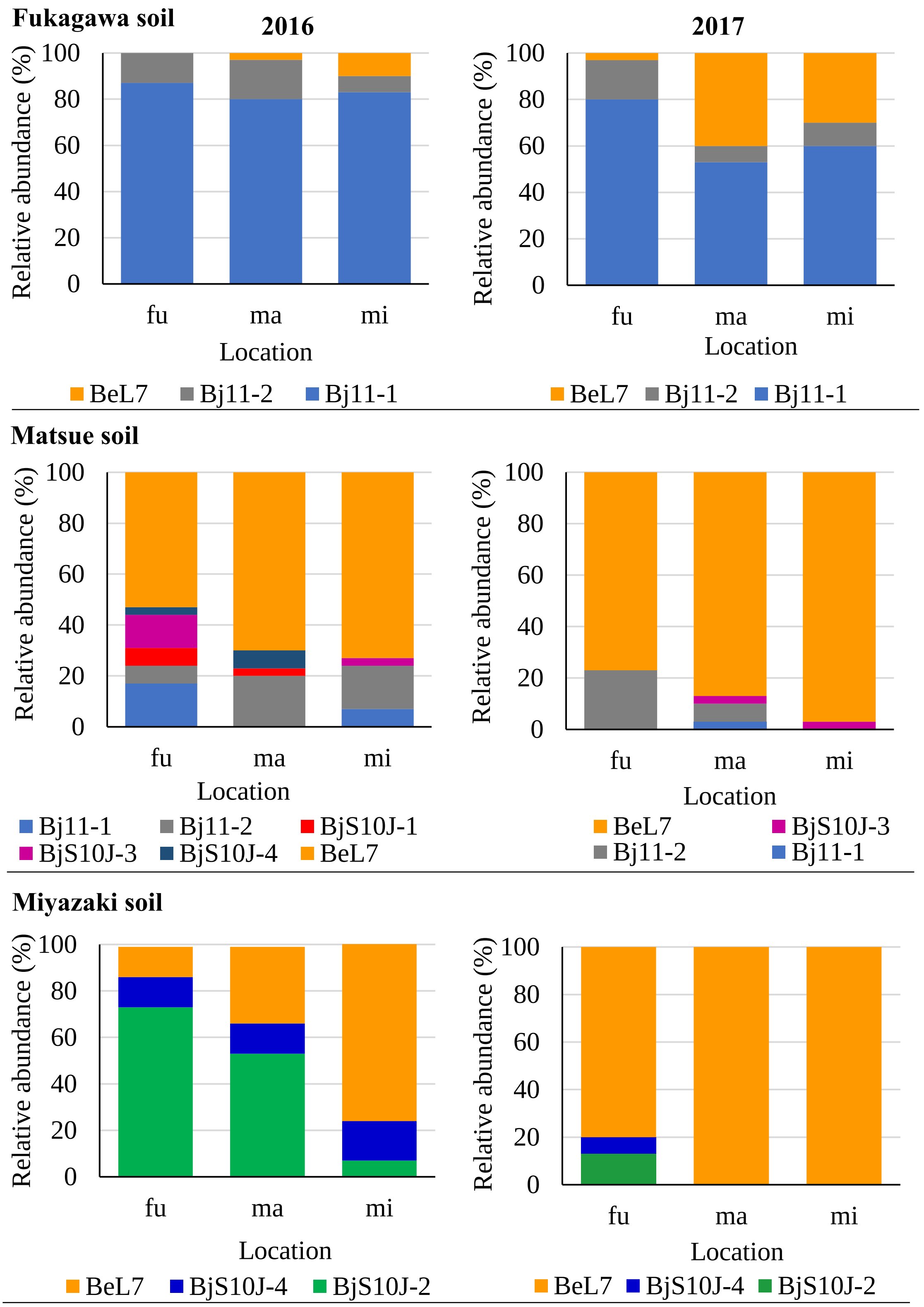


Figure 4. Relative abundance of the soybean rhizobial strains (left panel for 2016 and right panel for 2017).

S1) and produce less nodules in all locations (Figure 1C). This result indicates that the lowering of nodules in Miyazaki soil might be due to the higher nitrogen in soil.

The phylogenetic analysis of the 16S rRNA genes of the *Bradyrhizobium* spp. isolates showed the three clusters, Bj11, BjS10J, and BeL which mostly corresponded to the three clusters in phylogeny of ITS sequences. As pHs of the soil samples were slightly acidic (Table S1, Puri et al., 2018), the dominant presence of *B. japonicum* and *B. elkanii* in the nodules is reasonable Li et al. (2011). The three clusters were phylogenetically comparable with the results of Saeki et al. (2004) and Willems et al. (2003) (data not shown). Willems et al. (2003) showed that each cluster had more than 95.5% similarity in ITS sequences, whereas the similarity within each cluster in this study ranged 95–97%, and those within the subclusters (Bj11-1-2 and BjS10J-1-4) were 98–99% (data not shown). These results suggest that the *Bradyrhizobium* spp. isolates in this study were phylogenetically positioned in the same groups as previously reported, and they were further grouped by physiological property (Bj11-1 and 2), phylogeny of 16S rRNA genes (BjS10J-1), and origin of soil (BjS10J-1, 2 and 3).

Topology of the phylogenetic trees of 16S rDNA and ITS region was almost the same except for BjS10J-1. The variable position of a subcluster of *B. japonicum* has been also reported in the report of Saeki et al. (2006) and Adhikari et al. (2012). The ITS nucleotide sequence similarity of the BjS10J-1 strains was more than 98% with those of the Bj11 strains, while 88 to 90% with those of the other BjS10J strains having the same 16S rRNA gene sequences. Horizontal gene transfer among them would be one of the possible reasons for the discrepancy of their topologies.

Each cluster of BjS10J was characterized by their origins, on the other hand, BeL7 were originated form all soils having undistinguishable gene sequences, and Bj11 was isolated only from Fukagawa and Matsue soils. These results suggested that the range of distribution of the strains differed among the groups. As the wide range of distributions were generally reported in the previous studies (Adhikari et al., 2012; Risal et al., 2010), the limited range of distribution of the BjS10J strains suggested that their presence might depend on soil characteristics.

It has been well known that the species-specific distribution of soybean-nodulating rhizobia in the field is temperature-depended (Saeki et al., 2006; Shiro et al., 2013; Adhikari et al., 2012) and that the temperature effect is mainly due to their infection preference (Shiro et al. 2016) and/or proliferation in soil (Kluson et al., 1986; Suzuki et al., 2014). But it is uncertain which factor, temperature-dependent infection or proliferation in soil, contributes to the temperature-dependent distribution of the rhizobia in nodules.

In the case of Fukagawa soil, *B. japonicum* was dominant in the nodules in the high latitude Fukagawa location, and the dominancy was maintained for two years (Table 3 and Figure 4). This tendency is the same as the temperature-dependent nodule dominancy of *B. japonicum* as previously reported (Saeki et al., 2006; Shiro et al., 2013; Adhikari et al., 2012). When Fukagawa soil was moved to the warmer Matsue and Miyazaki locations, the nodule composition of *B. japonicum* and *B. elkanii* was not changed, suggesting originally lower population of *B. elkanii* in Fukagawa soil. If *B. elkanii* was present in Fukagawa soil to a certain extent and low temperature prevented their infection, then resulting in their lower nodule dominancy, the nodule dominancy of *B. elkanii* would increase when the temperature increased in the sub-tropical study locations. In the second year, however, the nodule dominancy of *B. elkanii* increased in Matsue and Miyazaki locations, suggesting increase of soil population of *B. elkanii* in the warmer environment. Regarding *B. japonicum* Bj11 in Fukagawa soil, the composition of Bj11-2 maintained in both years in Matsue and Miyazaki locations, while that of Bj11-1 decreased in the second year, suggesting higher sensitivity of Bj11-1 to high temperature.

In the case of Matsue soil, the dominancy of *B. elaknii* was observed in Matsue and Miyazaki locations (Table 3 and Figure 4). The similar temperature-dependent nodule occupancy of *B. elaknii* has been reported (Saeki et al., 2006; Shiro et al., 2013; Adhikari et al., 2012). The dominancy of *B. elkanii* increased in both study locations in the second year. When this soil was moved to the cooler Fukagawa location, the dominancy of *B. japonicum* increased in the first year, suggesting that *B. japonicum* originally presented in Matsue soil and their nodule dominancy increased due to lower temperature. The composition of *B. elkanii* increased in the second year, supposing decrease in the population of *B. japonicum* and/or increase in that of *B. elkanii*. Although the average minimum and maximun temperatures during the cultivation period were similar between both years, those during one month after sowing, when frequent nodulation would be expected, seemed to be a little higher in the second year (Table 1). It was supposed that the higher temperature in the second year might cause the increase in the relative dominancy of *B. elaknii*. Among the *B. japonicum* strains in the first year, Bj11-2 was dominant in Matsue and Miyazaki locations, while Bj11-1 and BjS10J in Fukagawa location. Along with the decrease of Bj11-1 and BjS10J in the second year, relative dominancy of Bj11-2 increased. The transition of the *B. japonicum* strains might be due the difference in sensitivity to high temperature among them.

In Miyazaki soil, BeL7 was dominant and BjS10J-4 was minor in Miyazaki location in 2016, while in Fukagawa and Matsue locations, BjS10J-2 appeared dominantly (Table 3 and Figure 4), suggesting that *B. japonicum* was originally present in Miyazaki soil and their nodulation increased due to the lower temperature in the cooler environments. In the second year, however, BeL7 recovered to 80–100%. A little higher temperature during one month after sowing in the second year might be the reason (Table 1), but the temperature in Fukagawa location in 2017 was lower than that in Matsue location in 2016, therefore, only the change in temperature could not explain the increase of BjS10J-2 and BeL7 in Matsue (2016) and Fukagawa (2017) locations, respectively. The difference in rainfall that changed at all study locations each year might be another possible reason (Table 1). As the nodule occupancy of BeL7 increased in Matsue and Miyazaki soils in 2017, but not in Fukagawa soil in Fukagawa location, BeL7 in the different soils might have different properties for competitive relationship with the coexisting Bj strains.

2.5. Conclusions

Various conditions of soil storage until the next year in the study location might differentiated in environmental conditions even in the same temperature conditions as outdoors. Potentially other environmental factors and their correlation with temperature might also affect the microorganisms. In addition, rainfall might affect the soil conditions between outdoor and indoor storage during the winter. Although the effects could not be verified, the shift of the nodule composition in the second year showed the same tendency in all soils and study locations, suggesting that the effects of the difference in the soil storage conditions did not seem to be serious on the composition of rhizobia. Fluctuating rainfall over two successive years in the study locations (Table 1) also suggests that difference in rainfall would not significantly affect the nodule composition.

By the novel methodology used in this study, we could assume that *B. japonicum* (Bj11-1) dominantly proliferated in Fukagawa soil and led to their dominant nodule composition, and that both *B. japonicum* (BjS10J-2) and *B. elkanii* (BeL7) existed in Miyazaki soil and the dominant nodule composition of *B. elkanii* (BeL7) was due to their temperature-dependent infection.

Table S1. Soil property of the study sites (Puri et al., 2018).

Location	**Soil type**[a]	**pH**	**NH_4-N (mg/kg)**	**P_2O_5 (mg/kg)**	**K_2O (mg/kg)**	**Total C (g/kg)**
Fukagawa	Andisol	6.0	16	472	369	5.2
Matsue	Inceptisol	6.2	12	288	86	1.2
Miyazaki	Andisol	6.4	22	160	220	4.4

[a] Based on USDA classification

Table S2. Ingredients of yeast mannitol agar (YMA) media.

Ingredients	**Amount (L^{-1})**
Mannitol	10.0g
K_2HPO_4	0.5g
Yeast extract	.04g
$Mg_2SO_4.7H_2O$	.02g
NaCl	0.1g
$CaCO_3$	0.01g
Agar*	15.0g
pH (adjusted)	7.0

*without Agar denoted as liquid YM media (Chapter 3: liquid culture experiment).

Source: Vincent, 1970.

Table S3. PCR ingredients for amplification of 16S rRNA and 16S-23S rRNA ITS region and cycle sequencing.

16S-23S rRNA ITS region		Cycle sequencing	
Ingredients	**Amount (µL)**	**Ingredients**	**Amount (µL)**
Reaction buffer(10X) (GENETBIO)	1.0	Big dye sequencing buffer v.1.1 (5X) Applied biosystem	1.75
dNTPs mixture (2.5mM) (GENETBIO)	0.25	-	-
forward primer (12.5µM) [a]	0.4	forward primer (3.5µM) [a]	0.5
reverse primer (12.5µM) [a]	0.4	-	-
Taq DNA polymerase (GENETBIO)	0.25	Big dye terminator v.1.1 (2.5X) Applied biosystem	0.5
DNA template/culture	[b]	DNA template	0.5
MilliQ water	7.7	MilliQ water	6.75
Total	10		10

[a] fD1 and rP2 (Weisburg et al., 1991) and 1512F and 23R (Hirashi et al., 1997) for 16S rRNA and 16S-23S ITS, respectively.

[b] A small amount of colony was directly used as template.

Table S4. PCR running conditions.

Reaction	Gene amplification	Cycle sequence
Pre-run	94°C, 3 min	96°C, 2 min
Denaturation	94°C, 30 sec	96°C, 10 sec
Annealing	50°C, 30 sec	50°C, 5 sec
Extension	72°C, 1 min	60°C, 4 min
Cycle number	30	30
Final run	72°C, 5 min	72°C, 5 min

Table S5. Primer used for gene amplification in this experiment.

Gene	Primer name	Sequence (5′ → 3′)	Reference
16S rRNA	Fwd: fd1	AGAGTTTGATCCTGGCTCAG	Weisburg et al., 1991
	Rev: rp2	ACGGCTACCTTGTTACGACTT	
16S-23R rRNA ITS region	Fwd: 1512f	GTCGTAACAAGGTAGCCGT	Hirashi et al., 1997
	Rev: 23R	TGCCAAGGCATCCACC	

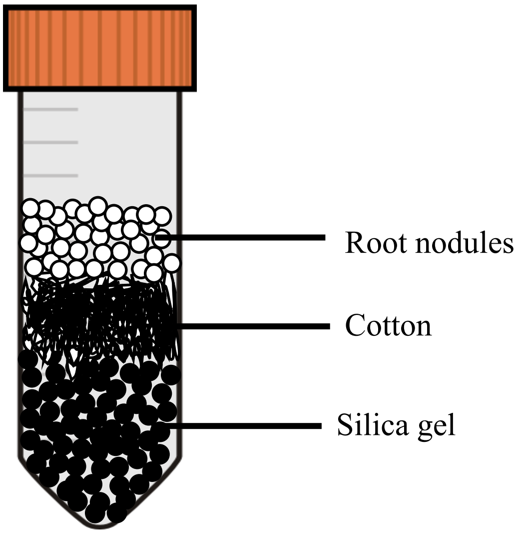


Figure S1. Preservation of soybean root nodules in a vial containing silica gel and cotton.

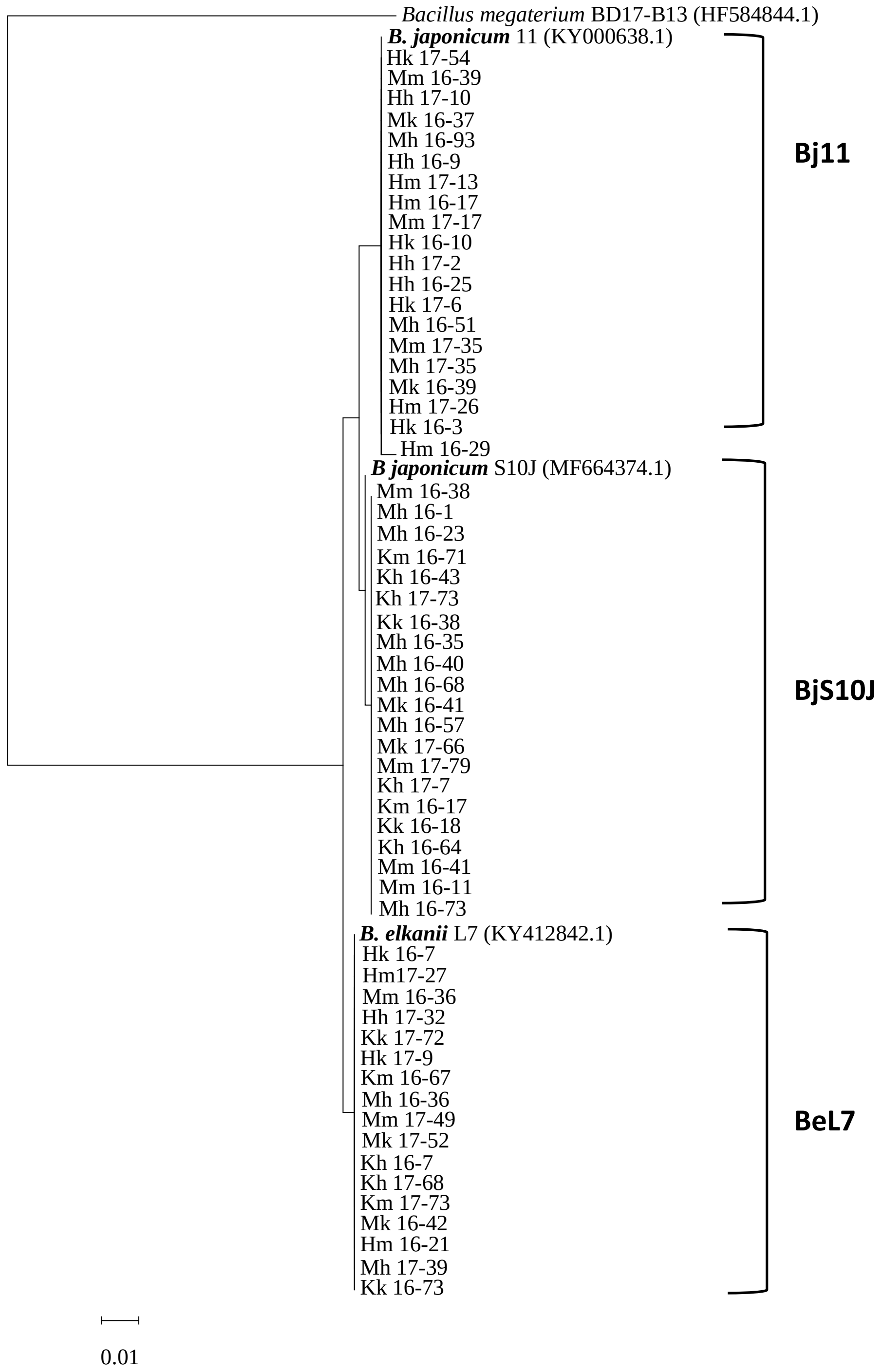


Figure S2. Phylogenetic tree of the 16S rRNA gene regions of the soybean rhizobial strains isolated in this study with reference strains. The isolates were designated by the soil [Fukagawa (H), Matsue (M) and Miyazaki (K)], the study location (h, m, and k), year of the cultivation, and the strain number. The scale bar indicates the number of substitutions per site.

Appendix 1. Closest candidate of rhizobial strain based on 16S rRNA gene sequence from soybean nodules in 2016 by BLAST search in NCBI database.

Appendix 1.1. In Fukagawa soil and Fukagawa location.

Replication	Nodule number	Morphology	Growth status	Isolate for sequence	Closest candidate	Accession number
1	1	R-TL-W	Slow	3	*B. japonicum* strain Bj11	KY000638.1
				4	*B. japonicum* strain Bj11	KY000638.1
	2	R-TL-W	Slow	5	*B. japonicum* strain Bj11	KY000638.1
				6	*B. japonicum* strain Bj11	KY000638.1
	3	R-TL-W	Slow	7	*B. japonicum* strain Bj11	KY000638.1
				8	*B. japonicum* strain Bj11	KY000638.1
	4	R-TL-W	Slow	9	*B. japonicum* strain Bj11	KY000638.1
				10	*B. japonicum* strain Bj11	KY000638.1
	5	R-TL-W	Slow	11	*B. japonicum* strain Bj11	KY000638.1
				12	*B. japonicum* strain Bj11	KY000638.1
	6	R-TL-W	Slow	13	*B. japonicum* strain Bj11	KY000638.1
				14	*B. japonicum* strain Bj11	KY000638.1
	7	R-TL-W	Slow	17	*B. japonicum* strain Bj11	KY000638.1
				18	*B. japonicum* strain Bj11	KY000638.1
	8	R-TL-W	Slow	19	*B. japonicum* strain Bj11	KY000638.1
				20	*B. japonicum* strain Bj11	KY000638.1
	9	R-TL-W	Slow	21	*B. japonicum* strain Bj11	KY000638.1
				22	*B. japonicum* strain Bj11	KY000638.1
	10	R-TL-W	Slow	25	*B. japonicum* strain Bj11	KY000638.1
				26	*B. japonicum* strain Bj11	KY000638.1
2	1	R-TL-W	Slow	27	*B. japonicum* strain Bj11	KY000638.1
				28	*B. japonicum* strain Bj11	KY000638.1
	2	R-TL-W	Slow	29	*B. japonicum* strain Bj11	KY000638.1
				30	*B. japonicum* strain Bj11	KY000638.1
		R-TL-W	Slow	31	*B. japonicum* strain Bj11	KY000638.1
	3	R-TL-W	Slow	32	*B. japonicum* strain Bj11	KY000638.1
				33	*B. japonicum* strain Bj11	KY000638.1
	4	R-TL-W	Slow	34	*B. japonicum* strain Bj11	KY000638.1
				35	*B. japonicum* strain Bj11	KY000638.1
	5	R-TL-W	Slow	36	*B. japonicum* strain Bj11	KY000638.1
				37	*B. japonicum* strain Bj11	KY000638.1
	6	R-TL-W	Slow	38	*B. japonicum* strain Bj11	KY000638.1
				39	*B. japonicum* strain Bj11	KY000638.1
	7	R-TL-W	Slow	42	*B. japonicum* strain Bj11	KY000638.1
				43	*B. japonicum* strain Bj11	KY000638.1
	8	R-TL-W	Slow	44	*B. japonicum* strain Bj11	KY000638.1
				45	*B. japonicum* strain Bj11	KY000638.1
		R-TL-W	Slow	46	*B. japonicum* strain Bj11	KY000638.1
				47	*B. japonicum* strain Bj11	KY000638.1
	9	R-TL-W	Slow	48	*B. japonicum* strain Bj11	KY000638.1
				49	*B. japonicum* strain Bj11	KY000638.1
	10	R-TL-W	Slow	50	*B. japonicum* strain Bj11	KY000638.1
				51	*B. japonicum* strain Bj11	KY000638.1

Note: R=Round, TL=Translucent, and W=White.

Appendix 1.1. (Cont’d) In Fukagawa soil and Fukagawa location.

Replication	Nodule number	Morphology	Growth status	Isolate for sequence	Closest candidate	Accession number
3	1	R-TL-W	Slow	52	*B. japonicum* strain Bj11	KY000638.1
				53	*B. japonicum* strain Bj11	KY000638.1
	2	R-TL-W	Slow	54	*B. japonicum* strain Bj11	KY000638.1
				55	*B. japonicum* strain Bj11	KY000638.1
	3	R-TL-W	Slow	58	*B. japonicum* strain Bj11	KY000638.1
				59	*B. japonicum* strain Bj11	KY000638.1
	4	R-TL-W	Slow	60	*B. japonicum* strain Bj11	KY000638.1
				61	*B. japonicum* strain Bj11	KY000638.1
	5	R-TL-W	Slow	62	*B. japonicum* strain Bj11	KY000638.1
				63	*B. japonicum* strain Bj11	KY000638.1
	6	R-TL-W	Slow	64	*B. japonicum* strain Bj11	KY000638.1
				65	*B. japonicum* strain Bj11	KY000638.1
	7	R-TL-W	Slow	66	*B. japonicum* strain Bj11	KY000638.1
				67	*B. japonicum* strain Bj11	KY000638.1
	8	R-TL-W	Slow	68	*B. japonicum* strain Bj11	KY000638.1
				69	*B. japonicum* strain Bj11	KY000638.1
	9	R-T-W	Slow	70	*B. japonicum* strain Bj11	KY000638.1
				71	*B. japonicum* strain Bj11	KY000638.1
	10	R-TL-W	Slow	72	*B. japonicum* strain Bj11	KY000638.1
				73	*B. japonicum* strain Bj11	KY000638.1

Note: R=Round, TL=Translucent, and W=White.

Appendix 1.2. In Fukagawa soil and Matsue location.

