Diversity and endemism of soybean rhizobia in the Philippines

（フィリピンにおけるダイズ根粒菌の分布と多様性に関する研究）

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Chapter 1

General Introduction

Rhizobia is a collective term for diazotrophic bacteria that has the ability to form nodules on the roots of leguminous plants and fix atmospheric nitrogen and renders it available for plant's use. In taxonomical classification, the bacterium *Rhizobium* belongs to the phylum of Proteobacteria, class Alphaproteobacteria, order Rhizobiales, and family Rhizobiaceae. Under the Bacteria domain, the phylum Proteobacteria is considered as the largest bacterial group and is named after the Greek god Proteus, which can assume various shapes, as to indicate the great diversity of the species under this phylum (Stackerbrandt et al., 1988). They are gram-negative with an outer membrane that consist largely of lipopolysaccharides, and most of them have flagella for locomotion and can be aerobic or anaerobic. This phylum have six classes: a) alphaproteobacteria, b) betaproteobacteria, c) gammaproteobacteria, d) deltaproteobacteria, e) epsilonproteobacteria, and f) zetaproteobacteria. The α-proteobacteria includes important members such as the plant symbionts (rhizobacteria), animal and plant pathogens (agrobacteria, rickettsia), and C1 compounds metabolizers (methylobacteria) (Pini et al., 2011). The Rhizobiales include not only the plant symbionts such as *Rhizobium*, *Bradyrhizobium*, *Sinorhizobium/Ensifer*, *Azorhizobium*, *Allorhizobium*, and *Mesorhizobium* but also the obligate and facultative intracellular bacteria and animal and plant pathogens. The family of *Rhizobiaceae* Conn 1938 is composed of at least six genera with *Rhizobium* as the type genus. The genera includes the fast-growers and acid-substance producers *Rhizobium*, *Sinorhizobium*/*Ensifer*, and *Allorhizobium*/*Rhizobium*; the intermediate-to-fast-growers and acid producers

Mesorhizobium; the fast-growers and acid producers *Azorhizobium*; and the slow-growers and alkaline producers *Bradyrhizobium*.

The focus of this study is on plant symbionts which belong to the genera *Rhizobium*, *Bradyrhizobium*, and *Sinorhizobium/Ensifer*, and thus, will be referred to as rhizobia in this text.

Rhizobium (Latin: *rhiza* means root, *bios* means life) is the original genus of bacteria that can form nodules with leguminous plants, and first isolated and cultured by Martinus Beijerinck of Holland in 1888 from different legumes and named it as *Bacillus radicicola* (Hirsch 2009). It originally referred to only one species, *R. leguminosarum* (Frank 1889) until 1982 but, as of 2006, 16 species have been described under this genus (Willems 2006). In 1982, Jordan proposed the genus *Bradyrhizobium* (Greek: *bradus* means slow) from the original *Rhizobium japonicum* to differentiate the slow-growing and alkaline-substance formation characteristic of this genus. The genus *Sinorhizobium* was proposed by Chen et al. (1988) as fast-growing rhizobia that can nodulate soybean but was later found to be identical via 16S rDNA gene sequence with *Ensifer adhaerans* previously described by Casida (1982). On the basis of priority according to the *Bacteriological Code* (1990 Revision), it has been recommended that *Ensifer* would take priority than *Sinorhizobium* since the first was the oldest valid name. However, this change in name has been undergoing differences in opinions from experts in rhizobacteria. As Willems et al. (2003) pointed out, the name *Ensifer* which means "sword-bearer" does not have the same significance to the symbiotic, nitrogen-fixing bacteria, like the name *Sinorhizobium*, and there is already a larger number of species described under the *Sinorhizobium* than the *Ensifer*. In this thesis, I prefer to use *Sinorhizobium* over *Ensifer* on all the succeeding texts.

Soybean (*Glycine max* [L.] Merill) belongs to the family of Fabaceae or Leguminosae. It establishes a symbiotic relationship with rhizobia, known as the nitrogen-fixing bacteria. These bacteria have nitrogenase enzyme that can reduce atmospheric nitrogen (N_2 – unavailable for plant) to ammonia (NH_3 – available for plant). This process is called Biological Nitrogen Fixation (BNF), an energy-expensive process that can be summarized by this overall reaction (Cheng, 2008): $N_2 + 8H^+ + 8e^- + 16MgATP \rightarrow 2NH_3 + H_2 + 16MgADP + 16Pi$. Symbiotic relationship between soybean and rhizobia takes place when the bacteria in the soil colonizes the root of the plant thereby, forming nodules where nitrogen fixation occurs. The host plant initiates symbiosis by secreting flavonoids, which are then detected by the specific rhizobia (Nelson and Sadowsky, 2015). The plant provides the rhizobia with carbon and source of energy for its growth and functions while the rhizobia fix atmospheric N_2 and provide the plant with a source of reduced nitrogen (Sulieman and Tran 2014) needed for its growth and development. Soybean has nodulation regulatory genes that controls the plant's compatibility with the rhizobia, and these are called *Rj* (*rj*) genes. The genotypes of these genes namely: non-*Rj*, *rj*$_1$, *Rj*$_2$, *Rj*$_3$ and *Rj*$_4$ were confirmed to exist naturally (Williams and Lynch, 1954; Devine and Kuykendall, 1996). In a review article by Hayashi et al. (2012), it was stated that because a particular *Rj* genotype can exclude nodulation with indigenous *Bradyrhizobium* strains from a specific cluster, they can have practical importance in agriculture. This is particularly true as knowledge on *Rj* (*rj*) genes and their genotypes can help researchers to improve the efficiency of inoculation technique. By using specific *Bradyrhizobium* strains that exhibit effective nitrogen fixation activity which are compatible with the host plant, inoculation can be made successful considering all other factors are desirable.

1. Use of genetic markers on the ecological studies of rhizobia

In 1932, Fred et al. established the "cross-inoculation group" concept as the earliest classification of rhizobia based largely on host specificity with description of morphological and physiological properties. However, many studies that followed was able to recognize that this concept was not an appropriate classification scheme and thus, is now seldom nowadays. The use of molecular analysis in taxonomical identification and ecology of rhizobia since the early nineties such as the sequence analysis of the 16S ribosomal RNA genes and genetic fingerprinting methods via PCR led to the better understanding of the rhizobial phylogeny (Sahgal and Johri, 2003). Since then, the ecological niche of rhizobia has been better understood.

A study by Mollet et al. (1997) compared the use of 16S rRNA gene with the *rpoB* gene for the identification of 20 Enterobacteriaceae strains and reported that *rpoB* gene has better sensitivity in detecting interspecies divergence than the 16S rRNA gene. It was reported by Case et al. (2007) that the use of 16S rRNA gene in microbial ecology studies provided intragenomic heterogeneity that affected the phylogeny of organism due to the multiple copies of the gene that is often present in certain bacteria. Another study (Acinas et al., 2004) stated that even a 99% sequence identity in the 16S rRNA gene could reveal an extensive ribotype microdiversity with potentially important ecological differentiation.

As demonstrated in earlier reports (Mollet et al., 1997; Dahllof et al., 2000) the single-copy *rpoB* gene provided better resolution than the 16S rRNA gene especially at the subspecies level. Case et al. (2007) noted its importance for ecological analysis because of the following reasons: (i) its universal presence in all prokaryotes; (ii) the

presence of slowly and quickly evolving regions for the design of probes and primers of differing specificities; (iii) the housekeeping function makes it less susceptible to some forms of lateral gene transfer; and (iv) a size large enough to contain phylogenetic information, even after removal of regions that are difficult to align.

Analysis of the internal transcribed spacer (ITS) region between the 16S and 23S ribosomal RNA gene has been a very useful tool for the identification of *Rhizobium* species and even on the strain level for the past several years (Germano et al., 2006; Saeki, 2011; Saeki et al., 2004, 2006, 2008, 2013; Suzuki et al., 2008; Minami et al., 2009; Risal et al., 2010; Adhikari et al., 2012; Yamakawa and Saeki, 2013). However, difference may still exist even within bacteria of the same ITS type. Therefore, it is better to analyze other genes in addition to ITS region. Meanwhile, analysis of housekeeping genes have been a recently popular approach for a more distinct identification of microorganisms (Vinuesa et al., 2008; Degefu et al., 2013; Yan et al., 2014; Guimarães et al., 2015). The inclusion of one or more housekeeping genes along with ITS region and 16S rRNA gene in the identification of rhizobia proved to be a very helpful alternative. The *rpoB* housekeeping gene is a single-copy gene that encodes the β subunit of RNA polymerase and is the site of mutation in bacteria (Ahmad et al., 2002; McCammon et al., 2005; Suresh et al., 2006). According to a study conducted by Khamis et al. (2003) they were able to confirm that *rpoB* gene is probably polymorphic enough to replace or supplement the 16S rRNA gene for definitive identification of *Afipia* and *Bosea* bacteria, as the two closest bacteria by 16S rRNA gene comparisons, with 1 different position (0.1%), differ by at least 3% with *rpoB* gene. Another study (Ade´kambi et al., 2008) reported that the *rpoB* gene sequence similarity provides an efficient supplement to DNA-DNA hybridization (DDH) and average nucleotide identity (ANI) measurements to delineate bacterial species and

genera, including delineation of as-yet-uncultivated, non-sequenced organisms. Thus, analysis of the housekeeping genes, such as *rpo*B gene, can provide a more distinct identification and differentiation of rhizobial strains which have also been proven in some recent studies (Martens et al., 2008; Nzoue´ et al., 2009; Rivas et al., 2009; Aserse et al., 2012; Delamuta et al., 2012; Baraúna et al., 2014). Therefore, *rpoB* housekeeping gene was included in this study aside from the ITS region for RFLP treatment and sequence analysis.

2. Soybean production and research in the Philippines

The Philippines is a tropical archipelago located in the Southeast Asia with a land area of about 298 km^2. It is basically an agriculture country where the main source of income for most Filipinos is in agriculture-related activities. From the approximately 7M hectares used for agriculture, only about 1,000 ha. are allocated to soybean. This is due to the low yield (≤1.0 ton/ha) of soybean and also low government support to soybean planters. In 2016, soybean-related imports in the Philippines was at the top second place next to wheat and meslin (http://countrystat.psa.gov.ph). In spite of the nutritional benefits and agricultural-related advantages of planting soybean, it remains as a lesser-priority crop in the country. However, recent trend in Philippines agriculture has geared towards a sustainable and healthy production system. The Government's program entitled "Philippine Soybean Development Program" launched in 2011 was able to increase the production area from ≈1,000 ha. to a record high of 5,280 has. But the problem remains unsolved; soybean yield is still low at about 1.0 ton/ha.

Thus, projects and studies on how to increase soybean yield in a sustainable manner prompted researchers to venture into development of plant varieties with high-yielding ability. It is known that soybean does not require high application of chemical fertilizers,

so it is a good rotation crop in between rice and corn, which demand high chemical inputs. However, breeding of high-yielding soybean varieties alone does not guarantee that it would attain its optimal yield. Other factors such as soil management and cultural practices has to be improved too.

3. Diversity of soybean rhizobia across a variety of regions

An extensive information on this family was written by Alves et al. (2014) citing Norris (1965) with a statement that although rhizobia might grow in any region, temperate regions are dominated by the fast-growing and acid producing strains while tropical regions are dominated by the slow-growing and alkaline-producing strains.

Majority of the studies about the diversity and distribution of soybean rhizobia have been extensively conducted in temperate and sub-tropical regions of Japan (Ikeda et al., 2008, 2010; Nguyen et al., 2010; Saeki et al., 2006, 2008, 2010; Shiro et al., 2012; Suzuki et al., 2008), USA (Fuhrmann 1990; Tang et al., 2012; Shiro et al., 2013; Saeki and Shiro 2014; Wongphatcharachai et al., 2015a, 2015b;), and China (Lin et al., 2007; Li et al., 2011; Man et al., 2008; Zhang et al., 2011; Yan et al., 2014; Zhao et al., 2014) but there are limited researches about this topic in tropical regions (Loureiro et al., 2007; Sharma et al., 2010; Ansari et al., 2013) particularly in the Philippines (Gamo et al., 1990). Tropical rhizobia represent a key component for the sustainability of tropical soils; and the genus *Bradyrhizobium*, which is considered to be the ancestral of all nitrogen-fixing rhizobial species, probably originated in the tropics (Delamuta et al., 2012). Even in the subtropical and tropical regions of China, which is said to be the center of diversification of *G. max*, Man et al. (2008) stated that the diversity of soybean rhizobia has not yet been clearly described. Therefore, this study would be an

interesting foundation for future researches about the diversity and endemism of soybean rhizobia in subtropical and tropical regions.

Comparing the results obtained by Saeki and Shiro (2014) on the diversity of soybean bradyrhizobia on the temperate regions of the United States and Japan, higher diversity were found on temperate regions such as Brazil (Loureiro et al., 2007; Guimarães et al., 2015; Ribeiro et al., 2015) and Philippines (Mason et al., 2017, 2018). Therefore, it is not an overstatement to say that the soybean bradyrhizobia are very interesting organisms as they can adapt to a wide variety of agricultural, geographical, and environmental conditions and their mechanism of adaptation also evolve with their ecological niche.

4. Diversity and endemism of soybean rhizobia in the Philippines

In this thesis, which contains two published papers (**Chapter I**, **Chapter II**), five molecular markers were used to detect the genomic variations that allowed an accurate identification of rhizobial species and strains as well as to indicate the diversity and ecological niche of each species. The major aim of this study is to investigate the diversity and endemism of soybean rhizobia in the Philippines. This was done with an ultimate goal to help increase the production of local soybean by using the indigenous rhizobia as inoculant. In the Philippines, soybean is not a priority crop so very few research were conducted in this topic and this research is the pioneer work that represented the country from north to south. The lack of published information about soybean rhizobia in the country became the greatest challenge in this study. In order to achieve the aim, this study was divided into four parts.

The first part focused on comparing the diversity of *B. elkanii* species between Southern Japan and Central Luzon, Philippines. In this report, the genomic variations

were detected by employing the Polymerase Chain reaction – Restriction Fragment Length Polymorphism (PCR-RFLP) with four restriction enzymes *HaeIII*, *HhaI*, *MspI* and *XspI* and sequence analysis of the 19 representative strains from Kumamoto and Okinawa, Southern Japan and Nueva Ecija, Philippines for the 16S rRNA gene, Internal Transcribed Spacer (ITS) region between the 16S and 23S rRNA gene, *rpoB* housekeeping gene, and the symbiotic genes *nifD* and *nodD1*. Based from the results, we were able to detect that the endemism and diversity of *B. elkanii* seemed to be influenced by the difference in temperature and the similarity in soil pH. Clearly, the diversity of *B. elkanii* strains in the three locations indicated that temperate regions have different rhizobia ecology than the tropical region.

For the second part, the research focused on the diversity of soybean bradyrhizobia in 11 locations at the Philippines. From the 771 isolates, we confirmed 424 bradyrhizobia by their morphological growth on YM media and through the unique fragment patterns via PCR-RFLP treatment with the four restriction enzymes. From these distinctive band patterns, 31 representative strains were selected for sequence analysis of the three target genes which were the 16S rRNA gene, ITS region, and *rpoB* gene. In this report, high diversity of bradyrhizobia were detected and according to the correlation analysis between the isolates and the agro-environmental gradients considered, the most influential factors for the diversity and distribution of soybean bradyrhizobia in the country were the length of flooding period, followed by other soil properties such as soil pH, nutrient content, and soil type. Additionally, the unique nucleotide sequence from the 16S rRNA gene, ITS region, and *rpoB* gene of some indigenous strains indicated the presence of novel species in the Philippines which could be studied in the future.

For the third part, the 12 most dominant indigenous strains for specific locations were selected and evaluated for their symbiotic efficiency and nitrogen-fixation ability for their potential use as soybean inoculant in the Philippines. Two soybean cultivars from the Philippines and three soybean cultivars from Japan were used as the host plants and were inoculated singly with the 12 indigenous strains. A proven efficient strain, the *B. diazoefficiens* USDA110 was used as the positive control and an un-inoculated treatment as the negative control. According to the results, all the indigenous strains in the Philippines are suggested to be type A strains in terms of nodulation ability. Based from the results, the most efficient microsymbionts of Philippines soybean cultivars was the *B. elkanii* IS-2, followed by the strains of potential novel *Bradyrhizobium* sp., *B. japonicum*, and *B. diazoefficiens*. This trend is in contrast with the Japanese cultivars, wherein the most efficient micro-symbiont was the *B. diazoefficiens* SK-5.

The fourth part consists of the data that were gathered from both the nodulating and the non-nodulating rhizobia. It has been reported that non-nodulating rhizobia exists widely in the rhizosphere of leguminous plants and even coexist with the nodulating rhizobia which sometimes help the rhizobia to extend it host range (Pongsilp et al., 2002; Denison and Kiers, 2004; Liu et al., 2010; Sachs et al., 2008, 2010; Wu et al., 2011). In this part, we also reported the presence of those rhizobia that were not able to nodulate the soybean plant.

In summary, the major aim of this study was achieved and we were able to obtain some potential novel species and strains which could be further used for research in the Philippines. This result will be very useful not only for the research institution but also for soybean farmers in the country. As for our prospective, we plan to use the indigenous strains for field trials in soybean farms to evaluate their feasibility and usefulness as inoculant in Philippine condition. This study was able to confirm that the

diversity and endemism of soybean rhizobia form a tropical country like the Philippines is indeed high and different from the temperate regions, like Japan.

Chapter 2

Genetic diversity of indigenous soybean-nodulating *Bradyrhizobium Elkanii* isolated from Kumamoto and Okinawa, Southern Japan and Nueva Ecija, Philippines

Introduction

Soybean (*Glycine max* [L.] Merrill.) is a high protein legume plant (40%) which can be used as food, animal feed and as an industrial raw material like biodiesel. This legume can establish a symbiotic relationship with the nitrogen-fixing bacteria, which are collectively known as rhizobia. As reported from recent literatures, there are more than 100 species of rhizobia which were isolated from legumes and other sources (Gyaneshwar et al., 2011; Peix et al., 2015) and currently, the major soybean-nodulating rhizobia that were identified are as follows: *Bradyrhizobium japonicum, B. diazoefficiens, B. elkanii,* and *Sinorhizobium*/*Ensifer fredii* (Jordan, 1982; Scholla and Elkan, 1984; Kuykendall et al., 1992; Young, 2003; Delamuta et al., 2013). For soybean, some cultivars possess nodulation regulatory genes which are known as *Rj* genes, and the genotypes which have been confirmed to exist in nature are non-*Rj, rj1, Rj2, Rj3*, and *Rj4* (Devine and Kuykendall, 1996).

In contrast with Japan, wherein soybean plays an important role in daily cuisine, soybean has very minimal role in Filipinos' diet since 90% of local production and importation are used for animal feed (Manuel et al., 1986). Nevertheless, the production of soybean in both countries cannot meet its local demand as reflected in the amount of soybean importation (Manuel et al., 1986; Wang, 2016). Aside from using chemical fertilizers, one way to increase the soybean yield per unit area is by performing an inoculation. Inoculating a useful rhizobia into soils with low N content may lead to an increase in the yield of soybean as revealed by numerous studies (Alves et al., 2003; Njira et al., 2013; Alam et al., 2015; Sanz-Sáez et al., 2015). However, many soybean inoculation does not succeed because of many factors and one of these factors is the competition between the introduced and the indigenous rhizobia in the soil (Yamakawa

et al., 2003). Therefore, it is essential to first understand the ecology of the indigenous rhizobia in the soil prior to inoculation.

Previous investigations about the diversity and distribution of indigenous soybean rhizobia in Japan was able to identify that the *B. japonicum* strains were dominant in the northern region of the country whereas *B. elkanii* strains were dominant in the southern region (Suzuki et al., 2008; Saeki et al., 2006, 2008, 2010, 2013). It was then stated that temperature was the most prominent factor for its prevalence (Saeki and Shiro, 2014). However, there is no published studies about the indigenous soybean rhizobia which have been done in the Philippines as of the writing of this manuscript.

The analysis about soybean rhizobial diversity in Japan was conducted mainly by analyzing the 16S rRNA gene and the 16S-23S rRNA gene ITS (Saeki et al., 2006, 2008, 2013; Suzuki et al., 2008; Minami et al., 2009; Saeki and Shiro, 2014). But, a major disadvantage of the 16S rRNA gene for taxonomic studies is that it is often a multiple-copy gene (Vos et al., 2012) and it contains little resolution below the species level (Germano et al., 2006; Martens et al., 2008). Instead, the ITS region and the *rpoB* housekeeping gene were able to provide a better discriminatory power up to the species level and below (Martens et al., 2008; Vinuesa et al., 2008; Delamuta et al., 2012; Vos et al., 2012; Degefu et al., 2013; Yan et al., 2014; Guimarães et al., 2015). So, it is generally better to employ other molecular marker in addition to the ITS region to provide a better genetic classification of rhizobial strains.

Yet, taxonomical studies of rhizobia do not necessarily reflect their symbiotic qualities, or their host range, which is crucial requirement of a useful inoculant. Thus, sequence analysis of the symbiotic genes *nifD* (encoded the α subunit of dinitrogenase) and *nodD1* (nodulation regulation protein) was also conducted. It was reported in previous studies that the genes in the symbiosis island might not show diversity even

among related species in rhizobial genera commonly due to the horizontal gene transfer as directed by their location (Minamisawa et al., 2002; Barcellos et al., 2007; Ramirez-Bahena et al., 2009; Ling et al., 2016). Yet, the role of NodD regulator proteins (including *nodD1*) in activating the transcription of nod genes is known to be a key factor that influences the competitiveness of rhizobia (Maj et al., 2010) due to its assumed specific interaction with flavonoids (Redmond et al., 1986; Zaat et al., 1989). Hence, it is an important genetic marker to be included for the evaluation of potential inoculant strains. Meanwhile, a report about the role of *nifD* in partner quality for *Rhizobium* stated that there might be a causal relationship between the locus and measures of partner quality (Gordon et al., 2016) which in turn, could influence the mutualism between the host plant and the rhizobia for an effective N fixation.

Many studies about the diversity and distribution of soybean rhizobia were conducted in both the temperate and subtropical regions of Japan (Ikeda et al., 2008, 2010; Nguyen et al., 2010; Saeki et al., 2006, 2008, 2010; Suzuki et al., 2008; Shiro et al., 2012) but a limited research about this topic have been conducted in the tropical regions (Loureiro et al., 2007; Sharma et al., 2010; Ansari et al., 2013). The tropical rhizobia represent a vital constituent for the sustainability of tropical soils; and the *Bradyrhizobium* genus, which is considered to be the ancestral of all the nitrogen-fixing rhizobial species, was believed to be originated from the tropics (Delamuta et al., 2012). A report stated that even in the subtropical and tropical regions of China, which is considered to be the center of diversification of *G. max*, the diversity of soybean rhizobia has not yet been clearly described (Man et al., 2008). Hence, this study would serve a helpful groundwork for the future research and studies about the diversity and distribution of soybean rhizobia in sub-tropical and tropical regions. The major aim of this study is to determine the possible endemism and genetic diversity of the soybean-

nodulating *B. elkanii* species between the temperate region of Kumamoto and sub-tropical region of Okinawa, Japan and the tropical region of Nueva Ecija, Philippines. Also, this study should be able to provide the first report in the Philippines and in Kumamoto, Japan.

Materials and Methods

1. Collection of soil and analysis of soil pH and electrical conductivity (EC)

The soil samples were collected from the three field sites (Kumamoto: Kumamoto Prefectural Agricultural Research Center, Goshi, Kumamoto, Japan and Okinawa: University of the Ryukyu, Nishihara, Okinawa, Japan and Nueva Ecija: Central Luzon State University, Nueva Ecija, Philipppines) which were previously planted with soybean and/or other legumes. The soil surface was cleared with litters before a bar of soil with dimension of 20 cm depth and 2 to 3 cm thickness was obtained. The soil bar approximately weighed 1 kg each. Then, 0.5 kg of the soil sample was air dried and pulverized for the analysis of soil pH and EC by the water extraction method (1:2.5 soil: water for pH and 1:5 soil: water for EC) and the remaining 0.5 kg of the soil was freshly used to cultivate soybean inside the growth chamber.

For the soil pH water extraction method (soil: water, 1:2.5), ten grams of the air-dried soil was mixed with a 25mL of distilled water in a 50mL capacity falcon tube. Then, it was placed firmly on a mechanical shaker with continuous agitation for approximately 1 hour. The pH meter was calibrated with three pH buffer solutions (4.0, 7.0, 10.0) prior to usage. The pH meter's probe was immersed into the falcon tube containing the mixed soil and water then, the pH value was recorded after about 30 seconds of immersion. This step was done thrice to obtain an average reading with thorough mixing before each immersion.

For the analysis of EC, the same method was used (soil: water, 1:5). The EC meter was calibrated with 0.01N KCl at 25°C. Five (5) grams of dried soil was mixed with 25mL of water in a falcon tube and placed in a mechanical shaker with continuous agitation for 1 hour. The probe of EC meter was immersed into the soil mixture and measurement was recorded at dS/m. Similar with the pH, measurement was done thrice and the average was recorded as the final reading.

The data of the annual average temperature from Nueva Ecija, Philippines was obtained from Philippine Atmospheric Geophysical and Astronomical Services Administration (PAGASA) Central Luzon State University (CLSU) station while the temperature data from Kumamoto and Okinawa, Japan were obtained from the Japan Meteorological Agency website at http://www.data.jma.go.jp/. All data used in this study were averages from this last decade (2006-2016).