Replication	Nodule number	Morphology	Growth status	Isolate for sequence	Closest candidate	Accession number
1	1	R-TL-W	Slow	1	*B. japonicum* strain Bj12	KY000639.1
				2	*B. japonicum* strain Bj12	KY000639.1
	2	R-TL-W	Slow	3	*B elkanii* strain SK-2	LC386884.1
				4	*B elkanii* strain SK-2	LC386884.1
	3	R-TL-W	Slow	5	*B. japonicum* strain Bj12	KY000639.1
				6	*B. japonicum* strain Bj11	KY000638.1
			Slow*	9	*B elkanii* strain SK-2	LC386884.1
				10	*B elkanii* strain SK-2	LC386884.1
	4	R-TL-W	Slow	11	*B. japonicum* strain Bj11	KY000638.1
				12	*B. japonicum* strain Bj11	KY000638.1
			Slow*	13	*B. japonicum* strain SEMIA 5080	AF234889.2
				14	*B. japonicum* strain SEMIA 5080	AF234889.2
	5	R-TL-W	Slow	15	*B. japonicum* strain Bj11	KY000638.1
				16	*B. japonicum* strain Bj11	KY000638.1
	6	R-TL-W	Slow	17	*B. japonicum* strain Bj11	KY000638.1
				18	*B. japonicum* strain Bj11	KY000638.1
			Slow*	21	*B elkanii* strain SK-2	LC386884.1
				22	*B elkanii* strain SK-2	LC386884.1
	7	R-TL-W	Slow	23	*B. japonicum* strain Bj11	KY000638.1
				24	*B. japonicum* strain Bj11	KY000638.1
	8	R-TL-W	Slow	25	*B. japonicum* strain Bj11	KY000638.1
				26	*B. japonicum* strain Bj11	KY000638.1
	9	R-TL-W	Slow	27	*B. japonicum* strain Bj11	KY000638.1
				28	*B. japonicum* strain Bj11	KY000638.1
			Slow*	31	*B elkanii* strain SK-2	LC386884.1
				32	*B elkanii* strain SK-2	LC386884.1
	10	R-TL-W	Slow	33	*B. japonicum* strain Bj11	KY000638.1
				34	*B. japonicum* strain Bj11	KY000638.1

Note:

- R=Round, TL=Translucent, and W=White.
- * Co-nodulation *of B. elkanii* with *B. japonicum* and less than 6 colonies of *B. elkanii* were on the plate produce relatively larger polysaccharide than *B. japonicum.*

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Appendix 1.2. (Cont'd) In Fukagawa soil and Matsue location.

Replication	Nodule number	Morphology	Growth status	Isolate for sequence	Closest candidate	Accession number
2	1	R-TL-W	Slow	35	*B. japonicum* strain Bj11	KY000638.1
				36	*B. japonicum* strain Bj11	KY000638.1
	2	R-TL-W	Slow	37	*B. japonicum* strain Bj11	KY000638.1
				38	*B. japonicum* strain Bj11	KY000638.1
	3	R-TL-W	Slow	41	*B. japonicum* strain Bj11	KY000638.1
				42	*B. japonicum* strain Bj11	KY000638.1
	4	R-TL-W	Slow	43	*B. japonicum* strain Bj11	KY000638.1
				44	*B. japonicum* strain Bj11	KY000638.1
	5	R-TL-W	Slow	47	*B. japonicum* strain Bj11	KY000638.1
				48	*B. japonicum* strain Bj11	KY000638.1
	6	R-TL-W	Slow	49	*B. japonicum* strain Bj11	KY000638.1
				50	*B. japonicum* strain Bj11	KY000638.1
	7	R-TL-W	Slow	53	*B. japonicum* strain Bj11	KY000638.1
				54	*B. japonicum* strain Bj11	KY000638.1
	8	R-TL-W	Slow	55	*B. japonicum* strain Bj11	KY000638.1
				56	*B. japonicum* strain Bj11	KY000638.1
	9	R-TL-W	Early grower	57	*B. japonicum* strain Bj11	KY000638.1
				58	*B. japonicum* strain Bj11	KY000638.1
	10	R-TL-W	Slow	59	*B. japonicum* strain Bj11	KY000638.1
				60	*B. japonicum* strain Bj11	KY000638.1
3	1	R-TL-W	Slow	61	*B. japonicum* strain Bj11	KY000638.1
				62	*B. japonicum* strain Bj11	KY000638.1
	2	R-TL-W	Slow	63	*B. japonicum* strain Bj11	KY000638.1
				64	*B. japonicum* strain Bj11	KY000638.1
	3	R-TL-W	Slow	65	*B. japonicum* strain Bj11	KY000638.1
				66	*B. japonicum* strain Bj11	KY000638.1
	4	R-TL-W	Early grower	67	*B. japonicum* strain Bj11	KY000638.1
				68	*B. japonicum* strain Bj11	KY000638.1
	5	R-TL-W	Slow	71	*B. japonicum* strain Bj11	KY000638.1
				72	*B. japonicum* strain Bj11	KY000638.1
	6	R-TL-W	Slow	73	*B. japonicum* strain Bj11	KY000638.1
				74	*B. japonicum* strain Bj11	KY000638.1
	7	R-TL-W	Slow	75	*B. japonicum* strain Bj11	KY000638.1
				76	*B. japonicum* strain Bj11	KY000638.1
	8	R-TL-W	Slow	77	*B. japonicum* strain Bj11	KY000638.1
				78	*B. japonicum* strain Bj11	KY000638.1
	9	R-TL-W	Slow	79	*B. japonicum* strain Bj11	KY000638.1
				80	*B. japonicum* strain Bj11	KY000638.1
	10	R-TL-W	Slow	81	*B. japonicum* strain Bj11	KY000638.1
				82	*B. japonicum* strain Bj11	KY000638.1

Note:

- R=Round, TL=Translucent, and W=White.
- The type strain *B. japonicum* Bj11, *B. japonicum* Bj12, *B. japonicum* BjSEMIA *5080* are identical to each other's within the study length.

Appendix 1.3. In Fukagawa soil and Miyazaki location.

Replication	Nodule number	Morphology	Growth status	Isolate for sequence	Closest candidate	Accession number
1	1	R-TL-W	Slow	3	*B. japonicum* strain Bj11	KY000638.1
				4	*B. japonicum* strain Bj11	KY000638.1
	2	R-TL-W	Slow	5	*B. japonicum* strain Bj11	KY000638.1
				6	*B. japonicum* strain Bj11	KY000638.1
	3	R-TL-W	Slow	7	*B. elkanii* strain L7	KY412842.1
				8	*B. elkanii* strain L7	KY412842.1
	4	R-TL-W	Slow	9	*B. japonicum* strain Bj11	KY000638.1
				10	*B. japonicum* strain Bj11	KY000638.1
	5	R-TL-W	Slow	11	*B. japonicum* strain Bj11	KY000638.1
				12	*B. japonicum* strain Bj11	KY000638.1
	6	R-TL-W	Slow	17	*B. japonicum* strain Bj11	KY000638.1
				18	*B. japonicum* strain Bj11	KY000638.1
	7	R-TL-W	Slow	23	*B. japonicum* strain Bj11	KY000638.1
				24	*B. japonicum* strain Bj11	KY000638.1
	8	R-TL-W	Slow	28	*B. japonicum* strain Bj11	KY000638.1
				29	*B. japonicum* strain Bj11	KY000638.1
	9	R-TL-W	Slow	33	*B. japonicum* strain Bj11	KY000638.1
				36	*B. japonicum* strain Bj11	KY000638.1
	10	R-TL-W	Slow	39	*B. japonicum* strain Bj11	KY000638.1
				40	*B. japonicum* strain Bj11	KY000638.1
2	1	R-TL-W	Slow	13	*B. japonicum* strain Bj11	KY000638.1
				14	*B. japonicum* strain Bj11	KY000638.1
	2	R-TL-W	Slow	15	*B. japonicum* strain Bj11	KY000638.1
				16	*B. japonicum* strain Bj11	KY000638.1
	3	R-TL-W	Slow	17	*B. elkanii* strain L7	KY412842.1
				18	*B. elkanii* strain L7	KY412842.1
	4	R-TL-W	Slow	19	*B. japonicum* strain Bj11	KY000638.1
				20	*B. japonicum* strain Bj11	KY000638.1
	5	R-TL-W	Slow	24	*B. japonicum* strain Bj12	KY000639.1
				25	*B. japonicum* strain Bj11	KY000638.1
	6	R-TL-W	Slow	27	*B. japonicum* strain Bj12	KY000639.1
				28	*B. japonicum* strain Bj11	KY000638.1
	7	R-TL-W	Slow	31	*B. japonicum* strain Bj12	KY000639.1
				32	*B. japonicum* strain Bj11	KY000638.1
	8	R-TL-W	Slow	33	*B. japonicum* strain Bj11	KY000638.1
				34	*B. japonicum* strain Bj11	KY000638.1
	9	R-TL-W	Slow	37	*B. elkanii* strain L7	KY412842.1
				38	*B. elkanii* strain L7	KY412842.1
	10	R-TL-W	Slow	39	*B. japonicum* strain Bj12	KY000639.1
				40	*B. japonicum* strain Bj11	KY000638.1

Note: R=Round, TL=Translucent, and W=White.

Appendix 1.3. (Cont'd) In Fukagawa soil and Miyazaki location.

Replication	Nodule number	Morphology	Growth status	Isolate for sequence	Closest candidate	Accession number
3	1	R-TL-W	Slow	24	*B. japonicum* strain Bj11	KY000638.1
				25	*B. japonicum* strain Bj11	KY000638.1
	2	R-TL-W	Slow	33	*B. japonicum* strain Bj11	KY000638.1
				34	*B. japonicum* strain Bj11	KY000638.1
	3	R-TL-W	Slow	35	*B. japonicum* strain Bj11	KY000638.1
				36	*B. japonicum* strain Bj11	KY000638.1
	4	R-TL-W	Slow	37	*B. japonicum* strain Bj11	KY000638.1
				38	*B. japonicum* strain Bj11	KY000638.1
	5	R-TL-W	Slow	55	*B. japonicum* strain Bj11	KY000638.1
				58	*B. japonicum* strain Bj11	KY000638.1
	6	R-TL-W	Slow	63	*B. japonicum* strain Bj12	KY000639.1
				64	*B. japonicum* strain Bj11	KY000638.1
	7	R-TL-W	Slow	65	*B. japonicum* strain Bj11	KY000638.1
				66	*B. japonicum* strain Bj11	KY000638.1
	8	R-TL-W	Slow	67	*B. japonicum* strain Bj11	KY000638.1
				68	*B. japonicum* strain Bj11	KY000638.1
	9	R-TL-W	Slow	69	*B. japonicum* strain Bj11	KY000638.1
				70	*B. japonicum* strain Bj11	KY000638.1
	10	R-TL-W	Slow	73	*B. japonicum* strain Bj12	KY000639.1
				74	*B. japonicum* strain Bj12	KY000639.1

Note:

- R=Round, TL=Translucent, and W=White.
- The type strain *B. japonicum* Bj11 and *B. japonicum* Bj12 are identical to each other's within the study length.

Appendix 1.4. In Matsue soil and Fukagawa location.

Replication	Nodules number	Morphology	Growth status	Isolates for sequence	Closest candidate	Accession number
1	1	R-TL-W	Slow	1	*B. japonicum* strain S10J	MF664374.1
				2	*B. japonicum* strain S10J	MF664374.1
	2	R-TL-W	Slow	7	*B. elkanii* strain L7	KY412842.1
				8	*B. elkanii* strain L7	KY412842.1
	3	R-TL-W	Slow	9	*B. japonicum* strain S10J	MF664374.1
				10	*B. japonicum* strain S10J	MF664374.1
	4	R-TL-W	Slow	33	*B. elkanii* strain L7	KY412842.1
				34	*B. elkanii* strain L7	KY412842.1
	5	R-TL-W	Slow	33	*B. elkanii* strain L7	KY412842.1
				34	*B. elkanii* strain L7	KY412842.1
	6	R-TL-W	Slow	33	*B. elkanii* strain L7	KY412842.1
				34	*B. elkanii* strain L7	KY412842.1
	7	R-TL-W	Slow	22	*B. japonicum* strain S10J	MF664374.1
				23	*B. japonicum* strain S10J	MF664374.1
	8	R-TL-W	Slow	35	*B. elkanii* strain L7	KY412842.1
				36	*B. elkanii* strain L7	KY412842.1
	9	R-TL-W	Slow	37	*B. elkanii* strain L7	KY412842.1
				38	*B. elkanii* strain L7	KY412842.1
	10	R-TL-W	Slow	39	*B. elkanii* strain L7	KY412842.1
				40	*B. elkanii* strain L7	KY412842.1
2	1	R-TR-W	Slow	35	*B. japonicum* strain S10J	MF664374.1
				36	*B. japonicum* strain S10J	MF664374.1
	2	R-TR-W	Slow	39	*B. elkanii* strain L7	KY412842.1
				40	*B. elkanii* strain L7	KY412842.1
	3	R-TR-W	Slow	41	*B. elkanii* strain L7	KY412842.1
				42	*B. elkanii* strain L7	KY412842.1
	4	R-TL-W	Slow	43	*B. elkanii* strain L7	KY412842.1
				44	*B. elkanii* strain L7	KY412842.1
	5	R-TL-W	Slow	45	*B. japonicum* strain S10J	MF664374.1
				46	*B. japonicum* strain S10J	MF664374.1
		R-TL-W	Slow	47	*B. japonicum* strain S10J	MF664374.1
				48	*B. japonicum* strain S10J	MF664374.1
	6	R-TL-W	Slow	51	*B. japonicum* strain S10J	MF664374.1
				52	*B. japonicum* strain S10J	MF664374.1
	7	R-TL-W	Slow	55	*B. japonicum* strain S10J	MF664374.1
				56	*B. japonicum* strain S10J	MF664374.1
	8	R-TL-W	Slow	59	*B. japonicum* strain S10J	MF664374.1
				60	*B. japonicum* strain S10J	MF664374.1
	9	R-TL-W	Slow	63	*B. japonicum* strain S10J	MF664374.1
				64	*B. japonicum* strain S10J	MF664374.1
	10	R-TL-W	Early grower	67	*B. elkanii* strain L7	KY412842.1
				68	*B. elkanii* strain L7	KY412842.1

Note: R=Round, TL=Translucent, TR=Transparent, and W=White.

Appendix 1.4. (Cont'd) In Matsue soil and Fukagawa location.

Replication	Nodule number	Morphology	Growth status	Isolates for sequence	Closest candidate	Accession number
3	1	R-TL-W	Slow	69	*B. japonicum* strain Bj11	KY000638.1
				70	*B. japonicum* strain Bj11	KY000638.1
		R-TL-W	Slow	73	*B. japonicum* strain S10J	MF664374.1
				74	*B. japonicum* strain S10J	MF664374.1
	2	R-TL-W	Slow	75	*B. japonicum* strain Bj11	KY000638.1
				76	*B. japonicum* strain Bj11	KY000638.1
	3	R-TL-W	Slow	79	*B. japonicum* strain S10J	MF664374.1
				80	*B. japonicum* strain S10J	MF664374.1
	4	R-TL-W	Slow	83	*B. japonicum* strain Bj11	KY000638.1
				84	*B. japonicum* strain Bj11	KY000638.1
		R-TL-W	Slow	87	*B. japonicum* strain S10J	MF664374.1
				88	*B. japonicum* strain S10J	MF664374.1
	5	R-TL-W	Slow	89	*B. elkanii* strain L7	KY412842.1
				90	*B. elkanii* strain L7	KY412842.1
	6	R-TL-W	Slow	91	*B. elkanii* strain L7	KY412842.1
				92	*B. elkanii* strain L7	KY412842.1
	7	R-TL-W	Slow	93	*B. elkanii* strain SK-2	LC386884.1
				94	*B. elkanii* strain SK-2	LC386884.1
	8	R-TL-W	Slow	95	*B. elkanii* strain TSBF1366	KC283158.1
				96	*B. elkanii* strain TSBF1366	KC283158.1
	9	R-TL-W	Slow	97	*B. japonicum* strain Bj11	KY000638.1
				98	*B. japonicum* strain Bj11	KY000638.1
	10	R-TL-W	Slow	101	*B. elkanii* strain TSBF1366	KC283158.1
				102	*B. elkanii* strain TSBF1366	KC283158.1

Note:

- R=Round, TL=Translucent, and W=White.
- The type strains *B. elkanii* BeL7, *B. elkanii* BeTSBF 1366 and *B. elkanii Be*SK-2 are identical to each other's within the study length.

Appendix 1.5. In Matsue soil and Matsue location.

Replication	Nodule number	Morphology	Growth status	Isolates for sequence	Closest candidate	Accession number
1	1	R-TL-W	Slow	9	*B. elkanii* strain L7	KY412842.1
				10	*B. elkanii* strain L7	KY412842.1
	2	R-TL-W	Slow	2	*B. elkanii* strain ATCC 49852	NR_114610.1
				3	*B. elkanii* strain ATCC 49852	NR_114610.1
	3	R-TL-W	Slow	4	*B. elkanii* strain L7	KY412842.1
				5	*B. elkanii* strain L7	KY412842.1
	4	R-TL-W	Slow	6	*B. elkanii* strain L7	KY412842.1
				7	*B. elkanii* strain L7	KY412842.1
	5	R-TL-W	Slow	11	*B. japonicum* strain S10J	MF664374.1
				12	*B. japonicum* strain S10J	MF664374.1
		R-TL-W	Slow	13	*B. japonicum* strain S10J	MF664374.1
				14	*B. japonicum* strain S10J	MF664374.1
	6	R-TL-W	Slow	17	*B. elkanii* strain L7	KY412842.1
				18	*B. elkanii* strain L7	KY412842.1
	7	R-TL-W	Slow	19	*B. elkanii* strain SK-2	LC386884.1
				20	*B. elkanii* strain SK-2	LC386884.1
		R-TL-W	Slow	21	*B. japonicum* strain S10J	MF664374.1
				22	*B. japonicum* strain S10J	MF664374.1
	8	R-TL-W	Early grower	23	*B. elkanii* strain L7	KY412842.1
				24	*B. elkanii* strain L7	KY412842.1
	9	R-TL-W	Slow	29	*B. elkanii* strain SK-2	LC386884.1
				30	*B. elkanii* strain SK-2	LC386884.1
	10	R-TL-W	Early grower	31	*B. elkanii* strain L7	KY412842.1
				32	*B. elkanii* strain L7	KY412842.1
2	1	R-TL-W	Slow	31	*B. elkanii* strain L7	KY412842.1
				32	*B. elkanii* strain L7	KY412842.1
	2	R-TL-W	Slow	33	*B. japonicum* strain S10J	MF664374.1
				34	*B. japonicum* strain S10J	MF664374.1
	3	R-TL-W	Slow	35	*B. japonicum* strain Bj11	KY000638.1
				36	*B. japonicum* strain Bj11	KY000638.1
	4	R-TL-W	Slow	37	*B. japonicum* strain Bj11	KY000638.1
				38	*B. japonicum* strain Bj11	KY000638.1
	5	R-TL-W	Slow	39	*B. elkanii* strain L7	KY412842.1
				40	*B. elkanii* strain L7	KY412842.1
	6	R-TL-W	Slow	41	*B. elkanii* strain SK-2	LC386884.1
				42	*B. elkanii* strain SK-2	LC386884.1
	7	R-TL-W	Slow	43	*B. elkanii* strain SK-2	LC386884.1
				44	*B. elkanii* strain SK-2	LC386884.1
	8	R-TL-W	Slow	45	*B. japonicum* strain Bj11	KY000638.1
				46	*B. japonicum* strain Bj11	KY000638.1
	9	R-TL-W	Slow	47	*B. japonicum* strain S10J	MF664374.1
				48	*B. japonicum* strain S10J	MF664374.1
	10	R-TL-W	Slow	51	*B. elkanii* strain SK-2	LC386884.1
				52	*B. elkanii* strain SK-2	LC386884.1

Note: R=Round, TL=Translucent, and W=White.

Appendix 1.5. (Cont'd) In Matsue soil and Matsue location.

Replication	Nodule number	Morphology	Growth status	Isolates for sequence	Closest candidate	Accession number
3	1	R-TL-W	Slow	53	*B. elkanii* strain SK-2	LC386884.1
				54	*B. elkanii* strain SK-2	LC386884.1
	2	R-TL-W	Slow	55	*B. elkanii* strain L7	KY412842.1
				56	*B. elkanii* strain L7	KY412842.1
	3	R-TL-W	Slow	58	*B. elkanii* strain L7	KY412842.1
				59	*B. elkanii* strain L7	KY412842.1
	4	R-TL-W	Slow	62	*B. elkanii* strain L7	KY412842.1
				63	*B. elkanii* strain L7	KY412842.1
	5	R-TL-W	Slow	64	*B. elkanii* strain L7	KY412842.1
				65	*B. elkanii* strain L7	KY412842.1
	6	R-TL-W	Slow	66	*B. japonicum* strain Bj11	KY000638.1
				67	*B. japonicum* strain Bj11	KY000638.1
	7	R-TL-W	Slow	68	*B. elkanii* strain L7	KY412842.1
				69	*B. elkanii* strain L7	KY412842.1
	8	R-TL-W	Slow	70	*B. japonicum* strain Bj11	KY000638.1
				71	*B. japonicum* strain Bj11	KY000638.1
	9	R-TL-W	Slow	72	*B. elkanii* strain SK-2	LC386884.1
				73	*B. elkanii* strain SK-2	LC386884.1
	10	R-TL-W	Slow	74	*B. japonicum* strain Bj11	KY000638.1
				75	*B. japonicum* strain Bj11	KY000638.1

Note:

- R=Round, TL=Translucent, and W=White.
- the type strain *B. elkanii* BeL7, *B. elkanii* BeATCC 49852 and *B. elkanii* BeSK-2 are identical to each other's within the study length.

Appendix 1.6. In Matsue soil and Miyazaki location.

Replication	Nodule number	Morphology	Growth status	Isolates for sequence	Closest candidate	Accession number
1	1	R-TL-W	Slow	1	*B. japonicum* strain Bj11	KY000638.1
				2	*B. japonicum* strain Bj11	KY000638.1
		R-TL-W	Slow*	3	*B. elkanii* strain L7	KY412842.1
				4	*B. elkanii* strain L7	KY412842.1
	2	R-TL-W	Slow*	5	*B. japonicum* strain Bj11	KY000638.1
				6	*B. japonicum* strain Bj11	KY000638.1
		R-TL-W	Slow	9	*B. japonicum* strain Bj11	KY000638.1
				10	*B. japonicum* strain Bj11	KY000638.1
	3	R-TL-W	Slow*	11	*B. elkanii* strain L7	KY412842.1
				12	*B. elkanii* strain L7	KY412842.1
		R-TL-W	Slow	13	*B. japonicum* strain Bj11	KY000638.1
				14	*B. japonicum* strain Bj11	KY000638.1
	4	R-TL-W	Slow*	15	*B. elkanii* strain L7	KY412842.1
				16	*B. elkanii* strain L7	KY412842.1
		R-TL-W	Slow	17	*B. japonicum* strain Bj11	KY000638.1
				18	*B. japonicum* strain Bj11	KY000638.1
	5	R-TR-W	Slow	21	*B. japonicum* strain Bj11	KY000638.1
				22	*B. japonicum* strain Bj11	KY000638.1
	6	R-TL-W	Slow*	23	*B. elkanii* strain L7	KY412842.1
				24	*B. elkanii* strain L7	KY412842.1
		R-TL-W	Slow	27	*B. japonicum* strain Bj11	KY000638.1
				28	*B. japonicum* strain Bj11	KY000638.1
	7	R-TL-W	Slow*	29	*B. elkanii* strain L7	KY412842.1
				30	*B. elkanii* strain L7	KY412842.1
		R-TL-W	Slow	35	*B. japonicum* strain Bj11	KY000638.1
				36	*B. japonicum* strain Bj11	KY000638.1
	8	R-TL-W	Slow	37	*B. japonicum* strain Bj11	KY000638.1
				38	*B. japonicum* strain Bj11	KY000638.1
	9	R-TL-W	Slow	39	*B. japonicum* strain Bj11	KY000638.1
				40	*B. japonicum* strain Bj11	KY000638.1
	10	R-TL-W	Slow*	41	*B. elkanii* strain L7	KY412842.1
				42	*B. elkanii* strain L7	KY412842.1
		R-TL-W	Slow	45	*B. japonicum* strain Bj11	KY000638.1
				46	*B. japonicum* strain Bj11	KY000638.1

Note:

- R=Round, TL=Translucent, TR=Transparent, and W=White.
- *Co-nodulation of *B. elkanii* with *B. japonicum* and less than 6 colonies of *B. elkanii* were on the plate produce relatively larger polysaccharide than *B. japonicum.*

Appendix 1.6. (Cont'd) In Matsue soil and Miyazaki location.