2. Isolation of rhizobia using different cultivars of soybean

Three soybean cultivars of three different *Rj* genotypes namely, Bragg (BM) or Akishirome (AK) as non-*Rj*, CNS (CM) or Bonminori (BO) as Rj_2Rj_3, and Hill (HM) or Fukuyutaka (FK) as Rj_4 were used to isolate the indigenous soybean rhizobia. Each soybean cultivar was planted in a 1 L culture pots (n = 3). The culture pots were filled with vermiculite containing N-free nutrient solution (Saeki et al., 2000) at 40% (vol/vol) water content then, were autoclaved at 121 °C for 20 min. Soybean seeds were surface-sterilized by soaking in 70% ethanol for 30 s then, in a diluted sodium hypochlorite solution (0.25% available chlorine) for 3 min. Afterwards, the seeds were washed with sterile distilled water for about 6 – 8 times. Then, a 2 – 3g of soil sample was placed on the vermiculite at a depth of 2 – 3cm, the seeds were then sown on the soil using a sterile forceps, and the pot was weighed and recorded. Afterwards, the

plants were grown for 28 days in a growth chamber at the following conditions (day, 28 °C for 16 h; night, 23 °C for 8 h), and were supplied weekly with sterile distilled water until the initial weight of the pot was reached.

After 4 weeks, 24 random nodules that have a size of more than 2mm were collected from the soybean roots per *Rj* genotype and were sterilized by soaking them in 70% ethanol for 3 min and in a diluted sodium hypochlorite solution (0.25% available chlorine) for 30 min; then washed with sterile distilled water (6 – 8 times). Then, the nodule was individually homogenized in a sterile distilled water, streaked onto a yeast extract mannitol agar (YMA; Vincent, 1970) plate, and incubated in the dark at 28 °C for about 1 week. The YMA was prepared using the following reagents (NACALAI TESQUE) for every litre of the solution: K_2HPO_4 0.5g, $MgSO_4.7H_2O$ 0.2g, NaCl 0.1g, Yeast Extract 0.4g, and Mannitol 10.0g (Vincent 1970) then, were mixed by magnetic stirrer in 1L capacity beaker. The solution was transferred in 1L capacity graduated cylinder and was filled with distilled water up to the volume needed. The pH was adjusted to 6.8 then, 15.0g of Agar powder (Wako Pure Chemical Industries, Ltd., Japan) and 10mL Congo Red (250mg/100mL EtOh) 10mL were added. This was sterilized in an autoclave (HA-240 MIV HIRAYAMA) for 20mins at 121°C and transferred into the petri dish at a rate of 18mL per dish. It was left inside the clean bench for about 15mins to solidify and then covered and sealed with a parafilm before storing to the sterilized boxes at room temperature.

Re-streaking twice on the same petri dish (YMA with CR) was done aseptically in order to ensure the growth of a single colony. The petri dish was then kept in a dark chamber at 28°C until growth of a single colony was visible (about 5-8 days). The single colony was then observed for its morphological characteristics to make sure whether it belonged to *B. japonicum* or *B. elkanii*. After observation, isolation of single colony

was done aseptically by streak-plate technique inside the clean bench. This procedure was done similarly to all the samples. Thereafter, s single colony was streaked onto YMA plate containing 0.002% (wt/wt) bromothymol blue (Keyser et al., 1982) to determine the genus then, incubated as described above.

3. Nodulation ability of the isolates

Aside from the morphological characteristics, the primary 16S rRNA gene and ITS region RFLP analysis of all the collected isolates were used as the basis to select the representative isolates. These strains were then tested for their capability to form nodules on the host soybean by inoculation test with the three *Rj* genotypes of soybean cultivars used in this study. Each of the isolate was cultured in YM broth (Vincent, 1970) using a 2.0mL microtube and were placed inside a dark shaker with continuous agitation at 28 °C for 1 week. Afterwards, the cultures were diluted with sterile distilled water to approximately 10^6 cells ml^{-1}. Then the, soybean seeds were sown as described above but without soil and inoculated with a 1 ml aliquot of each isolate per seed and replicated thrice. The formation of nodule was assessed after 4 weeks in the growth chamber under similar conditions as mentioned above. The control pots (un-inoculated) for both the Japanese (AK, BO, FK) and the US (BM, CM, HM) cultivars were also prepared under similar conditions.

The nodule number and its dry weight for each *Rj* genotype as well as the dry weight of shoot were gathered for the symbiotic analysis. Oven drying was done at 70 °C for 48 h. Then, dried shoot was finely ground into a 2 mm size prior to the Nitrogen analysis. The Total N was analyzed by an automatic high sensitive NC Analyzer Sumigraph NC-220F (Sumika Chemical Analysis Service. Ltd., Tokyo, Japan). The amount of N fixed was computed from the difference between the shoot N

content of the isolates with that of the control plants. The symbiotic efficiency of the isolates was computed using the following formula: (mg N fixed/mg dry nodule) × 100 which was adapted from Risal et al. (2010). All the statistical analyses in this study were conducted by employing the R software (v. 3.3.2) and the means of three replicates were compared by the Tukey's HSD test at $P < 0.05$. The comparison among means were conducted only between each isolate within the same *Rj* genotype and not between each *Rj* genotype.

4. Incubation of the pure colony and extraction of DNA

When the growth of a pure single colony was sufficient in size to be picked-up by a wire loop, the incubation using a culture broth was performed. First, HEPES-MES (HM) broth was prepared with the following reagents (NACALAI TESQUE, Kyoto Japan) in 1 litre of solution: $Na_2HPO_4.12H_2O$ 0.3153g, Na_2SO_4 0.25g, NH_4Cl 0.32g, $MgSO_4.7H_2O$ 0.18g, $FeCl_3.6H_2O$ 0.0067g, $CaCl_2.2H_2O$ 0.013g, HEPES 1.3g, MES 1.1g, Yeast Extract 0.25g, L. Arabinose 1.0g (Cole and Elkan 1973; Sameshima et al., 2003). These reagents were mixed thoroughly by a magnetic stirrer in a 1L capacity beaker. Afterwards, distilled water was added up to1L with and the pH was adjusted to 6.8. The solution was sterilized as described above with the YMA plates. Then, 1 mL of the sterilized HM broth was transferred into a 2mL micro tube inside the clean bench.

From the petri dish containing YMA with Congo Red, a single colony was picked-up by the sterile wire loop and was transferred to 2mL micro tubes containing 1mL of HM broth. It was incubated in the dark at a shaker (Invitro Shaker; Taitec, Japan), 120rpm, 28°C for about 1 week or until a sufficient turbidity can be seen. This procedure was followed with all the samples.

After the incubation, the bacteria cells cultured in the HM broth were collected by centrifugation and were washed with sterile distilled water. Extraction of the DNA was done by using BL buffer as described (Minami et al., 2009) from the method reported by Hiraishi et al. (1995). In summary, the master mix was prepared by transferring the following reagents per sample in a micro tube: 50µL of BL buffer (40mM Tris-HCl, 1% Tween20, 0.5% Nonidet® P-40, 1mM EDTA;pH=8.0), 10µL of proteinase K (1mg/mL; Sigma-Aldrich, Germany), and 40µL of sterilized milliQ water. For each sample, 100µL of the prepared master mix was transferred into the 2mL micro tube containing the collected cell pellet. It was flashed shortly in a centrifuge, and was put in incubators at 60°C (MG-1200, EYELA) for 20mins and 95°C (MG-2000, EYELA) for 5mins, respectively. Afterwards, samples were centrifuged at 15,000xg (KUBOTA-3500) at 4°C for 10mins to remove unwanted debris. Then, 80µL of the supernatant liquid was collected and transferred to a new 1.5mL micro tube. This is the DNA template and preservation was done at -20°C until further analysis. Similar method was done for the DNA extraction of reference strains.

5. PCR amplification of 16S rRNA gene, ITS region, *rpoB* gene and symbiotic genes *nifD* and *nodD1*

The amplification of the 16S rRNA gene, ITS region and *rpoB* gene were carried out after the extraction of crude DNA using the previously designed primer sets (Saeki et al., 2006) listed in Table 2.1. The master mix was prepared by transferring the following reagents (TaKaRa Bio, Co. Ltd., Otsu, Japan) per DNA sample in a micro tube: sterilized milliQ water 22.25µL, 10x*ExTaq* buffer 3.0µL, 2.5mM dNTP mix 2.4µL, Reverse and forward primer (10µM) 0.6µL each. Lastly, *ExTaq* DNA polymerase 0.12µL was flashed shortly before adding to the micro tube containing the

master mix. Eight (8) well PCR (0.2mL capacity) tubes were placed in metal block inside the icebox and 29µL of master mix was transferred to each well. Then 1µL of the DNA template was added to each well.

It was covered tightly with the corresponding PCR tube cap, and mixed by spinning shortly until the bubbles disappear. The PCR tubes were then placed in the PCR Thermal Cycler (TaKaRa Co. Ltd.) with the following conditions for the 16S rRNA gene: pre-run at 94°C for 5mins; followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 30secs, and extension at 72°C for 1min. The final extension was set at 72°C for 10mins and were kept at 4°C until further utilization.

Table 2.1. List of primers used for the PCR amplification of the 16S rRNA, 16S-23S rRNA ITS region, *rpoB, nifD* and *nodD1* genes.

Primer Set	Primer sequence 5' – 3'	Tm (°C)	Reference
16S-F	AGAGTTTGATCCTGGCTCAG	61.0	Weisberg *et al*., 1991
16S-R2	CGGCTACCTTGTTACGACTT	60.7	Weisberg *et al*., 1991
Bra-ITS-F	GACTGGGGTGAAGTCGTAAC	61.0	Saeki *et al*., 2006
Bra-ITS-R1	ACGTCCTTCATCGCCTC	61.5	Saeki *et al*., 2006
rpoB-83F	CCTSATCGAGGTTCACAGAAGGC	66.8	Martens *et al*., 2008
rpoB-1540R	AGCTGCGAGGAACCGAAG	65.7	Martens *et al*., 2008
nifD-F1	GAGGTGCTGAAGGTCT	60.3	Mason *et al*., 2017
nifD-R1	CTTCCTTGATCTTGTC	57.3	Mason *et al*., 2017
nodD1-F2	CGGAATCAGGCTAAAG	58.5	Mason *et al*., 2017
nodD1-R1	GGTAAAATCGATTGTT	57.1	Mason *et al*., 2017

The amplification of the ITS region followed the PCR reaction with the following conditions: pre-run at 94°C for 5mins; followed by 30 cycles of denaturation at 94°C for 30secs, annealing at 55°C for 30secs, and extension at 72°C for 1min. Final extension was set at 72°C for 10mins and indefinite preservation at 4°C. For the amplification of the *rpoB* housekeeping gene, the PCR conditions were as follows: pre-run at 94°C for 5mins; followed by 30 cycles of denaturation at 94°C for 30secs, annealing at 60°C for 1min, and extension at 72°C for 1min. Final extension was set at 72°C for 5mins and indefinite preservation at 4°C.

For the symbiotic genes *nifD* and *nodD1* genes, the primers were used were also listed in Table 2.1. Similar preparation with the above mentioned procedure of the master mix and the PCR reaction mixture was followed. The PCR cycle was the same with the ITS region except that the annealing temperature was increased to 57 °C for 1 min.

6. Confirmation of the amplified PCR product

Confirmation of the amplified PCR product was carried out by submerged gel electrophoresis. Agarose gel was prepared by melting 1% of agarose powder (PhilKoreaTech., Korea) with 1.0xTAE (40mM Tris, 20mM Acetic acid, 1nM EDTA) in a microwave oven. The same 1.0 x TAE was used as the buffer in the casting chamber. The melted agarose powder was then poured into the casting plate with the comb placed firmly on the plate, covered with aluminum foil, and left to solidify at room temperature for about 30mins. Two (2.0)µL of 10xLoading buffer (1% SDS, 50% Glycerol, 0.05% Bromophenol Blue; TaKaRa Bio) was mixed with 2.5µL of PCR product in a parafilm, then the mixed solution was loaded into the well of the agarose gel. At the first and last well of the gel, 2.0µL of 100bp DNA Ladder RTU (GeneDirex, Taiwan) was loaded on

each well to serve as the marker of the fragment sizes. Electrophoresis was set at 100v for about 20mins, and stained for 20mins with Ethidium Bromide (0.5μg/mL; Nippon Gene Tokyo, Japan). The amplified products' fragment sizes were visualized using LAS-4000 (FUJIFILM Tokyo, Japan).

This procedure was similar for the amplified product of the 16S rRNA gene, ITS region, *rpoB* gene, and the symbiotic genes.

7. RFLP analysis

The RFLP analysis of the 16S rRNA gene and the ITS region were performed using the four restriction enzymes namely: *HaeIII, HhaI, MspI* and *XspI* (TaKaRa Bio) whereas for *rpoB* gene, three restriction enzymes, *HaeIII, MspI* and *AluI* (TaKaRa Bio) were used. Then, the *Bradyrhizobium* USDA strains *B. japonicum* 4, 6^{T}, 38, 122, 123, 124, 129, 135, *B. diazoefficien*s 110 ^{T}, *B. elkanii* 31, 46, 76^{T}, 94, and 130 and *B. liaoningense* 3622^{T} (Saeki et al., 2004) were used as reference strains for the RFLP analysis of the 16S rRNA gene, ITS region and *rpoB* gene. A 2.5 μL aliquot of the PCR product was digested with the restriction enzymes at 37 °C for 16 h in a 10 μL reaction mixture. The restriction fragments were separated on 3 or 4% agarose gels in 0.5 x TBE buffer (Tris 108g, Boric Acid 55g, 0.5M EDTA 40mL (NACALAI TESQUE): pH 8.0) by means of a submerged gel electrophoresis against a 50bp DNA ladder (GeneDirex) as the marker and visualized with ethidium bromide.

8. Selection of representative isolates for sequence analysis

After collecting all the isolates that formed nodules with soybean, *Bradyrhizobium* species were differentiated from each other. This was done first by observing the differences in the colony morphology then confirmed by primary RFLP analysis of the

16S rRNA gene and ITS region (Fig.2.3). Since almost all isolates collected from Nueva Ecija, Philippines belonged to Be76 cluster, then only those isolates from Kumamoto and Okinawa, Japan, which also belonged to Be76 cluster were considered. Then, RFLP analysis of ITS region and *rpoB* gene were conducted for the selected isolates and based from the band pattern, random samples were further selected for sequence analysis of 16S rRNA, ITS region, *rpoB*, *nifD* and *nodD1* genes.

9. Sequence analysis

The PCR amplified products were purified according to the protocol of NucleoSpin® Gel and PCR Clean-up (Macherey-Nagel, Germany). The DNA concentration of the purified product was determined by using the NanoDrop 2000 Spectrophotometer (Thermo Scientific, U.S.A.). Then, preparation of the samples for the sequence analysis from the purified DNA followed the protocol for the premixed template and primer of the company (EUROFINS GENOMICS). After the preparation, the samples were sent to the company for sequence analysis. The sequence primers (Table 2.1) used were all designed for this study and were calculated using the OligoEvaluatorTM by Sigma- Aldrich® Co. LLC.

10. Sequence alignment and construction of phylogenetic trees

To search the homology of sequences, the Basic Local Alignment Search Tool (BLAST) program in DNA Databank of Japan (DDBJ) was used. Those sequences of type strains with a 100% similarity with our isolates were retrieved from the BLAST database. The alignment also included the sequences of *Bradyrhizobium* genospecies for the 16S rRNA gene and the ITS region which were previously determined (Saeki et al., 2004; van Berkum and Fuhrmann, 2000). Then, ClustalW was used to perform the

alignment of the sequences. The phylogeny was determined by the Neighbor-Joining (Saitou and Nei, 1987) method for the 16S rRNA, ITS region, *rpoB, nifD* and *nodD1* genes. The genetic distances were calculated using the Kimura 2-parameter model (Kimura, 1980) in the Molecular Evolutionary Genetic Analysis (MEGA v7) software (Kumar et al., 2016). The phylogenetic trees were bootstrapped with a 1000 replications of each sequence for the evaluation of the reliability of the tree topology.

Table 2.2. List of primers used for the sequence analysis of the 16S rRNA, 16S-23S rRNA ITS region, *rpoB, nifD* and *nodD1* genes.

Primer Set	Primer sequence 5' – 3'	Tm (°C)	Reference
16S-520F	GCAGCCGCGGTAATAC	60.36	Mason et al., 2017
16S-1050F	ATGGCTGTCGTCAGCTC	54.60	Mason et al., 2017
16S-630R	TCTACGAATTTCACCTCTACACTC	60.20	Mason et al., 2017
ITS-AraFF	TAGCTCAGCTGGGAGAGC	61.00	Mason et al., 2017
ITS-lleFF	GAGGTCGGAAGTTCAAGTC	58.80	Mason et al., 2017
ITS-AraR	GAACCGACGACCTCATG	60.21	Mason et al., 2017
rpoB-454F	GTCTCGCAGATGCACC	58.80	Mason et al., 2017
rpoB-1050F	CATCGACCACGTCAATG	59.60	Mason et al., 2017
rpoB-590R	CCTTGGCGTCGAACTC	60.50	Mason et al., 2017
nifD-F2	G T G CCAAGCAYCTCAA	57.70	Mason et al., 2017
nifD-R2	CGAAGAAGTTGTACTC	60.10	Mason et al., 2017
nodD1-F3	CAGGCTAAAGCCCTGC	59.70	Mason et al., 2017
nodD1-R2	GGTTCAAGGGACTTGA	58.20	Mason et al., 2017

All the nucleotide sequences determined in this study were deposited in DDBJ under accession numbers LC167347 to LC167402; LC167474 to LC167485;

LC168752 to LC168753; LC217878 to LC217896 and LC218023 to LC218041 at http://www.ddbj.nig.ac.jp/ and listed in Table 2.3 below.

Table 2.3. List of accession numbers for selected *Bradyrhizobium* USDA reference strains and isolates from the sequence analysis of 16S rRNA gene, 16S-23S rRNA gene ITS region, *rpoB* housekeeping gene and symbiotic genes *nif*D and *nod*D1.

Reference strain	Accession No.	Isolate	Accession No.				
	*rpo*B		*rpo*B	ITS	16S	*nif*D	*nod*D1
B. elkanii USDA31	LC167347	HBO 14	LC167367	LC167385	LC167474	LC217878	LC218023
B. elkanii USDA46	LC167348	HBO 16	LC167368	LC167386	LC167475	LC217879	LC218024
B. elkanii USDA61	LC167349	HBO 21	LC167369	LC167387	nd	LC217880	LC218025
B. elkanii USDA76^T	LC167350	HFK 2	LC167370	LC167388	nd	LC217881	LC218026
B. elkanii USDA94	LC167351	HFK 10	LC167371	LC167389	LC167476	LC217882	LC218027
B. elkanii USDA130	LC167352	HFK 12	LC167372	LC167390	nd	LC217883	LC218028
B. japonicum USDA4	LC167353	OAK 7	LC167373	LC167391	nd	LC217884	LC218029
B. japonicum USDA6^T	LC167354	OAK 10	LC167374	LC167392	LC167477	LC217885	LC218030
B. japonicum USDA38	LC167355	OAK 11	LC168752	LC168753	nd	LC217886	LC218031
B. japonicum USDA62	LC167356	OBO 4	LC167375	LC167393	LC167478	LC217887	LC218032
B. diazoefficiens USDA110^T	LC167357	OFK 6	LC167376	LC167394	nd	LC217888	LC218033
B. japonicum USDA115	LC167358	OFK 8	LC167377	LC167395	LC167479	LC217889	LC218034
B. japonicum USDA122	LC167359	OFK 9	LC167378	LC167396	LC167480	LC217890	LC218035
B. japonicum USDA123	LC167360	PBM 1	LC167379	LC167397	LC167481	LC217891	LC218036
B. japonicum USDA124	LC167361	PBM 3	LC167380	LC167398	LC167482	LC217892	LC218037
B. japonicum USDA125	LC167362	PCM 3	LC167381	LC167399	nd	LC217893	LC218038
B. japonicum USDA127	LC167363	PCM 5	LC167382	LC167400	LC167483	LC217894	LC218039
B. japonicum USDA129	LC167364	PHM 1	LC167383	LC167401	LC167484	LC217895	LC218040
B. japonicum USDA135	LC167365	PHM 4	LC167384	LC167402	LC167485	LC217896	LC218041
B. liaoningense USDA3622^T	LC167366						

nd – not determined

Results

1. Soil pH and EC

The soils which were collected from the Kumamoto, Japan and Nueva Ecija, Philippines were both slightly acidic at 6.23 and 6.21 soil pH, respectively while Okinawa soil was very strongly acidic (4.79). The EC (dS/m) for Kumamoto, Okinawa, and Nueva Ecija were as follows: 0.088, 0.072 and 0.046, respectively, which were all within the acceptable range of EC for soybean (Bernstein et al., 1955). The annual

average temperature from Nueva Ecija, Philippines and Kumamoto and Okinawa, Japan were 26.8°C, 15.8°C and 23.3°C, respectively.

2. Isolation of indigenous rhizobia and selection of the representative isolates

From the three locations, a total of 216 isolates were obtained (72 isolates per location) and their nodulation capability were confirmed through a single-strain inoculation test. The number of isolates which belonged to the Be76 cluster which were collected from the 216 samples were 21, 42, and 71 from Kumamoto, Okinawa, and Nueva Ecija, respectively. The rest of the isolates belonged to the *B. japonicum* USDA6^{T} and *B. diazoefficiens* USDA110^{T} and other minor *B. elkanii* strains. Therefore, we selected only 20 isolates which belonged to Be76 cluster from each location for the final RFLP analysis of the ITS region and the *rpoB* gene which totaled to 60 isolates (Table 2.4). The samples which are marked with black circle symbol (•) in the table below were the random samples used in the current study.

Afterwards, 6 isolates from each location where randomly selected according to the different *rpoB* gene type that was observed from the RFLP analysis. However, since some samples from Okinawa showed a similar ITS-*rpoB* type from the RFLP analysis with both Kumamoto and Nueva Ecija, we selected 7 isolates from this location to represent the difference. Thus, 19 isolates were used for the sequence analysis of the ITS, *rpoB, nifD* and *nodD1* whereas 12 isolates were used for 16S rRNA gene.

Table 2.4. Summary of the RFLP analysis in Kumamoto, Okinawa, and C. Luzon, Philippines.

	Kumamoto				Okinawa				N. Ecija, C. Luzon			
	strains	ITS	*rpoB*		strains	ITS	*rpoB*		strains	ITS	*rpoB*
1	KHA1	76	76	1	OAK1	76	76	1	•PBM1	76	*46
2	KHA3	76	76	2	OAK2	76	76	2	PBM2	76	*46
3	KHA6	76	76	3	OAK3	76	*46	3	•PBM3	76	*46
4	KHA15	76	76	4	OAK4	76	*46	4	PBM4	76	*46
5	KHA20	76	76	5	•OAK7	76	*46	5	PBM5	76	*46
6	HBO1	76	76	6	•OAK10	76	*46	6	PBM6	76	*46
7	HBO2	76	76	7	OAK11	76	76	7	PBM7	76	*46
8	HBO6	76	76	8	OAK12	76	*46	8	PBM8	76	*46
9	HBO7	76	76	9	OAK14	76	*46	9	PCM1	76	*46
10	•HBO14	76	76	10	OAK16	76	*46	10	PCM2	76	*46
11	•HBO16	76	76	11	OFK5	76	*46	11	•PCM3	76	*46
12	•HBO21	76	76	12	•OFK6	76	*46	12	PCM4	76	*46
13	HBO24	76	76	13	•OFK8	76	*46	13	•PCM5	76	*46
14	•HFK 2	76	76	14	•OFK9	76	*46	14	PCM6	76	*46
15	•HFK 10	76	76	15	OFK24	76	*46	15	•PHM 1	76	*46
16	KHF6 6	76	76	16	•OBO4	76	*46	16	PHM2	76	*46
17	KHF6 8	76	76	17	OBO9	76	*46	17	PHM3	76	*46
18	KHF6 9	76	76	18	OBO21	76	*46	18	•PHM4	76	*46
19	KHF6 10	76	76	19	OBO23	76	*46	19	PHM5	76	*46
20	•KHF612	76	76	20	OBO24	76	*46	20	PHM6	76	*46

* indicates different *rpoB* type from ITS region type

3. Nodulation test and symbiotic analysis

The oven dry weight of the shoot and nodules were obtained as well as the nodule number for the three *Rj* genotypes of the soybean cultivars that we used (Table 2.5). All the isolates were able to nodulate both the non-*Rj* and Rj_4 genotypes soybean plants. However, there were four (4) Kumamoto isolates (HFK2, HFK10, HFK12, HBO21) and 2 out 7 Okinawa isolates (OAK10, OFK6) which did not form any nodule with the Rj_2Rj_3 plants. In contrast, all the isolates from Nueva Ecija were able to form nodules with all the *Rj* genotypes used in this study. Accordingly, we were able to confirm that the control plants did not produce any nodule which eliminated the chance of contamination in this report.

The highest number of nodules that were produced were obtained from the plants inoculated with the isolates OBO4, OFK8, PBM1 and PHM4 regardless of the *Rj* genotypes which were significantly different than the other isolates. Meanwhile, the isolates HFK2, HFK10 and HBO21 showed comparably high number of nodules for both non-*Rj* and Rj_4 genotypes.