Replication	Nodule number	Morphology	Growth status	Isolates for sequence	Closest candidate	Accession number
2	1	R-TL-W	Slow	1	*B. japonicum* strain S10J	MF664374.1
				2	*B. japonicum* strain S10J	MF664374.1
		R-TL-W	Slow	3	*B. japonicum* strain Bj11	KY000638.1
				4	*B. japonicum* strain Bj11	KY000638.1
	2	R-TL-W	Slow	5	*B. elkanii* strain L7	KY412842.1
				6	*B. elkanii* strain L7	KY412842.1
	3	R-TL-W	Slow	9	*B. elkanii* strain L7	KY412842.1
				10	*B. elkanii* strain L7	KY412842.1
	4	R-TL-W	Slow	11	*B. elkanii* strain L7	KY412842.1
				12	*B. elkanii* strain L7	KY412842.1
	5	R-TL-W	Slow	13	*B. elkanii* strain L7	KY412842.1
				14	*B. elkanii* strain L7	KY412842.1
	6	R-TL-W	Slow	15	*B. elkanii* strain L7	KY412842.1
				16	*B. elkanii* strain L7	KY412842.1
	7	R-TL-W	Slow	17	*B. elkanii* strain L7	KY412842.1
				18	*B. elkanii* strain L7	KY412842.1
	8	R-TL-W	Slow	21	*B. japonicum* strain Bj11	KY000638.1
				22	*B. japonicum* strain Bj11	KY000638.1
	9	R-TL-W	Slow	23	*B. elkanii* strain SEMIA 6432	FJ025110.1
				24	*B. elkanii* strain SEMIA 6432	FJ025110.1
	10	R-TL-W	Slow	25	*B. elkanii* strain L7	KY412842.1
				26	*B. elkanii* strain L7	KY412842.1
3	1	R-TL-W	Slow	1	*B. elkanii* strain SEMIA 6432	FJ025110.1
				2	*B. elkanii* strain SEMIA 6432	FJ025110.1
	2	R-TL-W	Slow	5	*B. japonicum* strain Bj11	KY000638.1
				6	*B. japonicum* strain Bj11	KY000638.1
	3	R-TL-W	Slow*	7	*B. elkanii* strain SEMIA 6432	FJ025110.1
				8	*B. elkanii* strain SEMIA 6432	FJ025110.1
		R-TL-W	Slow	9	*B. japonicum* strain Bj11	KY000638.1
				10	*B. japonicum* strain Bj11	KY000638.1
	4	R-TL-W	Early grower	11	*B. elkanii* strain SEMIA 6432	FJ025110.1
				12	*B. elkanii* strain SEMIA 6432	FJ025110.1
		R-TL-W	Slow	15	*B. japonicum* strain Bj11	KY000638.1
				16	*B. japonicum* strain Bj11	KY000638.1
	5	R-TL-W	Slow	17	*B. japonicum* strain Bj11	KY000638.1
				18	*B. japonicum* strain Bj11	KY000638.1
	6	R-TL-W	Early grower	19	*B. elkanii* strain SEMIA 6432	FJ025110.1
				20	*B. elkanii* strain SEMIA 6432	FJ025110.1
	7	R-TL-W	Early grower	25	*B. elkanii* strain SEMIA 6432	FJ025110.1
				26	*B. elkanii* strain SEMIA 6432	FJ025110.1
	8	R-TL-W	Slow	33	*B. elkanii* strain SEMIA 6432	FJ025110.1
				34	*B. elkanii* strain SEMIA 6432	FJ025110.1
	9	R-TL-W	Slow	35	*B. elkanii* strain L7	KY412842.1
				36	*B. elkanii* strain L7	KY412842.1
	10	R-TL-W	Slow	37	*B. elkanii* strain L7	KY412842.1
				38	*B. elkanii* strain L7	KY412842.1

Note:

- R=Round, TL=Translucent, and W=White, The type strain *B. elkanii* BeL7 and *B. elkanii* Be SEMIA 6432 are identical to each other's within the study length.
- *Co-nodulation of *B. elkanii* with *B. japonicum* and less than 5 colonies of *B. elkanii* were on the plate produce relatively larger polysaccharide than *B. japonicum.*

Appendix 1.7. In Miyazaki soil and Fukagawa location.

Replication	Nodule Number	Morphology	Growth status	Isolate for sequence	Closest candidate	Accession number
1	1	R-TL-W	Slow	1	*B. japonicum* strain S10J	MF664374.1
				2	*B. japonicum* strain S10J	MF664374.1
		R-TL-W	Slow*	3	*B. elkanii* strain L7	KY412842.1
				4	*B. elkanii* strain L7	KY412842.1
	2	R-TL-W	Slow	5	*B. japonicum* strain S10J	MF664374.1
				6	*B. japonicum* strain S10J	MF664374.1
	3	R-TL-W	Slow	7	*B. elkanii* strain L7	KY412842.1
				8	*B. elkanii* strain L7	KY412842.1
	4	R-TL-W	Slow	9	*B. japonicum* strain S10J	MF664374.1
				10	*B. japonicum* strain S10J	MF664374.1
	5	R-TL-W	Slow	11	*B. japonicum* strain S10J	MF664374.1
				12	*B. japonicum* strain S10J	MF664374.1
	6	R-TL-W	Slow	13	*B. japonicum* strain S10J	MF664374.1
				14	*B. japonicum* strain S10J	MF664374.1
	7	R-TL-W	Slow	15	*B. japonicum* strain S10J	MF664374.1
				16	*B. japonicum* strain S10J	MF664374.1
	8	R-TL-W	Slow	17	*B. japonicum* strain S10J	MF664374.1
				18	*B. japonicum* strain S10J	MF664374.1
	9	R-TL-W	Slow	19	*B. japonicum* strain S10J	MF664374.1
				20	*B. japonicum* strain S10J	MF664374.1
	10	R-TL-W	Slow	21	*B. japonicum* strain S10J	MF664374.1
				22	*B. japonicum* strain S10J	MF664374.1
2	1	R-TL-W	Slow	23	*B. elkanii* strain L7	KY412842.1
				26	*B. elkanii* strain L7	KY412842.1
	2	R-TL-W	Slow	27	*B. japonicum* strain S10J	MF664374.1
				28	*B. japonicum* strain S10J	MF664374.1
	3	R-TL-W	Slow	31	*B. japonicum* strain S10J	MF664374.1
				32	*B. japonicum* strain S10J	MF664374.1
	4	R-TL-W	Slow	35	*B. japonicum* strain S10J	MF664374.1
				36	*B. japonicum* strain S10J	MF664374.1
	5	R-TL-W	Slow	39	*B. japonicum* strain S10J	MF664374.1
				40	*B. japonicum* strain S10J	MF664374.1
		R-TL-W	Slow*	41	*B. elkanii* strain L7	KY412842.1
				42	*B. elkanii* strain L7	KY412842.1
	6	R-TL-W	Slow	43	*B. japonicum* strain S10J	MF664374.1
				44	*B. japonicum* strain S10J	MF664374.1
	7	R-TL-W	Slow	47	*B. japonicum* strain S10J	MF664374.1
				48	*B. japonicum* strain S10J	MF664374.1
	8	R-TL-W	Slow	49	*B. japonicum* strain S10J	MF664374.1
				50	*B. japonicum* strain S10J	MF664374.1
	9	R-TL-W	Slow	53	*B. japonicum* strain S10J	MF664374.1
				54	*B. japonicum* strain S10J	MF664374.1
	10	R-TL-W	Slow	57	*B. japonicum* strain S10J	MF664374.1
				58	*B. japonicum* strain S10J	MF664374.1

Note:

- R=Round, TL=Translucent, and W=White.
- *Co-nodulation of *B. elkanii* with *B. japonicum* and less than 7 colonies of *B. elkanii* were on the plate produce relatively larger polysaccharide than *B. japonicum.*

Appendix 1.7. (Cont'd) In Miyazaki soil and Fukagawa location.

Replication	Nodule number	Morphology	Growth status	Isolate for sequence	Closest candidate	Accession number
3	1	R-TL-W	Slow	59	*B. japonicum* strain S10J	MF664374.1
				60	*B. japonicum* strain S10J	MF664374.1
	2	R-TL-W	Slow	61	*B. japonicum* strain S10J	MF664374.1
				62	*B. japonicum* strain S10J	MF664374.1
	3	R-TL-W	Slow	63	*B. japonicum* strain S10J	MF664374.1
				64	*B. japonicum* strain S10J	MF664374.1
	4	R-TL-W	Slow*	71	*B. elkanii* strain L7	KY412842.1
				72	*B. elkanii* strain L7	KY412842.1
		R-TL-W	Slow	73	*B. japonicum* strain S10J	MF664374.1
				74	*B. japonicum* strain S10J	MF664374.1
	5	R-TL-W	Slow	75	*B. japonicum* strain S10J	MF664374.1
				76	*B. japonicum* strain S10J	MF664374.1
	6	R-TL-W	Slow	77	*B. japonicum* strain S10J	MF664374.1
				78	*B. japonicum* strain S10J	MF664374.1
		R-TL-W	Slow*	79	*B. elkanii* strain L7	KY412842.1
				80	*B. elkanii* strain L7	KY412842.1
	7	R-TL-W	Slow	81	*B. elkanii* strain L7	KY412842.1
				82	*B. elkanii* strain L7	KY412842.1
	8	R-TL-W	Slow	83	*B. japonicum* strain S10J	MF664374.1
				84	*B. japonicum* strain S10J	MF664374.1
	9	R-TL-W	Slow	85	*B. elkanii* strain L7	KY412842.1
				86	*B. elkanii* strain L7	KY412842.1
	10	R-TL-W	Slow	87	*B. japonicum* strain S10J	MF664374.1
				88	*B. japonicum* strain S10J	MF664374.1

Note:

- R=Round, TL=Translucent, and W=White.
- *Co-nodulation of *B. elkanii* with *B. japonicum* and less than 4 colonies of *B. elkanii* were on the plate produce relatively larger polysaccharide than *B. japonicum.*

Appendix 1.8. In Miyazaki soil and Matsue location.

Replication	Nodule number	Morphology	Growth status	Isolate for sequence	Closest candidate	Accession number
1	1	R-TL-W	Slow	1	*B. japonicum* strain S10J	MF664374.1
				2	*B. japonicum* strain S10J	MF664374.1
	2	R-TL-W	Slow	5	*B. japonicum* strain S10J	MF664374.1
				6	*B. japonicum* strain S10J	MF664374.1
	3	R-TL-W	Slow	7	*B. japonicum* strain S10J	MF664374.1
				8	*B. japonicum* strain S10J	MF664374.1
	4	R-TL-W	Slow	9	*B. elkanii* strain L7	KY412842.1
				10	*B. elkanii* strain L7	KY412842.1
	5	R-TL-W	Slow	11	*B. japonicum* strain S10J	MF664374.1
				12	*B. japonicum* strain S10J	MF664374.1
	6	R-TL-W	Slow	13	*B. japonicum* strain S10J	MF664374.1
				14	*B. japonicum* strain S10J	MF664374.1
				16	*B. japonicum* strain S10J	MF664374.1
	7	R-TL-W	Slow	17	*B. japonicum* strain S10J	MF664374.1
				18	*B. japonicum* strain S10J	MF664374.1
				20	*B. japonicum* strain S10J	MF664374.1
	8	R-TL-W	Slow	23	*B. japonicum* strain S10J	MF664374.1
				24	*B. japonicum* strain S10J	MF664374.1
	9	R-TL-W	Slow	25	*B. japonicum* strain S10J	MF664374.1
				26	*B. japonicum* strain S10J	MF664374.1
				28	*B. japonicum* strain S10J	MF664374.1
	10	R-TL-W	Slow	29	*B. japonicum* strain S10J	MF664374.1
				30	*B. japonicum* strain S10J	MF664374.1
2	1	R-TR-W	Slow	31	*B. japonicum* strain S10J	MF664374.1
				32	*B. japonicum* strain S10J	MF664374.1
	2	R-TL-W	Slow	33	*B. elkanii* strain L7	KY412842.1
				34	*B. elkanii* strain L7	KY412842.1
	3	R-TL-W	Slow	37	*B. japonicum* strain S10J	MF664374.1
				38	*B. japonicum* strain S10J	MF664374.1
	4	R-TL-W	Slow	39	*B. japonicum* strain S10J	MF664374.1
				40	*B. japonicum* strain S10J	MF664374.1
	5	R-TL-W	Slow	41	*B. elkanii* strain L7	KY412842.1
				42	*B. elkanii* strain L7	KY412842.1
	6	R-TL-W	Slow	45	*B. elkanii* strain L7	KY412842.1
				46	*B. elkanii* strain L7	KY412842.1
	7	R-TL-W	Slow	49	*B. elkanii* strain L7	KY412842.1
				50	*B. elkanii* strain L7	KY412842.1
	8	R-TL-W	Slow	51	*B. elkanii* strain L7	KY412842.1
				52	*B. elkanii* strain L7	KY412842.1
	9	R-TL-W	Slow	53	*B. elkanii* strain L7	KY412842.1
				54	*B. elkanii* strain L7	KY412842.1
	10	R-TL-W	Slow	55	*B. japonicum* strain S10J	MF664374.1
				56	*B. japonicum* strain S10J	MF664374.1

Note: R=Round, TL=Translucent, Tr=Transparent, and W=White.

Appendix 1.8. (Cont'd) In Miyazaki soil and Matsue location.

Replication	Nodule number	Morphology	Growth status	Isolate for sequence	Closest candidate	Accession number
3	1	R-TL-W	Slow	57	*B. japonicum* strain S10J	MF664374.1
				58	*B. japonicum* strain S10J	MF664374.1
	2	R-TL-W	Slow	61	*B. japonicum* strain S10J	MF664374.1
				62	*B. japonicum* strain S10J	MF664374.1
	3	R-TL-W	Slow	63	*B. elkanii* strain L7	KY412842.1
				64	*B. elkanii* strain L7	KY412842.1
	4	R-TL-W	Slow	65	*B. elkanii* strain UFLA05-11	KT694160.1
				66	*B. elkanii* strain UFLA05-11	KT694160.1
	5	R-TL-W	Slow	67	*B. elkanii* strain TSBF1061	KC283148.1
				68	*B. elkanii* strain TSBF1061	KC283148.1
	6	R-TL-W	Slow	69	*B. japonicum* strain S10J	MF664374.1
				70	*B. japonicum* strain S10J	MF664374.1
	7	R-TL-W	Slow	71	*B. japonicum* strain S10J	MF664374.1
				72	*B. japonicum* strain S10J	MF664374.1
	8	R-TL-W	Slow	73	*B. japonicum* strain S10J	MF664374.1
				74	*B. japonicum* strain S10J	MF664374.1
	9	R-TL-W	Slow	75	*B. japonicum* strain S10J	MF664374.1
				76	*B. japonicum* strain S10J	MF664374.1
	10	R-TL-W	Slow	77	*B. japonicum* strain S10J	MF664374.1
				78	*B. japonicum* strain S10J	MF664374.1

Note:

- R=Round, TL=Translucent, and W=White.
- The type strain *B. elkanii* BeUFLA05-11, *B. elkanii* BeTSBF 1061 and *B. elkanii* BeL7 are identical to each other's within the study length.

Appendix 1.9. In Miyazaki soil and Miyazaki location.

Replication	Nodules number	Morphology	Growth status	Isolate for sequence	Closest candidate	Accession number
1	1	R-TL-W	Slow	2	*B. japonicum* strain S10J	MF664374.1
				3	*B. japonicum* strain S10J	MF664374.1
	2	R-TL-W	Slow	5	*B. japonicum* strain S10J	MF664374.1
				6	*B. japonicum* strain S10J	MF664374.1
	3	R-TL-W	Slow	7	*B. elkanii* strain L7	KY412842.1
				12	*B. elkanii* strain L7	KY412842.1
	4	R-TL-W	Slow	13	*B. japonicum* strain S10J	MF664374.1
				15	*B. japonicum* strain S10J	MF664374.1
	5	R-TL-W	Slow	16	*B. japonicum* strain S10J	MF664374.1
				18	*B. japonicum* strain S10J	MF664374.1
	6	R-TL-W	Slow	19	*B. elkanii* strain L7	KY412842.1
				21	*B. elkanii* strain L7	KY412842.1
	7	R-TL-W	Slow	26	*B. elkanii* strain L7	KY412842.1
				27	*B. elkanii* strain L7	KY412842.1
	8	R-TL-W	Slow	22	*B. elkanii* strain L7	KY412842.1
				23	*B. elkanii* strain L7	KY412842.1
	9	R-TL-W	Slow	24	*B. elkanii* strain L7	KY412842.1
				25	*B. elkanii* strain L7	KY412842.1
	10	R-TR-W	Slow	29	*B. elkanii* strain L7	KY412842.1
				30	*B. elkanii* strain L7	KY412842.1
2	1	R-TR-W	Slow	33	*B. elkanii* strain L7	KY412842.1
				34	*B. elkanii* strain L7	KY412842.1
	2	R-TL-W	Slow	37	*B. elkanii* strain L7	KY412842.1
				37	*B. elkanii* strain L7	KY412842.1
	3	R-TL-W	Slow	2	*B. elkanii* strain L7	KY412842.1
				3	*B. elkanii* strain L7	KY412842.1
	4	R-TL-W	Slow	4	*B. elkanii* strain L7	KY412842.1
				5	*B. elkanii* strain L7	KY412842.1
	5	R-TL-W	Slow	7	*B. elkanii* strain L7	KY412842.1
				8	*B. elkanii* strain L7	KY412842.1
	6	R-TL-W	Slow	9	*B. elkanii* strain L7	KY412842.1
				10	*B. elkanii* strain L7	KY412842.1
	7	R-TL-W	Slow	13	*B. elkanii* strain L7	KY412842.1
				14	*B. elkanii* strain L7	KY412842.1
	8	R-TL-W	Slow	16	*B. elkanii* strain L7	KY412842.1
				17	*B. elkanii* strain L7	KY412842.1
	9	R-TL-W	Slow	18	*B. elkanii* strain L7	KY412842.1
				19	*B. elkanii* strain L7	KY412842.1
	10	R-TL-W	Slow	22	*B. elkanii* strain L7	KY412842.1
				23	*B. elkanii* strain L7	KY412842.1

Note: R=Round, TL=Translucent, Tr=Transparent, and W=White.

Appendix 1.9. (Cont'd) In Miyazaki soil and Miyazaki location.

Replication	Nodules number	Morphology	Growth status	Isolates for sequence	Closest candidate	Accession number
3	1	R-TL-W	Slow	27	*B. elkanii* strain L7	KY412842.1
				28	*B. elkanii* strain L7	KY412842.1
	2	R-TL-W	Slow	29	*B. elkanii* strain L7	KY412842.1
				30	*B. elkanii* strain L7	KY412842.1
	3	R-TL-W	Slow	31	*B. elkanii* strain L7	KY412842.1
				32	*B. elkanii* strain L7	KY412842.1
	4	R-TL-W	Slow	34	*B. elkanii* strain L7	KY412842.1
				35	*B. elkanii* strain L7	KY412842.1
	5	R-TL-W	Slow	36	*B. japonicum* strain S10J	MF664374.1
				38	*B. japonicum* strain S10J	MF664374.1
	6	R-TL-W	Slow	42	*B. japonicum* strain S10J	MF664374.1
				43	*B. japonicum* strain S10J	MF664374.1
	7	R-TL-W	Slow	51	*B. japonicum* strain S10J	MF664374.1
				52	*B. japonicum* strain S10J	MF664374.1
	8	R-TL-W	Slow	55	*B. elkanii* strain L7	KY412842.1
				56	*B. elkanii* strain L7	KY412842.1
	9	R-TL-W	Slow	60	*B. elkanii* strain L7	KY412842.1
				61	*B. elkanii* strain L7	KY412842.1
	10	R-TL-W	Slow	62	*B. elkanii* strain L7	KY412842.1
				63	*B. elkanii* strain L7	KY412842.1

Note: R=Round, TL=Translucent, and W=White.

Appendix 2. Closest candidate of rhizobial strain based on 16S rRNA gene sequence from soybean nodules in 2017 by BLAST search in NCBI database.

Appendix 2.1. In Fukagawa soil and Fukagawa location.

Replication	Nodule number	Morphology	Growth tatus	Isolates for sequencing	Closest candidate	Accession number
1	1	R-TL-W	Slow	1	*B. japonicum* strain Bj11	KY000638.1
				2	*B. japonicum* strain Bj11	KY000638.1
	2	R-TL-W	Slow	3	*B. japonicum* strain Bj11	KY000638.1
				4	*B. japonicum* strain Bj11	KY000638.1
	3	R-TL-W	Slow	5	*B. japonicum* strain Bj11	KY000638.1
				6	*B. japonicum* strain Bj11	KY000638.1
	4	R-TL-W	Slow	7	*B. japonicum* strain Bj11	KY000638.1
				8	*B. japonicum* strain Bj11	KY000638.1
	5	R-TL-W	Slow	9	*B. japonicum* strain Bj11	KY000638.1
				10	*B. japonicum* strain Bj11	KY000638.1
	6	R-TL-W	Slow	11	*B. japonicum* strain Bj11	KY000638.1
				12	*B. japonicum* strain Bj11	KY000638.1
	7	R-TL-W	Slow	13	*B. japonicum* strain Bj11	KY000638.1
				14	*B. japonicum* strain Bj11	KY000638.1
	8	R-TL-W	Slow	15	*B. japonicum* strain Bj11	KY000638.1
				16	*B. japonicum* strain Bj11	KY000638.1
	9	R-TL-W	Slow	17	*B. japonicum* strain Bj11	KY000638.1
				18	*B. japonicum* strain Bj11	KY000638.1
	10	R-TL-W	Slow	19	*B. japonicum* strain Bj11	KY000638.1
				20	*B. japonicum* strain Bj11	KY000638.1
2	1	R-TL-W	Slow	21	*B. japonicum* strain Bj11	KY000638.1
				22	*B. japonicum* strain Bj11	KY000638.1
	2	R-TL-W	Slow	23	*B. japonicum* strain Bj11	KY000638.1
				24	*B. japonicum* strain Bj11	KY000638.1
		R-TL-W	Slow	25	*B. elkanii* strain L7	KY412842.1
				26	*B. elkanii* strain L7	KY412842.1
	3	R-TL-W	Slow	27	*B. japonicum* strain Bj11	KY000638.1
				28	*B. japonicum* strain Bj11	KY000638.1
	4	R-TL-W	Slow	29	*B. japonicum* strain Bj11	KY000638.1
				30	*B. japonicum* strain Bj11	KY000638.1
	5	R-TL-W	Slow	31	*B. japonicum* strain Bj12	KY000638.1
				32	*B. japonicum* strain Bj11	KY000638.1
	6	R-TL-W	Slow	33	*B. japonicum* strain Bj11	KY000638.1
				34	*B. japonicum* strain Bj11	KY000638.1
	7	R-TL-W	Slow	35	*B. japonicum* strain Bj12	KY000638.1
				36	*B. japonicum* strain Bj12	KY000638.1
	8	R-TL-W	Slow	37	*B. elkanii* strain TSBF1371	KC283161.1
				38	*B. elkanii* strain L7	KY412842.1
		R-TL-W	Slow	39	*B. japonicum* strain Bj11	KY412842.1
				40	*B. japonicum* strain Bj11	KY000638.1
	9	R-TL-W	Slow	41	*B. japonicum* strain Bj11	KY000638.1
				42	*B. japonicum* strain Bj11	KY000638.1
	10	R-TL-W	Slow	43	*B. japonicum* strain Bj11	KY000638.1
				44	*B. japonicum* strain Bj11	KY000638.1

Note: R=Round, TL=Translucent, and W=White.

Appendix 2.1. (Cont'd) In Fukagawa soil and Fukagawa location.