The amount of N fixed in the shoot showed significant differences among the isolates (Fig. 2.1A). For the non-*R*j genotype, the isolates HFK2, OBO4 and PHM1 fixed the highest N; the isolates HBO14 and PHM have the highest fixed N for the Rj_2Rj_3; and for the Rj_4, the isolate HFK2 fixed the highest amount of N. Some isolates did not form nodules with soybean cultivars which harbor the Rj_2Rj_3 (HFK2, HFK10, HFK12, HBO21, OAK10, OFK6) and it indicated the absence of symbiosis (Fig. 2.1B). Generally, a higher amount of N fixed and symbiotic efficiency were observed with the plants that produced a higher number of nodules for a particular *Rj* genotype.

Table 2.5. Shoot and nodule parameters of the 19 representative *B. elkanii* isolates employing three *Rj* genotypes. The mean comparison was conducted in triplicates only between isolates within the same *Rj* genotype.

Isolate	Shoot DW (g/plant)			Shoot N content (mg/plant)			N fixed (mg/plant)			Nodule (no./plant)			Nodule DW (mg/plant)			Symbiotic efficiency		
	non-*Rj*	*Rj*$_2$*Rj*$_3$	*Rj*$_4$	non-*Rj*	*Rj*$_2$*Rj*$_3$	*Rj*$_4$	non-*Rj*	*Rj*$_2$*Rj*$_3$	*Rj*$_4$	non-*Rj*	*Rj*$_2$*Rj*$_3$	*Rj*$_4$	non-*Rj*	*Rj*$_2$*Rj*$_3$	*Rj*$_4$	non-*Rj*	*Rj*$_2$*Rj*$_3$	*Rj*$_4$
HFK2	0.16	0.14	0.16	23.7	17.5	26.2	8.5a	<0.0d	10.6a	8a	0c	11a	16.2c	-	24.5a	52.6a	0.0h	43.3a
HFK10	0.12	0.15	0.14	17.6	18.2	20.5	2.4c	<0.0d	4.9d	10a	0c	9a	21.2a	-	17.2cd	11.2j	0.0h	28.5b
HFK12	0.11	0.19	0.13	15.2	19.1	17.6	0.02d	<0.0d	2.0de	9a	0c	6b	15.3c	-	13.6e	0.14l	0.0h	14.4c
HBO14	0.15	0.17	0.14	20.1	18.1	19.3	4.9b	8a	3.7d	6ab	11a	8a	11.1d	23.9a	15.3d	44.0b	33.5b	24.1b
HBO16	0.15	0.17	0.14	20.3	22.2	18.9	5.1b	2.1bc	3.4de	8a	6b	7ab	14.1c	10.2e	13.8e	36.3d	20.3d	24.3b
HBO21	0.18	0.14	0.12	22.0	19.4	17.7	6.8ab	<0.0d	2.1de	9a	0c	10a	18.5b	-	21.1b	37.0d	0.0h	10.1d
OBO4	0.15	0.18	0.16	23.1	24.1	22.2	7.9a	4.0b	6.6c	11a	11a	9a	21.4a	21.2b	17.3cd	37.0d	18.7de	38.4a
OAK7	0.14	0.15	0.09	18.6	22.2	10.8	3.4b	2.1bc	<0.0e	11a	10a	4b	21.3a	24.1a	9.4f	15.8i	8.8f	0.0e
OAK10	0.12	0.15	0.11	16.0	17.0	15.8	0.8c	<0.0d	0.2e	7ab	0c	9a	14.3c	-	17.0cd	5.5k	0.0h	1.3e
OAK11	0.15	0.18	0.14	20.2	24.2	18.6	5.1b	4.1b	3.0de	8a	8ab	9a	15.3c	15.9	17.3cd	33.0e	25.7c	17.2c
OFK6	0.11	0.20	0.15	16.6	21.7	19.4	1.4c	1.6c	3.9d	10a	0c	7ab	20.7a	-	14.5d	6.8k	0.0h	26.5b
OFK8	0.11	0.17	0.16	16.5	21.7	22.7	1.3c	1.6c	7.1c	9a	10a	9a	18.7b	19.6bc	17.5cd	6.8k	8.0f	40.3a
OFK9	0.14	0.16	0.12	19.1	20.7	18.9	3.9b	0.6c	3.3de	9a	8ab	11a	16.4c	15.0c	23.3a	23.6g	4.1g	14.2c
PBM1	0.10	0.12	0.18	12.0	13.2	21.2	4.5b	2.7b	6.7c	8a	10a	11a	12.0d	19.9bc	21.9b	37.4cd	13.3e	30.6b
PBM3	0.08	0.12	0.15	8.7	12.2	15.7	1.2c	1.7c	1.2e	3b	4b	3b	4.2e	9.0e	8.2f	29.2f	18.6de	14.8c
PCM3	0.09	0.15	0.16	9.8	15.2	17.2	2.3c	4.7b	2.7de	7ab	6b	9a	13.6c	11.9d	17.1cd	16.6i	39.2a	16.0c
PCM5	0.11	0.13	0.16	14.1	15.2	16.7	6.6ab	4.6b	2.2de	11a	11a	5b	17.0bc	21.7b	8.1f	38.8c	21.2d	27.1b
PHM1	0.11	0.15	0.18	14.8	17.2	23.1	7.3a	6.6a	8.7b	10a	8ab	10a	19.1ab	15.8c	19.1c	38.0c	41.9a	45.4a
PHM4	0.09	0.14	0.18	11.7	16.2	23.7	4.2b	5.7b	9.3ab	11a	10a	12a	21.1a	21.5b	23.0a	19.8h	26.3c	40.3a
Cont. 1	0.12	0.16	0.12	15.2	20.1	15.6	-	-	-	-	-	-	-	-	-	-	-	-
Cont. 2	0.07	0.11	0.14	7.5	10.6	14.4	-	-	-	-	-	-	-	-	-	-	-	-

Different letters indicate significant difference ($p<0.05$, Tukey's HSD). DW – dry weight; Cont. 1 – Japanese cultivars; Cont. 2 – USA cultivars.

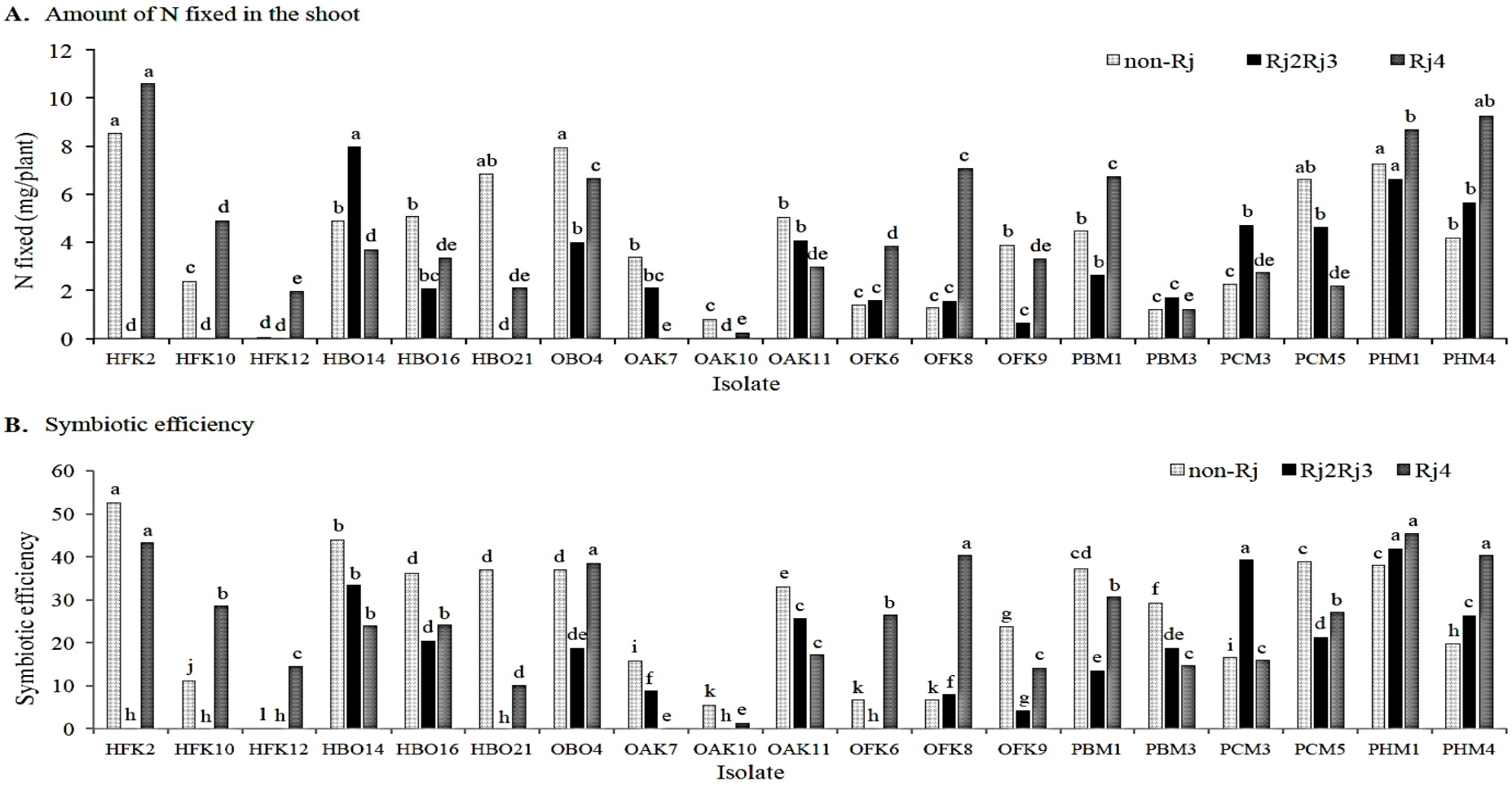


Figure 2.1. Amount of N fixed in the shoot (**A**) and symbiotic efficiency (**B**) of the 19 representative *B. elkanii* isolates employing three *Rj* genotypes. Mean comparison was conducted in triplicates only between isolates within the same *Rj* genotype.

4. RFLP treatment of the ITS region and *rpoB* gene

The phylogenetic trees of the preliminary RFLP analysis of the 60 *B. elkanii* isolates from the three locations that elucidated the clusters are shown in Figure 2.2A for the ITS region and at Figure 2.2A for the *rpoB* gene. All the 60 *B. elkanii* isolates belonged to Be76 cluster for the ITS region-RFLP analysis but were separated into two clusters (Be76 and Be46) for the *rpoB* gene-RFLP analysis. The RFLP band patterns of all the 60 isolates for ITS region were similar to each other on all the four restriction enzymes (*HaeIII, HhaI, MspI* and *XspI*) which indicate that they all belonged to only one ITS type. Therefore, only the band patterns of 1 isolate per location (HBO14 – Kumamoto, OAK10 – Okinawa and PBM1 – Nueva Ecija) and *B. elkanii* reference strains were shown and it was clear that all isolates belonged to Be76 cluster (Fig. 2.3).

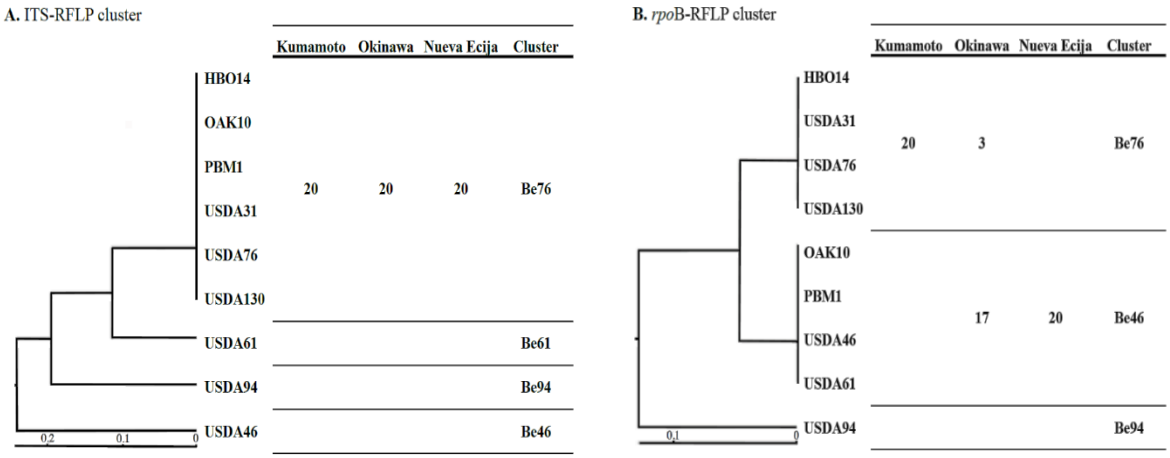


Figure 2.2. Cluster analysis of the 60 *B. elkanii* isolates and *B. elkanii* USDA reference strains based on the primary PCR-RFLP analysis of **(A)** ITS region and **(B)** *rpo*B gene.

	HB014	OAK10	PBM1	USDA31	USDA46	USDA61	USDA76	USDA94	USDA130
HaeIII								344	
	338	338	338	338			338		338
					325	325			
	218	218	218	218	218	218	218	218	218
	204	204	204	204		204	204	204	204
					193				
	124	124	124	124	124	124	124	124	124
HhaI								233	
	227	227	227	227	227	227	227		227
	203	203	203	203	203	203	203	203	203
								136	
	130	130	130	130			130		130
					123	123			
								111	
	107	107	107	107	107	107	107	107	107
					100				
	82	82	82	82		82	82		82
	54	54	54	54		54	54		54
MspI								276	
	270	270	270	270	270	270	270		270
	252	252	252	252	252	252	252	252	252
	165	165	165	165		165	165	165	165
					154				
	144	144	144	144			144	144	144
					131	131			
	99	99	99	99	99	99	99	99	99
XspI								760	
	754	754	754	754			754		754
					741	741			
	176	176	176	176		176	176	176	176
					165				

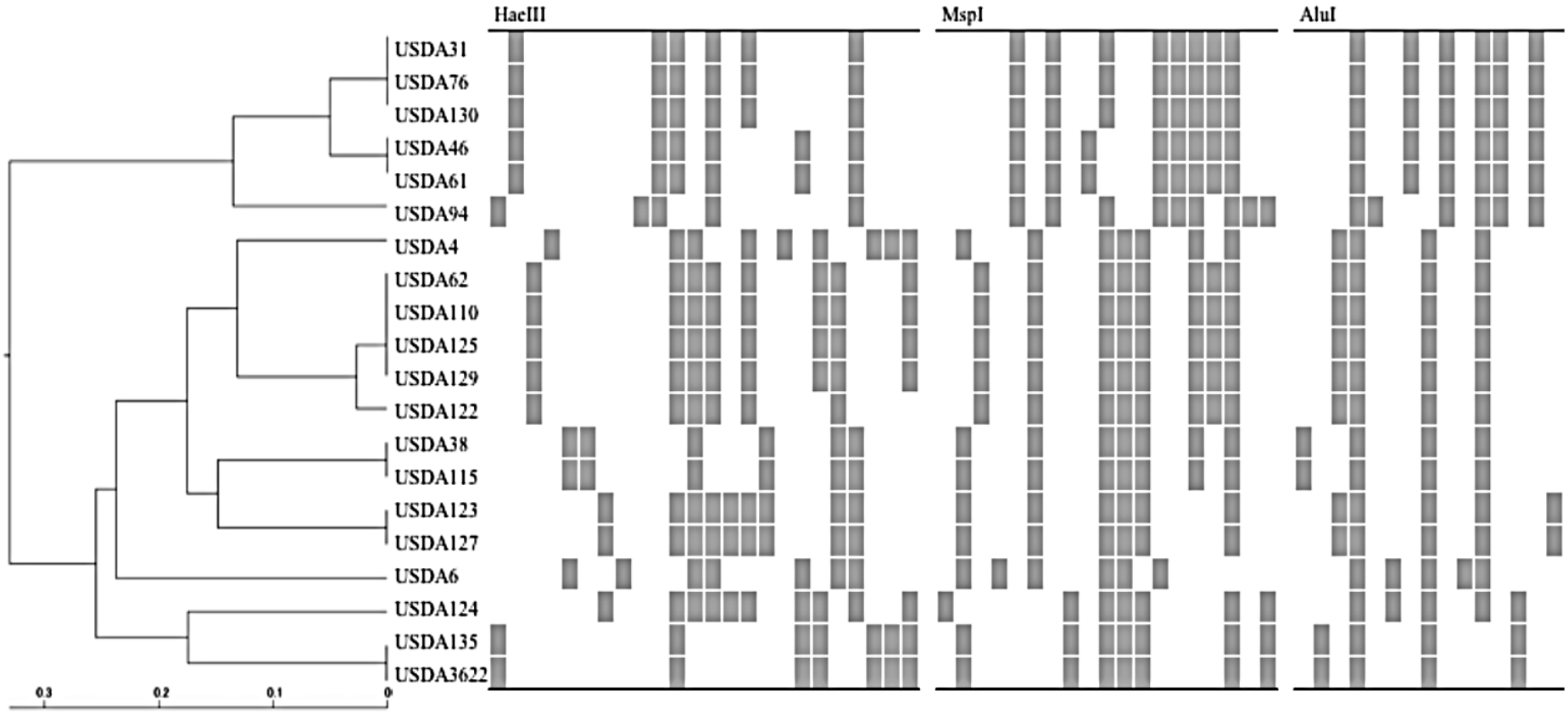
Figure 2.3. Schematic representation of the RFLP band patterns based on the 16S-23S rRNA gene ITS region PCR-RFLP analysis of representative isolate per location and 6 *B. elkanii* reference strains. Sizes (bp) are indicated in the column. Smaller fragment sizes were not shown due to difficulty in recognition on the gel.

On the other hand, the RFLP band patterns of the 60 isolates for *rpoB* gene digested with the 3 restriction enzymes (*HaeIII, MspI* and *AluI*) showed two distinct band patterns which were similar to the clusters of Be76 and Be46 (data not shown). All the Kumamoto isolates and three isolates from Okinawa (represented by OAK11) have identical band patterns with the Be76 cluster whereas, all the Nueva Ecija isolates and the remaining Okinawa isolates have an identical band patterns with the Be46

cluster (data not shown). So, only one isolate per location which represented the band patterns clearly were chosen and plotted against the *B. elkanii* reference strains (Fig. 2.4) indicating the two distinct band patterns for the *rpoB* gene. These results showed that although the RFLP analysis of ITS region indicated that all the isolates have Be76 ITS type, the analysis of *rpoB* gene indicated that Okinawa (except for three isolates represented by OAK11) and Nueva Ecija samples have different *rpoB* type than their ITS type.

	HB014	OAK10	PBM1	USDA31	USDA46	USDA61	USDA76	USDA94	USDA130
HaeIII								662	
	654	654	654	654	654	654	654		654
								293	
	215	215	215	215	215	215	215	215	215
	198	198	198	198	198	198	198		198
	145	145	145	145	145	145	145	145	145
	133			133			133		133
		95	95		95	95			
	81	81	81	81	81	81	81	81	81
	49	49	49	49	49	49	49	49	49
MspI	279	279	279	279	279	279	279	279	279
	239	239	239	239	239	239	239	239	239
		197	197		197	197			
	176			176			176	176	176
	144	144	144	144	144	144	144	144	144
	141	141	141	141	141	141	141	141	141
	132	132	132	132	132	132	132	132	132
	126	126	126	126	126	126	126		126
	123	123	123	123	123	123	123	123	123
								66	
								60	
AluI	451	451	451	451	451	451	451	451	451
								402	
	357	357	357	357	357	357	357		357
	201	201	201	201	201	201	201	201	201
	157	157	157	157	157	157	157	157	157
	144	144	144	144	144	144	144	144	144
	102	102	102	102	102	102	102	102	102

Figure 2.4. Schematic representation of the RFLP band patterns based on gel electrophoresis of the *rpoB* housekeeping gene PCR-RFLP analysis of the 3 representative isolates and 6 *B. elkanii* reference strains. Sizes (bp) are indicated in the column. Smaller fragment sizes were not shown due to difficulty in recognition on the gel.


HaeIII
MspI
AluI
USDA31
USDA76
USDA130
USDA46
USDA61
USDA94
USDA4
USDA62
USDA110
USDA125
USDA129
USDA122
USDA38
USDA115
USDA123
USDA127
USDA6
USDA124
USDA135
USDA3622
0.3
0.2
0.1
0

5. Sequence analysis of 16S rRNA, ITS region and *rpoB* gene

The phylogenetic tree for the ITS region showed that all the isolates were grouped into Be76 cluster which included *B. elkanii* USDA76^{T}, 31 and 130 with bootstrap support of 43 to 99% (Fig. 2.6). Other *Bradyrhizobium* strains obtained from BLAST database which included *Bradyrhizobium* sp. CB1809, *Bradyrhizobium* sp. Glm-3, *Bradyrhizobium* sp. WB1, *B. elkanii* LMG 6134, B. elkanii NBRC 14791 and *B. elkanii* UM19 showed 100% sequence homology with *B. elkanii* USDA76^{T} whereas the *B. elkanii* LMG 6135 and *B. elkanii* MAS8 showed a 99–100% sequence homology with *B. elkanii* USDA31 and 130. For simplicity, we refer to this cluster as Be76 cluster. This result was similar with RFLP analysis of ITS region and indicated that all the isolates belonged to cluster Be76.

Meanwhile, the sequence analysis of the *rpoB* gene revealed three (3) distinct groups under Be46 and Be76 clusters (Fig. 2.7). Group I is composed of five (5) isolates from Okinawa and Group II is composed of all the six (6) isolates from Nueva Ecija along with one (1) isolate from Okinawa (OAK7). These two groups belong to Be46 cluster. Group III is composed of all the six (6) isolates from Kumamoto along with one (1) isolate from Okinawa (OAK11) and it belongs to Be76 cluster.

In addition, Figure 2.7 showed the first phylogeny of the *Bradyrhizobium* USDA strains with specific serogroups from the *rpoB* gene sequence analysis. This phylogeny is similar with the band patterns of the *rpoB* gene which were obtained from RFLP treatment which indicated its usefulness (Fig. 2.5). On the other hand, the phylogenetic tree of the 16S rRNA gene of the 12 representative isolates clearly separated the groups of *B. elkanii* strains from *B. japonicum* and *S. fredii* (Fig. 2.8).

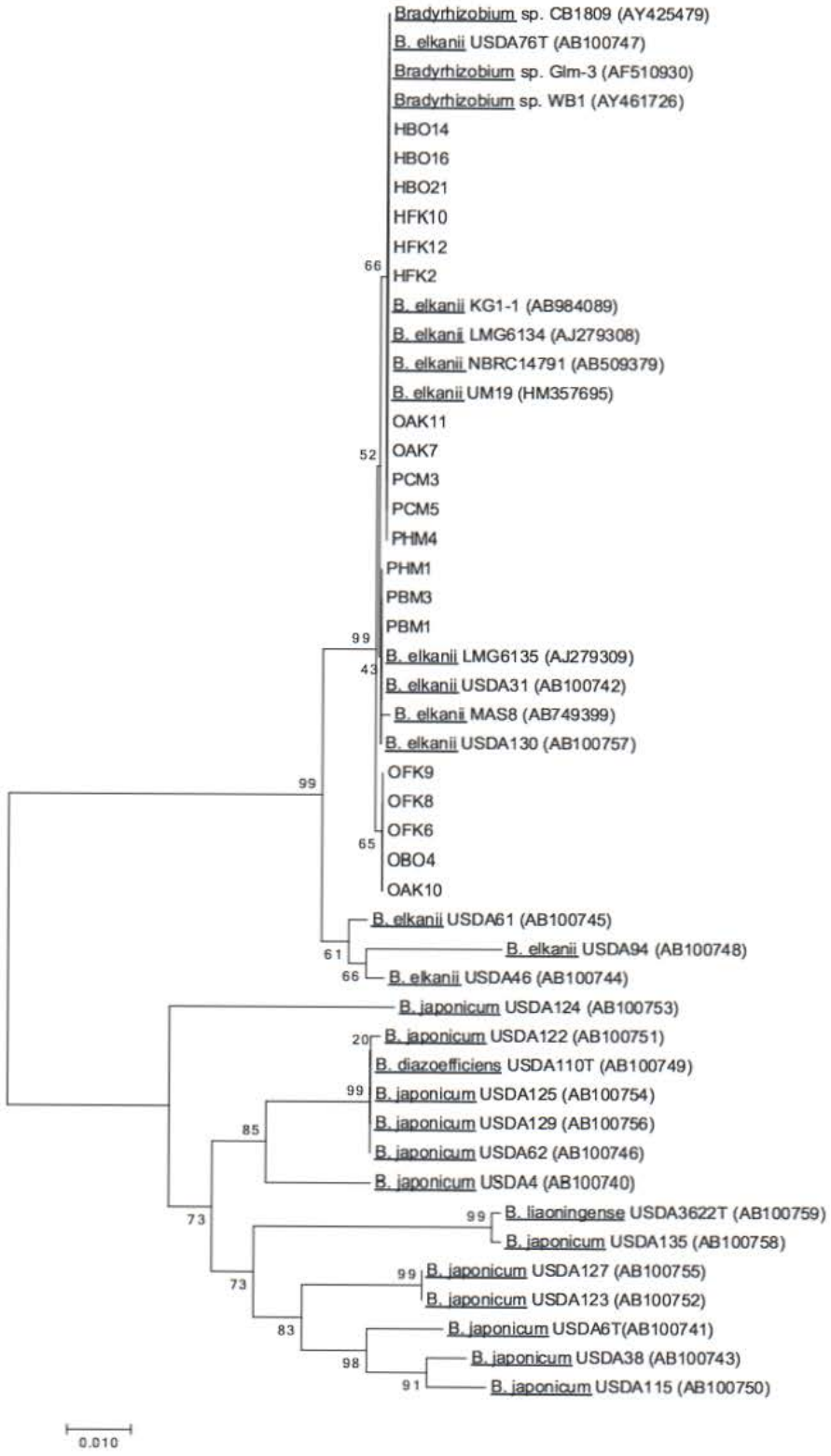


Figure 2.6. Phylogenetic tree based on the sequence analysis of 16S-23S rRNA gene internal transcribed spacer (ITS) region. The tree was constructed with the Neighbor-Joining method using the Kimura 2-parameter (K2P) distance correlation model and 1000 bootstrap replications in MEGA v.7 software. The first letter of isolates' name indicates the location as follows: **H** - Kumamoto; **O** - Okinawa; **P** - C. Luzon, Philippines.

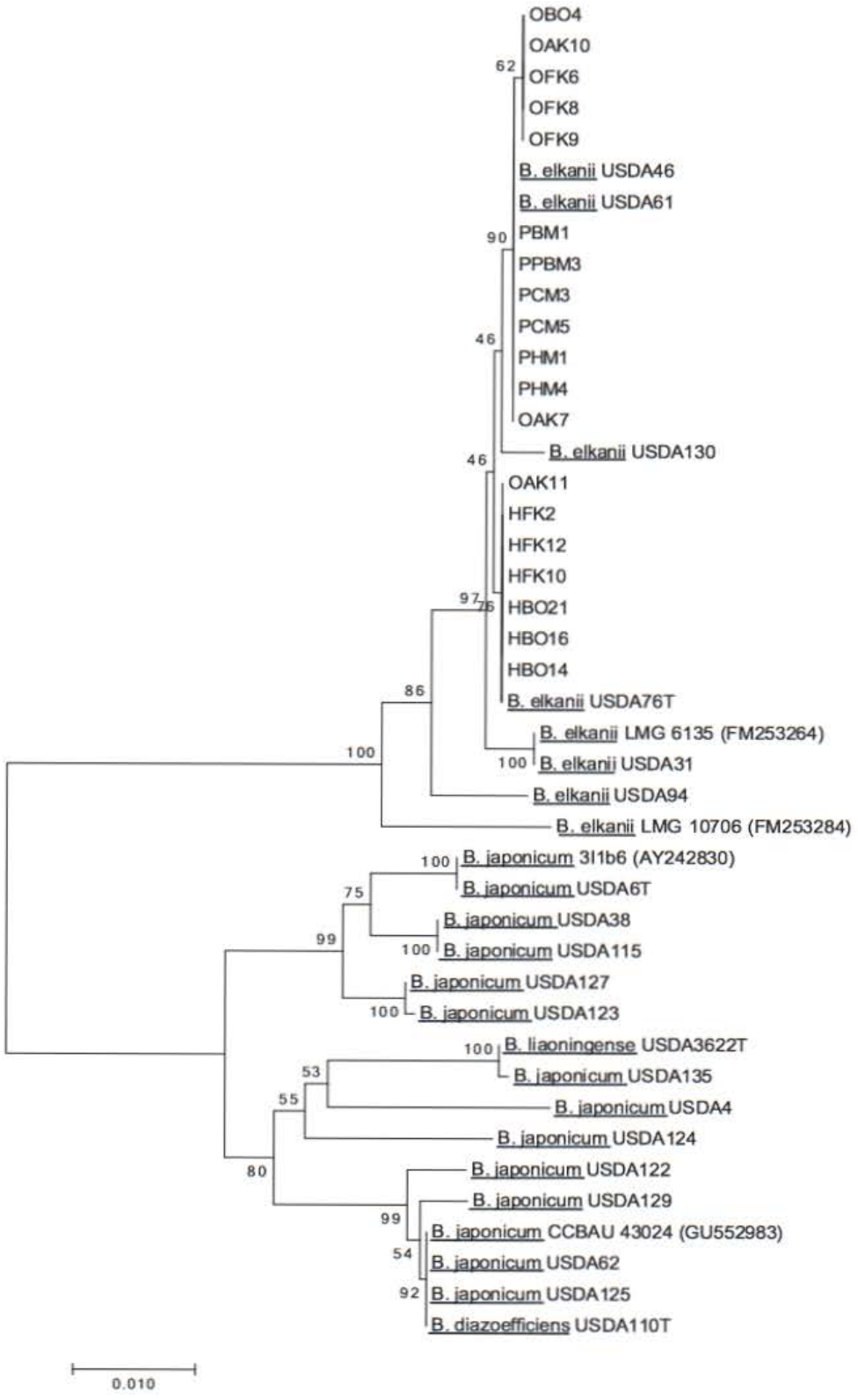


Figure. 2.7. Phylogenetic tree based on the sequence analysis of the *rpoB* housekeeping gene. The tree was constructed with the Neighbor-Joining method using the Kimura 2-parameter (K2P) distance correlation model and 1000 bootstrap replications in MEGA v.7 software. The first letter of isolates' name indicates the location as follows: **H** - Kumamoto; **O** - Okinawa; **P** - C. Luzon, Philippines.

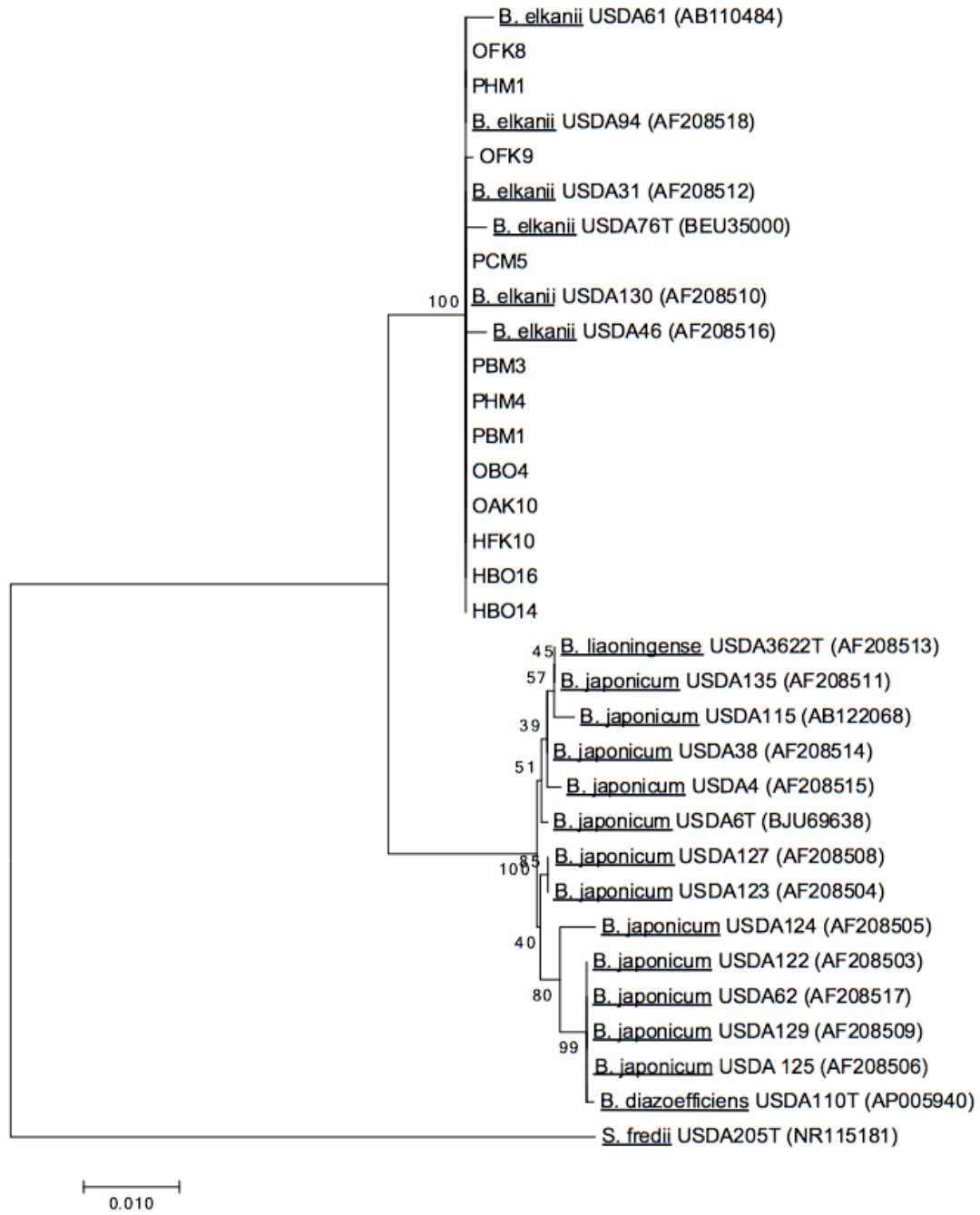


Figure 2.8. Phylogenetic tree based on the sequence analysis of the 16S rRNA gene. The tree was constructed with the Neighbor-Joining method using the Kimura 2-parameter (K2P) distance correlation model and 1000 bootstrap replications in MEGA v.7 software. The first letter of isolates' name indicates the location as follows: **H** - Kumamoto; **O** - Okinawa; **P** - C. Luzon, Philippines.

6. Sequence analysis of the symbiotic genes *nifD* and *nodD1*

For the symbiotic genes, the 19 representative isolates which were used for the ITS region and *rpoB* gene were also classified phylogenetically based from the DNA fragments of the *nifD* and *nodD1* genes (Fig. 2.9). It can be seen from the phylogenetic trees of both the *nifD* (Fig. 2.9A) and the *nodD1* (Fig. 2.9B) that there was no diversity among the isolates. All the isolates from the three locations have a homogenous nucleotide (nt) sequences for both the *nifD* (785 nt) and the *nodD1* (717 nt). It is clear that all the isolates were grouped under the *B. elkanii* cluster in *nifD* wherein a separation of the *B. elkanii* and the *B. japonic*um strains was also distinguished. For the *nodD1*, all the isolates showed a 100% similarity with the *B. elkanii* USDA94 and *B. elkanii* M13 which were differentiated from the *B. japonic*um and *B. diazoefficiens* strains. As of the writing of this report, we were not able to find other *B. elkanii* strains in the DDBJ database for the *nodD1* gene which had at least 97% similarity with any of our isolates. In relation to this, we used the *B. elkanii* USDA94 *nodD2* gene nucleotide sequence as the outgroup.

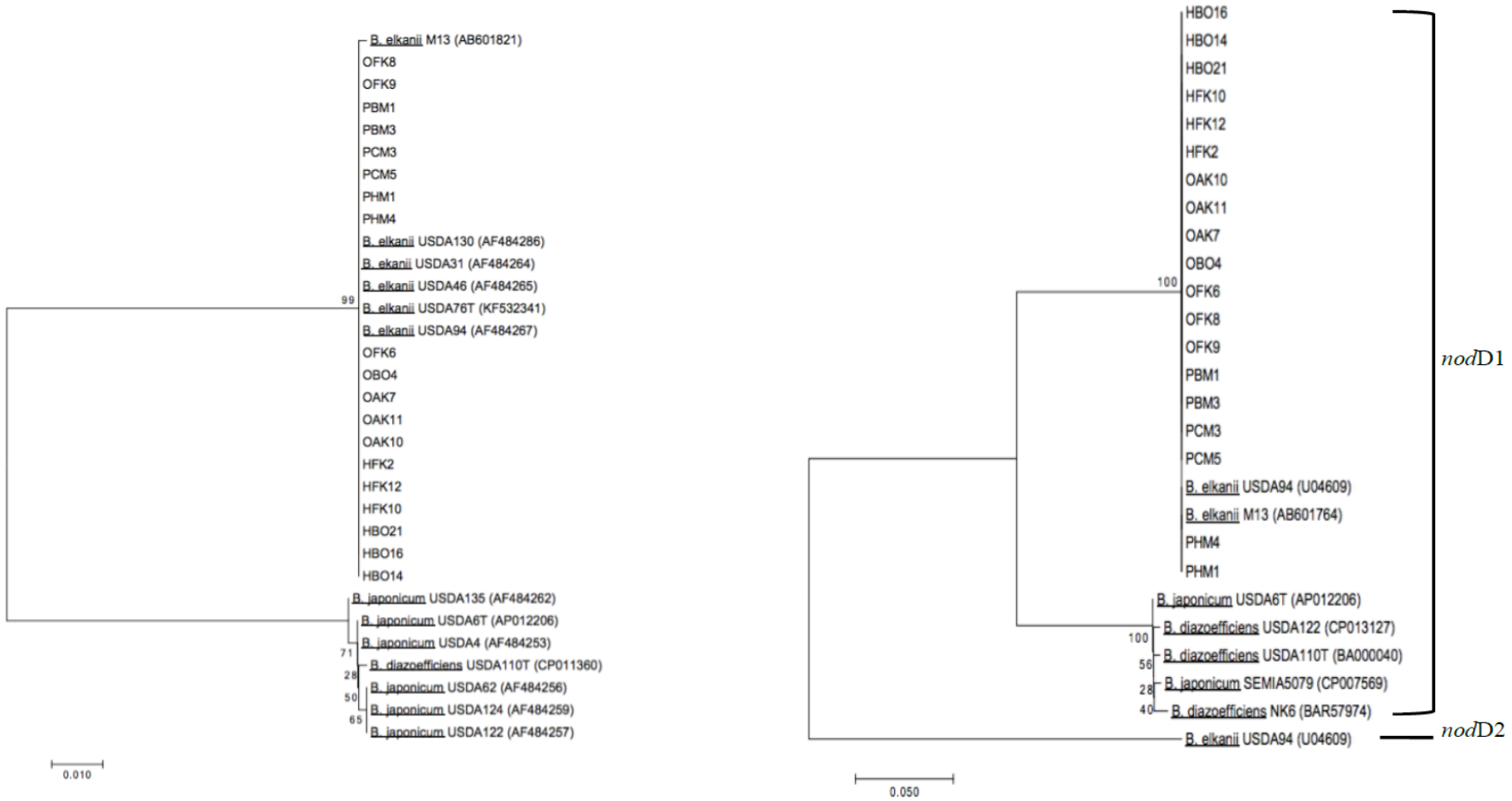


Figure 2.9. Phylogenetic tree based on the sequence analysis of **(A)** *nif*D gene and **(B)** *nod*D1 gene. The tree was constructed with the Neighbor-Joining method using the Kimura 2-parameter (K2P) distance correlation model and 1000 bootstrap replications in MEGA v.7 software. The first letter of isolates' name indicates the location as follows: **H** - Kumamoto; **O** - Okinawa; **P** - C. Luzon, Philippines.

7. Comparison of the RFLP and sequence analysis

The results from the RFLP and sequence analysis were summarized in Table 2.4 and it can be noted that from the 19 representative isolates used in this study, the isolates with ITS-*rpoB* types of 31′-46′ and 31–46 might be potentially endemic in Okinawa, Japan and Nueva Ecija, Philippines, respectively. Also, some isolates possessed an ITS type which was different from its *rpoB* type and these isolates were found in Okinawa, Japan and Nueva Ecija, Philippines.

Comparing the RFLP treatment and the sequence analysis, the genetic diversity of the isolates were better discriminated with the sequence analysis than by the RFLP analysis. There were two ITS-*rpoB* types from Nueva Ecija that were obtained from this report which were Be31-Be46 and Be76-Be46 whereas, three ITS-*rpoB* types were observed from Okinawa as follows: Be76-Be76', Be76-Be46, and Be31′-Be46'. On the other hand, all the Kumamoto isolates possessed a similar ITS-*rpoB* types which was Be76-Be76'. The diversity among the isolates as obtained from the ITS region and the *rpoB* gene was observed to be the highest in Okinawa, followed by Nueva Ecija then, Kumamoto. Meanwhile, no diversity was observed for both the *nifD* and *nodD1* genes but both have similar results with the 16S rRNA gene showing a high homogeneity with the *B. elkanii* USDA strains and the *B. elkanii* M13 strain, which was isolated from *Vigna radiata* plant in Nepal.

Table 2.6. Difference in ITS-*rpo*B type of the isolates against the two closest *B. elkanii* reference strains as detected by the sequence analysis of the 16S-23S rRNA gene ITS region and the *rpoB* housekeeping gene.

Strain	Location	16S rRNA gene	RFLP cluster		Sequence cluster		ITS-*rpo*B type
			ITS region	*rpoB* gene	ITS region	*rpoB* gene	
HBO 14	Kumamoto	*B. elkanii*	Be76	Be76	Be76	Be76’	76-76’
HBO 16	Kumamoto	*B. elkanii*	Be76	Be76	Be76	Be76’	76-76’
HBO 21	Kumamoto		Be76	Be76	Be76	Be76’	76-76’
HFK 2	Kumamoto		Be76	Be76	Be76	Be76’	76-76’
HFK 10	Kumamoto	*B. elkanii*	Be76	Be76	Be76	Be76’	76-76’
HFK 12	Kumamoto		Be76	Be46	Be76	Be76’	76-76’
OBO 4	Okinawa	*B. elkanii*	Be76	Be46	Be31’	Be46’	31’-46’
OAK 7	Okinawa		Be76	Be46	Be76	Be46	76-46
OAK 10	Okinawa	*B. elkanii*	Be76	Be46	Be31’	Be46’	31’-46’
OAK 11	Okinawa		Be76	Be76	Be76	Be76’	76-76’
OFK 6	Okinawa		Be76	Be46	Be31’	Be46’	31’-46’
OFK 8	Okinawa	*B. elkanii*	Be76	Be46	Be31’	Be46’	31’-46’
OFK 9	Okinawa	*B. elkanii*	Be76	Be46	Be31’	Be46’	31’-46’
PBM 1	Nueva Ecija	*B. elkanii*	Be76	Be46	Be31	Be46	31-46
PBM 3	Nueva Ecija	*B. elkanii*	Be76	Be46	Be31	Be46	31-46
PCM 3	Nueva Ecija		Be76	Be46	Be31	Be46	31-46
PCM 5	Nueva Ecija	*B. elkanii*	Be76	Be46	Be76	Be46	76-46
PHM 1	Nueva Ecija	*B. elkanii*	Be76	Be46	Be76	Be46	76-46
PHM 4	Nueva Ecija	*B. elkanii*	Be76	Be46	Be76	Be46	76-46
B. elkanii USDA 76^T			Be76	Be76	Be76	Be76	76-76
B. elkanii USDA 46			Be46	Be46	Be46	Be46	46-46

Note: The prime symbol **(‘)** - indicates a slight variation in the nucleotide sequence of the isolate from that of the reference strain.

Discussion

1. Genetic diversity of the indigenous soybean rhizobia as detected by the RFLP analysis

Several studies conducted in this laboratory have revealed that Okinawa, Japan was dominated by the species of *B. elkanii* particularly the Be76 cluster (Saeki et al., 2006, 2008) and in our present study, we obtained a similar result. Meanwhile, this is the first report that was able to identify the indigenous soybean-nodulating *B. elkanii* strains from Nueva Ecija, Philippines and Kumamoto, Japan.

As previously reports stated, the diversity of soybean rhizobia is influenced by several factors such as soil acidity, salinity, geographic location and environmental gradients (Suzuki et al., 2008; Zhang et al., 2011; Adhikari et al., 2012; Shiro et al., 2013; Yan et al., 2014; Zhao et al., 2014; Htwe et al., 2015). Similarly, it could be said that in this particular report, the most influential factors might be the temperature and soil pH. It can be noted that since the salinity level for all the locations was almost similar, thus it is assumed that salinity was not directly related to the diversity of the soybean rhizobia in this case.

For the strongly acidic soils where the Okinawa isolates were obtained, the RFLP analysis showed that all isolates with Be76 ITS type were divided into Be76 and Be46 type in the *rpoB* gene. In case of the Nueva Ecija isolates, all the Be76 ITS type became Be46 type in the *rpoB* gene. Thus, this difference could be attributed to the temperature gradient since Okinawa, Japan is considered as a subtropical region whereas Nueva Ecija, Philippines is a tropical region. On the other hand, the Kumamoto isolates which were obtained from a temperate region that possessed the Be76 ITS type remained as Be76 type with the *rpoB* gene. Since Kumamoto, Japan and Nueva Ecija, Philippines have both the same degree of soil acidity, the main difference between the two location

is temperature, which might have caused the change in the *rpoB* type. The effect of the different temperature regimes on the changes of population occupancy of bradyrhizobia in Japan was evaluated earlier and it was revealed that the cluster Be76 was dominant in the middle (25 °C) and high (35 °C) temperatures (Saeki et al., 2010). Therefore, our results seem to support the idea that Be76 is a dominant cluster at higher temperatures which can be found in the subtropical and tropical climates of the three locations in this study.

By analyzing the polymorphisms of the *rpoB* gene, possible endemic strains of soybean rhizobia in Okinawa and Nueva Ecija isolates were detected which were not distinguished in the ITS region. Also, the existence of strains with similar ITS type but completely different *rpoB* type as detected from Okinawa and Nueva Ecija isolates were reported. This observation could be due to the influence of the temperature and soil acidity, although a more detailed analysis should be conducted to verify this. The observed inconsistency between the ITS and *rpoB* type within the same strains could be likely due to the recombination events which is widespread within the bacteria, and is more often to the members of the same microbiological species (Didelot and Maiden, 2010). As recently investigated for *Rhizobium* species associated with *Phaseolus vulgaris*, higher nucleotide diversity is introduced from recombination events rather than mutation (Carrascal et al., 2016).

2. Genetic diversity of indigenous soybean rhizobia as revealed by the sequence analysis of the ITS region and *rpoB* gene

The usefulness of the sequence analysis of the ITS region to distinguish *Bradyrhizobium* species even at the strain level is presented in Figure 2.6. However, the ITS region failed to detect a clear endemism and genetic diversity of the indigenous

soybean rhizobia as seen in this study. Considering the previous studies on the diversity of soybean rhizobia in temperate regions as earlier cited, one possible explanation is that the evolution rate of the ITS region of soybean rhizobia in the subtropical and tropical regions was not similarly influenced by the environmental gradients, particularly temperature. Thus, we have elucidated that for subtropical and tropical regions, the use of the ITS region alone was not enough to detect a genetic diversity among the *B. elkanii* strains.

Meanwhile, the phylogenetic tree from the sequence analysis of the *rpoB* gene (Fig. 2.7) provided a higher resolution among the strains and was able to reveal that the genetic diversity of the indigenous soybean rhizobial isolates varied geographically and is believed that this variation could be due to the temperature and soil pH. The high discriminatory power of the *rpoB* gene was already proven in several reports which merit its use for detecting genetic divergence (Vinuesa et al., 2008; Rivas et al., 2009; Degefu et al., 2013; Yan et al., 2014; Guimarães et al., 2015). Also, the existence of some Okinawa isolates (OAK7 and OAK11) in Nueva Ecija and Kumamoto groups indicated that the distribution of soybean rhizobia could be affected by the change in temperature and soil pH. It was previously stated that the occurrence of recombination events is affected by the physical proximity, genetic distance and environmental changes (Didelot and Maiden, 2010) and in this present report, it might be the differences in the temperature and soil pH. Thus, it was demonstrated that the *rpoB* gene is a useful genetic marker for the analysis of diversity and detection of potential endemic strains of soybean rhizobia in these three locations, particularly for *B. elkanii*.

3. Genetic diversity of indigenous soybean rhizobia based from the symbiotic genes

In contrast with the ITS region and *rpoB* gene, the sequence analysis of *nifD* and *nodD1* genes did not provide diversity among the isolates. The high nucleotide similarity (99–100%) observed between the isolates and *B. elkani*i strains for both *nifD* and *nodD1* and its congruence with the 16S rRNA gene phylogeny might suggest that the evolution of symbiotic genes from the isolates of Kumamoto and Okinawa, Japan and Nueva Ecija, Philippines have progressed comparably with their conserved genes. Some previous studies (Minamisawa et al., 2002; Barcellos et al., 2007; Ling et al., 2016) stated that the horizontal gene transfer do occur for symbiotic genes in rhizobial genera that commonly causes the conformity in the phylogenetic analyses. Although we cannot say that this is also the case with our results because we did not perform an experiment that will support this. However, our result is also similar with an earlier report (Risal et al., 2010) which stated the similarity of phylogenies obtained from the conserved 16S rRNA gene region and the symbiotic genes *nifD* and *nodD1* for the Nepalese isolates. Thus, we suggest that the symbiotic genes *nifD* and *nodD1* may not appropriately indicate a genetic diversity, particularly for *B. elkan*ii. This idea is supported by a previous study which stated that even distinct rhizobial species can share similar symbiotic genes and it might be because they are located in an easily interchangeable elements like the symbiosis island (Ramirez-Bahena et al., 2009). Another plausible explanation for the similar phylogenies that we obtained from *nifD* and *nodD1* genes might be due to the gene exchange and internal genetic rearrangements that could have occurred after the co-transfer of nod and nif genes as previously reported (Laguerre et al., 2001).

On the contrary, the incongruent phylogenies of *nifD* and *nodD1* with that of the *rpoB* gene is possibly due to the lateral gene transfer as previously observed (Martinez-Romero and Caballero-Mellado, 1996; Laguerre et al., 2001; Tian et al., 2010). Hence,

there are cases wherein symbiotic genes, particularly nodulation genes, have independent phylogenies from other taxonomic markers such as chromosomal genes (Tian et al., 2010). In this study, we analyzed the *nodD* sequence as a representative gene of the common nod gene. The common nod genes which include the *nod* D, A, B, C are concerned with the construction of the based structure of Nod factor. The Nod factor is related with host specificity between the rhizobia and leguminous species, and not with the compatibility between the rhizobia and *Rj*-genotype varieties. The responsible gene in the bradyrhizobia for incompatibility with *Rj*-genotype soybean is not yet clarified with some candidate genes (Tsurumaru et al., 2008; Yasuda et al., 2016). For the incompatibility with Rj_2-genotype soybean, it was reported by Tsurumaru et al. (2008) that some bradyrhizobial mutants could break the incompatibility with Rj_2-genotype, and the breaking genes were not common nod genes. Though the responsible gene for the incompatibility is not elucidated as of the moment, the gene may be important not only for the compatibility but also for genomic diversity.