Replication	Nodule number	Morphology	Growth status	Isolates for sequencing	Closest candidate	Accession number
3	1	R-TL-W	Slow	45	*B. japonicum* strain Bj11	KY000638.1
				46	*B. japonicum* strain Bj11	KY000638.1
	2	R-TL-W	Slow	47	*B. japonicum* strain Bj12	KY000639.1
				48	*B. japonicum* strain Bj11	KY000638.1
	3	R-TL-W	Slow	49	*B. japonicum* strain Bj11	KY000638.1
				50	*B. japonicum* strain Bj11	KY000638.1
	4	R-TL-W	Slow	51	*B. japonicum* strain Bj11	KY000638.1
				52	*B. japonicum* strain Bj11	KY000638.1
	5	R-TL-W	Slow	53	*B. japonicum* strain Bj11	KY000638.1
				54	*B. japonicum* strain Bj11	KY000638.1
	6	R-TL-W	Slow	55	*B. japonicum* strain Bj11	KY000638.1
				56	*B. japonicum* strain Bj11	KY000638.1
	7	R-TL-W	Slow	57	*B. japonicum* strain Bj11	KY000638.1
				58	*B. japonicum* strain Bj11	KY000638.1
	8	R-TL-W	Slow	59	*B. japonicum* strain Bj11	KY000638.1
				60	*B. japonicum* strain Bj11	KY000638.1
	9	R-TL-W	Slow	61	*B. japonicum* strain Bj11	KY000638.1
				62	*B. japonicum* strain Bj11	KY000638.1
	10	R-TL-W	Slow	63	*B. japonicum* strain Bj11	KY000638.1
				64	*B. japonicum* strain Bj11	KY000638.1

Note:

- R=Round, TL=Translucent, and W=White.
- The type strain *B. japonicum* Bj11 and *B. japonicum* Bj12; BeL7 and *B. elkanii* BeTSBF 1371 are identical to each other's within the study length.

Appendix 2.2. In Fukagawa soil and Matsue location.

Replication	Nodule number	Morphology	Growth status	Isolates for sequencing	Closest candidate	Accession number
1	1	R-TL-W	Slow	5	*B. elkanii* strain Cte-504	MH938235.1
				6	*B. elkanii* strain Cte-504	MH938235.1
	2	R-TL-W	Slow	13	*B. japonicum* strain NARS-B10	MF817963.1
				14	*B. japonicum* strain NARS-B10	MF817963.1
	3	R-TL-W	Slow	18	*B. japonicum* strain NARS-B10	MF817963.1
				19	*B. japonicum* strain NARS-B10	MF817963.1
	4	R-TL-W	Slow	20	*B. japonicum* strain NARS-B10	MF817963.1
				21	*B. japonicum* strain NARS-B10	MF817963.1
	5	R-TL-W	Slow	25	*B. japonicum* strain NARS-B10	MF817963.1
				26	*B. japonicum* strain NARS-B10	MF817963.1
	6	R-TL-W	Slow	29	*B. japonicum* strain NARS-B10	MF817963.1
				30	*B. japonicum* strain NARS-B10	MF817963.1
	7	R-TL-W	Slow	31	*B. elkanii* strain Cte-504	MH938235.1
				32	*B. elkanii* strain Cte-504	MH938235.1
	8	R-TL-W	Slow	37	*B. japonicum* strain NARS-B10	MF817963.1
				38	*B. japonicum* strain NARS-B10	MF817963.1
	9	R-TL-W	Slow	39	*B. japonicum* strain NARS-B10	MF817963.1
				40	*B. japonicum* strain NARS-B10	MF817963.1
	10	R-TL-W	Slow	41	*B. japonicum* strain NARS-B10	MF817963.1
				42	*B. japonicum* strain NARS-B10	MF817963.1
2	1	R-TL-W	Slow	43	*B. japonicum* strain NARS-B10	MF817963.1
				44	*B. japonicum* strain NARS-B10	MF817963.1
	2	R-TL-W	Slow	47	*B. elkanii* strain Cte-504	MH938235.1
				48	*B. elkanii* strain Cte-504	MH938235.1
	3	R-TL-W	Slow	49	*B. japonicum* strain NARS-B10	MF817963.1
				50	*B. japonicum* strain NARS-B10	MF817963.1
	4	R-TL-W	Slow	59	*B. japonicum* strain NARS-B10	MF817963.1
				60	*B. japonicum* strain NARS-B10	MF817963.1
	5	R-TL-W	Slow	61	*B. japonicum* strain NARS-B10	MF817963.1
				62	*B. japonicum* strain NARS-B10	MF817963.1
	6	R-TL-W	Slow	63	*B. japonicum* strain NARS-B10	MF817963.1
				64	*B. japonicum* strain NARS-B10	MF817963.1
	7	R-TL-W	Slow	65	*B. japonicum* strain NARS-B10	MF817963.1
				66	*B. japonicum* strain NARS-B10	MF817963.1
	8	R-TL-W	Slow	67	*B. japonicum* strain NARS-B10	MF817963.1
				68	*B. japonicum* strain NARS-B10	MF817963.1
	9	R-TL-W	Slow	69	*B. japonicum* strain NARS-B10	MF817963.1
				70	*B. japonicum* strain NARS-B10	MF817963.1
	10	R-TL-W	Slow	73	*B. japonicum* strain NARS-B10	MF817963.1
				74	*B. japonicum* strain NARS-B10	MF817963.1

Note: R=Round, TL=Translucent, and W=White.

Appendix 2.2. (Cont'd) In Fukagawa soil and Matsue location.

Replication	Number of nodules	Morphology	Growth status	Isolates for sequencing	Closest isolates	Accession number
3	1	R-TL-W	Slow	75	*B. japonicum* strain Bj11	KY000638.1
				76	*B. japonicum* strain Bj11	KY000638.1
	2	R-TL-W	Slow	77	*B. elkanii* strain SS01	LC385715.1
				78	*B. elkanii* strain SS01	LC385715.1
	3	R-TL-W	Slow	79	*B. elkanii* strain SS01	LC385715.1
				80	*B. elkanii* strain SS01	LC385715.1
	4	R-TL-W	Slow	81	*B. elkanii* strain SS01	LC385715.1
				82	*B. elkanii* strain TSBF 1060	KC283148.1
	5	R-TL-W	Slow	83	*B. elkanii* strain SS01	LC385715.1
				84	*B. elkanii* strain TSBF 1060	KC283148.1
	6	R-TL-W	Slow	85	*B. elkanii* strain TSBF 1060	KC283148.1
				86	*B. elkanii* strain TSBF 1060	KC283148.1
	7	R-TL-W	Slow	93	*B. elkanii* strain Cte-504	MH938235.1
				94	*B. elkanii* strain Cte-504	MH938235.1
	8	R-TL-W	Slow	97	*B. elkanii* strain Cte-504	MH938235.1
				98	*B. elkanii* strain Cte-504	MH938235.1
	9	R-TL-W	Slow	99	*B. elkanii* strain Cte-504	MH938235.1
				100	*B. elkanii* strain Cte-504	MH938235.1
	10	R-TL-W	Slow	103	*B. elkanii* strain Cte-504	MH938235.1
				104	*B. elkanii* strain Cte-504	MH938235.1

Note:

- R=Round, TL=Translucent, and W=White
- The type strain *B. elkanii* BeL7, BeSS01, BeTSBF 1060, BeTSBF 1376 and *B. elkanii* BeCte-504 are identical to each other's within the study length. And *B. japonicum* Bj11 and *B. japonicum* BjNARS-B10 are also identical within the study length.

Appendix 2.3. In Fukagawa soil and Miyazaki location.

Replication	Nodule number	Morphology	Growth status	Isolates for sequencing	Closest isolates	Accession number
1	1	R-TL-W	Slow	1	*B. elkanii* strain Cte-504	MH938235.1
				2	*B. elkanii* strain Cte-504	MH938235.1
	2	R-TL-W	Slow	5	*B. japonicum* strain NARS-B10	MF817963.1
				6	*B. japonicum* strain NARS-B10	MF817963.1
	3	R-TL-W	Slow	7	*B. elkanii* strain Cte-504	MH938235.1
				8	*B. elkanii* strain Cte-504	MH938235.1
	4	R-TL-W	Slow	9	*B. japonicum* strain NARS-B10	MF817963.1
				10	*B. japonicum* strain NARS-B10	MF817963.1
	5	R-TL-W	Slow	11	*B. japonicum* strain NARS-B10	MF817963.1
				12	*B. japonicum* strain NARS-B10	MF817963.1
	6	R-TL-W	Slow	13	*B. elkanii* strain Cte-504	MH938235.1
				14	*B. elkanii* strain Cte-504	MH938235.1
	7	R-TL-W	Slow	15	*B. japonicum* strain NARS-B10	MF817963.1
				16	*B. japonicum* strain NARS-B10	MF817963.1
	8	R-TL-W	Slow	17	*B. elkanii* strain Cte-504	MH938235.1
				18	*B. elkanii* strain Cte-504	MH938235.1
	9	R-TL-W	Slow	19	*B. japonicum* strain NARS-B10	MF817963.1
				20	*B. japonicum* strain NARS-B10	MF817963.1
	10	R-TL-W	Slow	21	*B. japonicum* strain NARS-B10	MF817963.1
				22	*B. japonicum* strain NARS-B10	MF817963.1
2	1	R-TL-W	Slow	27	*B. japonicum* strain NARS-B10	MF817963.1
				28	*B. japonicum* strain NARS-B10	MF817963.1
	2	R-TL-W	Slow	31	*B. elkanii* strain Cte-504	MH938235.1
				32	*B. elkanii* strain Cte-504	MH938235.1
	3	R-TL-W	Slow	33	*B. japonicum* strain NARS-B10	MF817963.1
				34	*B. japonicum* strain NARS-B10	MF817963.1
	4	R-TL-W	Slow	37	*B. japonicum* strain NARS-B10	MF817963.1
				38	*B. japonicum* strain NARS-B10	MF817963.1
	5	R-TL-W	Slow	39	*B. japonicum* strain NARS-B10	MF817963.1
				40	*B. japonicum* strain NARS-B10	MF817963.1
	6	R-TL-W	Slow	41	*B. japonicum* strain NARS-B10	MF817963.1
				42	*B. japonicum* strain NARS-B10	MF817963.1
	7	R-TL-W	Slow	45	*B. japonicum* strain NARS-B10	MF817963.1
				46	*B. japonicum* strain NARS-B10	MF817963.1
	8	R-TL-W	Slow	47	*B. japonicum* strain NARS-B10	MF817963.1
				48	*B. japonicum* strain NARS-B10	MF817963.1
	9	R-TL-W	Slow	49	*B. japonicum* strain NARS-B10	MF817963.1
				50	*B. japonicum* strain NARS-B10	MF817963.1
	10	R-TL-W	Slow	53	*B. japonicum* strain NARS-B10	MF817963.1
				54	*B. japonicum* strain NARS-B10	MF817963.1

Note: R=Round, TL=Translucent, and W=White.

Appendix 2.3. (Cont'd) In Fukagawa soil and Miyazaki location.

Replication	Nodule number	Morphology	Growth status	Isolates for sequencing	Closest isolates	Accession number
3	1	R-TL-W	Slow	66	*B. elkanii* strain Cte-504	MH938235.1
				67	*B. elkanii* strain Cte-504	MH938235.1
	2	R-TL-W	Slow	68	*B. japonicum* strain NARS-B10	MF817963.1
				69	*B. japonicum* strain NARS-B10	MF817963.1
	3	R-TL-W	Slow	70	*B. japonicum* strain NARS-B10	MF817963.1
				71	*B. japonicum* strain NARS-B10	MF817963.1
	4	R-TL-W	Slow	72	*B. elkanii* strain Cte-504	MH938235.1
				73	*B. elkanii* strain Cte-504	MH938235.1
	5	R-TL-W	Slow	75	*B. japonicum* strain NARS-B10	MF817963.1
				75	*B. japonicum* strain NARS-B10	MF817963.1
	6	R-TL-W	Slow	76	*B. elkanii* strain Cte-504	MH938235.1
				77	*B. elkanii* strain Cte-504	MH938235.1
	7	R-TL-W	Slow	78	*B. japonicum* strain NARS-B10	MF817963.1
				79	*B. japonicum* strain NARS-B10	MF817963.1
	8	R-TL-W	Slow	80	*B. elkanii* strain Cte-504	MH938235.1
				81	*B. elkanii* strain Cte-504	MH938235.1
	9	R-TL-W	Slow	82	*B. japonicum* strain NARS-B10	MF817963.1
				83	*B. japonicum* strain NARS-B10	MF817963.1
	10	R-TL-W	Slow	84	*B. japonicum* strain NARS-B10	MF817963.1
				85	*B. japonicum* strain NARS-B10	MF817963.1

Note:

- R=Round, TL=Translucent, and W=White.
- The type strain *B. elkanii* BeL7 and *B. elkanii* BeCte-504 are identical to each other's within the study length. And *B. japonicum* Bj11 and *B. japonicum* BjNARS-B10 are also identical within the study length.

Appendix 2.4. In Matsue soil and Fukagawa location.

Replication	Nodule number	Morphology	Growth status	Isolates for sequence	Closest isolates	Accession number
1	1	R-TL-W	Slow	1	*B. elkanii* strain Cte-504	MH938235.1
				2	*B. elkanii* strain Cte-504	MH938235.1
	2	R-TL-W	Slow	11	*B. elkanii* strain Cte-504	MH938235.1
				12	*B. elkanii* strain Cte-504	MH938235.1
	3	R-TL-W	Slow	13	*B. elkanii* strain Cte-504	MH938235.1
				14	*B. elkanii* strain Cte-504	MH938235.1
	4	R-TL-W	Slow	21	*B. elkanii* strain Cte-504	MH938235.1
				22	*B. elkanii* strain Cte-504	MH938235.1
	5	R-TL-W	Slow	23	*B. elkanii* strain Cte-504	MH938235.1
				24	*B. elkanii* strain Cte-504	MH938235.1
	6	R-TL-W	Slow	27	*B. elkanii* strain Cte-504	MH938235.1
				28	*B. elkanii* strain Cte-504	MH938235.1
	7	R-TL-W	Slow	31	*B. elkanii* strain Cte-504	MH938235.1
				32	*B. elkanii* strain Cte-504	MH938235.1
	8	R-TL-W	Slow	35	*B. japonicum* strain Bj11	KY000638.1
				36	*B. japonicum* strain Bj11	KY000638.1
	9	R-TL-W	Slow	39	*B. elkanii* strain Cte-504	MH938235.1
				40	*B. elkanii* strain Cte-504	MH938235.1
	10	R-TL-W	Slow	53	*B. japonicum* strain Bj11	KY000638.1
				54	*B. japonicum* strain Bj11	KY000638.1
2	1	R-TL-W	Slow	59	*B. japonicum* strain Bj11	KY000638.1
				60	*B. japonicum* strain Bj11	KY000638.1
	2	R-TL-W	Slow	71	*B. japonicum* strain Bj11	KY000638.1
				72	*B. japonicum* strain Bj11	KY000638.1
	3	R-TL-W	Slow	73	*B. elkanii* strain Cte-504	MH938235.1
				74	*B. elkanii* strain Cte-504	MH938235.1
	4	R-TL-W	Slow	75	*B. elkanii* strain Cte-504	MH938235.1
				76	*B. elkanii* strain Cte-504	MH938235.1
	5	R-TL-W	Slow	77	*B. elkanii* strain Cte-504	MH938235.1
				78	*B. elkanii* strain Cte-504	MH938235.1
	6	R-TL-W	Slow	81	*B. elkanii* strain Cte-504	MH938235.1
				82	*B. elkanii* strain Cte-504	MH938235.1
	7	R-TL-W	Slow	85	*B. elkanii* strain Cte-504	MH938235.1
				86	*B. elkanii* strain Cte-504	MH938235.1
	8	R-TL-W	Slow	89	*B. elkanii* strain Cte-504	MH938235.1
				90	*B. elkanii* strain Cte-504	MH938235.1
	9	R-TL-W	Slow	91	*B. elkanii* strain Cte-504	MH938235.1
				92	*B. elkanii* strain Cte-504	MH938235.1
	10	R-TL-W	Slow	95	*B. japonicum* strain Bj11	KY000638.1
				96	*B. japonicum* strain Bj11	KY000638.1

Note: R=Round, TL=Translucent, and W=White.

Appendix 2.4. (Cont’d) In Matsue soil and Fukagawa location.

Replication	Nodule number	Morphology	Growth status	Isolates for sequencing	Closest isolates	Accession number
3	1	R-TL-W	Slow	101	*B. elkanii* strain Cte-504	MH938235.1
				102	*B. elkanii* strain Cte-504	MH938235.1
	2	R-TL-W	Slow	103	*B. japonicum* strain Bj11	KY000638.1
				104	*B. japonicum* strain Bj11	KY000638.1
	3	R-TL-W	Slow	105	*B. elkanii* strain Cte-504	MH938235.1
				106	*B. elkanii* strain Cte-504	MH938235.1
	4	R-TL-W	Slow	107	*B. elkanii* strain Cte-504	MH938235.1
				108	*B. elkanii* strain Cte-504	MH938235.1
	5	R-TL-W	Slow	109	*B. elkanii* strain Cte-504	MH938235.1
				110	*B. elkanii* strain Cte-504	MH938235.1
	6	R-TL-W	Slow	111	*B. elkanii* strain Cte-504	MH938235.1
				112	*B. elkanii* strain Cte-504	MH938235.1
	7	R-TL-W	Slow	113	*B. elkanii* strain Cte-504	MH938235.1
				114	*B. elkanii* strain Cte-504	MH938235.1
	8	R-TL-W	Slow	115	*B. elkanii* strain Cte-504	MH938235.1
				116	*B. elkanii* strain Cte-504	MH938235.1
	9	R-TL-W	Slow	117	*B. elkanii* strain Cte-504	MH938235.1
				118	*B. elkanii* strain Cte-504	MH938235.1
	10	R-TL-W	Slow	119	*B. japonicum* strain Bj11	KY000638.1
				120	*B. japonicum* strain Bj11	KY000638.1

Note:

- R=Round, TL=Translucent, and W=White.
- The type strain *B. elkanii* BeL7 and *B. elkanii* BeCte-504 are identical to each other’s within the study length.

Appendix 2.5. In Matsue soil and Matsue location.

Replication	Nodule number	Morphology	Growth status	Isolates for sequencing	Closest isolates	Accession number
1	1	R-TL-W	Slow	3	*B. elkanii* strain Cte-504	MH938235.1
				4	*B. elkanii* strain Cte-504	MH938235.1
	2	R-TL-W	Slow	5	*B. elkanii* strain Cte-504	MH938235.1
				6	*B. elkanii* strain Cte-504	MH938235.1
	3	R-TL-W	Slow	21	*B. elkanii* strain Cte-504	MH938235.1
				22	*B. elkanii* strain Cte-504	MH938235.1
	4	R-TL-W	Slow	7	*B. japonicum* strain Bj11	KY000638.1
				8	*B. japonicum* strain Bj11	KY000638.1
		R-TL-W	Slow*	21	*B. elkanii* strain Cte-504	MH938235.1
				22	*B. elkanii* strain Cte-504	MH938235.1
	5	R-TL-W	Slow	9	*B. elkanii* strain Cte-504	MH938235.1
				10	*B. elkanii* strain Cte-504	MH938235.1
	6	R-TL-W	Slow	23	*B. elkanii* strain Cte-504	MH938235.1
				24	*B. elkanii* strain Cte-504	MH938235.1
	7	R-TL-W	Slow	11	*B. elkanii* strain Cte-504	MH938235.1
				12	*B. elkanii* strain Cte-504	MH938235.1
	8	R-TL-W	Slow	15	*B. elkanii* strain Cte-504	MH938235.1
				16	*B. elkanii* strain Cte-504	MH938235.1
	9	R-TL-W	Slow	17	*B. japonicum* strain Bj11	KY000638.1
				18	*B. japonicum* strain Bj11	KY000638.1
	10	R-TL-W	Slow	17	*B. japonicum* strain Bj11	KY000638.1
				18	*B. japonicum* strain Bj11	KY000638.1
2	1	R-TL-W	Slow	27	*B. elkanii* strain Cte-504	MH938235.1
				28	*B. elkanii* strain Cte-504	MH938235.1
	2	R-TL-W	Slow	29	*B. elkanii* strain Cte-504	MH938235.1
				30	*B. elkanii* strain Cte-504	MH938235.1
	3	R-TL-W	Slow	31	*B. elkanii* strain Cte-504	MH938235.1
				32	*B. elkanii* strain Cte-504	MH938235.1
	4	R-TL-W	Slow	33	*B. elkanii* strain Cte-504	MH938235.1
				34	*B. elkanii* strain Cte-504	MH938235.1
	5	R-TL-W	Slow	35	*B. elkanii* strain Cte-504	MH938235.1
				36	*B. elkanii* strain Cte-504	MH938235.1
	6	R-TL-W	Slow	39	*B. elkanii* strain Cte-504	MH938235.1
				40	*B. elkanii* strain Cte-504	MH938235.1
	7	R-TL-W	Slow	49	*B. elkanii* strain Cte-504	MH938235.1
				50	*B. elkanii* strain Cte-504	MH938235.1
	8	R-TL-W	Slow	51	*B. elkanii* strain Cte-504	MH938235.1
				52	*B. elkanii* strain Cte-504	MH938235.1
	9	R-TL-W	Slow	53	*B. elkanii* strain Cte-504	MH938235.1
				54	*B. elkanii* strain Cte-504	MH938235.1
	10	R-TL-W	Slow	55	*B. elkanii* strain Cte-504	MH938235.1
				56	*B. elkanii* strain Cte-504	MH938235.1

Note: R=Round, TL=Translucent, and W=White.

Appendix 2.5. (Cont’d) In Matsue soil and Matsue location.

Replication	Nodule number	Morphology	Growth status	Isolates for sequencing	Closest isolates	Accession number
3	1	R-TL-W	Slow	59	*B. elkanii* strain Cte-504	MH938235.1
				60	*B. elkanii* strain Cte-504	MH938235.1
	2	R-TL-W	Slow	61	*B. elkanii* strain Cte-504	MH938235.1
				62	*B. elkanii* strain Cte-504	MH938235.1
	3	R-TL-W	Slow	57	*B. elkanii* strain Cte-504	MH938235.1
				58	*B. elkanii* strain Cte-504	MH938235.1
	4	R-TL-W	Slow	67	*B. elkanii* strain Cte-504	MH938235.1
				68	*B. elkanii* strain Cte-504	MH938235.1
	5	R-TL-W	Slow	69	*B. elkanii* strain Cte-504	MH938235.1
				70	*B. elkanii* strain Cte-504	MH938235.1
	6	R-TL-W	Slow	71	*B. elkanii* strain Cte-504	MH938235.1
				72	*B. elkanii* strain Cte-504	MH938235.1
	7	R-TL-W	Slow	79	*B. japonicum* strain S10J	MF664374.1
				80	*B. japonicum* strain S10J	MF664374.1
	8	Fused	Slow	75	*B. elkanii* strain Cte-504	MH938235.1
				76	*B. elkanii* strain Cte-504	MH938235.1
	9	R-TL-W	Slow	77	*B. elkanii* strain Cte-504	MH938235.1
				78	*B. elkanii* strain Cte-504	MH938235.1
	10	R-TL-W	Slow	77	*B. elkanii* strain Cte-504	MH938235.1
				78	*B. elkanii* strain Cte-504	MH938235.1

Note:

- R=Round, TL=Translucent, and W=White
- The type strain *B. elkanii* BeL7 and *B. elkanii* BeCte-504 are identical to each other’s within the study length.

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Appendix 2.6. In Matsue soil and Miyazaki location.