The nucleotide divergence from the sequence analysis of the *Bradyrhizobium* USDA strains and the selected isolates are presented in Figures 2. 10 – 2.13.

4. Symbiotic performance of the indigenous soybean *B. elkanii*

An important feature of a useful inoculant is its efficiency in symbiosis with the host. Here, we observed that most of the isolates have a broad range of host, which is a positive characteristic for a potential inoculant, particularly for the isolates from Nueva Ecija wherein 100% possessed this quality. The fact that the phylogeny of the *nodD1* did not show any differences among the isolates, it is deemed possible that this nodulation regulator protein had no or little correlation with the *Rj* genotypes although it might have influenced the broad host-range in some isolates.

In this study, there is no isolate which was host-specific that could be generally due to the role of *nodD*1 as a nodulation regulator. These isolates also showed different symbiotic efficiency, which were significantly different between the isolates within the same *Rj* genotype. Although this result was not indicated in the phylogeny of the *nifD* gene, it might be possible that the *nifD* gene had no direct or little relationship with the *Rj* genotypes. Nevertheless, it is worthy to note that some isolates from the Philippines (PBM1, PCM5, PHM1 and PHM4) and Southern Japan (HBO14, HBO16, OBO4, OAK11) maybe further studied for their potential as inoculant in relation to host-range and symbiotic efficiency.

5. Genetic diversity and observation of some endemic rhizobia

For more than a decade, our research group has already established that for the diversity studies on soybean rhizobia for temperate regions, the 16S-23S rRNA ITS region provided high diversity (Saeki et al., 2006, 2008, 2010; Shiro et al., 2013). However, for the subtropical and tropical regions, it is suggested that the *rpoB* gene should at least be included in addition to the ITS region as one of the molecular markers. Also, it is proposed that the following *Bradyrhizobium* USDA reference strains (*B. japonicum* USDA 4, 6^{T}, 38, 122, 123, 124, 129, 135, *B. diazoefficiens* USDA 110 ^{T}, *B. elkanii* USDA 31, 46, 76^{T}, 94, and 130 and *B. liaoningense* USDA 3622^{T}) be used in the analysis of the *rpoB* gene for soybean rhizobia. This study was able to distinguish that the ITS-*rpoB* type of *B. elkanii* isolates from Kumamoto and Okinawa, Japan and Nueva Ecija, Philippines were not 100% identical to the two closest reference strains, which were USDA 76^{T} and USDA46.

In this study, the presence of possible endemic strains of *B. elkanii* that nodulate the soybean in Okinawa, Japan and Nueva Ecija, Philippines was observed and that the

genetic diversity of the isolates studied might have varied with the difference in temperature and similarity in soil pH as revealed by the sequence analysis of the *rpoB* gene. It is also observed that the symbiotic genes *nifD* and *nodD1* were possibly not correlated with the compatibility of the *Rj* genotypes used in this study, although a more detailed analysis is recommended to confirm this statement.

This study was able to provide the following significant results: 1), the production of the first phylogenetic tree of the *Bradyrhizobium* USDA reference strains for *rpoB* gene with specific serogroups; 2), pioneer study that reported the existence of different *rpoB* gene type from the ITS type within the same strain for *B. elkanii*; and last, the first study that detected and reported the presence of possible endemic soybean rhizobia in Nueva Ecija, Philippines and Okinawa, Japan. Furthermore, the strains that have possible broad range of host compatibility and could be considered as efficient microsymbionts of soybean were identified in the three locations that could be studied for their efficiency and effectiveness as suitable inoculants in the future. The information obtained in this research might help the inoculation strategy to be more successful particularly in Nueva Ecija, Philippines and Kumamoto, Japan since the indigenous soybean rhizobia have been identified. It is suggested that more locations should be considered particularly in the Philippines that could represent the whole country.

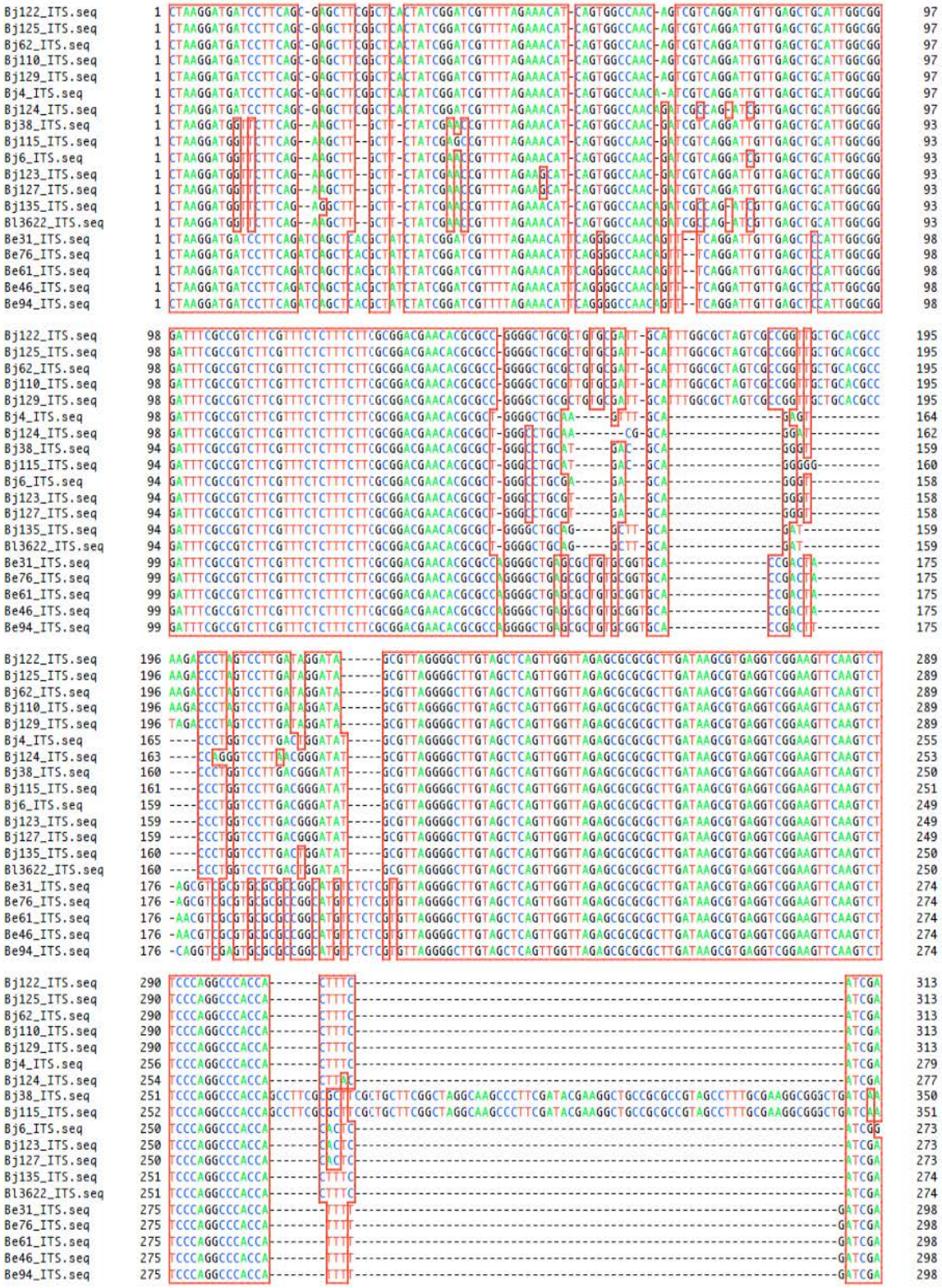


Figure 2.10. Alignment result of ITS region of *Bradyrhizobium* USDA strains.

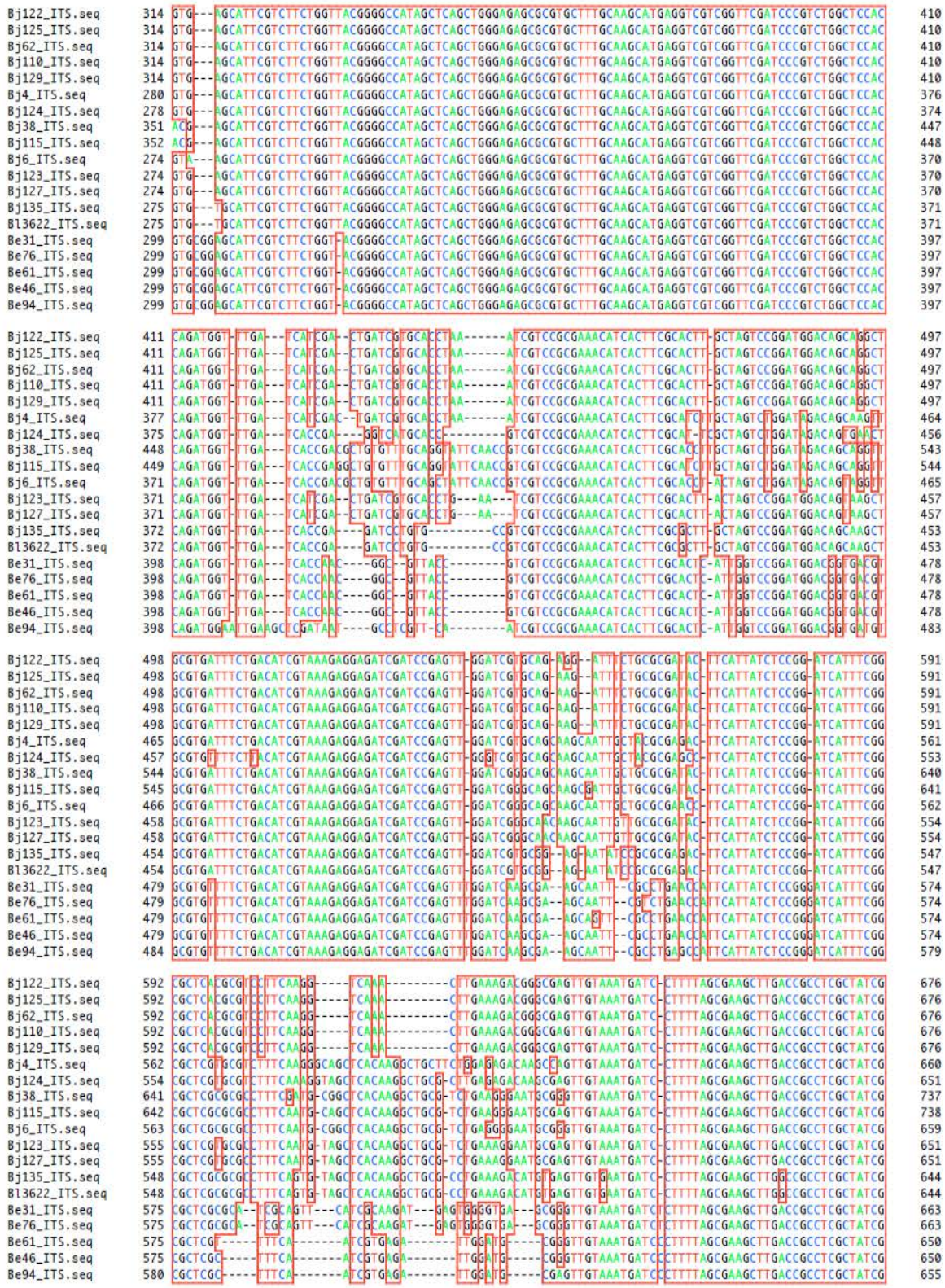


Figure 2.10 (continued). Alignment result of ITS region of *Bradyrhizobium* USDA strains.

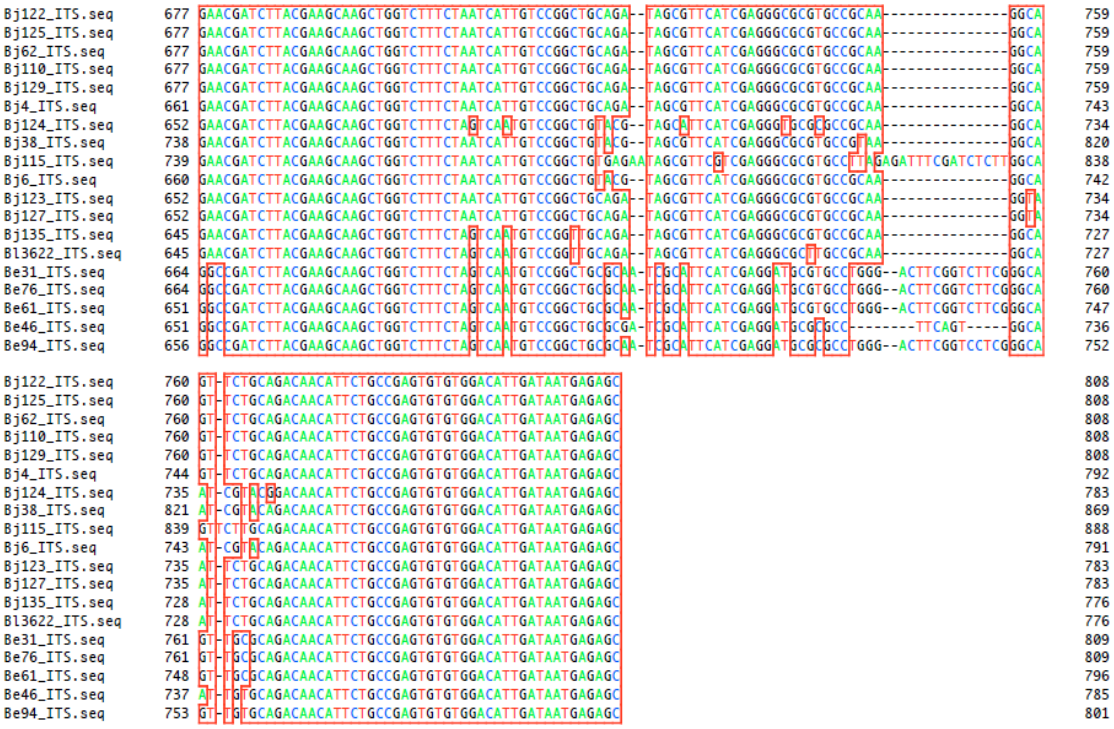


Figure 2.10 (continued). Alignment result of ITS region of *Bradyrhizobium* USDA strains.

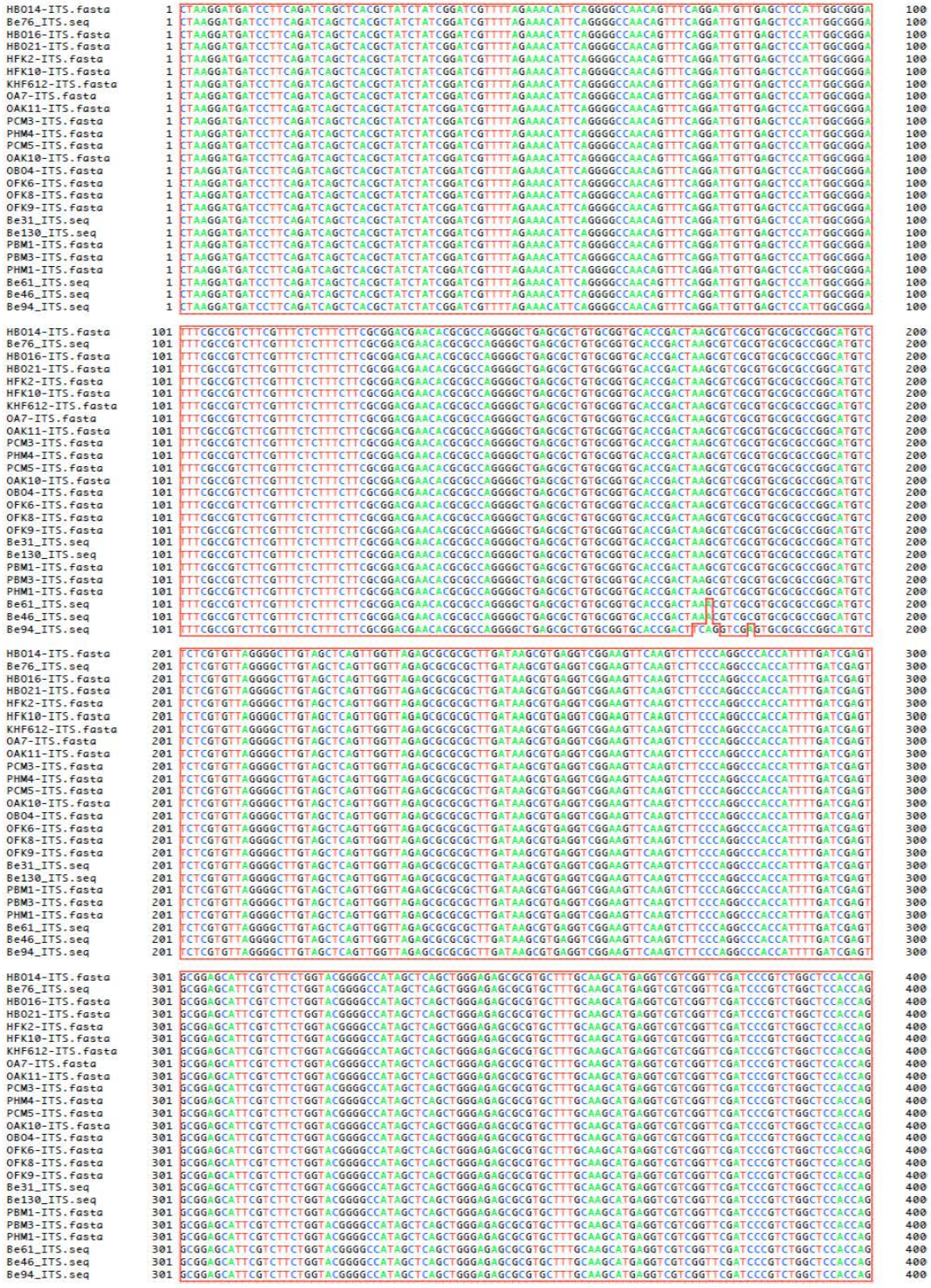


Figure 2.11. Alignment result of ITS region of *B. elkanii* USDA strains and isolates.

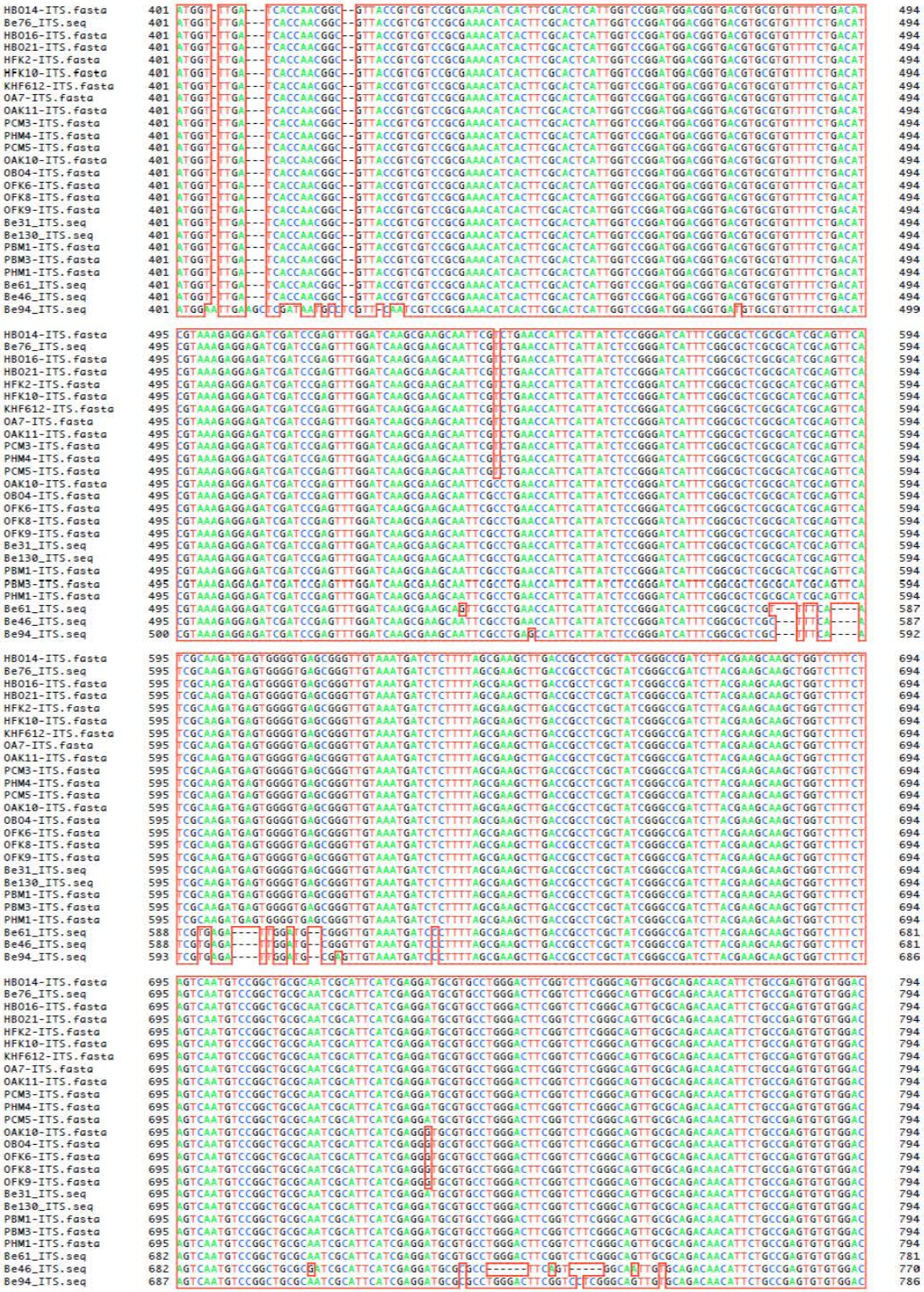


Figure 2.11 (continued). Alignment result of ITS region of *B. elkanii* USDA strains and isolates.

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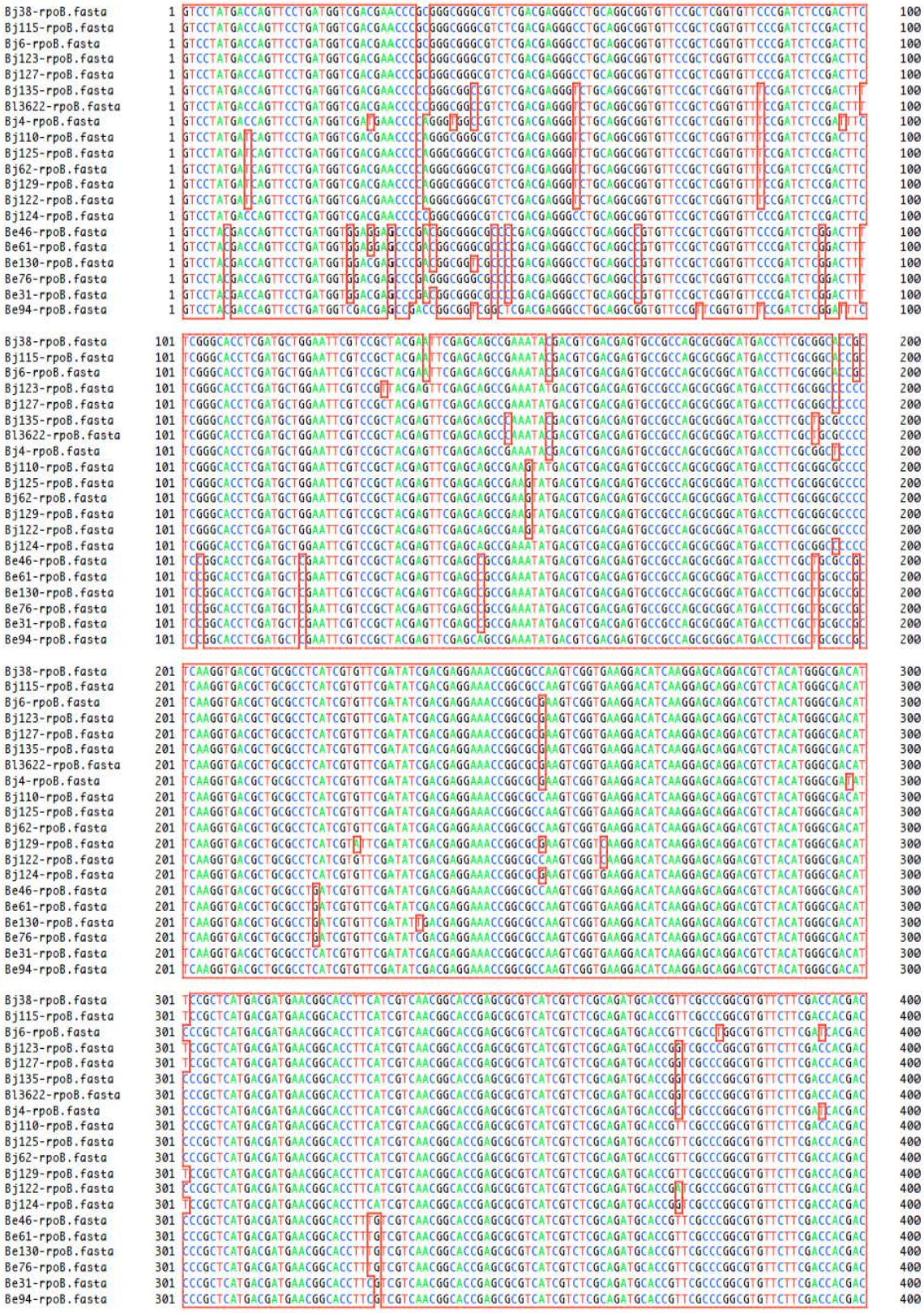

Figure 2.12. Alignment result of *rpoB* gene of *Bradyrhizobium* USDA strains.

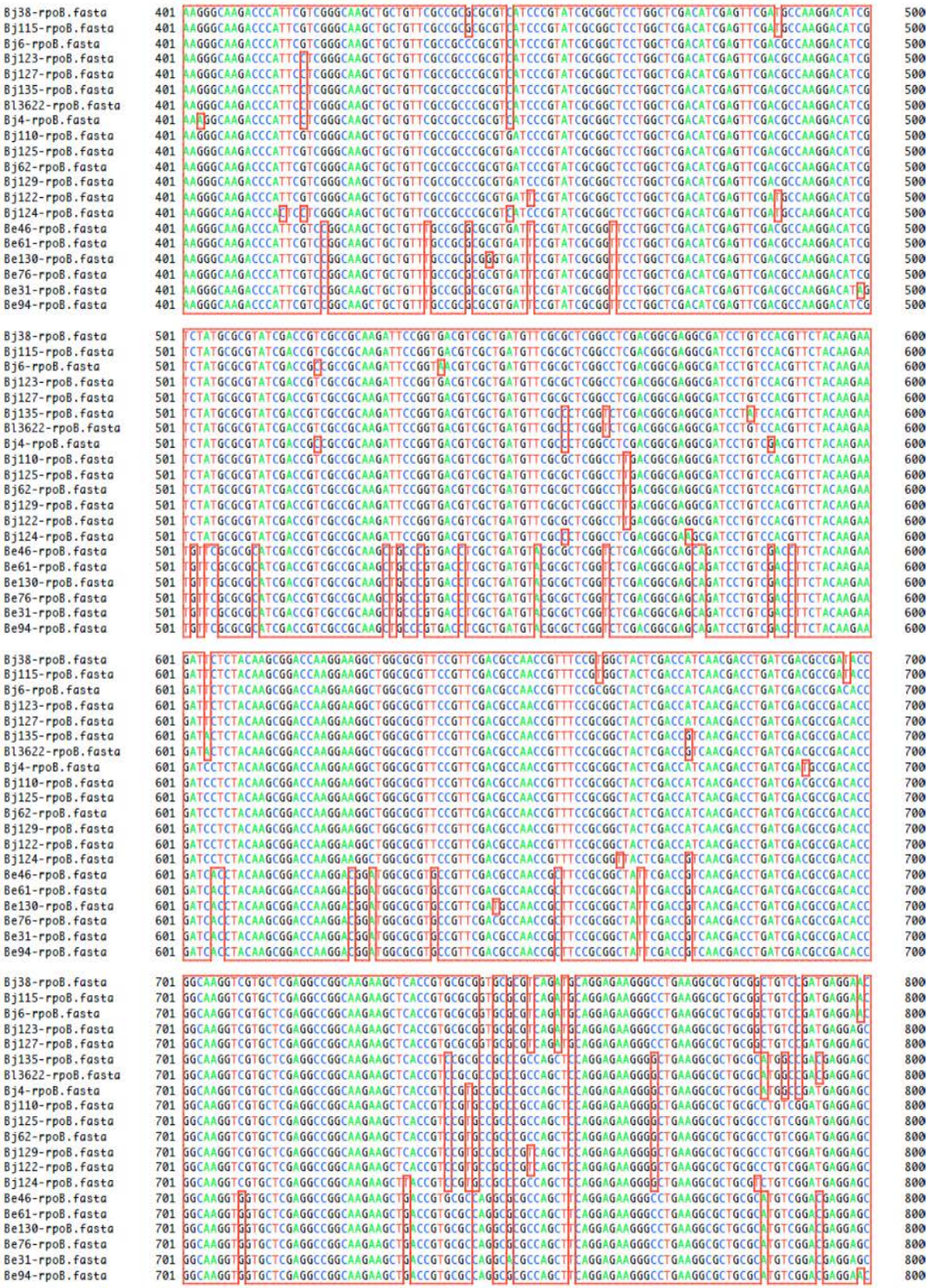

Figure 2.12 (continued). Alignment result of *rpoB* gene of *Bradyrhizobium* USDA strains.

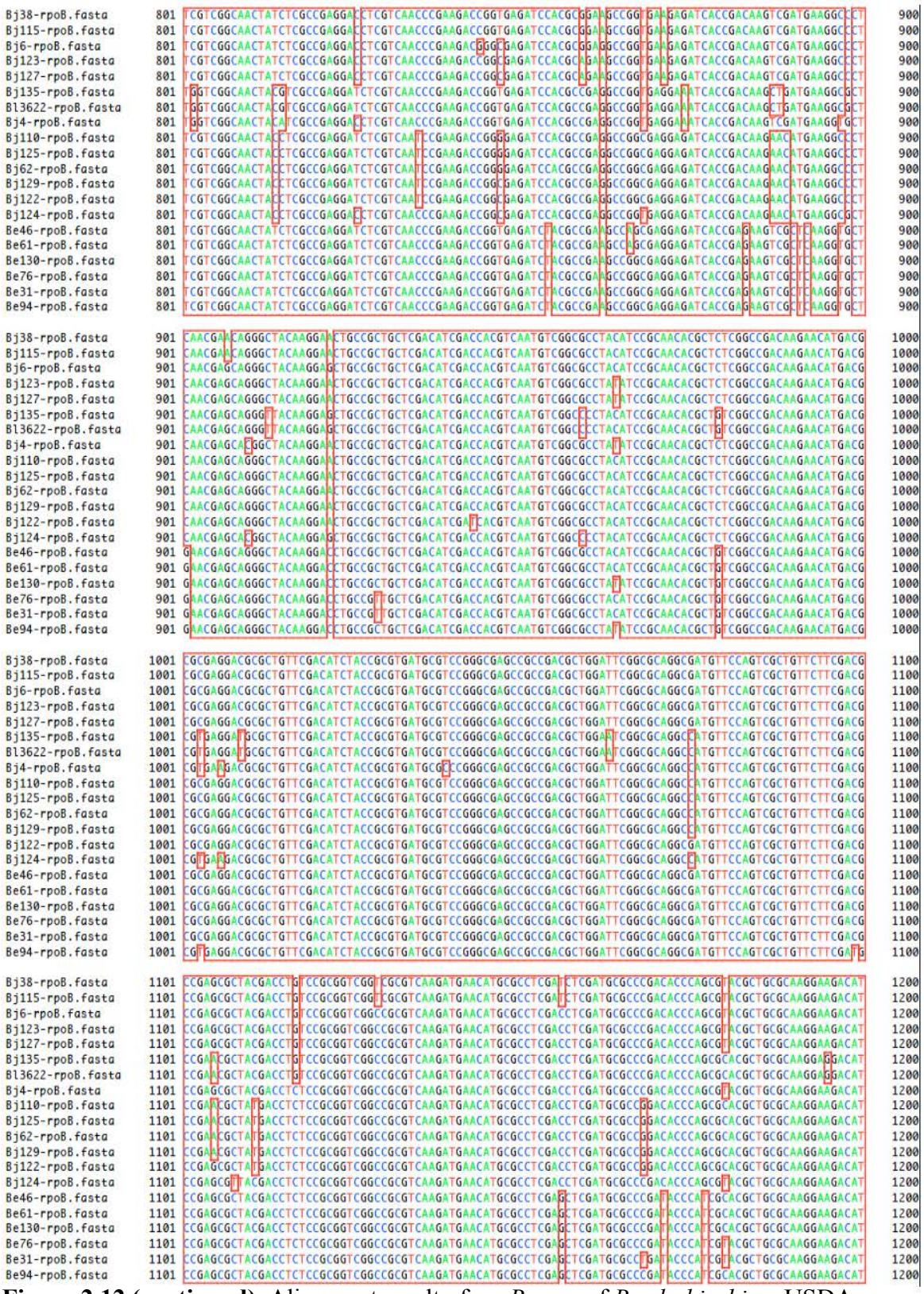

Figure 2.12 (continued). Alignment result of *rpoB* gene of *Bradyrhizobium* USDA strains.

Figure 2.12 (continued). Alignment result of *rpoB* gene of *Bradyrhizobium* USDA strains.

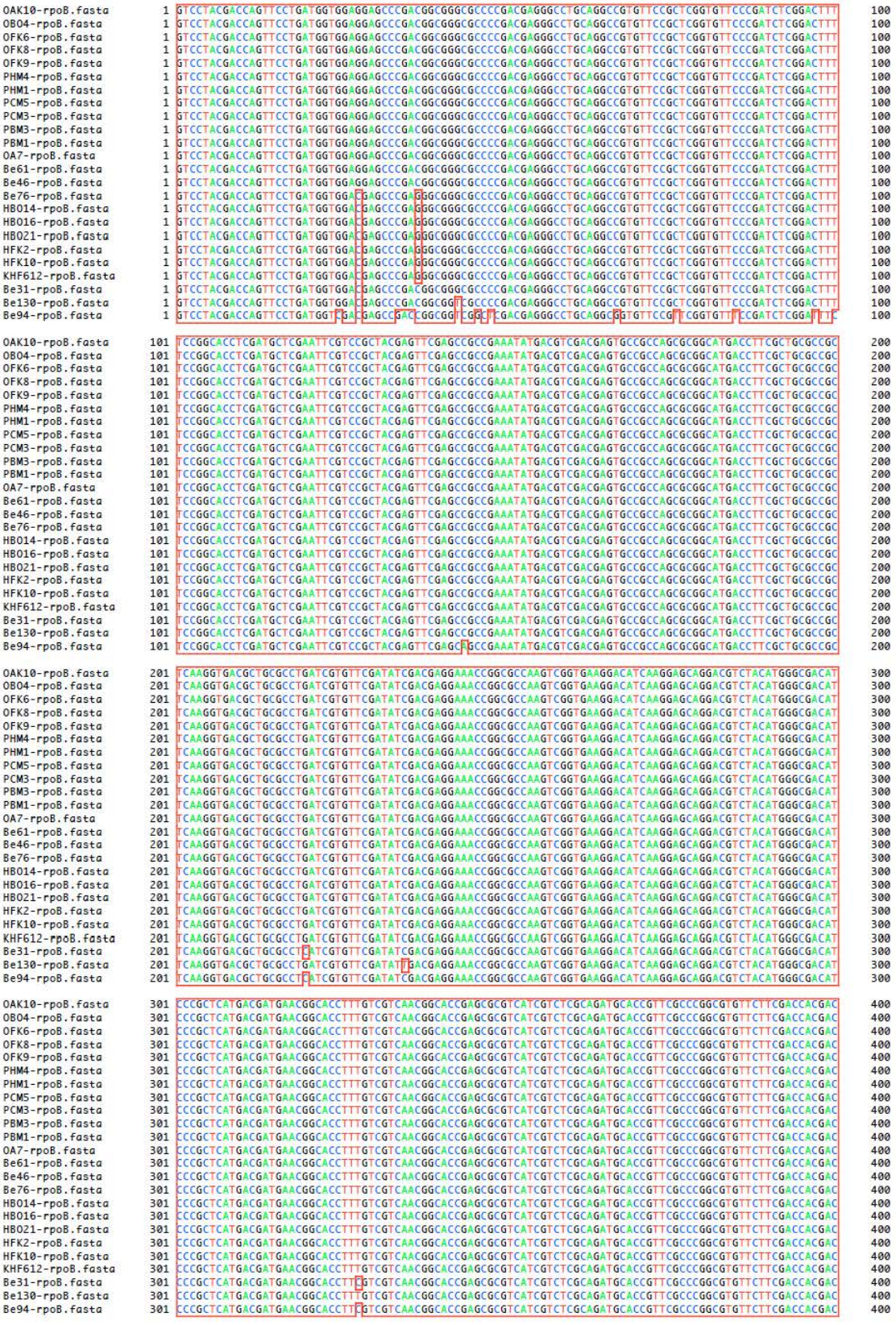

Figure 2.13. Alignment result of *rpoB* gene of *B. elkanii* USDA strains and isolates.

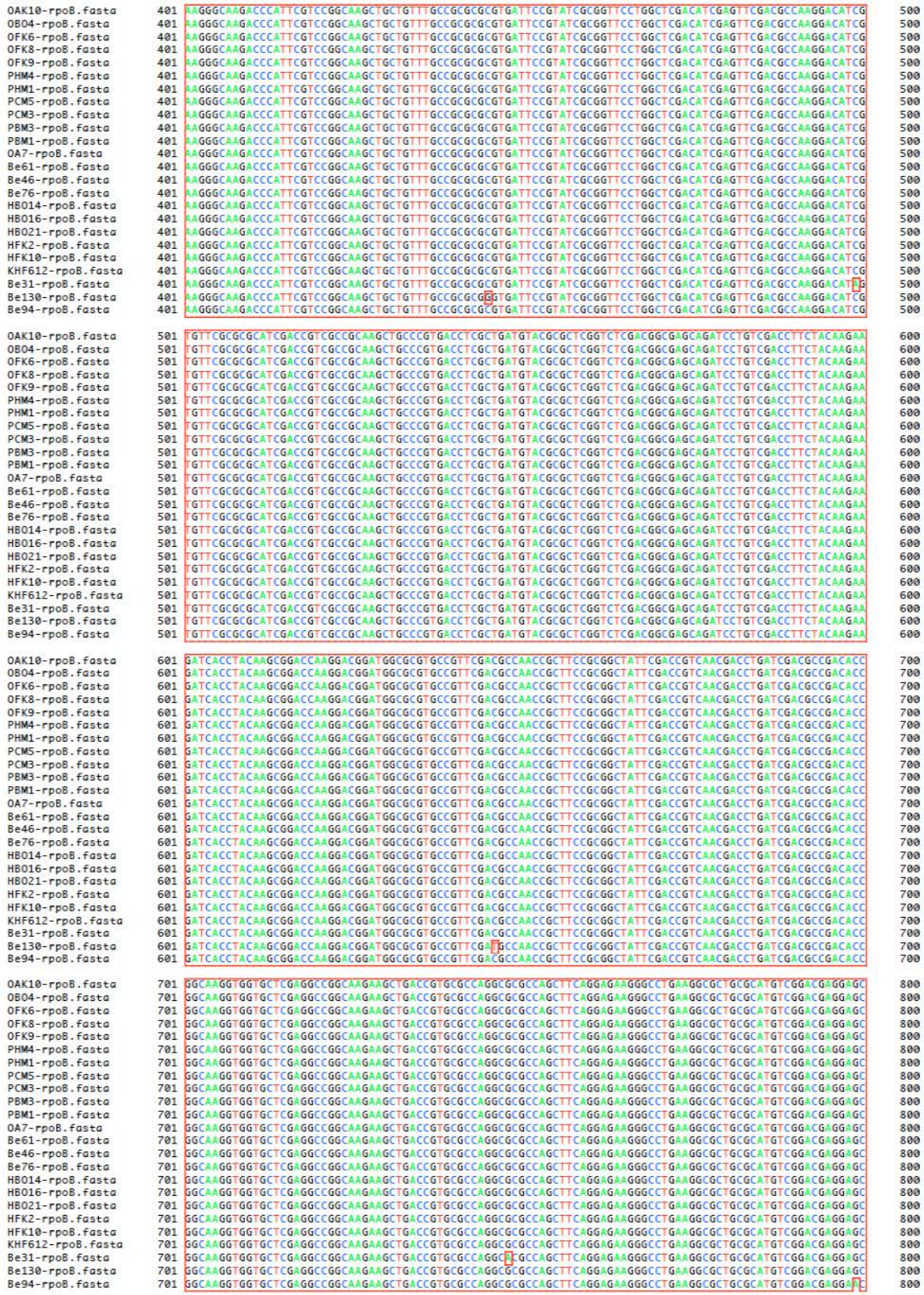

Figure 2.13 (continued). Alignment result of *rpoB* gene of *B. elkanii* USDA strains and isolates.

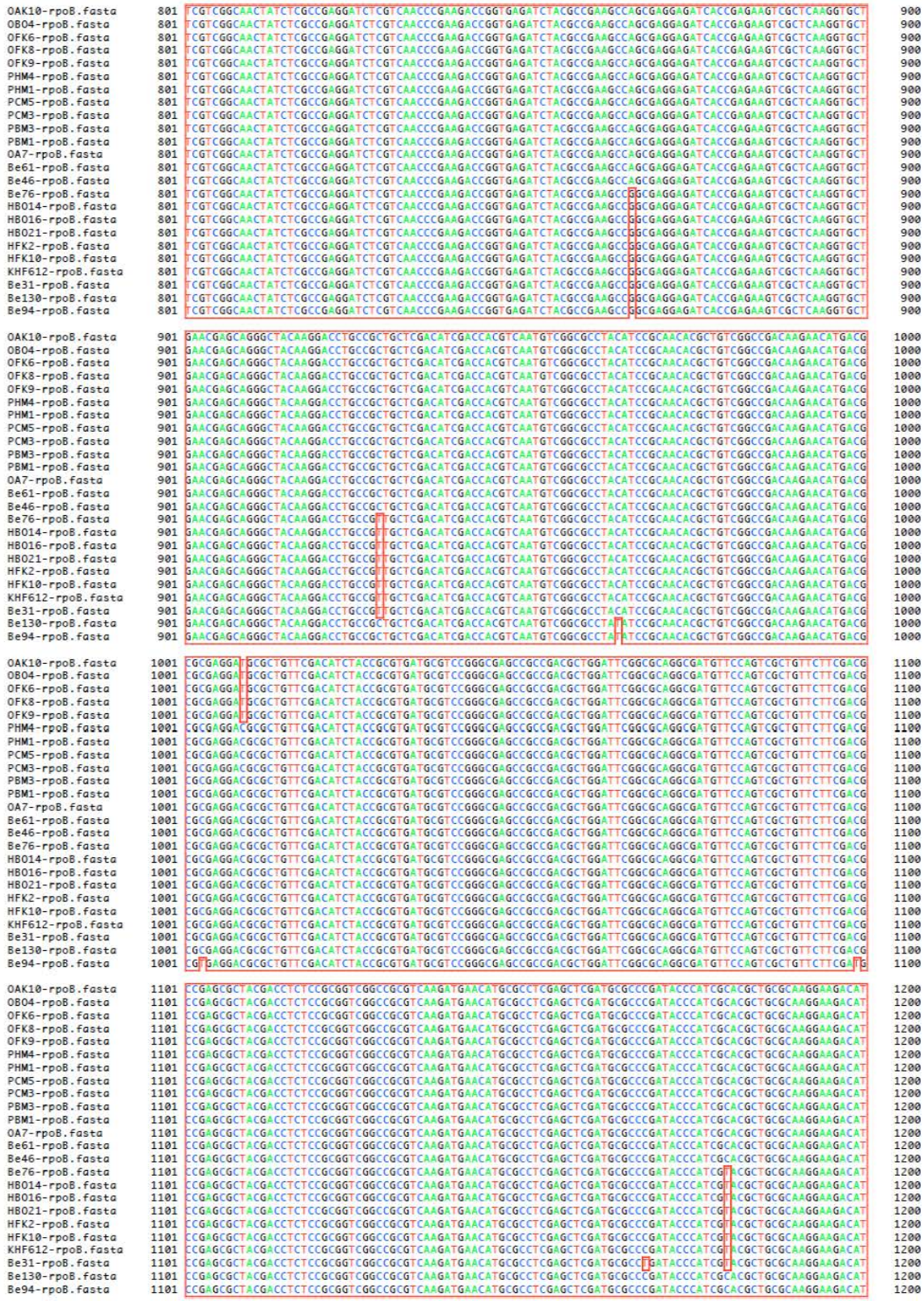

Figure 2.13 (continued). Alignment result of *rpoB* gene of *B. elkanii* USDA strains and isolates.

Figure 2.13 (continued). Alignment result of *rpoB* gene of *B. elkanii* USDA strains and isolates.

Summary

To understand the factors that influence the diversity of soybean-nodulating rhizobia is an important aspect before doing inoculation. Since studies about this topic in tropical regions are limited, particularly in the Philippines, this could lay the foundation for related research on *Bradyrhizobium elkanii*. In this study, we conducted the Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) and sequence analysis of the 16S rRNA gene, internal transcribed spacer (ITS) region and *rpoB* housekeeping gene to know the genetic diversity of *B. elkanii* in different regions. Additionally, the sequence analysis of symbiotic *nifD* and *nodD1* genes was performed. The analysis of the *rpoB* gene revealed a higher genetic diversity than the ITS region, and some possible endemic *B. elkanii* strains were identified. Meanwhile, there was no variation among the strains in both *nifD* and *nodD1* phylogenies. Through the analysis of the *rpoB* gene, some variations in the ITS-*rpoB* type of the *B. elk*anii strains were differentiated with that of the two closest reference strains. Some potential soybean inoculants which possess good symbiotic efficiency regardless of the *Rj* genotypes used were also identified which suggested a broad host-range capability of the selected strains. Thus, this study was able to demonstrate how the genetic diversity of soybean-nodulating *B. elkanii* strains in the subtropical Japan and tropical Philippines might be influenced by the temperature and soil pH and, provided some insights between the symbiotic genes and the *Rj* genotypes.

Chapter 3

Genetic diversity and distribution of indigenous soybean-nodulating bradyrhizobia in the Philippines as influenced by flooding and other soil properties

Introduction

For soybean (*Glycine max* [L.] Merrill), *Bradyrhizobium* is known as its major micro-symbiont that could form nodules through symbiosis. The symbiotic relationship between the rhizobia and a leguminous plant is a highly specific, complicated and energy-exhaustive process (Wang et al., 2012) which may improve the crop's yield. The *Bradyrhizobium* genus is considered as the predominant rhizobia in the tropics (Delamuta et al., 2015) and tropical regions have diverse environmental gradients that could influence the diversity of organisms. Because of this, a high diversity of soybean-nodulating rhizobia were reported to exist in tropical regions (Delamuta et al., 2012) than the temperate regions. As of this time, a diverse species of bradyrhizobia were described as micro-symbionts of soybean such as *Bradyrhizobium japonicum*, *B. elkanii*, *B. yuanmingense*, *B. liaoningense*, *B. huanghuaihaiense* and *B. diazoefficiens* (Jordan, 1982; Kuykendall et al., 1992; Xu et al., 1995; Saeki et al., 2006; Appunu et al., 2008; Zhang et al., 2012; Delamuta et al., 2013). Also, several reports stated that soil pH, salinity, climate, nutrients, and cultural management could influence the diversity and distribution of soybean bradyrhizobia (Loureiro et al., 2007; Grossman et al., 2011; Adhikari et al., 2012; Shiro et al., 2013; Yan et al., 2014; Chibeba et al., 2017; Mason et al., 2017; Saeki et al., 2017). A productive and fertile soil harbors a high diversity of microorganisms so, a vast and accurate information about this could lead to a better crop productivity.

The Philippines, as a tropical country is characterized by high temperature, humidity and abundant rainfall. It only has two distinct seasons based from rainfall: (1) dry season – from December to May and (2) rainy season – from June to November. The average temperature in the country is almost similar at 25.5 – 27.5°C throughout the year except for Benguet, which exists in high altitude. The country's agricultural

production system has been chemically-dependent with rice or corn mono-cropping that the soil became acidic and unproductive. In the Philippines, rice is the major agricultural product. But, a recent trend in agriculture industry recognized the role of soybean in nutrition and soil fertility restoration. This prompted the creation of a Research and Development Roadmap for Soybean to increase the area and volume of production. This also encouraged researchers to improve the inoculation techniques to support the government's endeavor.

However, inoculation sometimes fail due to several reasons such as the incompatibility between the macro and micro-symbiont (Yamakawa et al., 2003; Hayashi et al., 2012; Yamakawa and Saeki, 2013), competition between the indigenous and inoculated rhizobia (Schumpp and Deakin, 2010; Ji et al., 2017) and the agro-environmental factors mentioned above that influence the distribution and diversity of rhizobia. Therefore, it is a must that prior to inoculation, information on the distribution and diversity of indigenous rhizobia in the soil should be acquired.

In our previous report, (Mason et al., 2017) it was stated that in the Philippines, there was no information about the indigenous soybean bradyrhizobia except for one location that was used in that study, which is Nueva Ecija. Since the Philippines is composed of more than 7,000 islands surrounded by bodies of water with considerable variation in agro-environmental conditions, it is hypothesized that a diverse species of bradyrhizobia can be collected and identified. Therefore, this study aimed to (a) identify the indigenous soybean bradyrhizobia in the Philippines soil and (b) determine the factors that influence its distribution and diversity. The output of this study could help to improve the current status of soybean production in the country and to better understand the biogeography of tropical bradyrhizobia.