Replication	Nodule number	Morphology	Growth status	Isolates for sequencing	Closest isolates	Accession number
1	1	R-TL-W	Slow	1	*B. elkanii* strain Cte-504	MH938235.1
				2	*B. elkanii* strain Cte-504	MH938235.1
	2	R-TL-W	Slow	3	*B. elkanii* strain Cte-504	MH938235.1
				4	*B. elkanii* strain Cte-504	MH938235.1
	3	R-TL-W	Slow	5	*B. elkanii* strain Cte-504	MH938235.1
				6	*B. elkanii* strain Cte-504	MH938235.1
	4	R-TL-W	Slow	7	*B. elkanii* strain Cte-504	MH938235.1
				8	*B. elkanii* strain Cte-504	MH938235.1
	5	R-TL-W	Slow	9	*B. elkanii* strain Cte-504	MH938235.1
				10	*B. elkanii* strain Cte-504	MH938235.1
	6	R-TL-W	Slow	11	*B. elkanii* strain Cte-504	MH938235.1
				12	*B. elkanii* strain Cte-504	MH938235.1
	7	R-TL-W	Slow	19	*B. elkanii* strain Cte-504	MH938235.1
				20	*B. elkanii* strain Cte-504	MH938235.1
	8	R-TL-W	Slow	21	*B. elkanii* strain Cte-504	MH938235.1
				22	*B. elkanii* strain Cte-504	MH938235.1
	9	R-TL-W	Slow	25	*B. elkanii* strain Cte-504	MH938235.1
				26	*B. elkanii* strain Cte-504	MH938235.1
	10	R-TL-W	Slow	29	*B. elkanii* strain Cte-504	MH938235.1
				30	*B. elkanii* strain Cte-504	MH938235.1
2	1	R-TL-W	Slow	33	*B. elkanii* strain Cte-504	MH938235.1
				34	*B. elkanii* strain Cte-504	MH938235.1
	2	R-TL-W	Slow	35	*B. elkanii* strain Cte-504	MH938235.1
				36	*B. elkanii* strain Cte-504	MH938235.1
	3	R-TL-W	Slow	37	*B. elkanii* strain Cte-504	MH938235.1
				38	*B. elkanii* strain Cte-504	MH938235.1
	4	R-TL-W	Slow	41	*B. elkanii* strain Cte-504	MH938235.1
				42	*B. elkanii* strain Cte-504	MH938235.1
	5	R-TL-W	Slow	45	*B. elkanii* strain Cte-504	MH938235.1
				46	*B. elkanii* strain Cte-504	MH938235.1
	6	R-TL-W	Slow	47	*B. elkanii* strain Cte-504	MH938235.1
				48	*B. elkanii* strain Cte-504	MH938235.1
	7	R-TL-W	Slow	49	*B. elkanii* strain Cte-504	MH938235.1
				50	*B. elkanii* strain Cte-504	MH938235.1
	8	R-TL-W	Slow	51	*B. elkanii* strain Cte-504	MH938235.1
				52	*B. elkanii* strain Cte-504	MH938235.1
	9	R-TL-W	Slow	53	*B. elkanii* strain Cte-504	MH938235.1
				54	*B. elkanii* strain Cte-504	MH938235.1
	10	R-TL-W	Slow	*57*	*B. elkanii* strain Cte-504	MH938235.1
				58	*B. elkanii* strain Cte-504	MH938235.1

Note: R=Round, TL=Translucent, and W=White.

Appendix 2.6. (Cont'd) In Matsue soil and Miyazaki location.

Replication	Nodule number	Morphology	Growth status	Isolates for sequencing	Closest isolates	Accession number
3	1	R-TL-W	Slow	61	*B. elkanii* strain Cte-504	MH938235.1
				62	*B. elkanii* strain Cte-504	MH938235.1
	2	R-TL-W	Slow	65	*B. japonicum* strain S10J	MH938235.1
				66	*B. japonicum* strain S10J	MH938235.1
	3	R-TL-W	Slow	67	*B. elkanii* strain Cte-504	MH938235.1
				68	*B. elkanii* strain Cte-504	MH938235.1
	4	R-TL-W	Slow	69	*B. elkanii* strain Cte-504	MH938235.1
				70	*B. elkanii* strain Cte-504	MH938235.1
	5	R-TL-W	Slow	71	*B. elkanii* strain Cte-504	MH938235.1
				72	*B. elkanii* strain Cte-504	MH938235.1
	6	R-TL-W	Slow	75	*B. elkanii* strain Cte-504	MH938235.1
				76	*B. elkanii* strain Cte-504	MH938235.1
	7	R-TL-W	Slow	77	*B. elkanii* strain Cte-504	MH938235.1
				78	*B. elkanii* strain Cte-504	MH938235.1
	8	R-TL-W	Slow	79	*B. elkanii* strain Cte-504	MH938235.1
				80	*B. elkanii* strain Cte-504	MH938235.1
	9	R-TL-W	Slow	81	*B. elkanii* strain Cte-504	MH938235.1
				82	*B. elkanii* strain Cte-504	MH938235.1
	10	R-TL-W	Early grower	83	*B. elkanii* strain Cte-504	MH938235.1
				84	*B. elkanii* strain Cte-504	MH938235.1
		R-TL-W	Slow	85	*B. elkanii* strain Cte-504	MH938235.1
				86	*B. elkanii* strain Cte-504	MH938235.1

Note:

- R=Round, TL=Translucent, and W=White.
- The type strain *B. elkanii* BeL7 and *B. elkanii* BeCte-504 are identical to each other's within the study length.

Appendix 2.7. In Miyazaki soil and Fukagawa location.

Replication	Nodule number	Morphology	Growth status	Isolates for sequencing	Closest isolates	Accession number
1	1	R-TL-W	Slow	1	*B. elkanii* strain Cte-504	MH938235.1
				2	*B. elkanii* strain Cte-504	MH938235.1
	2	R-TL-W	Slow	3	*B. japonicum* strain NARS-B3	MF817965.1
				4	*B. japonicum* strain NARS-B3	MF817965.1
	3	R-TL-W	Slow	7	*B. japonicum* strain NARS-B3	MF817965.1
				8	*B. japonicum* strain NARS-B3	MF817965.1
	4	R-TL-W	Slow*	11	*B. elkanii* strain Cte-504	MH938235.1
				12	*B. elkanii* strain Cte-504	MH938235.1
		R-TL-W	Slow	13	*B. japonicum* strain NARS-B3	MF817965.1
				14	*B. japonicum* strain NARS-B3	MF817965.1
	5	R-TL-W	Slow	15	*B. elkanii* strain Cte-504	MH938235.1
				16	*B. elkanii* strain Cte-504	MH938235.1
	6	R-TL-W	Slow	17	*B. elkanii* strain Cte-504	MH938235.1
				18	*B. elkanii* strain Cte-504	MH938235.1
	7	R-TL-W	Slow	22	*B. japonicum* strain NARS-B3	MF817965.1
				23	*B. japonicum* strain NARS-B3	MF817965.1
	8	R-TL-W	Slow	26	*B. elkanii* strain Cte-504	MH938235.1
				27	*B. elkanii* strain Cte-504	MH938235.1
	9	R-TL-W	Slow	28	*B. elkanii* strain Cte-504	MH938235.1
				29	*B. elkanii* strain Cte-504	MH938235.1
	10	R-TL-W	Slow	30	*B. elkanii* strain Cte-504	MH938235.1
				31	*B. elkanii* strain Cte-504	MH938235.1
2	1	R-TL-W	Slow	35	*B. japonicum* strain S10J	MF664374.1
				36	*B. japonicum* strain S10J	MF664374.1
	2	R-TL-W	Slow	37	*B. elkanii* strain Cte-504	MH938235.1
				38	*B. elkanii* strain Cte-504	MH938235.1
	3	R-TL-W	Slow	43	*B. japonicum* strain NARS-B3	MF817965.1
				44	*B. japonicum* strain NARS-B3	MF817965.1
	4	R-TL-W	Slow	45	*B. elkanii* strain Cte-504	MH938235.1
				46	*B. elkanii* strain Cte-504	MH938235.1
	5	R-TL-W	Slow	49	*B. elkanii* strain Cte-504	MH938235.1
				50	*B. elkanii* strain Cte-504	MH938235.1
	6	R-TL-W	Slow	55	*B. elkanii* strain Cte-504	MH938235.1
				56	*B. elkanii* strain Cte-504	MH938235.1
	7	R-TL-W	Slow	59	*B. elkanii* strain Cte-504	MH938235.1
				60	*B. elkanii* strain Cte-504	MH938235.1
	8	R-TL-W	Slow	63	*B. elkanii* strain Cte-504	MH938235.1
				64	*B. elkanii* strain Cte-504	MH938235.1
	9	R-TL-W	Slow	67	*B. elkanii* strain Cte-504	MH938235.1
				68	*B. elkanii* strain Cte-504	MH938235.1
	10	R-TL-W	Slow	73	*B. japonicum* strain NARS-B3	MF817965.1
				74	*B. japonicum* strain NARS-B3	MF817965.1

Note:

- R=Round, TL=Translucent, and W=White.
- *Co-nodulation of *B. elkanii* with *B. japonicum* and less than 4 colonies of *B. elkanii* were on the plate produce relatively larger polysaccharide than *B. japonicum.*

Appendix 2.7. (Cont'd) In Miyazaki soil and Fukagawa location.

Replication	Nodule number	Morphology	Growth status	Isolates for sequencing	Closest isolates	Accession number
3	1	R-TL-W	Slow	75	*B. elkanii* strain Cte-504	MH938235.1
				76	*B. elkanii* strain Cte-504	MH938235.1
	2	R-TL-W	Slow	77	*B. elkanii* strain Cte-504	MH938235.1
				78	*B. elkanii* strain Cte-504	MH938235.1
	3	R-TL-W	Slow	79	*B. elkanii* strain Cte-504	MH938235.1
				80	*B. elkanii* strain Cte-504	MH938235.1
	4	R-TL-W	Slow	81	*B. elkanii* strain Cte-504	MH938235.1
				82	*B. elkanii* strain Cte-504	MH938235.1
	5	R-TL-W	Slow	83	*B. elkanii* strain Cte-504	MH938235.1
				84	*B. elkanii* strain Cte-504	MH938235.1
	6	R-TL-W	Slow	85	*B. elkanii* strain Cte-504	MH938235.1
				86	*B. elkanii* strain Cte-504	MH938235.1
	7	R-TL-W	Slow	87	*B. elkanii* strain Cte-504	MH938235.1
				88	*B. elkanii* strain Cte-504	MH938235.1
	8	R-TL-W	Slow	89	*B. elkanii* strain Cte-504	MH938235.1
				90	*B. elkanii* strain Cte-504	MH938235.1
	9	R-TL-W	Slow	91	*B. elkanii* strain Cte-504	MH938235.1
				92	*B. elkanii* strain Cte-504	MH938235.1
	10	R-TL-W	Slow	95	*B. elkanii* strain Cte-504	MH938235.1
				96	*B. elkanii* strain Cte-504	MH938235.1

Note:

- R=Round, TL=Translucent, and W=White.
- The type strain *B. elkanii* BeL7 and *B. elkanii* BeCte-504 are identical to each other's within the study length. And *B. japonicum* BjS10J and *B. japonicum* BjNARS-B3 are also identical within the stud length.

Appendix 2.8. In Miyazaki soil and Matsue location.

Replication	Nodule number	Morphology	Growth status	Isolates for sequencing	Closest isolates	Accession number
1	1	R-TL-W	Slow	1	*B. elkanii* strain Cte-504	MH938235.1
				3	*B. elkanii* strain Cte-504	MH938235.1
	2	R-TL-W	Slow	5	*B. elkanii* strain Cte-504	MH938235.1
				6	*B. elkanii* strain Cte-504	MH938235.1
	3	R-TL-W	Slow	9	*B. elkanii* strain Cte-504	MH938235.1
				11	*B. elkanii* strain Cte-504	MH938235.1
	4	R-TL-W	Slow	14	*B. elkanii* strain Cte-504	MH938235.1
				17	*B. elkanii* strain Cte-504	MH938235.1
	5	R-TL-W	Slow	20	*B. elkanii* strain Cte-504	MH938235.1
				21	*B. elkanii* strain Cte-504	MH938235.1
	6	R-TL-W	Slow	22	*B. elkanii* strain Cte-504	MH938235.1
				23	*B. elkanii* strain Cte-504	MH938235.1
	7	R-TL-W	Slow	24	*B. elkanii* strain Cte-504	MH938235.1
				25	*B. elkanii* strain Cte-504	MH938235.1
	8	R-TL-W	Slow	26	*B. elkanii* strain Cte-504	MH938235.1
				27	*B. elkanii* strain Cte-504	MH938235.1
	9	R-TL-W	Slow	32	*B. elkanii* strain Cte-504	MH938235.1
				33	*B. elkanii* strain Cte-504	MH938235.1
	10	R-TL-W	Slow	34	*B. elkanii* strain Cte-504	MH938235.1
				35	*B. elkanii* strain Cte-504	MH938235.1
2	1	R-TL-W	Slow	46	*B. elkanii* strain Cte-504	MH938235.1
				47	*B. elkanii* strain Cte-504	MH938235.1
	2	R-TL-W	Slow	50	*B. elkanii* strain Cte-504	MH938235.1
				51	*B. elkanii* strain Cte-504	MH938235.1
	3	R-TL-W	Slow	54	*B. elkanii* strain Cte-504	MH938235.1
				55	*B. elkanii* strain Cte-504	MH938235.1
	4	R-TL-W	Slow	58	*B. elkanii* strain Cte-504	MH938235.1
				59	*B. elkanii* strain Cte-504	MH938235.1
	5	R-TL-W	Slow	62	*B. elkanii* strain Cte-504	MH938235.1
				63	*B. elkanii* strain Cte-504	MH938235.1
	6	R-TL-W	Slow	66	*B. elkanii* strain Cte-504	MH938235.1
				67	*B. elkanii* strain Cte-504	MH938235.1
	7	R-TL-W	Slow	68	*B. elkanii* strain Cte-504	MH938235.1
				69	*B. elkanii* strain Cte-504	MH938235.1
	8	R-TL-W	Slow	70	*B. elkanii* strain Cte-504	MH938235.1
				71	*B. elkanii* strain Cte-504	MH938235.1
	9	R-TL-W	Slow	72	*B. elkanii* strain Cte-504	MH938235.1
				73	*B. elkanii* strain Cte-504	MH938235.1
	10	R-TL-W	Slow	76	*B. elkanii* strain Cte-504	MH938235.1
				77	*B. elkanii* strain Cte-504	MH938235.1

Note: R=Round, TL=Translucent, and W=White.

Appendix 2.8. (Cont'd) In Miyazaki soil and Matsue location.

Replication	Nodule number	Morphology	Growth Status	Isolates for sequencing	Closest isolates	Accession Number
3	1	R-TL-W	Slow	80	*B. elkanii* strain Cte-504	MH938235.1
				81	*B. elkanii* strain Cte-504	MH938235.1
	2	R-TL-W	Slow	86	*B. elkanii* strain Cte-504	MH938235.1
				87	*B. elkanii* strain Cte-504	MH938235.1
	3	R-TL-W	Slow	93	*B. elkanii* strain Cte-504	MH938235.1
				94	*B. elkanii* strain Cte-504	MH938235.1
	4	R-TL-W	Slow	97	*B. elkanii* strain Cte-504	MH938235.1
				98	*B. elkanii* strain Cte-504	MH938235.1
	5	R-TL-W	Slow	101	*B. elkanii* strain Cte-504	MH938235.1
				102	*B. elkanii* strain Cte-504	MH938235.1
	6	R-TL-W	Slow	103	*B. elkanii* strain Cte-504	MH938235.1
				104	*B. elkanii* strain Cte-504	MH938235.1
	7	R-TL-W	Slow	107	*B. elkanii* strain Cte-504	MH938235.1
				108	*B. elkanii* strain Cte-504	MH938235.1
	8	R-TL-W	Slow	109	*B. elkanii* strain Cte-504	MH938235.1
				110	*B. elkanii* strain Cte-504	MH938235.1
	9	R-TL-W	Slow	111	*B. elkanii* strain Cte-504	MH938235.1
				112	*B. elkanii* strain Cte-504	MH938235.1
	10	R-TL-W	Slow	113	*B. elkanii* strain Cte-504	MH938235.1
				114	*B. elkanii* strain Cte-504	MH938235.1

Note:

- R=Round, TL=Translucent, and W=White.
- The type strain *B. elkanii Be*L7 and *B. elkanii* BeCte-504 are identical to each other's within the study length.

Appendix 2.9. In Miyazaki soil and Miyazaki location.

Replication	Nodule number	Morphology	Growth status	Isolates for sequencing	Closest isolates	Accession number
1	1	R-TL-W	Slow	1	*B. elkanii* strain Cte-504	MH938235.1
				2	*B. elkanii* strain Cte-504	MH938235.1
	2	R-TL-W	Slow	3	*B. elkanii* strain Cte-504	MH938235.1
				4	*B. elkanii* strain Cte-504	MH938235.1
	3	R-TL-W	Slow	7	*B. elkanii* strain Cte-504	MH938235.1
				8	*B. elkanii* strain Cte-504	MH938235.1
	4	R-TL-W	Slow	9	*B. elkanii* strain Cte-504	MH938235.1
				10	*B. elkanii* strain Cte-504	MH938235.1
	5	R-TL-W	Slow	11	*B. elkanii* strain Cte-504	MH938235.1
				12	*B. elkanii* strain Cte-504	MH938235.1
	6	R-TL-W	Slow	13	*B. elkanii* strain Cte-504	MH938235.1
				14	*B. elkanii* strain Cte-504	MH938235.1
	7	R-TL-W	Slow	15	*B. elkanii* strain Cte-504	MH938235.1
				16	*B. elkanii* strain Cte-504	MH938235.1
	8	R-TL-W	Slow	17	*B. elkanii* strain Cte-504	MH938235.1
				18	*B. elkanii* strain Cte-504	MH938235.1
	9	R-TL-W	Slow	19	*B. elkanii* strain Cte-504	MH938235.1
				20	*B. elkanii* strain Cte-504	MH938235.1
	10	R-TL-W	Slow	23	*B. elkanii* strain Cte-504	MH938235.1
				24	*B. elkanii* strain Cte-504	MH938235.1
2	1	R-TL-W	Slow	27	*B. elkanii* strain Cte-504	MH938235.1
				28	*B. elkanii* strain Cte-504	MH938235.1
	2	R-TL-W	Slow	29	*B. elkanii* strain Cte-504	MH938235.1
				30	*B. elkanii* strain Cte-504	MH938235.1
	3	R-TL-W	Slow	31	*B. elkanii* strain Cte-504	MH938235.1
				32	*B. elkanii* strain Cte-504	MH938235.1
	4	R-TL-W	Slow	33	*B. elkanii* strain Cte-504	MH938235.1
				34	*B. elkanii* strain Cte-504	MH938235.1
	5	R-TL-W	Slow	35	*B. elkanii* strain Cte-504	MH938235.1
				36	*B. elkanii* strain Cte-504	MH938235.1
	6	R-TL-W	Slow	37	*B. elkanii* strain Cte-504	MH938235.1
				38	*B. elkanii* strain Cte-504	MH938235.1
	7	R-TL-W	Slow	39	*B. elkanii* strain Cte-504	MH938235.1
				40	*B. elkanii* strain Cte-504	MH938235.1
	8	R-TL-W	Slow	41	*B. elkanii* strain Cte-504	MH938235.1
				42	*B. elkanii* strain Cte-504	MH938235.1
	9	R-TL-W	Slow	43	*B. elkanii* strain Cte-504	MH938235.1
				44	*B. elkanii* strain Cte-504	MH938235.1
	10	R-TL-W	Slow	45	*B. elkanii* strain Cte-504	MH938235.1
				46	*B. elkanii* strain Cte-504	MH938235.1

Note: R=Round, TL=Translucent, and W=White.

Appendix 2.9. (Cont'd) In Miyazaki soil and Miyazaki location.

Replication	Nodule number	Morphology	Growth status	Isolates for sequencing	Closest isolates	Accession number
3	1	R-TL-W	Slow	51	*B. elkanii* strain Cte-504	MH938235.1
				52	*B. elkanii* strain Cte-504	MH938235.1
	2	R-TL-W	Slow	53	*B. elkanii* strain Cte-504	MH938235.1
				54	*B. elkanii* strain Cte-504	MH938235.1
	3	R-TL-W	Slow	55	*B. elkanii* strain Cte-504	MH938235.1
				56	*B. elkanii* strain Cte-504	MH938235.1
	4	R-TL-W	Slow	57	*B. elkanii* strain Cte-504	MH938235.1
				58	*B. elkanii* strain Cte-504	MH938235.1
	5	R-TL-W	Slow	61	*B. elkanii* strain Cte-504	MH938235.1
				62	*B. elkanii* strain Cte-504	MH938235.1
	6	R-TL-W	Slow	63	*B. elkanii* strain Cte-504	MH938235.1
				64	*B. elkanii* strain Cte-504	MH938235.1
	7	R-TL-W	Slow	65	*B. elkanii* strain Cte-504	MH938235.1
				66	*B. elkanii* strain Cte-504	MH938235.1
	8	R-TL-W	Slow	67	*B. elkanii* strain Cte-504	MH938235.1
				68	*B. elkanii* strain Cte-504	MH938235.1
	9	R-TL-W	Slow	69	*B. elkanii* strain Cte-504	MH938235.1
				70	*B. elkanii* strain Cte-504	MH938235.1
	10	R-TL-W	Slow	71	*B. elkanii* strain Cte-504	MH938235.1
				72	*B. elkanii* strain Cte-504	MH938235.1

Note:

- R=Round, TL=Translucent, and W=White.
- The type strain *B. elkanii* BeL7 and *B. elkanii* BeCte-504 are identical to each other's within the study length.

CHAPTER 3

Growth and Competitive Infection Behaviors of *Bradyrhizobium japonicum* and *Bradyrhizobium elkanii* at Different Temperature

3.1. Introduction

Soybean-nodulating bacteria have distributed worldwide (Hymowitz, 1970; Hymowitz and Singh, 1987) and established important symbiotic relationships with the host plant to fix atmospheric nitrogen (Hungria et al., 2015). *Bradyrhizobium japonicum* and *B. elkanii* are reported as the major soybean nodulating rhizobia (Jordan, 1982; Kyukendall et al., 1992) and their nodulation behaviours in fields still need to be clarified in relation to environmental conditions because their nodulation and nitrogen fixation are known to be highly dependent on environmental conditions (Hungria and Vargas, 2000). In the previous studies, latitudinal characteristic nodulation of *B. japonicum* and *B. elkanii* have been reported in Japan (Hafiz et al., 2021; Saeki et al., 2006), the United States (Shiro et al., 2013) and Nepal (Adhikari et al., 2012), in which *B. japonicum* and *B. elkanii* dominate in soybean nodules in northern and southern regions, respectively. These results suggest that the temperature of the soybean growing location contributes to the nodule composition of *B. japonicum* and *B. elkanii*.

To elucidate the possible reason, laboratory competitive inoculation experiments have been conducted at different temperatures. Kluson et al. (1986) reported that *B. japonicum* strains dominated in nodules at lower temperatures, while *B. elkanii* strains did at higher temperature. Suzuki et al. (2014) examined the relative population of *B. japonicum* and *B. elkanii* strains in the rhizosphere of soybean and their nodule compositions at different temperatures and revealed that the *B. japonicum* strain dominated in nodules at lower temperature even though the relative populations of both strains were similar in the rhizosphere, while at higher temperature, the *B. elkanii* strains dominated in nodules due to their larger relative population in the rhizosphere. Shiro et al. (2016) reported that the nodule occupancy of *B. elkanii* increased at higher temperatures, whereas that of *B. japonicum* increased at lower temperatures, corresponding to their temperature-dependent *nodC* gene expressions. These results suggest that the temperature-dependent infections and proliferations in soils was considered as possible reasons for the temperature-dependent nodule compositions of rhizobia in the fields. However, it has been uncertain which factor, namely, temperature-dependent infection or proliferation in soil, contributes to the temperature-dependent distribution of rhizobia in nodules.

For elucidating which factor is more involved in the soybean nodule composition under local climatic conditions, Hafiz et al. (2021) examined the changes in the nodule composition when soil samples were used for soybean cultivation under the different climatic conditions from the original locations, and found that the *B. japonicum* strains nodulated dominantly in the Fukagawa location (temperate continental climate) and the dominancy of *B. japonicum* did not change when soybean was cultivated in the Matsue and Miyazaki locations (humid sub-tropical climate) using the Fukagawa soil. The results suggest that the *B. japonicum* strains proliferated dominantly in the Fukagawa soil leading to their nodule dominancy because *B. elkanii* did not appear in the Matsue and Miyazaki locations. On the other hand, the *B. elkanii* strains dominated in the Miyazaki soil and location while the *B. japonicum* strains dominated when soybean was cultivated in the Fukagawa location using the Miyazaki soil, suggesting that the temperature-dependent infection would lead to the nodule dominancy of the *B. elkanii* and *B. japonicum* strains in the Miyazaki and Fukagawa locations, respectively.

In addition, in the Fukagawa soil and location, phylogenetic sub-group *B. japonicum* Bj11-1, which was characterized as a slow grower, dominated the nodules compared to another sub-group *B. japonicum* Bj11-2, which was characterized as a fast grower Hafiz et al. (2021), suggesting that infection preference might determine the nodule composition among the *B. japonicum* strains rather than their growth properties. In the Miyazaki soil and location, it was suggested that both *B. japonicum* and *B. elkanii* strains proliferated, and that the species-specific nodule compositions under the different local climatic conditions might be due to the temperature-dependent growth and infection properties of the *Bradyrhizobium* strains Hafiz et al. (2021).

These hypotheses presented in the previous study Hafiz et al. (2021) should be confirmed by *in vitro* growth and inoculation experiments under the controlled temperatures using the *B. japonicum* and *B. elkanii* strains isolated from the corresponding soils and locations. In this study, we compared growth and infection behaviors at different temperatures of the *B. japonicum* and *B. elkanii* strains isolated from the soybean nodules cultivated in the Fukagawa and Miyazaki soils, and elucidated the reason why the species-specific nodule compositions are present in the Fukagawa and Miyazaki soils and locations.