Materials and Methods

1. Soil Collection and analysis

The soil samples used in this study were collected as formerly described (Mason et al., 2017) from the eleven locations which were previously and/or currently planted with soybean and/or other legumes (Fig. 1). The locations that were characterized by two different periods of flooding condition (flooded means the soil usually contains gravitational water for a period of at least 10 months in one year while non-flooded means the soil usually contains gravitational water for a period of at most 5 months in one year). The location and basic information on the study sites are summarized in Table 3.1. From the one (1) kg composite soil, a 0.5 kg was air dried and pulverized for soil analyses that include pH (1:2.5 water extraction method) and electrical conductivity (EC: 1:5 water extraction method), Total C, Total N, Bray P, K (flame spectrophotometer) and soil texture then, the remaining 0.5 kg was freshly used for the soybean cultivation. For the soil texture analysis, the Hydrometer method was used as described (Bouyoucous, 1962), whereas C and N analysis was performed by an automatic high-sensitive NC Analyzer Sumigraph NC-220F (Sumika Chemical Analysis Service. Ltd., Tokyo, Japan). The data of the annual average temperature and rainfall were obtained from the Philippine Atmospheric Geophysical and Astronomical Services Administration (PAGASA) website (https://www1.pagasa.dost.gov.ph/) which were averages from the last two decades (2000-2017).

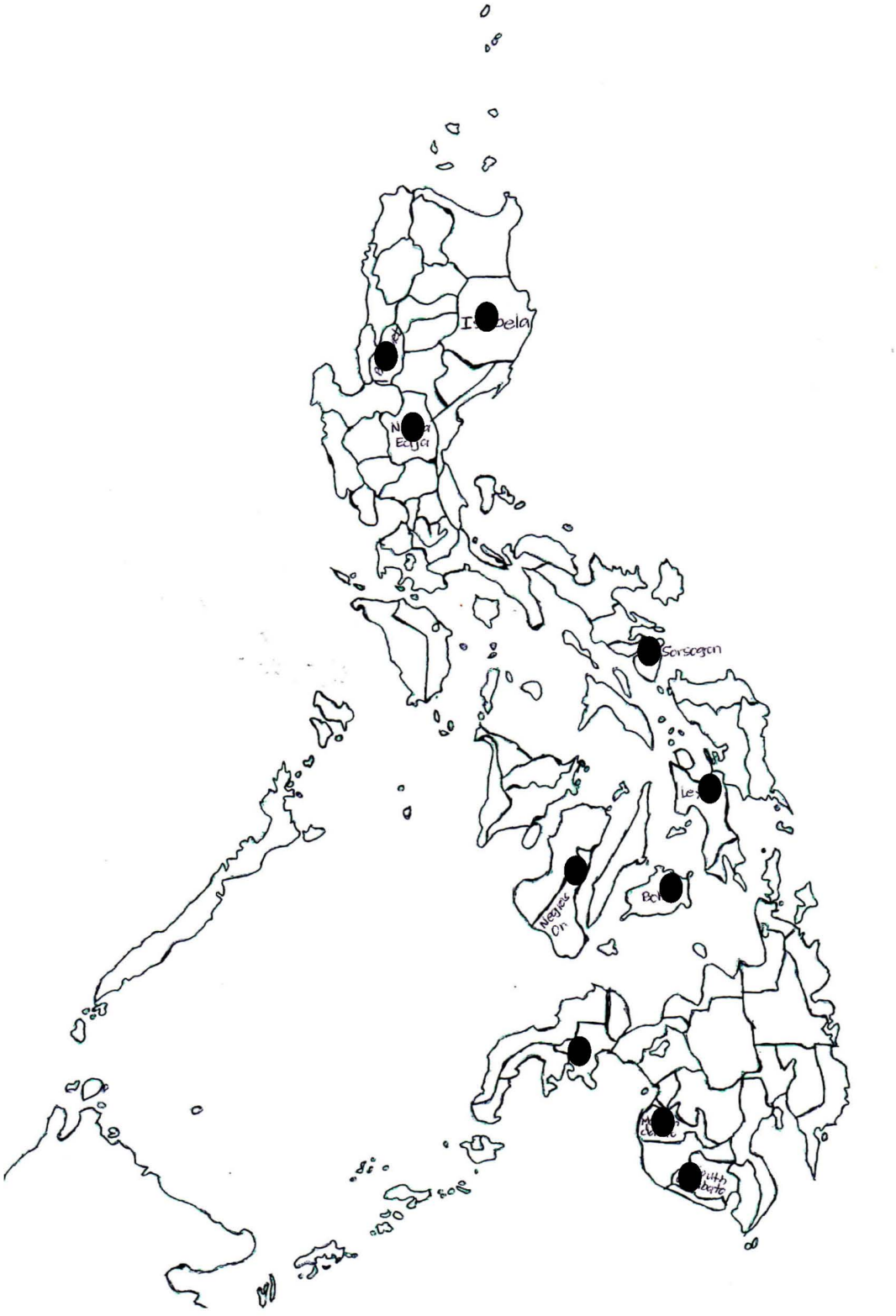

Figure 3.1. Map of the Philippines showing the location of the sampling points (●) in this study. The map was drawn manually by the author.

Table 3.1. Basic information on the agro-environmental gradients of the Philippines soil used in this study.

Location	Coordinate	pH	EC (dS/m)	C (%)	N (%)	C/N	Bray P ($mgkg^{-1}$)	K ($mgkg^{-1}$)	Sa (%)	Silt (%)	Clay (%)	Ave. Temp. (°C)	Flooding Period (month)
Ilagan, Isabela **(IS)**	17.30°N,122.01°E	5.90	0.08	1.34	0.13	10.3	1.86	51.80	28.0	34.5	37.5	26.9	5
Gamu, Isabela **(GI)**	17.08°N,121.79°E	5.52	0.15	1.85	0.17	10.9	2.30	58.60	28.2	33.8	38.0	27.0	10
Baguio, Benguet **(BA)**	16.40°N,120.60°E	5.22	0.20	3.10	0.24	12.9	22.22	51.00	19.0	41.4	39.6	19.3	10
Nueva Ecija1 **(NE1)**	15.74°N,120.93°E	6.21	0.05	1.37	0.13	10.5	6.74	73.90	28.7	34.6	36.7	26.9	5
Nueva Ecija2 **(NE2)**	15.74°N,120.93°E	5.81	0.12	2.36	0.22	10.7	21.63	49.40	27.4	34.7	37.9	26.9	10
Irosin, Sorsogon **(SO)**	12.72°N,124.04°E	5.26	0.15	1.92	0.22	8.7	2.57	55.80	28.9	33.6	37.5	27.1	10
Abuyog, Leyte **(LT)**	10.67°N,125.04°E	5.80	0.12	1.50	0.15	10.0	6.39	174.20	29.2	32.8	38.0	27.0	10
La Carlota, Negros Occidental **(NR)**	10.24°N,122.59°E	5.62	0.15	0.63	0.07	9.0	20.44	74.10	28.0	34.2	37.8	27.7	5
Ubay, Bohol **(BO)**	9.99°N,124.45°E	5.82	0.11	0.63	0.06	10.5	2.80	47.80	29.7	33.5	36.8	27.2	5
Sultan Kudarat, Maguindanao **(SK)**	6.51°N,124.42°E	6.64	0.14	2.48	0.19	13.1	4.53	59.60	24.0	34.1	41.9	27.3	10
Tupi, South Cotabato **(SC)**	6.34°N,124.97°E	5.52	0.15	1.36	0.14	9.7	31.18	47.20	19.5	42.5	38.0	25.4	10

Note: **Sa** – sand; Flooding period means the number of months that the land was in a submerged condition and contains gravitational water.

The procedure for the soil pH and EC determination was described in the materials and methods section of Chapter 2.

2. Cultivation of soybean and isolation of indigenous rhizobia

To isolate the indigenous soybean rhizobia, a commonly available local soybean cultivar with the local name of PSB-SY2 was used. The soybean seeds were surface-sterilized by soaking in 70% ethanol and sodium hypochlorite solution as formerly described (Saeki et al., 2006) and planted in a 1-liter culture pots (n = 4). Then, the culture pots were filled with vermiculite and a N-free nutrient solution was added (Saeki et al., 2004) at 40% (vol/vol) distilled water content then, were autoclaved for 20 min at 121°C. A 2 to 3 g soil sample was placed on the vermiculite with a sterile spatula at a depth of 2 to 3 cm, then the surface-sterilized seeds were sown on the soil. The pot was weighed and recorded after covering with aluminum foil. The aluminum foil was removed after 16 h or overnight. Afterwards, the plants were grown for 28 days inside the growth chamber (33°C for 16 h, day; 28°C for 8h, night), and were supplied weekly with sterile distilled water until the initial weight of the pot was reached.

A 15 to 20 nodules with a size of more than 2mm were randomly collected from the roots of each soybean plant after 4 weeks. These nodules were surface-sterilized with 70% ethanol and sodium hypochlorite solution as described previously (Suzuki et al., 2008). Each nodule was crushed in sterile distilled water inside a 1.5 mL microtube by using a sterilized toothpick and streaked on the yeast extract mannitol agar (YMA; Vincent, 1970) plate. It was then incubated for about 1 week in the dark at 28°C. A single colony was picked-up by a sterile wire loop and streaked onto YMA plate containing 0.002% (wt/wt) bromothymol blue (BTB; Keyser et al., 1982) to determine

the genus and incubated as described above. Thereafter, the pure single colonies were obtained by repeated streaking into YMA plates.

All the isolates obtained from the 11 locations were subjected to an inoculation test to determine their nodulation capability. Each isolate was cultured in YM broth culture (YMB; Vincent, 1970) for about 1 week at 28°C, and the cultures were diluted with sterile distilled water to approximately 10^6 cells ml^{-1}. Then, surface-sterilized soybean seeds were sown as described previously but without soil, and a 1-ml aliquot of each isolate per seed was inoculated with three replications. The plants were grown inside the growth chamber for 4 weeks then, nodule formation was assessed. A control pot (un-inoculated) was prepared under similar conditions.

3. DNA collection and extraction

The isolated pure single colony from the YMA plate was cultured in a HEPES-MES (HM) broth culture (Cole and Elkan, 1973; Sameshima et al., 2003) for 3 to 4 days at 28°C with continuous agitation at 120 rpm in a dark condition. Thereafter, the bacteria cells cultured in the HM broth were collected by centrifugation and washed with sterile distilled water. The extraction of the DNA was done by using BL buffer from the method reported previously (Hiraishi et al., 1995) which was described by Minami et al. (2009).

A more detailed procedure of the DNA extraction was discussed in the materials and methods section of the Chapter 2.

4. PCR-RFLP analysis of the 16S rRNA, ITS region and *rpoB* gene

The isolates that were able to nodulate the soybean plants were then used for the

PCR amplification. The amplification of the 16S rRNA, ITS region, and *rpoB* gene was conducted using the *Ex Taq* DNA polymerase (TaKaRa Bio, Otsu, Shiga, Japan) with primers and PCR cycle conditions previously used (Mason et al., 2017) and are listed in the Table 2.1 of this manuscript. Then, the RFLP analyses of the 16S rRNA and ITS region were performed using the restriction enzymes *HaeIII, HhaI, MspI* and *XspI* (TaKaRa Bio) whereas for the *rpoB* gene, enzymes *HaeIII, MspI* and AluI (TaKaRa Bio) were used. The same serogroups of the *Bradyrhizobium* USDA were used as th reference strains which are listed in the Materials and Methods section of Chapter 2. From the PCR product, a 5.0 µl aliquot was digested with the restriction enzymes overnight at 37°C in a 20 µl reaction mixture. A 3 or 4% agarose gels in TBE buffer was used for the submerged gel electrophoresis to separate the fragments and were visualized with a 1% ethidium bromide. A 50bp DNA ladder (GeneDirex) was used as the marker of the fragment sizes.

5. Sequence analysis of the three genes

Based from the OTUs which were obtained from the RFLP analysis of the 16S rRNA, ITS region and *rpoB* gene, 31 representative isolates were selected for sequence analysis. The amplified products from the PCR reaction of the representative isolates for the ITS region and *rpoB* gene were purified according to the protocol of NucleoSpin® Gel and PCR Clean-up (Macherey-Nagel, Germany). Then, the DNA concentration of the purified product was confirmed with the NanoDrop 2000 Spectrophotometer (Thermo Scientific, U.S.A.). After then, samples were prepared according to the protocol for a premixed template and primer of the manufacturer (EUROFINS GENOMICS) using the previously designed sequence primers (Mason et al., 2017) which are listed in the Table 2.2 and were sent to the company (EUROFINS).

Based from the similarity in the ITS region-*rpoB* gene type of the representative isolates, a few isolates were randomly selected to confirm the sequences of 16S rRNA gene.

6. Sequence alignment and construction of the phylogenetic trees

The Basic Local Alignment Search Tool (BLAST) program in DNA Databank of Japan (DDBJ) was used to determine the nucleotide homology of the isolates in this study. The sequences of type strains having a similarity with our isolates of at least 99% for the 16S rRNA, 96% for the ITS region and 98% for the *rpoB* gene were retrieved from the BLAST database. The phylogenetic trees also included the previously determined sequences of the 16S rRNA and ITS region *Bradyrhizobium* genospecies (Saeki et al., 2000; van Berkum and Fuhrmann, 2000). Then, the alignment of sequences obtained were performed using the ClustalW. The Neighbor-Joining (Saitou and Nei, 1987) method for the 16S rRNA, ITS region and *rpoB* gene was used to construct the phylogenetic trees. The genetic distances were then calculated using the Kimura 2-parameter model (Kimura, 1980) in the Molecular Evolutionary Genetic Analysis (MEGA v7) software (Kumar et al., 2016). Thereafter, the phylogenetic trees were bootstrapped with 1,000 replications of each sequence to evaluate the reliability of the tree topology. All the nucleotide sequences determined in this study were deposited in DDBJ at http://www.ddbj.nig.ac.jp/ and listed in Table 3.2.

7. Cluster analysis and diversity of the indigenous bradyrhizobia

Only those isolates with reproducible fragments longer than 50 bp in the electrophoresis gels were used for the cluster analysis. The genetic distance between pairs of isolates (D) was calculated using the equation $D_{AB} = 1\ [2N_{AB}/\ (N_A + N_B)]$, where N_{AB} represents the number of RFLP bands shared by strains A and B whereas N_A and

N_B represent the numbers of RFLP bands found only in strains A and B, respectively (Nei and Li, 1979; Sakai et al., 1998). Then, the diversity analysis was performed by the Shannon-Wiener diversity index as described previously (MacArthur, 1965; Pielou, 1969; Saeki et al., 2008) for the species.

To expound on the community structure of the dominant soybean bradyrhizobia, a multi-dimensional scaling (MDS) analysis using Bray-Curtis Index was employed using R software v. 3.4.0.

8. Principal component analysis (PCA)

To detect the relationship between the agro-environmental factors and the distribution of soybean bradyrhizobia in the Philippines, PCA method was performed in R software. The variables for the principal component include soil chemical properties, soil texture and some environmental data.

Table 3.2. List of accession numbers for the 31 indigenous representative isolates/strains of soybean-nodulating bradyrhizobia which were collected from the 11 locations in the Philippines. These were obtained from the sequence analysis of the 16S rRNA gene, 16S-23S rRNA gene ITS region and *rpoB* housekeeping gene.

No.	Isolate	Genus/Species name	Accession Number		
			16S rRNA	***rpoB* gene**	**ITS region**
1	IS-2	*B. elkanii*	LC386868	LC367064	LC367098
2	GI-4	*Bradyrhizobium* sp.	LC386869	LC367065	LC367099
3	GI-8	*B. elkanii*	nd	LC367066	LC367100
4	BA-24	*B. japonicum*	LC386870	LC367067	LC367101
5	BA-41	*B. japonicum*	LC386871	LC367068	LC367102
6	BA-42	*B. elkanii*	LC386872	LC367069	LC367103
7	NE1-6	*B. elkanii*	LC386873	LC367070	LC367104
8	NE1-19	*Bradyrhizobium* sp.	nd	LC415435	LC425436
9	NE1-34	*Bradyrhizobium* sp.	LC386874	LC367071	LC367105
10	NE1-65	*B. diazoefficiens*	LC386875	LC367072	LC367106
11	NE2-1	*B. elkanii*	LC386876	LC367073	LC367107
12	NE2-3	*Bradyrhizobium* sp.	nd	LC367074	LC367108
13	NE2-37	*Bradyrhizobium* sp.	LC386877	LC367075	LC367109
14	NE2-66	*B. japonicum*	nd	LC367076	LC367110
15	SO-1	*B. diazoefficiens*	LC386878	LC367077	LC367111
16	LT-3	*B. diazoefficiens*	LC386879	LC367078	LC367112
17	LT-36	*B. elkanii*	nd	LC367080	LC367114
18	NR-1	*B. japonicum*	nd	LC367081	LC367115
19	NR-2	*B. elkanii*	nd	LC367082	LC367116
20	NR-40	*B. japonicum*	LC386880	LC367083	LC367117
21	NR-48	*B. diazoefficiens*	LC386881	LC367084	LC367118
22	NR-60	*B. elkanii*	nd	LC367085	LC367119
23	BO-4	*B. elkanii*	LC386882	LC367086	LC367120
24	BO-15	*B. diazoefficiens*	nd	LC367087	LC367121
25	BO-52	*Bradyrhizobium* sp.	nd	LC367091	LC367125
26	SK-1	*B. elkanii*	LC386883	LC367092	LC367126
27	SK-2	*B. elkanii*	LC386884	LC367093	LC367127
28	SK-5	*B. diazoefficiens*	nd	LC367094	LC367128
29	SK-12	*B. yuanmingense*	LC386885	LC367095	LC367129
30	SC-3	*B. japonicum*	LC386886	LC367096	LC367130
31	SC-49	*B. elkanii*	nd	LC367097	LC367131

A summary of the activities conducted in this study is presented in Figure 3.2 for easier understanding.

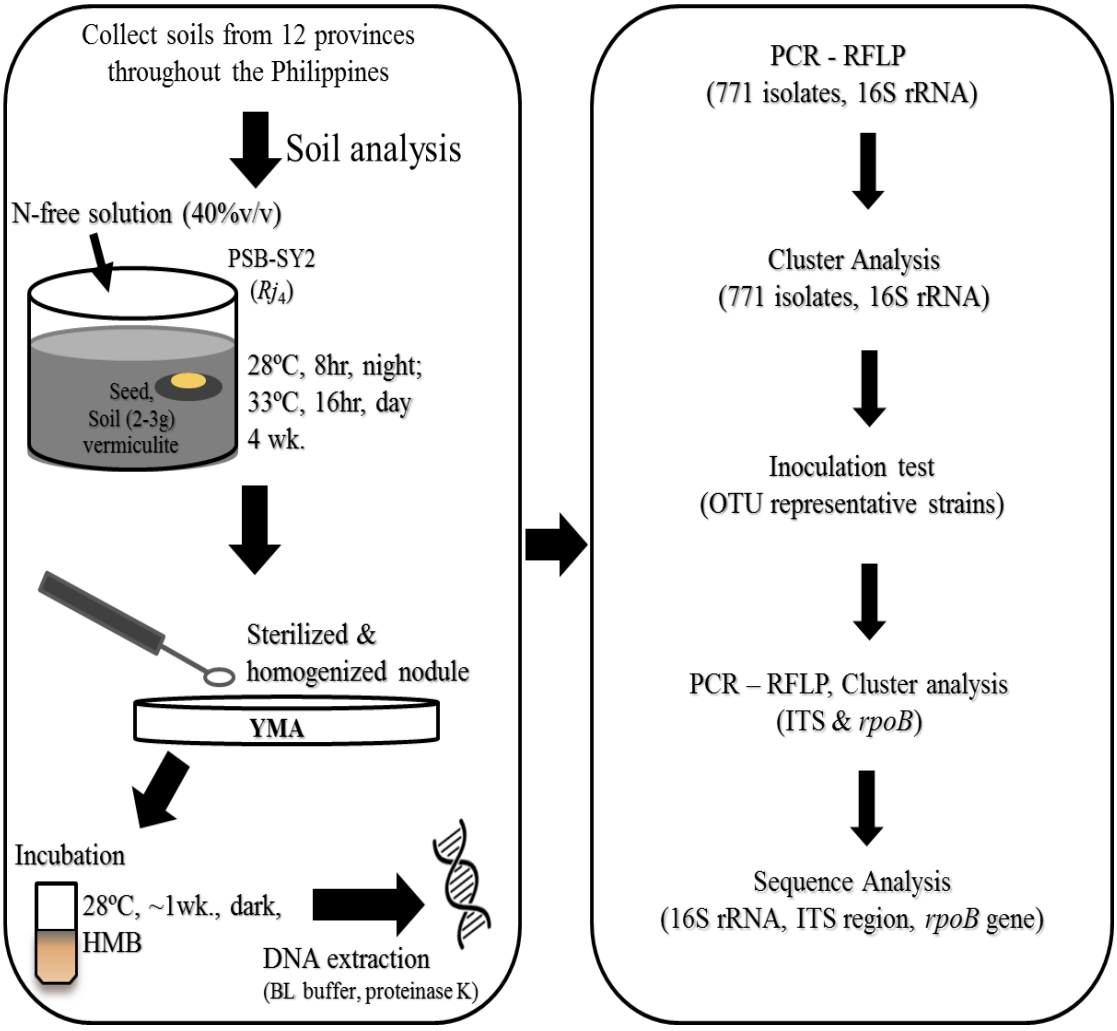

Figure 3.2. A summarized flowchart of activities that were conducted for this study.

Results

1. Isolation of the indigenous bradyrhizobia

A total of 771 isolates was obtained from the study sites with a range of 63 to 79 isolates per location. The samples were labeled with a combination of the abbreviation of the sampling site (IS - Ilagan; GI - Gamu; BA - Baguio; NE1 - 1st location in Nueva Ecija; NE2 - 2nd location in Nueva Ecija; SO - Sorsogon; LT - Leyte; BO - Bohol; NR

- Negros; SK - Sultan Kudarat; and SC - South Cotabato) and the number of the isolate (1-63 or 1-79) (e.g., for South Cotabato, SC – 1-74).

The inoculation test showed that the 424 isolates were capable to form nodules on the host plant and all these isolates has produced alkaline or neutral reaction in the YMA plates with BTB thus, were considered as *Bradyrhizobium* (Jordan, 1982). We also confirmed that the un-inoculated pots did not produce any nodules which eliminated the contamination in this experiment.

2. RFLP analysis of the 16S rRNA, ITS region and *rpoB* gene

The 424 isolates were all used for the RFLP treatment of the 16S rRNA, ITS region and *rpoB* gene. For the RFLP analysis of these three target genes, dendrograms were also constructed as a basis for the selection of representative strains to be used for the sequence analysis. The Tables 3.4, 3.5, and 3.6 showed the matrix of the genetic distances from the RFLP patterns of the 31 representative strains and the *Bradyrhizobium* USDA reference strains which were calculated by the abovementioned formula.

The differences in the fragment sizes and patterns through the RFLP treatment showed 31 OTUs which were used for the sequence analysis and the results are summarized in Table 3.3. It is evident that the genetic variations mostly occurred for Be clusters within the strains of *B. elkanii* 31, 46 and 76. The isolates which have similar ITS-*rpoB* type were excluded from the 16S rRNA gene sequence analysis.

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Table 3.3. Summary of the genetic variations detected from the 31 representative indigenous soybean-nodulating bradyrhizobia in the Philippines through the Restriction Fragment Length Polymorphism (RFLP) treatment and sequence analysis of the 16S rRNA gene, 16S-23S rRNA ITS region, and *rpoB* housekeeping gene

Rep. isolate	Isolate (no.)	Cluster (RFLP)			Cluster (sequence analysis)			ITS- *rpoB* type
		16S rRNA	ITS region	*rpoB* gene	16S rRNA	ITS region	*rpoB* gene	
IS-2	40	Be	Be76	Be46	*B.elkanii*	Be31	Be46	Be31-Be46
GI-4	30	Bj	Bj	Bj	*B. japonicum*	Br	Br	Br-Br
GI-8	6	Be	Be76	Be46	nd	Be31	Be46	Be31-Be46
BA-24	31	Bj	Bj6	Bj6	*B. japonicum*	Bj6’	Bj6	Bj6’-Bj6
BA-41	1	Bj	Bj124	Bj124	*B. japonicum*	Bj124’	Bj124’	Bj124’-Bj124’
BA-42	1	Be	Be76	Be76	*B.elkanii*	Be31	Be46	Be31-Be46
NE1-6	49	Be	Be76	Be46	*B.elkanii*	Be76’	Be46	Be76’-Be46
NE1-19	5	Br	Br	Br	nd	Br	Br	Br-Br
NE1-34	1	Bj	Bj	Bj	*B. japonicum*	BrNE	BrNE	BrNE-BrNE
NE1-65	1	Bj	Bj	Bj	*B. diazoefficiens*	Bd110	Br	Bd110-Br
NE2-1	3	Be	Be76	Be46	*B.elkanii*	Be76	Be46	Be76-Be46
NE2-3	5	Bj	Br	Br	nd	Br	Br	Br-Br
NE2-37	26	Br	Br	Br	*B. japonicum*	Br	Br	Br-Br
NE2-66	1	Bj	Bj	Bj	nd	Br	Bj124’	Br-Bj124’
SO-1	44	Bj	Bd110	Bd110	*B. diazoefficiens*	Bd110	Bd110	Bd110-Bd110
LT-3	42	Bj	Bd110	Bd110	*B. diazoefficiens*	Bd110	Bd110	Bd110-Bd110
LT-36	1	Be	Be76	Be46	nd	Be76’	Be46	Be76’-Be46
NR-1	4	Bj	Bj6	Bj6	nd	Bj6	Bj6	Bj6-Bj6
NR-2	22	Be	Be76	Be46	nd	Be76	Be46	Be76-Be46
NR-40	3	Bj	Bj124	Br	*B. japonicum*	Bj124’	Bj	Bj124’-Bj
NR-48	1	Bj	Bd110	Br	*B. diazoefficiens*	Bd110	Bd110’	Bd110-Bd110’
NR-60	1	Br	Be76	Br	nd	Be76	Br	Be76-Br
BO-4	24	Be	Be76	Be46	*B.elkanii*	Be31	Be46	Be31-Be46
BO-15	4	Bj	Bd110	Bd110	nd	Bd110	Bd110	Bd110-Bd110
BO-52	1	Bj	Bj	Br	nd	Br	Br	Br-Br
SK-1	4	Be	Be46	Be76	*B.elkanii*	Be46’	Be130	Be46’-Be130
SK-2	6	Be	Be94	Be94	*B.elkanii*	Be94’	Be94	Be94’-Be94
SK-5	29	Bj	Bd110	Bd110	nd	Bd110	Bd110	Bd110-Bd110
SK-12	4	Br	Br	Br	*B. yuanmingense*	BySK	BySK	BySK-BySK
SC-3	31	Bj	Bj6	Bj6	*B. japonicum*	Bj6	Bj6	Bj6-Bj6
SC-49	3	Be	Be76	Be46	nd	Be76	Be46	Be76-Be46
Total isolates	**424**							

Table 3.4. Table matrix of the genetic distance detected from the 31 representative indigenous soybean-nodulating bradyrhizobia in the Philippines through the Restriction Fragment Length Polymorphism (RFLP) treatment of the 16S rRNA gene.