3.2. Materials and Methods

3.2.1. Effect of Temperature on Growth of *Bradyrhizobium* spp. in Liquid Culture

The strains used are listed in Table 1. They were isolated from nodules of soybean cultivated in the Fukagawa and Miyazaki soils and study locations in 2016, and selected based on their phylogenetic characteristics based on the 16S rRNA and 16S-23S rRNA ITS gene sequences Hafiz et al. (2021).

Considering the temperature ranges during the soybean cultivation period in the study locations (Table 2), the temperatures were set at 15 °C (around average daily minimum temperature in the Fukagawa location), 20 °C (around average daily temperature in the Fukagawa location), 25 °C (around average daily maximum and minimum temperatures in the Fukagawa and Miyazaki locations, respectively), 30 °C (around average daily temperature in the Miyazaki location), and 35 °C (around average daily maximum temperature in the Miyazaki location).

Each strain was pre-incubated on yeast mannitol (YM) (Vincent, 1970) agar medium at 26 °C for 5–10 days, and a part of the colony was taken into 3 mL of YM liquid medium to adjust optical density at 660 nanometer wavelengths (OD_{660}) at 0.03, then incubated with shaking at 125 rpm for seven days while measuring their OD_{660} at 24-h intervals. All the experiments were done in triplicate.

Table 1. *Bradyrhizobium* strains used in this study.

Strain [a]	**Closest 16 rDNA**	**ITS Group [b]**	**Accession Number [c]**
Hh 16-9	*B. japonicum* Bj11	Bj11-1	LC582854, LC579849
Hh-16-25	*B. japonicum* Bj11	Bj11-2	LC582860, LC579855
Hk 16-7	*B. elkanii* L7	BeL7	LC582891, LC579886
Kh 16-43	*B. japonicum* S10J	BjS10J-2	LC582874, LC579869
Kh 16-64	*B. japonicum* S10J	BjS10J-4	LC582887, LC579882
Kh 16-7	*B. elkanii* L7	BeL7	LC582901, LC579896

[a] The strains were isolated from nodules of soybean cultivated using Fukagawa (H) and Miyazaki (K) soils at Fukagawa (h) and Miyazaki (k) study locations in 2016. The isolates were designated by soil, location, year, and strain number.

[b] Group based on gene sequence of 16S-23S rRNA internal transcribes spacer (ITS) region.

[c] Gene accession number of 16S rRNA and ITS sequences.

Table 2. Geographical and climatic characteristics of the study locations in Japan Hafiz et al. (2021).

Location	**Latitude (°N)**	**Longitude (°E)**	**Temperature (°C)** [a]	**Rainfall (mm)** [a]
Fukagawa	43.71	142.01	16–26/16–26 (14–24/17–27) [b]	432/243
Miyazaki	31.82	131.41	24–32/24–31 (25–32/25–33)	240/860

[a] Average daily minimum and maximum temperatures and total rainfall during the cultivation period in 2016/2017.

[b] Figures in parenthesis indicate those during one month after sowing. (https://www.jma.go.jp).

3.2.2. Effect of Temperature on Competitive Infection of *Bradyrhizobium* spp. in Soybean

For the competition experiment, each set consisting of three strains from each soil was used as follows: *B. japonicum* Hh 16-9 (Bj11-1), *B. japonicum* Hh 16-25 (Bj11-2) and *B. elkanii* Hk 16-7 (BeL7) from the Fukagawa soil; *B. japonicum* Kh 16-43 (Bj10J-2), *B. japonicum* Kh 16-64 (Bj10J-4) and *B. elkanii* Kh 16-7 (BeL7) from the Miyazaki soil.

The strains were cultured in YM liquid medium with shaking at 25 °C for seven days, then each cell density was adjusted to 10^9 colony forming unit (CFU)/mL with sterilized distilled water based on OD-CFU/mL correlated linear equations prepared for each strain (Figure S1). Each one milliliter aliquot of the culture was added onto sterilized vermiculite in a 400 mL Leonard jar (Leonard, 1943), which was supplemented with sterilized N-free nutrient solution (Murashige and Skoog, 1962) (Table S1). Three jars were prepared for each treatment. After mixing the inoculated vermiculite thoroughly, three soybean seeds, cv. Orihime (non-*Rj*) were sown in each Leonard jar and cultivated in a phytotron (LH-220S, NK system, Osaka, Japan) at 20/18 °C and 30/28 °C in 16/8 h (day/night) cycle with an occasional supply of the N-free nutrient solution. The soybean seeds were surface-sterilized prior to sowing with 70% ethanol for 30 s and then with 2.5% NaOCl solution for 3 min (Shiro et al., 2012), Seedlings were thinned to one plant per jar one week after germination. At three weeks after sowing, length and weight of shoot and root were measured, and number of nodules were counted. Then nodule composition of the inoculated strains was examined using randomly selected ten nodules per plant. Control plants without inoculation were prepared to check contamination, and the experiment was conducted in triplicate. Each nodule was surface sterilized with 70% ethanol for 30 s followed by washing with sterilized distilled water six times, then each nodule was crushed with 200 µL of sterilized MilliQ water for extraction of DNA (Saeki et al., 2005). The inoculated strain in each nodule was specified by PCR and nucleotide sequence of the 16S-23S rRNA internal transcribed spacer (ITS) region, according to the procedures described previously Hafiz et al. (2021).

3.2.3. Statistical Analysis

Statistical analysis of the soybean growth and nodule compositions of *Bradyrhizobium* spp. were performed using the MSTAT-C 6.1.4 software package (Freed, 2007). The data were subjected to Duncan's multiple range test after one-way ANOVA.

3.3. Results

3.3.1. Effect of Temperature on Growth of *Bradyrhizobium* spp. Strains in Liquid Culture

Effect of temperature on the proliferation of the *Bradyrhizobium* spp. strains are presented in Figure 1. The responses to different temperatures varied among the strains. At 15–20 °C, the growth rates of *B. japonicum* Bj11-1 and Bj11-2 were similar and higher than that of *B. elkanii* BeL7 in the Fukagawa strains, and the similar growth patterns were observed in *B. japonicum* BjS10J-2 and BjS10J-4, and *B. elakanii* BeL7 in the Miyazaki strains. At 25–35 °C, *B. elkanii* BeL7 proliferated better than the *B. japonicum* strains in the Fukagawa strains, and *B. japonicum* Bj11-1 did not proliferate at 35 °C. Similarly, in the Miyazaki strains, the growth rate of *B. elkanii* BeL7 increased at high temperatures, while those of the *B. japonicum* strains decreased at 30–35 °C, and *B. japonicum* BJS10J-2 did not proliferate at 35 °C.

For each strain, OD_{660} at 5 days of incubation was shown in Figure 2 and normalized as a relative % of OD_{660} to the maximum value in a range of temperatures examined. In the Fukagawa strains, the relative % of Bj11-1 and Bj11-2 were 93–100% at 15–20 °C, while those of BeL7 were 11–13%. The relative % of all strains were more than 80% at 25 °C. At higher temperatures, those of Bj11-1 and Bj11-2 decreased significantly at above 25 and 30 °C, respectively, while those of Bel7 were similar at 25–35 °C. In the Miyawaki strains, BjS10J-2 showed larger relative % than BjS10j-4 at lower temperatures, and those of BeL7 were less than 20%. At higher temperature, those of BjS10J-2, BjS10j-4 and Bel7 decreased significantly at above 20, 25 and 30 °C, respectively.

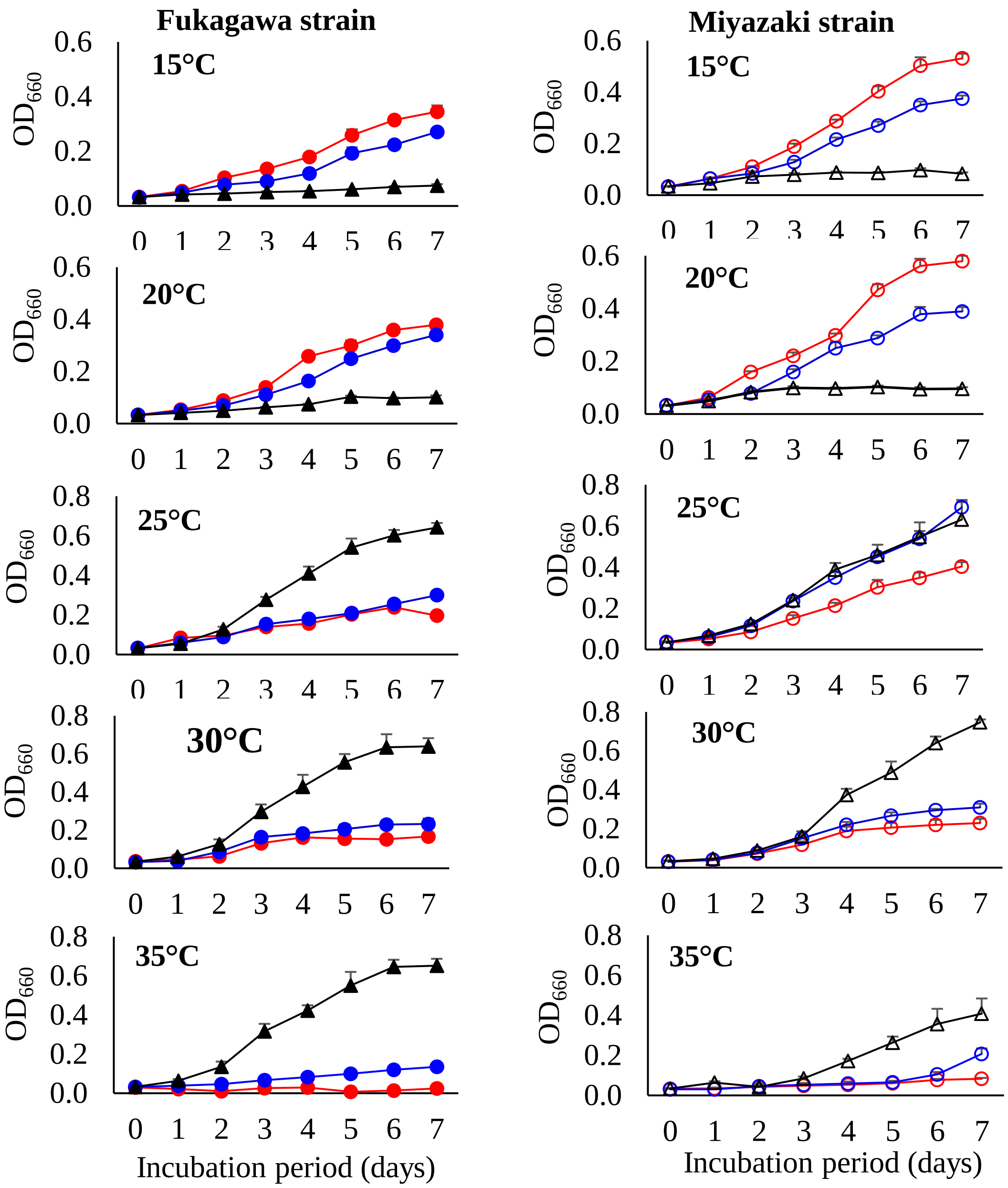

Figure 1. Effects of temperature on growth of *Bradyrhizobium* spp. strains in liquid culture. Fukagawa strains: *B. japonicum* Hh 16-9 (Bj11-1) (●), *B. japonicum* Hh 16-25 (Bj11-2) (●), B. *elkanii* Hk 16-7 (BeL7) (▲), Miyazaki strains: *B. japonicum* Kh 16-43 (BjS10J-2) (○), *B. japonicum* Kh 16-64 (BjS10J-4) (○), and *B. elkanii* Kh 16-7 (BeL7) (Δ). The bars represent the standard deviation (*n* = 3).

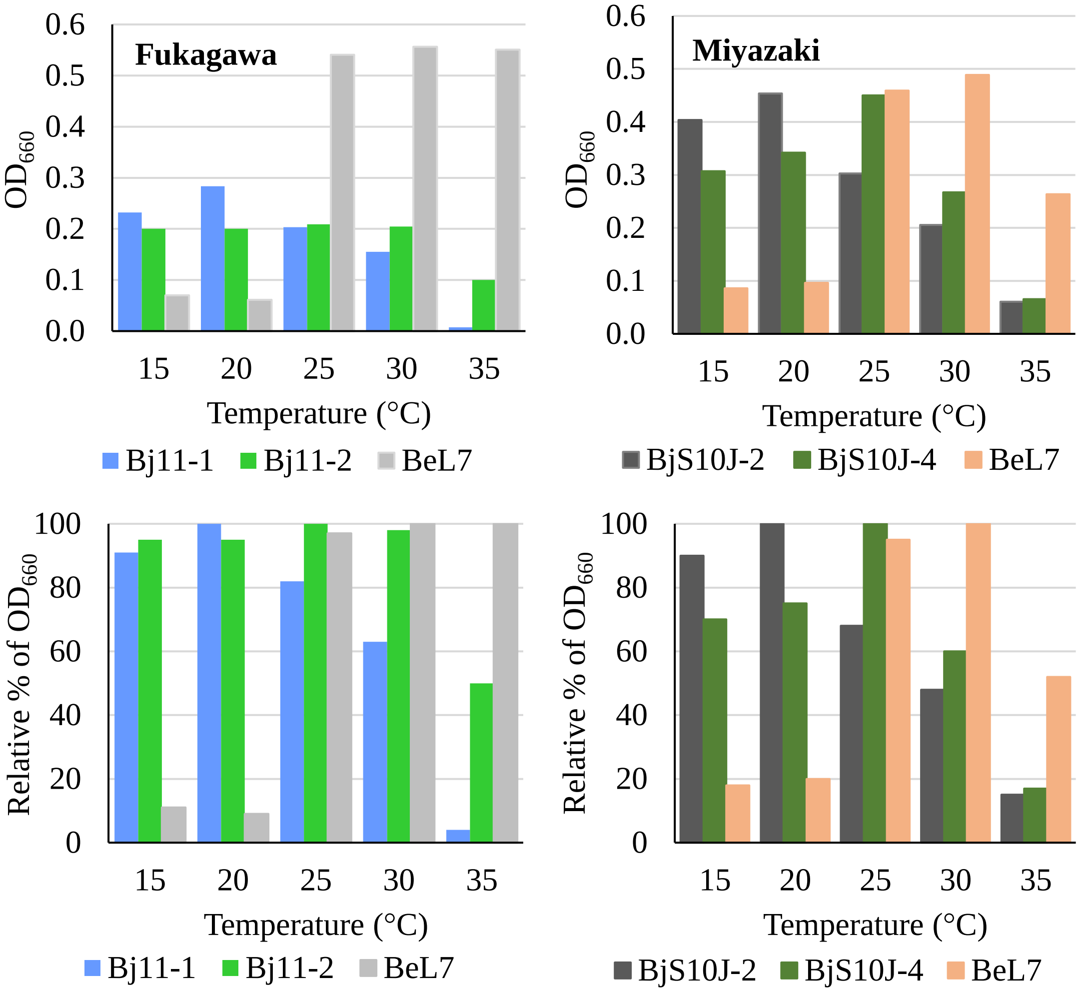

Figure 2. Effects of temperature on growth of *Bradyrhizobium* spp. strains in liquid culture. Upper: OD$_{660}$ at 5 days, Lower: Relative percentage of OD$_{660}$ to maximum for each strain. Fukagawa strains: *B. japonicum* Hh 16-9 (Bj11-1) (🟦), *B. japonicum* Hh 16-25 (Bj11-2) (🟩), B. *elkanii* Hk 16-7 (BeL7) (⬜), Miyazaki strains: *B. japonicum* Kh 16-43 (BjS10J-2) (■), *B. japonicum* Kh 16-64 (BjS10J-4) (🟩), and *B. elkanii* Kh 16-7 (BeL7) (🟧).

3.3.2. Effect of Temperature on Growth and Nodule Number of Soybean Inoculated with a Set of *Bradyrhizobium* spp. Strains

Effect of temperature on the growth and nodule number of soybean is presented in Figure 3. The shoot and root lengths, and the shoot and root weights were significantly higher at 30/28 °C than 20/18 °C in all treatments except for the root lengths of the soybeans inoculated with Miyazaki strains. While the nodule numbers were not significantly different between the different temperature conditions. The inoculation of the *Bradyrhizobium* spp. strains affected significantly on the shoot length and the root weight of soybean at 30/28 °C while the effects were not observed at 20/18 °C. Significant difference in the effects was not present between the Fukagawa and Miyazaki strains. No nodule was recorded in the control plants, indicating that there was no contamination in the experimental procedure.

3.3.3. Effect of Temperature on Soybean Nodule Composition of Inoculated *Bradyrhizobium* spp. Stains

The relative nodule composition of the inoculated *Bradyrhizobium* spp. strains is presented in Figure 4. Under the competitive conditions for the Fukagawa strains, only Bj11-1 formed the nodules at 20/18 °C, while only BeL7 did at 30/28 °C. For the Miyazaki strains, BjS10J-2 was dominant in the nodules at 20/18 °C with the minor presence of BjS10J-4. At high temperature (30/28 °C) BeL7 was dominant and BjS10J-4 was minor in the nodules. Mixed colonization of nodules with two or three strains in the same nodule would be possible, but minor signals were not visibly observed in the nucleotide chromatogram.

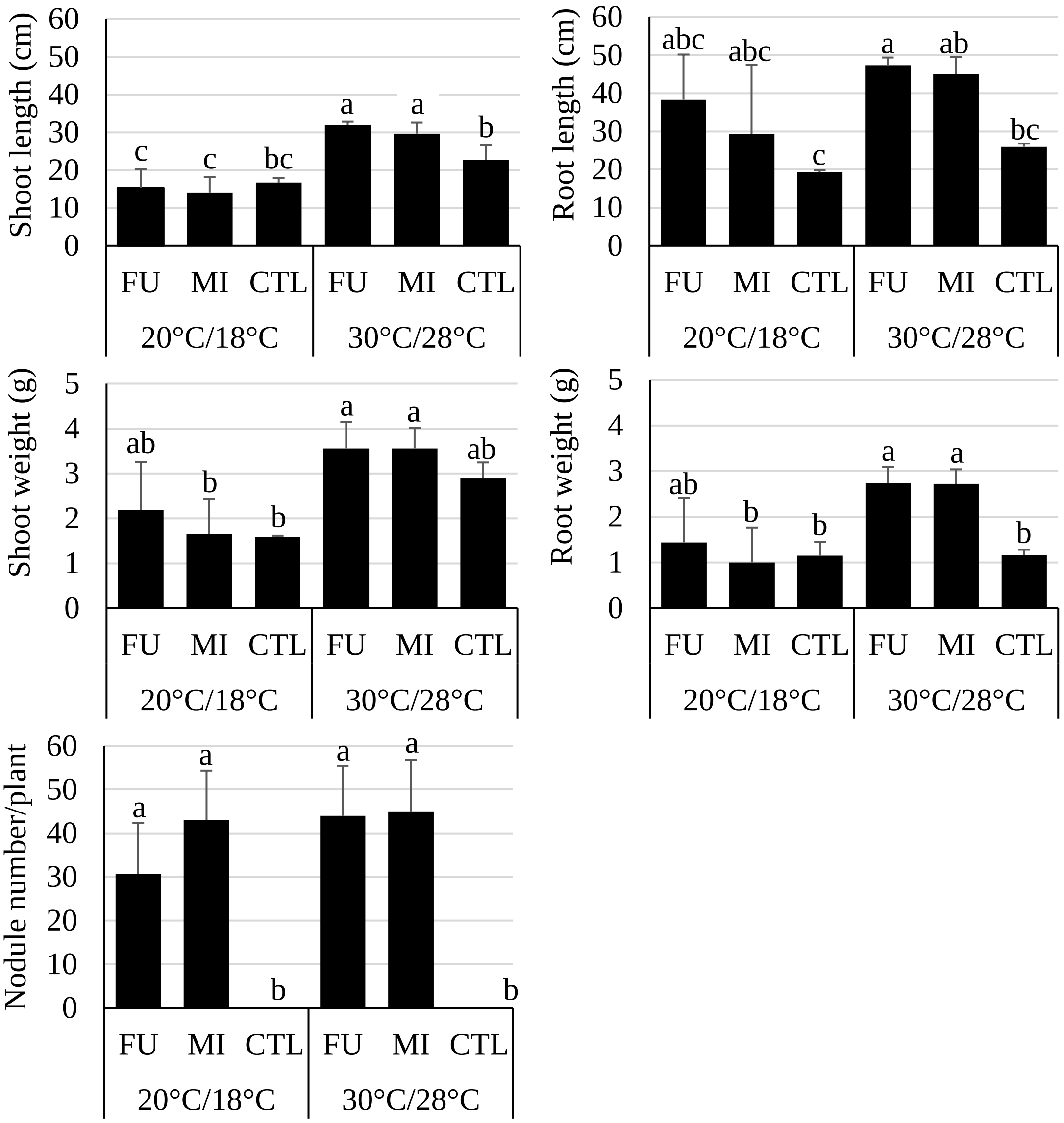

Figure 3. Effects of temperature on growth and nodule number of soybean inoculated with a mixture of the *Bradyrhizobium* spp. strains. Soybean was cultivated in a phytotron at 20/18 °C (day/night) and 30/28 °C at 16/8 h cycle. FU: *B. japonicum* Hh 16-9 (Bj11-1), *B. japonicum* Hh 16-25 (Bj11-2), and *B. elkanii* Hk 16-7 (BeL7), MI: *B. japonicum* Kh 16-43 (Bj10J-2), *B. japonicum* Kh 16-64 (Bj10J-4), and *B. elkanii* Kh 16-7 (BeL7). CTL: no inoculation. The bars represent the standard deviation (n = 3) and different letters indicate significant differences at $p < 0.05$ by Duncan's test.

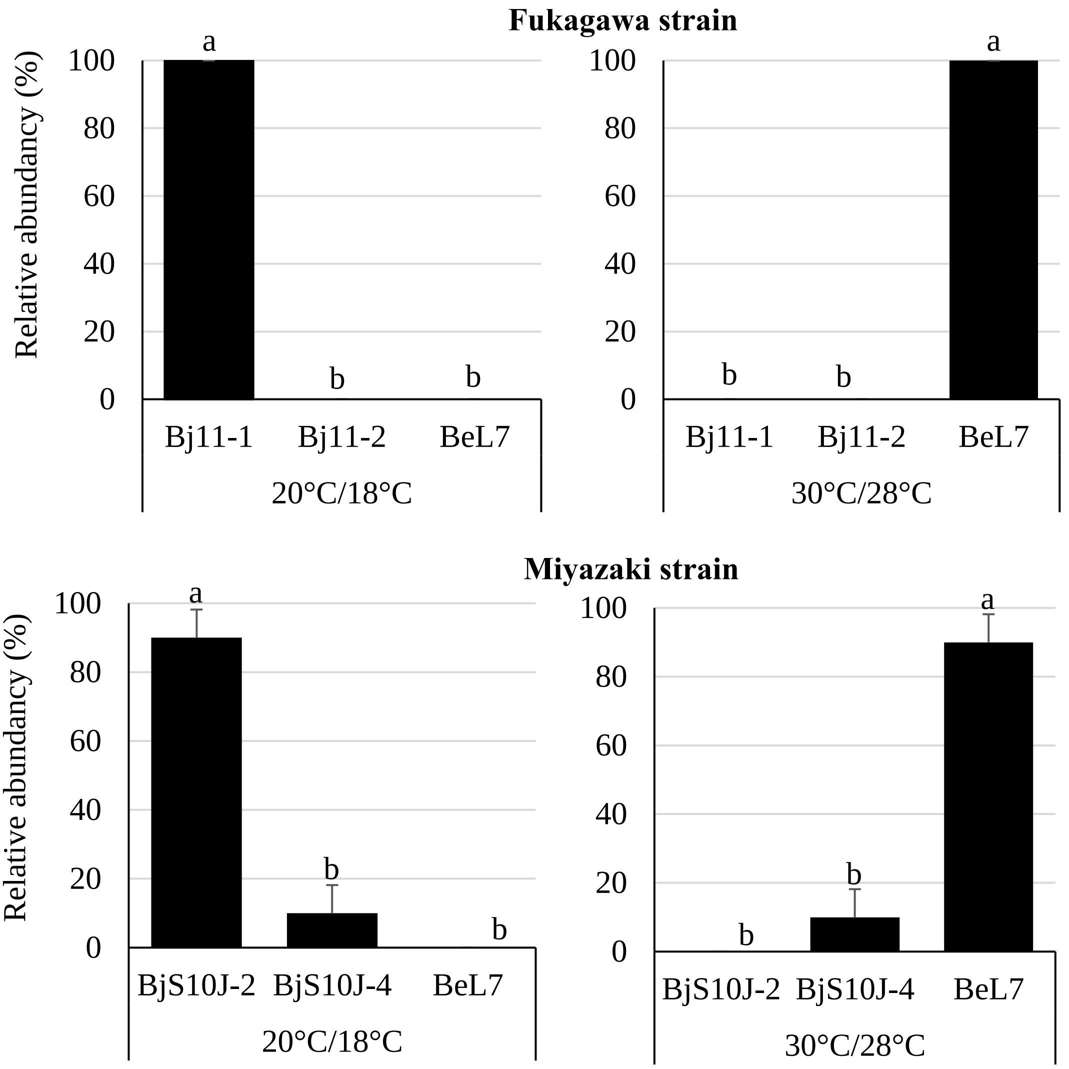

Figure 4. Effects of temperature on relative abundancy of inoculated *Bradyrhizobium* spp. strains in soybean. Soybean was cultivated in a phytotron at 20/18 °C (day/night) and 30/28 °C at 16/8 h cycle. Fukagawa strains: *B. japonicum* Hh 16-9 (Bj11-1), *B. japonicum* Hh 16-25 (Bj11-2), and *B. elkanii* Hk 16-7 (BeL7), Miyazaki strains: *B. japonicum* Kh 16-43 (BjS10J-2), *B. japonicum* Kh 16-64 (BjS10J-4), and *B. elkanii* Kh 16-7 (BeL7). The bars represent the standard deviation (n = 3) and different letters indicate significant differences at $p < 0.05$ by Duncan's test.

3.4. Discussion

Although the number is limited, the similar temperature-dependent growth tendency in liquid media of the two *Bradyrhizobium* species have been reported previously. Three *B. japonicum* strains grew better at 15 °C than 25 °C, and could not grow at 35 °C, while one *B. elkanii* strain grew better at 25–35 °C than 15 °C (Saeki et al., 2010). Kluson et al. (1986) also reported that optimum growth of two *B. elkanii* strains was around 25 °C while two *B. japonicum* strains grew best at 20 °C in the range of 20–35 °C. These results suggest that *B. japonicum* and *B. elkanii* have species-specific temperature preference in their proliferations. The tendencies are consistent with the previous results on the latitudinal characteristic nodulation of *B. japonicum* and *B. elkanii* in Japan (Hafiz et al., 2021; Saeki et al., 2006), the United States (Shiro et al., 2013) and Nepal (Adhikari et al., 2012).

In the infection experiment, we used the sterilized vermiculite to simplify the experimental conditions, the same population of the inoculants, and elimination of effects of indigenous soil microorganisms on the competition. Sterilization of soil samples by autoclaving could change its physicochemical conditions. Actually, population of the inoculated rhizobia decreased in the sterilized Fukagawa soil due to unknown reasons in a preliminary experiment (data not shown). Then, we could not use the soil samples in this study.

The better growth of soybean at higher temperature has been reported previously in the similar range of temperatures (Kluson et al., 1986; Stoyanova, 1996; Zhang and Smith, 1994). The number of nodules was temperature-independent in this study (Figure 3), while temperature-dependent nodule formation, that is, the higher temperature, the larger nodule number in the similar temperature range with this study, has been reported when *B. japonicum* strains were inoculated in laboratory experiments (Stoyanova, 1996; Montañez et al., 1995). In this study, the *B. japonicum* and *B. elkanii* strains were co-inoculated and the different strain was dominant among the inoculated strains in the nodules depending on the temperature (Figure 4), therefore, the nodule number would be dependent on nodulating properties of the dominant strains in the nodules rather than temperature.

The high nodule dominancy of *B. elkanii* BeL7 (Hk 16-7 and Kh 16-7) at high temperature (30/28 °C) is presumed to be due to the difference in temperature sensitivity between the *B. japonicum* and *B. elkanii* strains (Figure 2), in addition to the up-regulated expression of *nodC* in *B. elkanii* at high temperature compared with *B. japonicum* (Shiro et al., 2016). The temperature-dependent growth properties of the *Bradyrhizobium* spp. strains suggests high nodule dominancy of the *B. japonicum* strains at low temperature (20/18 °C). However, one of the two *B. japonicum* strains for each soil was dominant in the nodules even though their growth properties were similar (Figure 1). Difference in their expression levels of nodulation genes and response to isoflavones secreted from soybean roots might determine the nodule composition between them. The same temperature-dependent nodule composition; dominancy of B. *japonicum* and *B. elkanii* at low and high temperatures, respectively, has been reported in the other laboratory competitive studies (Kluson et al., 1986; Suzuki et al., 2014; Shiro et al., 2016).

Generally, composition of soybean rhizobia in a field soil has been estimated by nodule composition. Regarding the latitudinal characteristic nodule composition of soybean rhizobia (Hafiz et al., 2021; Saeki et al., 2006; Shiro et al., 2013; Adhikari et al., 2012), the competitive inoculation experiments have revealed that the nodule composition is affected by species-specific temperature-dependent infection and proliferation in soils (Kluson et al., 1986; Suzuki et al., 2014; Shiro et al., 2016). However, it is uncertain which factor contributes to the temperature-dependent nodule composition.

In our previous study (Hafiz et al., 2021), we selected three study locations of different local climatic conditions in Japan, and each soil sample of the study locations was used for soybean cultivation at all the study locations to examine the changes in the nodule compositions under the different local climatic conditions. As a result, we assumed that *B. japonicum* dominantly proliferate in the Fukagawa soil and lead to their dominant nodule composition because the nodule composition was not affected under warmer climatic conditions in Miyazaki location. To confirm our assumption, the competitive inoculation experiment was conducted using the rhizobial strains isolated from soybean nodules cultivated in Fukagawa soil, and the results showed that *B. japonicum* dominated nodules at lower temperature while *B. elkanii* did at higher temperature (Figure 4), supporting our assumption that *B. japonicum* dominantly proliferate in the Fukagawa soil because the dominancy of *B. elkanii* did not increase at higher temperature in Miyazaki location.

We also assumed that both *B. japonicum* and *B. elkanii* exist in the Miyazaki soil and the dominant nodule composition of *B. elkanii* is due to their preferred infection because the nodule composition was affected under cooler climatic conditions in Fukagawa location. In the competitive inoculation experiment using the Miyazaki rhizobial strains, *B. japonicum* and *B. elkanii* dominated nodules at lower and higher temperatures, respectively (Figure 4), also supporting our assumption that both *B. japonicum* and *B. elkanii* exist in the Miyazaki soil and their preferred infection determined the nodule composition.

3.5. Conclusions

The experiments performed in the liquid cultures revealed better growth of *B. japonicum* at lower temperatures and *B. elkanii* at higher temperatures, and therefore it can be assumed that temperature of soil affects rhizobia growth in rhizosphere and could be a reason for different competitive properties of *B. japonicum* and *B. elkanii* strains at different temperatures. In addition, competitive infection was suggested between the *B. japonicum* strains.

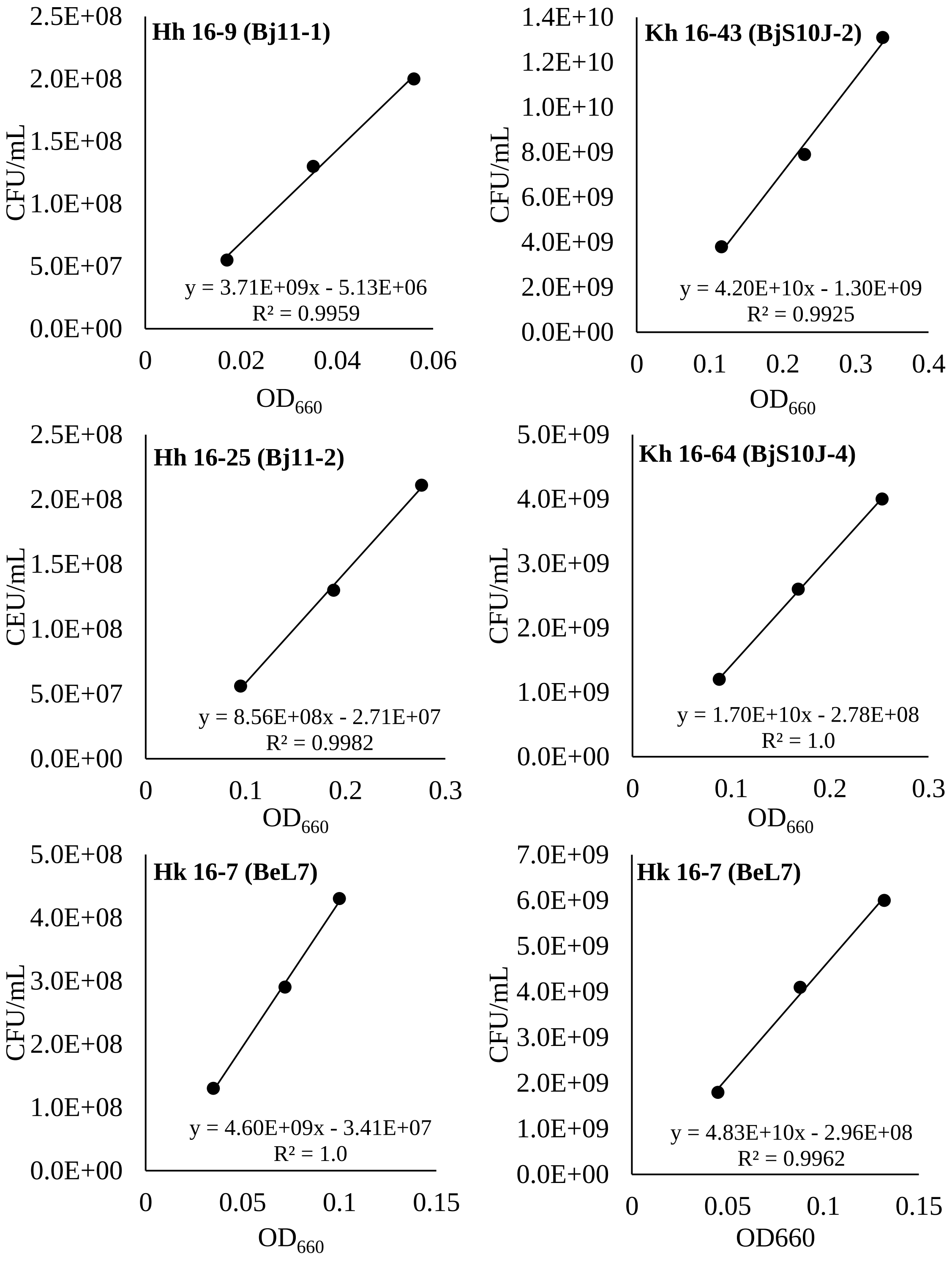

Figure S1. Standard curve of OD_{660} versus CFU/mL for different strains used in this study. In each equation y = CFU/mL and x= OD_{660} of the specific strain mentioned in the figure.

Table S1. Ingredients of nitrogen (N) free Murashige and Skoog (MS) media.

Ingredient	**Constituent**	**Final concentration (mgL^{-1})**
Macro nutrient	NH_4NO_3	0.0
	KCl	1400
	$CaCl_2.7H_2O$	440
	$MgSO_4.7H_2O$	370
	KH_2PO_4	170
Micronutrient	KI	0.83
	H_3BO_3	6.2
	$MnSO_4.4H_20$	22.3
	$ZnSO_4.7H_2O$	8.6
	$Na_2MoO_4.2H_2O$	0.25
	$CuSO_4.5H_2O$	0.025
	$CoCl_2.6H_2O$	0.025
	$Na_2.EDTA$	37.3
	$FeSO_4.7H_2O$	27.8
Vitamins and organics	Pyridoxine (HCl)	0.5
	Thiamine (HCl)	0.1
	Myoinositol	100
	Glycine	2.0
	Nicotinic acid	0.5
pH (adjusted)		5.78

Source: Murashige and Skoog, 1962.

References

Abdel-Wahab, A.M.; Abd-Alla, M.H. Effect of different rates of N-fertilizers on nodulation, nodule activities and growth of two field grown cvs. of soybean. *Fertil. Res.* **1996**, *43*, 37–41.

Adhikari, D.; Kaneto, M.; Itoh, K.; Suyama, K.; Pokharel, B.B.; Gaihre, Y.K. Genetic diversity of soybean-nodulating rhizobia in Nepal in relation to climate and soil properties. *Plant Soil* **2012**, 357, 131–145.

Afzal, A.; Bano, A.; Fatima, M. Higher soybean yield by inoculation with N-fixing and P-solubilizing bacteria. *Agron. Sust. Dev.* **2010**, *30*, 487–495.

Altschul, S.F.; Madden, T.L.; Schäffer, A.A.; Zhang, J.; Zhang, Z.; Miller, W.; Lipman, D.J. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* **1997**, 25, 3389–3402.

Ando, S.; Yokoyama, T. Phylogenetic analyses of *Bradyrhizobium* strains nodulating soybean (*Glycine max*) in Thailand with reference to the USDA strains of *Bradyrhizobium*. *Can. J. Microbiol.* **1999**, *45*, 639–645.

Andrews, M.; Andrews, M.E. Specificity in legume-rhizobia symbioses. *Int. J. Mol. Sci.* **2017**, *18*, 705.

Appunu, C.; N'Zoue, A.; Laguerre, G. Genetic diversity of native Bradyrhizobia isolated from soybeans (*Glycine max* L.) in different agricultural-ecological-climatic regions of India. *Appl. Environ. Microbiol.* **2008**. *74*, 5991–5996.

Appunu, C.; Sasirekha, N.; Prabavathi, V.R.; Nair, S. A significant proportion of indigenous rhizobia from India associated with soybean (*Glycine max* L.) distinctly belong to *Bradyrhizobium* and *Ensifer* genera. *Biol. Fertil. Soils* **2009**, *46*, 57–63.

Berrabah, F.; Ratet, P.; Gourion, B. Legume nodules: massive infection in the absence of defense induction. *Mol. Plant-Microbe Interact.* **2018**, *32,* 35–44.

Buhian, W.P.; Bensmihen, S. Mini-review: nod factor regulation of phytohormone signaling and homeostasis during rhizobia-legume symbiosis. *Front. Plant Sci.* **2018**, *9*, 1247.

Burén, S.; Rubio, L.M. State of the art in eukaryotic nitrogenase engineering. *FEMS Microbiol. Lett.* **2017**, *365*, 274.

Chebotar, V.K., Asis Jr., C. A.; Akao, S. Production of growth-promoting substances and high colonization ability of rhizobacteria enhance the nitrogen fixation of soybean when coinoculated with *Bradyrhizobium japonicum*. *Biol. Fertil. Soils* **2001**, *34*, 427–432.

Chen, L.S.; Figueredo, A.; Pedrosa, F.O.; Hungria, M. Genetic characterization of soybean rhizobia in Paraguay. *Appl. Environ. Microbiol.* **2000**, *66*, 5099–5103.

Chen, W.; Wang, E.; Wang, S.; Li, Y.; Chen, X.; Li, Y. Characteristics of Rhizobium tianshanense sp. nov., a moderately and slowly growing root nodule bacterium isolated from an arid saline environment in Xinjiang, People’s Republic of China. *Int. J. Syst. Bacteriol.* **1995**, 45, 153–159.

Chen, W.X.; Yan, G.H.; Li, J.L. Numerical taxonomic study of fast-growing soybean rhizobia and a proposal that *Rhizobium fredii* be assigned to *Sinorhizobium* gen. nov. *Int. J. Syst. Bacteriol.* **1988**, *38*, 392–397.

de Lajudie, P.M.; Andrews, M.; Ardley, J.; Eardly, B.; Jumas-Bilak, E.; Kuzmanovic, N.; Lassalle, F.; Lindström, K.; Mhamdi, R.; Martínez-Romero, E.; Moulin, L.; Mousavi, S.A.; Nesme, X.; Peix, A.; Puławska, J.; Steenkamp, E.; Stępkowski, T.; Tian, Chang-Fu., Vinuesa, P.; Wei, G.; Willems, A.; Zilli, J.; Young, P. Minimal standards for the description of new genera and species of rhizobia and agrobacteria. *Int. J. Syst. Evol. Microbiol.* **2019**, *69*, 1852-1863.

Dupare, B.U.; Billore, S.D.; Joshi, O.P.; Husain, S.M. Origin, domestication, introduction, and success of soybean in India. *Asian Agri-history* **2008**, *12*, 179–195.

Franssen, H.; Mylona, P.; Pawlowski, K.; Van De, S.K.; Heidstra, R.; Geurts, R.; Kozik, A.; Matvienko, M.; Yang-Wei, C.; Hadri, Az-Eddine.; Martinez-Abarca, F.; Bisseling, T. Plant genes involved in root-nodule development on legumes. *Phil. Trans. R. Soc. Lond. B.* **1995**, *350*, 101–107.

Freed, R. MSTAT: A software program for plant breeder. In *Principles of Plant Genetics and Breeding*, 2nd ed.; Acquaah, G., Ed.; Blackwell Publishing: Malden, MA, USA, **2007**; Volume 1, pp. 426–431.

Fuleky, G. Cultivated plants, primarily as food sources. EOLSS Publications: **2009** Volume I**,** pp. 1-372.

Garrity, G.; Bell, J.; Lilburn, T. Class I. Alphaproteobacteria class. nov. In Bergey's Manual of Systematic Bacteriology, Brenner, D., Krieg, N., Staley, J., Ed.; Springer US, New York, **2005**; pp. 1–574.

Hafiz, M.H.R.; Salehin, A.; Adachi, F.; Omichi, M.; Saeki, Y.; Yamamoto, A.; Hayashi, S.; Itoh, K. Latitudinal Characteristic Nodule Composition of Soybean-Nodulating Bradyrhizobia: Temperature-Dependent Proliferation in Soil or Infection? *Horticulturae* **2021**, *7*, 22.

Han, L.L.; Wang, T.T.; Han, T.X.; Liu, J.; Sui, X.H.; Chen, W.F.; Chen, W.X. Unique community structure and biogeography of soybean rhizobia in the saline-alkaline soils of Xinjiang, China. *Plant Soil* **2009**, *324*, 91–305.

Hiraishi, A.; Inagaki, K.; Tanimoto, Y.; Iwasaki, M.; Kishimoto, N.; Tanaka, I. Phylogenetic characterization of a new thermoacidophilic bacterium isolated from hot springs in Japan. *J. Gen. Appl. Microbiol.* **1997**, 43, 295–304.

Hirsch, A.M. Developmental biology of legume nodulation. *New Phytol.* **1992**. *122***,** 211–237.

Hungria, M.; Campo, R.J.; Mendes, I.C.; Graham, P. Contribution of biological nitrogen fixation to the N nutrition of grain crops in the tropics: the success of soybean [*Glycine max* L. Merr.] in South America. In: Singh, R.P., Shankar, N., Jaiwal, P.K., Ed.; Nitrogen Nutrition and Sustainable Plant Productivity. Studium Press, LLC, Houston, Texas, **2006**; pp. 43–93.

Hungria, M.; Vargas, M.A.T. Environmental factors affecting N2 fixation in grain legumes in the tropics, with an emphasis on Brazil. *F. Crop Res.* **2000**, 65, 151–164.

Hungria, M; Mendes, I.C.; de Bruijn, F. Nitrogen fixation with soybean: the perfect symbiosis? In *Biological Nitrogen Fixation*, vol. 2; De Bruijn F., Ed.; Wiley Publisher, NJ, USA, **2015**, pp. 1005-1019.

Hymowitz, T. On the domestication of soybean. *Econ. Bot.* **1970**, *24*, 408-421.

Hymowitz, T.; Harlan, J.R. Introduction of soybean to north America by Samuel Bowen in 1765. *Econ. Bot.* **1983**, *37*, 371–379.

Hymowitz, T.; Singh, R.J. Taxonomy and speciation. In *Soybeans, Improvement, Production and Uses*, 2nd. ed.: Wilcox J.R., Ed.; American Society of Agronomy, Madison, WI, USA, **1987**, pp. 23-48.

Jordan, D.C. Transfer of *Rhizobium japonicum* Buchanan 1980 to *Bradyrhizobium* gen. nov., a genus of slow-growing, root nodule bacteria from leguminous plants. *Int. J. Syst. Bacteriol*. **1982**, *32*, 136–139.

Khojely, D.M.; Ibrahim, S.E.; Sapey, E.; Han, T. History, current status, and prospects of soybean production and research in sub-Saharan Africa. *Crop J.* **2018**, *6*, 226–235.

Kluson, R.A.; Kenworthy, W.J.; Weber, D.F. Soil temperature effects on competitiveness and growth of *Rhizobium japonicum* and on *Rhizobium*-induced chlorosis of soybeans. *Plant Soil* **1986**, 95, 201–207.

Konno, S. Soybean Cultivation in Japan. *Jpn. Agril. Res. Quart.* **1970**, *5*, 21-27.

Konno, S. Soybean production in Japan. *Jpn. Agril. Res. Quart.* **1983**, *17*, 95-102.

Kumar, S.; Stecher, G.; Tamura, K. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Mol. Biol. Evol.* **2016**, *33*, 1870–1874.

Kyukendall, L.D.; Saxena, B.; Cevine, T.E.; Udell, S.E. Genetic diversity in *Bradyrhizobium japonicum* Jordan 1982 and a proposal for *Bradyrhizobium elkanii* sp. nov. *Can. J. Microbiol.* **1992**, *38*, 501–505.

Laranjo, M.; Alexandre, A.; Oliveira, S. Legume growth-promoting rhizobia: an overview on the *Mesorhizobium* genus. *Microbiol. Res.* **2014**, *169*, 2–17.

Larkin, M.A.; Blackshields, G.; Brown, N.P.; Chenna, R.; McGettigan, P.A.; McWilliam, H.; Valentin, F.; Wallace, I.M.; Wilm, A.; Lopez, R.; Thompson, J.D.; Gibson, T.J.; Higgins, D.G. Clustal W and Clustal X version 2.0. *Bioinformatics* **2007**, *23*, 2947–2948.

Leonard, L.T. A simple assembly for use in the testing of cultures of rhizobia. *J. Bacteriol.* **1943**, *45*, 523–527.

Li, Q.Q.; Wang, E.T.; Zhang, Y.Z.; Zhang, Y.M.; Tian, C.F.; Sui, X.H.; Chen, W.F.; Chen, W.X. Diversity and biogeography of rhizobia isolated from root nodules of *Glycine max* grown in Hebei province, China. *Microb. Ecol.* **2011**, *61*, 917–931.

Lindström, K.; Mousavi, S.A. Effectiveness of nitrogen fixation in rhizobia. *Microb. Biotec.* **2020**, *13*, 1314-1335.

Lloret, L.; Martinez-Romero, E. Evolución y filogenia de *Rhizobium*. *Latinoam. Microbiol.* **2005**, *47*: 43–60.

Long, S.R. *Rhizobium* symbiosis: Nod factors in perspective. *Plant Cell* **1996**, *8*,1885–1898.

Man, C.X.; Wang, H; Chen, W.F.; Sui, X.H.; Wang, E.T.; Chen, W.X. Diverse rhizobia associated with soybean grown in the subtropical and tropical regions of China. *Plant Soil* **2008**, *310*,77–87

McAdam, E.L.; Reid, J.B.; Foo, E. Gibberellins promote nodule organogenesis but inhibit the infection stages of nodulation. *J. Exp. Bot.* **2018**, *69*, 2117–2130.

Montañez, A.; Danso, S.K.A.; Hardarson, G. The effect of temperature on nodulation and nitrogen fixation by five *Bradyrhizobium japonicum* strains. *Appl. Soil Ecol.* **1995**, *2*, 165–174.

Moulin, L.; Béna, G.; Boivin-Masson, C.; Stężpkowski, T. Phylogenetic analyses of symbiotic nodulation genes support vertical and lateral gene co-transfer within the *Bradyrhizobium* genus. *Mol. Phylogenet. Evol*. **2004**, *30*, 720–732.

Mousavi, S.A. Revised taxonomy of the family Rhizobiaceae, and phylogeny of mesorhizobia nodulating *Glycyrrhiza* spp. Dissertationes Schola Doctoralis Scientiae Circumiectalis, Alimentariae, Biologicae. University of Helsinki, 2016. Pp.1-47. [PhD Thesis].

Murashige, T.; Skoog, F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *J. Plant Physiol.* **1962**, *15*, 473–497.

Oldroyd, G.E.D.; Downie, J.A. Coordinating nodule morphogenesis with rhizobial infection in legumes. *Annu. Rev. Plant. Biol*. **2008**, *59*, 519–546.

Peng, G.X.; Tan, Z.Y.; Wang, E.T.; Reinhold-Hurek, B.; Chen, W.F.; Chen, W.X. Identification of isolates from soybean nodules in Xinjiang Region as *Sinorhizobium xinjiangense* and genetic differentiation of *S. xinjiangense* from *Sinorhizobium fredii*. Int. J. Sys. *Evol. Microbiol.* **2002**, *52*, 457–462.

Polhill, R.M.; Raven, P.H. Advances in Legume Systematics. Royal Botanic Gardens: **1981**, Part 2, pp. 1-425.

Poole, P.; Ramachandran, V.; Terpolilli, J. Rhizobia: from saprophytes to endosymbionts. *Nat. Rev. Microbiol.* **2018**, *16,* 291-303.

Postgate J. Nitrogen Fixation. Cambridge University Press, Cambridge UK, 3rd Ed, **1998**, pp.1–112.

Puri, R.R.; Adachi, F.; Omichi, M.; Saeki, Y.; Yamamoto, A.; Hayashi, S.; Itoh, K. Culture-dependent analysis of endophytic bacterial community of sweet potato (*Ipomoea batatas*) in different soils and climates. *J. Adv. Microbiol.* **2018**, *13,* 1–12.

Risal, C.P.; Yokoyama, T.; Ohkama-Ohtsu, N.; Djedidi, S.; Sekimoto, H. Genetic diversity of native soybean bradyrhizobia from different topographical regions along the southern slopes of the Himalayan Mountains in Nepal. *Syst. Appl. Microbiol.* **2010**, *33,* 416–425.

Saeki, Y.; Aimi, N.; Hashimoto, M.; Tsukamoto, S.; Kaneko, A.; Yoshida, N.; Nagatomo, Y.; Akao, S. Grouping of *bradyrhizobium* USDA strains by sequence analysis of 168 rRDNA and 168-238 rDNA internal transcribed spacer region. *Soil Sci. Plant Nutr.* **2004**, *50,* 517–525.

Saeki, Y.; Aimi, N.; Tsukamoto, S.; Yamakawa, T.; Nagatomo, Y.; Akao, S. Diversity and geographical distribution of indigenous soybean-nodulating bradyrhizobia in Japan. *Soil Sci. Plant Nutr.* **2006**, *52*, 418–426.

Saeki, Y.; Kaneko, A.; Hara, T.; Suzuki, K.; Yamakawa, T.; Nguyen, M.T.; Nagatomo, Y.; Akao, S. Phylogenetic analysis of soybean-nodulating rhizobia isolated from alkaline soils in Vietnam. *Soil Sci. Plant Nutr.* **2005**, *51*, 1043–1052.

Saeki, Y.; Ozumi, S.; Yamamoto, A.; Umehara, Y.; Hayashi, M.; Sigua, G.C. Changes in population occupancy of bradyrhizobia under different temperature regimes. *Microbes Environ.* **2010**, *2*, 309–312.

Shiro, S.; Kuranaga, C.; Yamamoto, A.; Sameshima-Saito, R.; Saeki, Y. Temperature-dependent expression of NodC and community structure of soybean-nodulating bradyrhizobia. *Microbes Environ.* **2016**, *31*, 27–32.

Shiro, S.; Matsuura, S.; Saiki, R.; Sigua, G.C.; Yamamoto, A.; Umehara, Y.; Hayashi, M.; Saeki, Y. Genetic diversity and geographical distribution of indigenous soybean-nodulating bradyrhizobia in the United States. *Appl. Environ. Microbiol.* **2013**, *79*, 3610–3618.

Shiro, S.; Yamamoto, A.; Umehara, Y.; Hayashi, M.; Yoshida, N.; Nishiwaki, A.; Saeki, Y. Effect of Rj genotype and cultivation temperature on the community structure of soybean-nodulating bradyrhizobia. *Appl. Environ. Microbiol.* **2012**, *78*, 1243–1250.

Simpson, F.B.; Burris, R.H. A nitrogen pressure of 50 atmospheres does not prevent evolution of hydrogen by nitrogenase. *Science* **1984**, *224,*1095–1097.

Sprent, J.I. 60Ma of legume nodulation. What's new? What's changing? *J. Exp. Bot.* **2008**, *59*, 1081–1084.

Stoyanova, J. Growth, nodulation and nitrogen fixation in soybean as affected by air humidity and root temperature. *Biol. Plant* **1996**, *38*, 537–544.

Streeter, J.G. Failure of inoculant rhizobia to overcome the dominance of indigenous strains for nodule formation. *Can. J. Microbiol.* **1994**, *40*, 513–522.

Suzuki, K.; Oguro, H.; Yamakawa, T.; Yamamoto, A.; Akao, S.; Saeki, Y. Diversity and distribution of indigenous soybean-nodulating rhizobia in the Okinawa islands. *Jpn. Soil. Sci. Plant. Nutr.* **2008**, *54*, 237–246.

Suzuki, Y.; Adhikari, D.; Itoh, K.; Suyama, K. Effects of temperature on competition and relative dominance of *Bradyrhizobium japonicum* and *Bradyrhizobium elkanii* in the process of soybean nodulation. *Plant Soil* **2014**, *374,* 915–924.

Tan, Y.Z.; XU, X.D.; Wang, E.T.; Gao, J.I.; Martinez-Romero, E.; Chen, W.X. Phylogenetic and genetic relationships of *Mesorhizobium tianshanense* and related rhizobia. *Int. J. Syst. Bacteriol.* **1997**, *47,* 874–879.

USDA (United States Department of Agriculture), Soybean explorer, https://ipad.fas.usda.gov/cropexplorer/cropview/commodityView.aspx?startrow=11&cropid=2222000&sel_year=2020&rankby=Production.

van Berkum, P.; Fuhrmann, J.J. Evolutionary relationships among the soybean bradyrhizobia reconstructed from 16S rRNA gene and internally transcribed spacer region sequence divergence. *Int. J. Syst. Evol. Microbiol.* **2000**, *50,* 2165– 2172.

Vincent, J.M. A manual for practical study of root nodule bacteria. In *IBP Handbook No. 15*; Blackwell Scientific Publishers, Oxford, **1970**; p.164.

Vinuesa, P.; Leon-Barrios, M.; Silva, C.; Willema, A.; Jarabo-Lorenzo, A.; Perez-Galdona, R.; Warner, D.; Martinez-Romero, E. *Bradyrhizobium canariense* sp. nov., an acid-tolerant endosymbiont that nodulates endemic genistoid legumes (Papilionoideae:Genisteae) growing in the Canary Islands, along with *B. japonicum* bv. Genistearum, *Bradyrhizobium* genospecies α and *Bradyrhizobium* genospecies *β*. *Int. J. Syst. Evol. Microbiol.* **2005**, *55*, 569–575

Vinuesa, P.; Rojas-Jimenez K.; Contreas-Moreira, B.; Mahna, S.K.; Prasad, B.N.; Moe, H.; Selvaraju, S.B.; Theirfelder, H.; Werner, D. Multilocus sequence analysis for assessment of the biogeography and evolutionary genetics of four *Bradyrhizobium* species that nodulate soybeans in the Asiatic Continent. *Appl. Environ. Microbiol.* **2008**, *74*, 6987–6996.

Wasike, V.W.; Lesueur, D.; Wachira, F.N.; Mungai, N.W.; Mumera, L.M.; Sanginga, N.; Mburu, H.N.; Mugadi, D.; Wango, P.; Vanlauwe, B. Genetic diversity of indigenous *Bradyrhizobium* nodulating promiscuous soybean [*Glycine max* (L) Merr.] varieties in Kenya: impact of phosphorus and lime fertilization in two contrasting sites. *Plant Soil* **2009**, *322*, 151–163.

Weisburg, W.G.; Barns, S.M.; Pelletier, D.A.; Lane, D.J. 16S ribosomal DNA amplification for phylogenetic study. *J. Bacteriol.* **1991**, *173*, 697–703.

Willems, A.; Munive, A.; de Lajudie, P.; Gillis, M. In most *Bradyrhizobium* groups sequence comparison of 16S-23S rDNA internal transcribed spacer regions corroborates DNA-DNA hybridizations. *Syst. Appl. Microbiol.* **2003**, *26*, 203–210.

Xia, X.; Ma, C.; Dong, S.; Xu, Y.; Gong, Z. Effects of nitrogen concentrations on nodulation and nitrogenase activity in dual root systems of soybean plants. *Soil Sci. Plant Nutr.* **2017**, *63*, 470-482.

Xu, L.M.; Ge, C.; Cui, Z.; Li, J.; Fan, H. *Bradyrhizobium liaoningense* sp. nov., isolated from the root nodules of soybeans. *Int. J. Syst. Bacteriol.* **1995**, *45*, 706–711.

Yang, J.K.; Zhou, J.C. Diversity, phylogeny and host specificity of soybean and peanut bradyrhizobia. *Biol. Fertil. Soils.* **2008**, *44*, 843–851.

Yao, Z.Y.; Kan, F.L.; Wang, E.T.; Wei, G.H.; Chen, W.X. Characterization of rhizobia that nodulate legume species of the genus Lespedeza and description of *Bradyrhizobium yuanmingense* sp. nov. *Int. J. Syst. Evol. Microbiol.* **2002**, *52,* 2219–2230.

Yokoyama, T. Effects of temperature on competition for nodulation in phylogenetically different *Bradyrhizobium* strains. *Jpn. J. Soil. Sci. Plant. Nutr.* **2005**, *76*, 599–607.

Zhang, F.; Smith, D.L. Effects of low root zone temperatures on the early stages of symbiosis establishment between soybean [*Glycine max* (L.) Merr.] and *Bradyrhizobium japonicum*. *J. Exp. Bot.* **1994**, *45*, 1467–1473.

Zotarelli, L. Balanço de nitrogênio na rotação de culturas em sistemas de plantio direto e convencional na região de Londrina-PR. Universidade Federal Rural do Rio de Janeiro, Seropédica, Brazil. 2000. pp.1-128. [PhD Thesis].

SUMMARY (In English)

Soybean (*Glycine max* [L] Merr.) belongs to the Leguminosae family, and is an important grain legume. Several soybean-nodulating bacteria, which can fix atmospheric nitrogen symbiotically in the nodules of the host plant, have been reported worldwide. Effective nitrogen fixation mainly depends on the potential of the rhizobial strain and their competition for proliferation and infection among indigenous rhizobia in a soil. Because effective nitrogen fixation depends on the potential of an individual rhizobial strain, competition for proliferation and infection among the rhizobial strains in a soil is crucial subject. In addition, their behaviors need to be considered based on environmental conditions.

Bradyrhizobium japonicum and *B. elkanii* are the major soybean nodulating rhizobia and it has been reported that *B. japonicum* and *B. elkanii* dominate in nodules of soybean cultivated in latitudinally northern and southern fields, respectively. Previous reports suggest that temperature-dependent infection and proliferation in a soil determine their nodule compositions. But it has not been elucidated which factor is more responsible for the latitudinally characteristic nodule composition of the bradyrhizobia.

To examine the contribution of two factors in fields under the local climate conditions, we selected three study locations, Fukagawa with temperate continental climate, and Matsue and Miyazaki with humid sub-tropical climate in Japan. Each soil sample was transported to the other study locations, and soybean cv. Orihime (non-*Rj*) was pot-cultivated using three soils at three study locations for successive two years.

Three healthy soybean plants per pot were cultivated in triplicate for ca. 2–3 months without fertilization, and fresh plant weight and number of nodules were measured at harvest. Ten nodules were randomly selected from one plant for each replication, and rhizobial strains were isolated and phylogenetically characterized based on their partial 16S rRNA and 16S–23S rRNA ITS gene sequences, and the nodule composition of *Bradyrhizobium* spp. was determined.

The fresh plant weight and the nodule numbers were not significantly different among soils in both years in all locations with a few exceptions. Overall, the fresh plant weight increased from northern to southern study locations, whereas the nodule number showed the opposite tendency. Reduction in nodules at higher temperature might be due to strain-specific properties.

In this study, two *Bradyrhizobium japonicum* (Bj11 and BjS10J) and one *B. elkanii* (BeL7) were isolated, and Bj11 and BjS10J were phylogenetically sub-grouped into two (Bj11-1-2) and four clusters (BjS10J-1-4) based on their ITS sequence, respectively. Bj11-1 was characterized as slow grower and isolated primarily in the Fukagawa soil, while Bj11-2 was fast grower and isolated in the Fukagawa and Matsue soils. Regarding BjS10J, BjS10J-1 and BjS10J-3 were originated from the Matsue soil, while BjS10J-2 and BjS10J-4 were primarily isolated from the Miyazaki soil. *B. elkanii* L7 was ubiquitous in all soils.

In the Fukagawa soil, Bj11-1 dominated (87%) in the Fukagawa location, and the dominancy was not changed in the Matsue (80%) and Miyazaki (83%) locations. In the Matsue soil, the composition was similar in the Matsue and Miyazaki locations, in which

BeL7 was dominated (70–73%) and Bj11-2 was minor (17-20%). While in the Fukagawa location, BeL7 decreased to 53% along with the increase of Bj11-1 (17%) and BjS10J-3 (13%). In the Miyazaki soil, BeL7 dominated at 77%, and BeL7 decreased to 13% and 33% in the Fukagawa and Matsue locations, respectively, while BjS10J-2 (53-73%) and BjS10J-4 (13%) increased.

These results suggested that the *B. japonicum* strain preferably proliferated in the Fukagawa location, leading to its nodule dominancy because the nodule composition was not changed in the Matsue and Miyazaki locations. While in the Miyazaki location, *B. elkanii* dominated in the nodules, but the dominant strain changed to *B. japonicum* in the Matsue and Fukagawa locations, suggesting that the temperature-dependent infection would lead to the nodule dominancy of *B. elkanii* in the Miyazaki location. In the Matsue location, because the nodule composition partially changed in the Fukagawa location, both factors would be involved in the determination of the nodule composition. In the second year, BeL7 found to be increased in all soils and locations, which might be due to the little higher temperature during one month after sowing in the second year.

Growth and competitive infection behaviors of two sets of *Bradyrhizobium* spp. strains were examined at different temperatures to explain the strain-specific soybean nodulation in Fukagawa and Miyazaki by using the *Bradyrhizobium* spp. strains isolated from the corresponding soils and locations. Each set consisting of three strains was as follows: *B. japonicum* Hh 16-9 (Bj11-1), *B. japonicum* Hh 16-25 (Bj11-2) and *B. elkanii* Hk 16-7 (BeL7); *B. japonicum* Kh 16-43 (Bj10J-2), *B. japonicum* Kh 16-64 (Bj10J-4) and *B. elkanii* Kh 16-7 (BeL7), which were isolated from the soybean nodules cultivated in the Fukagawa and Miyazaki soils, respectively. The growth of each strain was evaluated in yeast mannitol (YM) liquid medium at 15, 20, 25, 30 and 35 °C with shaking at 125 rpm for one week while measuring their OD_{660} daily. In the competitive infection experiment, each set of the strains was inoculated in sterilized vermiculite followed by sowing surface sterilized soybean seeds, and they were cultivated at 20/18 °C and 30/28 °C in 16/8 h (day/night) cycle in a phytotron for three weeks. After three weeks plant length and weight of shoot and root were measured, and number of nodules were counted. Then nodule composition of the inoculated strains was examined using randomly selected ten nodules per plant. The nodule compositions were determined based on the partial 16S-23R rRNA ITS gene sequence of the DNA extracted from the nodules.

The optimum growth temperatures were at 15–20°C for all *B. japonicum* strains, while they were at 25–35 °C for all *B. elkanii* strains. The shoot and root lengths, and the shoot and root weights were significantly higher at 30/28 °C than 20/18 °C in all treatments except for the root lengths of the soybeans inoculated with the Miyazaki strains. While the nodule numbers were not significantly different between the different temperature conditions. In the Fukagawa strains, Bj11-1 and BeL7 dominated in the nodules at the low and high temperatures, respectively. In the Miyazaki strains, BjS10J-2 and BeL7 dominated at the low and high temperatures, respectively.

In the Fukagawa soil, because *B. elkanii* BeL7, which has higher ability to grow and nodulate than the *B. japonicum* strains (Bj11-1 and 2) at the high temperature, did not appear in the Matsue and Miyazaki locations, it was suggested that the *B. japonicum* strains preferably proliferated in the Fukagawa soil, and Bj11-1 nodulated more preferably than Bj11-2. In the Miyazaki soil, *B. elkanii* BeL7 dominated in the nodules, but the *B. japonicum* strains BjS10J-2 and BjS10J-4 increased in the Fukagawa and Matsue locations, suggesting that both *B. japonicum* and *B. elkanii* proliferate in the Miyazaki soil, and *B. elkanii* (BeL7) dominated in nodules due to their temperature-dependent infection.

SUMMARY (In Japanese)

ダイズ（*Glycine max* [L.] Merr）はマメ科に属し、重要な穀物植物ある。ダイズには根粒内で大気中の窒素を共生的に固定することができるダイズ根粒菌が世界中で報告されている。効率的な窒素固定は根粒菌の窒素固定能力に依存するため、土壌中の根粒菌間の増殖および感染における競争は重要である。また、根粒菌のこれらの挙動については、環境条件に基づいて解明する必要がある。

Bradyrhizobium japonicum と *B. elkanii* は、主要なダイズ根粒菌であり、*B. japonicum* と *B.elkanii* は、それぞれ、高緯度と低緯度の地域で栽培されるダイズの根粒で優占することが報告されている。これまでの報告では、温度に依存した土壌中での増殖と感染が根粒菌の根粒における根粒菌の組成を決定することが示唆されている。しかし、どちらの要因がより重要であるのかについては解明されていない。

栽培圃場の気候条件下におけるこれらの要因の寄与について調べるために、温帯大陸性気候の深川（北海道）、および湿潤亜熱帯気候の松江と宮崎の3ヵ所の調査場所を選択した。各土壌サンプルを他の調査場所に輸送し、3ヵ所の調査場所で3つの土壌を使用してダイズ品種オリヒメ（非*Rj*型）を2年連続でポット栽培した。ポットあたり3株のダイズを3ポットで施肥なしで2～3か月間栽培し、収穫時に植物の生重量と

根粒数を測定した。ポット毎に1つの植物からランダムに選択した10個の根粒から根粒菌を分離し、部分的な 16S rRNA および 16S-23S rRNA ITS 遺伝子塩基配列から *Bradyrhizobium* 属細菌の系統的特徴を明らかにした。それに基づいて根粒中における根粒菌の組成を決定した。

ダイズの生重量と根粒の数は、いくつかの例外を除いて、すべての場所と土壌の間で有意差はなかった。全体で見ると、生重量は北部（深川）から南部（宮崎）になるにつれて増加したが、根粒数はその反対の傾向を示した。南部になるにつれて根粒数が減少したのは、根粒菌固有の特性が原因である可能性が考えられた。

本研究では、2種類の *B. japonicum*（Bj11 と BjS10J）と1種類の *B. elkanii*（BeL7）が分離され、Bj11 と BjS10J はそれぞれ ITS 遺伝子塩基配列に基づいて系統的に2つ（Bj11-1-2）と4つのクラスター（BjS10J-1-4）に分類された。Bj11-1 は増殖が遅く、主に深川土壌で分離されたのに対し、Bj11-2 は増殖が速く、深川土壌と松江土壌で分離された。BjS10J に関しては、BjS10J-1 と BjS10J-3 は松江土壌に由来し、BjS10J-2 と BjS10J-4 は主に宮崎土壌から分離された。一方、*B. elkanii* BeL7 はすべての土壌に遍在していた。

深川土壌では、深川で Bj11-1 が優占（87%）し、松江（80%）と宮崎（83%）でダイズを栽培してもその優占は変化しなかった。松江土

壌では、松江と宮崎では根粒中の根粒菌組成が類似しており、BeL7が優占（70〜73%）し、Bj11-2（17〜20%）が検出された。一方、深川では、Bj11-1（17%）と BjS10J-3（13%）が増加し、BeL7 は 53%に減少した。宮崎土壌では、BeL7 が 77%と優占したが、深川と松江ではそれぞれ 13%、33%に減少し、BjS10J-2（53-73%）と BjS10J-4（13%）が増加した。

これらの結果は、深川土壌を用いて松江と宮崎で栽培した時に深川と同様に *B. japonicum* が優占したことから、*B. japonicum* が深川土壌で優占しているために、根粒で優占していることを示唆している。宮崎では、*B. elkanii* が根粒で優占したが、松江と深川では優占根粒菌が *B. japonicum* に変化したことから、温度依存的な感染により、*B. elkanii* が宮崎で優占していることが示唆された。松江では、根粒菌の組成がダイズを深川で栽培した時に部分的に変化したため、*B. elkanii* の土壌での優占と温度依存的な感染の両方の要因が根粒菌の組成に関与していることが示唆された。栽培2年目には、BeL7の割合がすべての土壌と場所で増加したが、2 年目は播種後 1 か月間の気温が 1 年目より少し高かったことが原因である可能性が考えられた。

深川と宮崎で特徴的であったダイズ根粒菌の根粒内組成の要因を解明するために、それぞれの土壌と場所から分離された根粒菌株を用いて

、異なる温度における *Bradyrhizobium* 属菌の増殖特性と感染における競合関係について検討した。深川で分離された根粒菌の組合せは、*B. japonicum* Hh16-9（Bj11-1）、*B. japonicum* Hh16-25（Bj11-2）および *B. elkanii* Hk16-7（BeL7）、宮崎で分離された根粒菌の組合せは、*B. japonicum* Kh16-43（Bj10J-2）、*B. japonicum* Kh16-64（Bj10J-4）および *B. elkanii* Kh16-7（BeL7）であった。各菌株の増殖における温度特性については、酵母エキス・マンニトール（YM）液体培地を用いて、15、20、25、30、35℃で 1 週間、振とう培養し、1 日毎に OD_{660} を測定した。競合的感染実験では、各組合せの菌株を滅菌バーミキュライトに接種した後、表面滅菌したダイズ種子を播種し、20/18℃および 30/28℃（16/8 時間、明/暗）に設定した人工気象器で 3 週間栽培した後、ダイズ地上部と地下部の長さと重量を測定し、根粒を計数した。次に、植物毎にランダムに選択した 10 個の根粒から DNA を抽出し、部分的な 16S-23S rRNA ITS 遺伝子の塩基配列に基づいて接種した根粒菌の根粒内組成を決定した。

B. japonicum 4 株と *B. elkanii* 2 株の増殖至適温度は、それぞれ 15～20℃および 25～35℃であった。宮崎株を接種したダイズの根長を除いて、すべての処理区において、ダイズ地上部と地下部の長さと重量は、20/18℃よりも 30/28℃で有意に大きかった。温度の違いで根粒数に有意な差は無かった。深川株では、Bj11-1 と BeL7 がそれぞれ低温条件と高

温条件で栽培したダイズの根粒で優占し、宮崎株では、BjS10J-2 と BeL7がそれぞれ低温条件と高温条件で栽培したダイズの根粒で優占した。

深川土壌では、高温条件で *B. japonicum* 株（Bj11-1 および 2）よりも増殖および根粒形成能が高い *B. elkanii* BeL7 が、松江および宮崎で栽培したダイズの根粒中に出現しなかったため、*B. japonicum* 株が深川土壌で優占的に増殖していること、また、*B. japonicum* Bj11-1 は Bj11-2 よりも根粒形成能が高いことが示唆された。宮崎土壌では、*B. elkanii* BeL7 が根粒で優占していたが、深川と松江では *B. japonicum* 株 BjS10J-2 と BjS10J-4 が優占したため、*B. japonicum* 株と *B. elkanii* 株の両者が宮崎土壌には生息し、*B. elkanii*（BeL7）が、温度依存的な感染のために根粒で優占していることが示唆された。

List of Publications

1. Hafiz, M.H.R., Salehin, A., Adachi, F., Omichi, M., Saeki, Y., Yamamoto, A., Hayashi, S. and Itoh, K. Latitudinal characteristic nodule composition of soybean-nodulating bradyrhizobia: temperature-dependent proliferation in soil or infection?. Horticulturae 7(2): 1-12 (https://doi.org/10.3390/horticulturae7020022) **(Chapter 2).**

2. Hafiz, M.H.R., Salehin, A. and Itoh, K. Growth and competitive infection behaviors of *Bradyrhizobium japonicum* and *Bradyrhizobium elkanii* at different temperatures. Horticulturae 7(3): 1-10 (https://doi.org/10.3390/horticulturae7030041) **(Chapter 3).**