Strain	31	46	61	76	94	130	4	6	38	110	115	122	123	124	129	135	3622	Ecoli	IS-2	GI-4	BA-41	BA-42	BA-24	NE1-6	NE1-34	NE1-65	NE2-1	NE2-37	SO-1	LT-3	NR-40	NR-48	BO-4	SK-1	SK-2	SK-12	SC-3
USDA31	0.00	0.00	0.00	0.00	0.00	0.00	0.08	0.08	0.08	0.08	0.08	0.10	0.08	0.13	0.10	0.08	0.08	0.69	0.06	0.17	0.20	0.08	0.17	0.08	0.08	0.20	0.08	0.59	0.20	0.23	0.23	0.20	0.10	0.08	0.00	0.17	0.17
USDA46	0.00	0.00	0.00	0.00	0.00	0.00	0.08	0.08	0.08	0.08	0.08	0.10	0.08	0.13	0.10	0.08	0.08	0.69	0.06	0.17	0.20	0.08	0.17	0.08	0.08	0.20	0.08	0.59	0.20	0.23	0.23	0.20	0.10	0.08	0.00	0.17	0.17
USDA61	0.00	0.00	0.00	0.00	0.00	0.00	0.08	0.08	0.08	0.08	0.08	0.10	0.08	0.13	0.10	0.08	0.08	0.69	0.06	0.17	0.20	0.08	0.17	0.08	0.08	0.20	0.08	0.59	0.20	0.23	0.23	0.20	0.10	0.08	0.00	0.17	0.17
USDA76	0.00	0.00	0.00	0.00	0.00	0.00	0.08	0.08	0.08	0.08	0.08	0.10	0.08	0.13	0.10	0.08	0.08	0.69	0.06	0.17	0.20	0.08	0.17	0.08	0.08	0.20	0.08	0.59	0.20	0.23	0.23	0.20	0.10	0.08	0.00	0.17	0.17
USDA94	0.00	0.00	0.00	0.00	0.00	0.00	0.08	0.08	0.08	0.08	0.08	0.10	0.08	0.13	0.10	0.08	0.08	0.69	0.06	0.17	0.20	0.08	0.17	0.08	0.08	0.20	0.08	0.59	0.20	0.23	0.23	0.20	0.10	0.08	0.00	0.17	0.17
USDA130	0.00	0.00	0.00	0.00	0.00	0.00	0.08	0.08	0.08	0.08	0.08	0.10	0.08	0.13	0.10	0.08	0.08	0.69	0.06	0.17	0.20	0.08	0.17	0.08	0.08	0.20	0.08	0.59	0.20	0.23	0.23	0.20	0.10	0.08	0.00	0.17	0.17
USDA4	0.08	0.08	0.08	0.08	0.08	0.08	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.04	0.02	0.00	0.00	0.79	0.13	0.08	0.11	0.16	0.08	0.16	0.00	0.11	0.16	0.63	0.11	0.13	0.13	0.11	0.18	0.16	0.06	0.11	0.08
USDA6	0.08	0.08	0.08	0.08	0.08	0.08	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.04	0.02	0.00	0.00	0.79	0.13	0.08	0.11	0.16	0.08	0.16	0.00	0.11	0.16	0.63	0.11	0.13	0.13	0.11	0.18	0.16	0.06	0.11	0.08
USDA38	0.08	0.08	0.08	0.08	0.08	0.08	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.04	0.02	0.00	0.00	0.79	0.13	0.08	0.11	0.16	0.08	0.16	0.00	0.11	0.16	0.63	0.11	0.13	0.13	0.11	0.18	0.16	0.06	0.11	0.08
USDA110	0.08	0.08	0.08	0.08	0.08	0.08	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.04	0.02	0.00	0.00	0.79	0.13	0.08	0.11	0.16	0.08	0.16	0.00	0.11	0.16	0.63	0.11	0.13	0.13	0.11	0.18	0.16	0.06	0.11	0.08
USDA115	0.08	0.08	0.08	0.08	0.08	0.08	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.04	0.02	0.00	0.00	0.79	0.13	0.08	0.11	0.16	0.08	0.16	0.00	0.11	0.16	0.63	0.11	0.13	0.13	0.11	0.18	0.16	0.06	0.11	0.08
USDA122	0.10	0.10	0.10	0.10	0.10	0.10	0.02	0.02	0.02	0.02	0.02	0.00	0.02	0.02	0.00	0.02	0.02	0.79	0.16	0.11	0.11	0.18	0.11	0.18	0.02	0.08	0.18	0.58	0.08	0.11	0.11	0.08	0.18	0.18	0.08	0.13	0.11
USDA123	0.08	0.08	0.08	0.08	0.08	0.08	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.04	0.02	0.00	0.00	0.79	0.13	0.08	0.11	0.16	0.08	0.16	0.00	0.11	0.16	0.63	0.11	0.13	0.13	0.11	0.18	0.16	0.06	0.11	0.08
USDA124	0.13	0.13	0.13	0.13	0.13	0.13	0.04	0.04	0.04	0.04	0.04	0.02	0.04	0.00	0.04	0.06	0.06	0.80	0.20	0.15	0.15	0.23	0.15	0.23	0.06	0.13	0.23	0.59	0.13	0.15	0.08	0.13	0.23	0.23	0.13	0.17	0.15
USDA129	0.10	0.10	0.10	0.10	0.10	0.10	0.02	0.02	0.02	0.02	0.02	0.00	0.02	0.04	0.00	0.02	0.02	0.79	0.16	0.11	0.11	0.18	0.11	0.18	0.02	0.08	0.18	0.58	0.08	0.11	0.11	0.08	0.18	0.18	0.08	0.13	0.11
USDA135	0.08	0.08	0.08	0.08	0.08	0.08	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.06	0.02	0.00	0.00	0.79	0.13	0.08	0.11	0.16	0.08	0.16	0.00	0.11	0.16	0.63	0.11	0.13	0.13	0.11	0.18	0.16	0.06	0.11	0.08
USDA3622	0.08	0.08	0.08	0.08	0.08	0.08	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.06	0.02	0.00	0.00	0.79	0.13	0.08	0.11	0.16	0.08	0.16	0.00	0.11	0.16	0.63	0.11	0.13	0.13	0.11	0.18	0.16	0.06	0.11	0.08
Ecoli	0.69	0.69	0.69	0.69	0.69	0.69	0.79	0.79	0.79	0.79	0.79	0.79	0.79	0.80	0.79	0.79	0.79	0.00	0.67	0.85	0.85	0.72	0.85	0.72	0.79	0.85	0.72	0.92	0.85	0.85	0.85	0.85	0.72	0.72	0.67	0.85	0.85
IS-2	0.06	0.06	0.06	0.06	0.06	0.06	0.13	0.13	0.13	0.13	0.13	0.16	0.13	0.20	0.16	0.13	0.13	0.67	0.00	0.17	0.13	0.02	0.17	0.08	0.15	0.13	0.02	0.59	0.20	0.23	0.23	0.13	0.04	0.02	0.06	0.17	0.17
GI-4	0.17	0.17	0.17	0.17	0.17	0.17	0.08	0.08	0.08	0.08	0.08	0.11	0.08	0.15	0.11	0.08	0.08	0.85	0.17	0.00	0.07	0.12	0.04	0.12	0.04	0.07	0.12	0.60	0.07	0.09	0.09	0.07	0.14	0.12	0.12	0.07	0.04
BA-41	0.20	0.20	0.20	0.20	0.20	0.20	0.11	0.11	0.11	0.11	0.11	0.11	0.11	0.15	0.11	0.11	0.11	0.85	0.13	0.07	0.00	0.06	0.09	0.16	0.09	0.00	0.06	0.61	0.09	0.11	0.11	0.00	0.06	0.06	0.16	0.11	0.09
BA-42	0.08	0.08	0.08	0.08	0.08	0.08	0.16	0.16	0.16	0.16	0.16	0.18	0.16	0.23	0.18	0.16	0.16	0.72	0.02	0.12	0.06	0.00	0.17	0.08	0.17	0.10	0.00	0.59	0.20	0.23	0.23	0.10	0.02	0.00	0.08	0.17	0.17
BA-24	0.17	0.17	0.17	0.17	0.17	0.17	0.08	0.08	0.08	0.08	0.08	0.11	0.08	0.15	0.11	0.08	0.08	0.85	0.17	0.04	0.09	0.17	0.00	0.16	0.08	0.11	0.16	0.63	0.02	0.04	0.13	0.11	0.18	0.16	0.16	0.02	0.00
NE1-6	0.08	0.08	0.08	0.08	0.08	0.08	0.16	0.16	0.16	0.16	0.16	0.18	0.16	0.23	0.18	0.16	0.16	0.72	0.08	0.12	0.16	0.08	0.16	0.00	0.17	0.20	0.08	0.59	0.20	0.23	0.23	0.20	0.10	0.08	0.08	0.17	0.17
NE1-34	0.08	0.08	0.08	0.08	0.08	0.08	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.06	0.02	0.00	0.00	0.79	0.15	0.04	0.09	0.17	0.08	0.17	0.00	0.11	0.16	0.63	0.11	0.13	0.13	0.11	0.18	0.16	0.06	0.11	0.08
NE1-65	0.20	0.20	0.20	0.20	0.20	0.20	0.11	0.11	0.11	0.11	0.11	0.08	0.11	0.13	0.08	0.11	0.11	0.85	0.13	0.07	0.00	0.10	0.11	0.20	0.11	0.00	0.08	0.58	0.08	0.11	0.11	0.00	0.08	0.08	0.18	0.13	0.11
NE2-1	0.08	0.08	0.08	0.08	0.08	0.08	0.16	0.16	0.16	0.16	0.16	0.18	0.16	0.23	0.18	0.16	0.16	0.72	0.02	0.12	0.06	0.00	0.16	0.08	0.16	0.08	0.00	0.59	0.20	0.23	0.23	0.10	0.02	0.00	0.08	0.17	0.17
NE2-37	0.59	0.59	0.59	0.59	0.59	0.59	0.63	0.63	0.63	0.63	0.63	0.58	0.63	0.59	0.58	0.63	0.63	0.92	0.59	0.60	0.61	0.59	0.63	0.59	0.63	0.58	0.59	0.00	0.46	0.46	0.46	0.46	0.46	0.46	0.46	0.52	0.52
SO-1	0.20	0.20	0.20	0.20	0.20	0.20	0.11	0.11	0.11	0.11	0.11	0.08	0.11	0.13	0.08	0.11	0.11	0.85	0.20	0.07	0.09	0.20	0.02	0.20	0.11	0.08	0.20	0.46	0.00	0.02	0.11	0.08	0.18	0.18	0.18	0.04	0.02
LT-3	0.23	0.23	0.23	0.23	0.23	0.23	0.13	0.13	0.13	0.13	0.13	0.11	0.13	0.15	0.11	0.13	0.13	0.85	0.23	0.09	0.11	0.23	0.04	0.23	0.13	0.11	0.23	0.46	0.02	0.00	0.13	0.11	0.21	0.21	0.21	0.06	0.04
NR-40	0.23	0.23	0.23	0.23	0.23	0.23	0.13	0.13	0.13	0.13	0.13	0.11	0.13	0.08	0.11	0.13	0.13	0.85	0.23	0.09	0.11	0.23	0.13	0.23	0.13	0.11	0.23	0.46	0.11	0.13	0.00	0.11	0.21	0.21	0.21	0.16	0.13
NR-48	0.20	0.20	0.20	0.20	0.20	0.20	0.11	0.11	0.11	0.11	0.11	0.08	0.11	0.13	0.08	0.11	0.11	0.85	0.13	0.07	0.00	0.10	0.11	0.20	0.11	0.00	0.10	0.46	0.08	0.11	0.11	0.00	0.08	0.08	0.18	0.13	0.11
BO-4	0.10	0.10	0.10	0.10	0.10	0.10	0.18	0.18	0.18	0.18	0.18	0.18	0.18	0.23	0.18	0.18	0.18	0.72	0.04	0.14	0.06	0.02	0.18	0.10	0.18	0.08	0.02	0.46	0.18	0.21	0.21	0.08	0.00	0.00	0.08	0.18	0.18
SK-1	0.08	0.08	0.08	0.08	0.08	0.08	0.16	0.16	0.16	0.16	0.16	0.18	0.16	0.23	0.18	0.16	0.16	0.72	0.02	0.12	0.06	0.00	0.16	0.08	0.16	0.08	0.00	0.46	0.18	0.21	0.21	0.08	0.00	0.00	0.08	0.17	0.17
SK-2	0.00	0.00	0.00	0.00	0.00	0.00	0.06	0.06	0.06	0.06	0.06	0.08	0.06	0.13	0.08	0.06	0.06	0.67	0.06	0.12	0.16	0.08	0.16	0.08	0.06	0.18	0.08	0.46	0.18	0.21	0.21	0.18	0.08	0.08	0.00	0.17	0.17
SK-12	0.17	0.17	0.17	0.17	0.17	0.17	0.11	0.11	0.11	0.11	0.11	0.13	0.11	0.17	0.13	0.11	0.11	0.85	0.17	0.07	0.11	0.17	0.02	0.17	0.11	0.13	0.17	0.52	0.04	0.06	0.16	0.13	0.18	0.17	0.17	0.00	0.19
SC-3	0.17	0.17	0.17	0.17	0.17	0.17	0.08	0.08	0.08	0.08	0.08	0.11	0.08	0.15	0.11	0.08	0.08	0.85	0.17	0.04	0.09	0.17	0.00	0.17	0.08	0.11	0.17	0.52	0.02	0.04	0.13	0.11	0.18	0.17	0.17	0.19	0.00

Table 3.5. Table matrix of the genetic distance detected from the 31 representative indigenous soybean-nodulating bradyrhizobia in the Philippines through the Restriction Fragment Length Polymorphism (RFLP) treatment of the 16S-23S rRNA gene ITS region.

Strain	31	46	61	76	94	130	4	6	38	62	110	115	122	123	124	125	127	129	135	3622	IS-2	GI-8	BA-42	NE2-1	NR-2	BO-4	SC-49	SK-1	NE1-6	LT-36	SK-2	GI-4	NE2-3	BO-52	NR-60	NE1-65	SO-1	LT-3	NR-48	BO-15	SK-5	NE1-34	BA-24	NR-1	SK-12	BA-41	SC-3	NE2-66	NR-40	NE1-19	NE2-37
USDA31	0.00	0.03	0.03	0.00	0.03	0.00	0.36	0.48	0.55	0.36	0.36	0.55	0.36	0.42	0.36	0.36	0.42	0.36	0.31	0.31	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.06	0.06	0.06	0.42	0.42	0.42	0.00	0.42	0.36	0.36	0.36	0.36	0.36	0.36	0.48	0.48	0.42	0.42	0.48	0.48	0.36	0.42	0.42
USDA46	0.03	0.00	0.06	0.06	0.00	0.06	0.38	0.50	0.57	0.38	0.38	0.57	0.38	0.44	0.33	0.38	0.44	0.38	0.33	0.33	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.00	0.13	0.13	0.09	0.38	0.38	0.38	0.06	0.38	0.38	0.38	0.38	0.38	0.38	0.38	0.50	0.50	0.44	0.44	0.50	0.50	0.29	0.38	0.38
USDA61	0.03	0.06	0.00	0.03	0.03	0.03	0.36	0.48	0.55	0.36	0.36	0.55	0.36	0.42	0.36	0.36	0.42	0.36	0.31	0.31	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.10	0.10	0.06	0.42	0.42	0.42	0.03	0.42	0.36	0.36	0.36	0.36	0.36	0.36	0.48	0.48	0.42	0.42	0.48	0.48	0.36	0.42	0.42
USDA76	0.00	0.06	0.03	0.00	0.03	0.00	0.36	0.48	0.55	0.36	0.36	0.55	0.36	0.42	0.36	0.36	0.42	0.36	0.31	0.31	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.06	0.06	0.06	0.42	0.42	0.42	0.00	0.42	0.36	0.36	0.36	0.36	0.36	0.36	0.48	0.48	0.42	0.42	0.48	0.48	0.36	0.42	0.42
USDA94	0.03	0.00	0.03	0.03	0.00	0.06	0.38	0.50	0.57	0.38	0.38	0.57	0.38	0.44	0.33	0.38	0.44	0.38	0.33	0.33	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.00	0.13	0.13	0.09	0.38	0.38	0.38	0.06	0.38	0.38	0.38	0.38	0.38	0.38	0.38	0.50	0.50	0.44	0.44	0.50	0.50	0.29	0.38	0.38
USDA130	0.00	0.06	0.03	0.00	0.06	0.00	0.36	0.48	0.55	0.36	0.36	0.55	0.36	0.42	0.36	0.36	0.42	0.36	0.31	0.31	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.06	0.06	0.06	0.42	0.42	0.42	0.00	0.42	0.36	0.36	0.36	0.36	0.36	0.36	0.48	0.48	0.42	0.42	0.48	0.48	0.36	0.42	0.42
USDA4	0.36	0.38	0.36	0.36	0.38	0.36	0.00	0.11	0.20	0.15	0.15	0.20	0.15	0.15	0.15	0.15	0.15	0.15	0.20	0.20	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.43	0.43	0.36	0.25	0.25	0.25	0.30	0.20	0.11	0.11	0.15	0.15	0.15	0.11	0.11	0.11	0.11	0.15	0.11	0.15	0.20	0.15	0.25
USDA6	0.48	0.50	0.48	0.48	0.50	0.48	0.11	0.00	0.07	0.23	0.23	0.07	0.23	0.03	0.14	0.23	0.03	0.23	0.28	0.28	0.45	0.45	0.45	0.45	0.45	0.45	0.45	0.45	0.52	0.52	0.45	0.33	0.33	0.33	0.45	0.28	0.19	0.19	0.23	0.23	0.23	0.10	0.00	0.00	0.07	0.07	0.00	0.10	0.19	0.23	0.33
USDA38	0.55	0.57	0.55	0.55	0.57	0.55	0.20	0.07	0.00	0.33	0.33	0.00	0.33	0.16	0.29	0.33	0.16	0.33	0.44	0.44	0.57	0.57	0.57	0.57	0.57	0.57	0.57	0.57	0.57	0.57	0.57	0.38	0.38	0.38	0.57	0.33	0.29	0.29	0.33	0.33	0.33	0.24	0.13	0.13	0.20	0.20	0.13	0.24	0.33	0.33	0.38
USDA62	0.36	0.38	0.36	0.36	0.38	0.36	0.15	0.23	0.33	0.00	0.00	0.33	0.00	0.24	0.44	0.00	0.24	0.00	0.33	0.33	0.38	0.38	0.38	0.38	0.38	0.38	0.38	0.38	0.44	0.44	0.33	0.44	0.44	0.44	0.38	0.09	0.00	0.00	0.00	0.00	0.00	0.29	0.29	0.29	0.29	0.33	0.29	0.33	0.50	0.33	0.44
USDA110	0.36	0.38	0.36	0.36	0.38	0.36	0.15	0.23	0.33	0.00	0.00	0.33	0.00	0.24	0.44	0.00	0.24	0.00	0.33	0.33	0.38	0.38	0.38	0.38	0.38	0.38	0.38	0.38	0.44	0.44	0.33	0.44	0.44	0.44	0.38	0.09	0.00	0.00	0.00	0.00	0.00	0.29	0.29	0.29	0.29	0.33	0.29	0.33	0.50	0.33	0.44
USDA115	0.55	0.57	0.55	0.55	0.57	0.55	0.20	0.07	0.00	0.33	0.33	0.00	0.33	0.16	0.29	0.33	0.16	0.33	0.44	0.44	0.57	0.57	0.57	0.57	0.57	0.57	0.57	0.57	0.57	0.57	0.57	0.38	0.38	0.38	0.57	0.33	0.29	0.29	0.33	0.33	0.33	0.24	0.13	0.13	0.20	0.20	0.13	0.24	0.33	0.33	0.38
USDA122	0.36	0.38	0.36	0.36	0.38	0.36	0.15	0.23	0.33	0.00	0.00	0.33	0.00	0.24	0.44	0.00	0.24	0.00	0.33	0.33	0.38	0.38	0.38	0.38	0.38	0.38	0.38	0.38	0.44	0.44	0.33	0.44	0.44	0.44	0.38	0.09	0.00	0.00	0.00	0.00	0.00	0.29	0.29	0.29	0.29	0.33	0.29	0.33	0.50	0.33	0.44
USDA123	0.42	0.44	0.42	0.42	0.44	0.42	0.15	0.03	0.16	0.24	0.24	0.16	0.24	0.00	0.21	0.21	0.00	0.21	0.21	0.21	0.42	0.42	0.42	0.42	0.42	0.42	0.42	0.42	0.48	0.48	0.42	0.42	0.42	0.42	0.42	0.26	0.17	0.17	0.21	0.21	0.21	0.17	0.06	0.06	0.13	0.13	0.06	0.17	0.26	0.31	0.42
USDA124	0.36	0.33	0.36	0.36	0.33	0.36	0.15	0.14	0.29	0.44	0.44	0.29	0.44	0.21	0.00	0.42	0.21	0.42	0.26	0.26	0.36	0.36	0.36	0.36	0.36	0.36	0.36	0.31	0.48	0.48	0.42	0.17	0.17	0.17	0.36	0.36	0.36	0.36	0.42	0.42	0.42	0.17	0.17	0.17	0.10	0.10	0.17	0.13	0.06	0.17	0.17
USDA125	0.36	0.38	0.36	0.36	0.38	0.36	0.15	0.23	0.33	0.00	0.00	0.33	0.00	0.21	0.42	0.00	0.24	0.00	0.33	0.33	0.38	0.38	0.38	0.38	0.38	0.38	0.38	0.38	0.44	0.44	0.33	0.44	0.44	0.44	0.38	0.09	0.00	0.00	0.00	0.00	0.00	0.29	0.29	0.29	0.29	0.33	0.29	0.33	0.50	0.33	0.44
USDA127	0.42	0.44	0.42	0.42	0.44	0.42	0.15	0.03	0.16	0.24	0.24	0.16	0.24	0.00	0.21	0.24	0.00	0.21	0.21	0.21	0.42	0.42	0.42	0.42	0.42	0.42	0.42	0.42	0.48	0.48	0.42	0.42	0.42	0.42	0.42	0.26	0.17	0.17	0.21	0.21	0.21	0.17	0.06	0.06	0.13	0.13	0.06	0.17	0.26	0.31	0.42
USDA129	0.36	0.38	0.36	0.36	0.38	0.36	0.15	0.23	0.33	0.00	0.00	0.33	0.00	0.21	0.42	0.00	0.21	0.00	0.33	0.33	0.38	0.38	0.38	0.38	0.38	0.38	0.38	0.38	0.44	0.44	0.33	0.44	0.44	0.44	0.38	0.09	0.00	0.00	0.00	0.00	0.00	0.29	0.29	0.29	0.29	0.33	0.29	0.33	0.50	0.33	0.44
USDA135	0.31	0.33	0.31	0.31	0.33	0.31	0.20	0.28	0.44	0.33	0.33	0.44	0.33	0.21	0.26	0.33	0.21	0.33	0.00	0.00	0.36	0.36	0.36	0.36	0.36	0.36	0.36	0.36	0.41	0.41	0.36	0.36	0.36	0.36	0.36	0.41	0.31	0.31	0.36	0.36	0.36	0.23	0.36	0.36	0.27	0.27	0.36	0.31	0.31	0.31	0.36
USDA3622	0.31	0.33	0.31	0.31	0.33	0.31	0.20	0.28	0.44	0.33	0.33	0.44	0.33	0.21	0.26	0.33	0.21	0.33	0.00	0.00	0.36	0.36	0.36	0.36	0.36	0.36	0.36	0.36	0.41	0.41	0.36	0.36	0.36	0.36	0.36	0.41	0.31	0.31	0.36	0.36	0.36	0.23	0.36	0.36	0.27	0.27	0.36	0.31	0.31	0.31	0.36
IS-2	0.00	0.06	0.03	0.00	0.06	0.00	0.30	0.45	0.57	0.38	0.38	0.57	0.38	0.42	0.36	0.38	0.42	0.38	0.36	0.36	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.06	0.06	0.06	0.42	0.42	0.42	0.00	0.42	0.36	0.36	0.36	0.36	0.36	0.36	0.48	0.48	0.42	0.42	0.48	0.48	0.36	0.42	0.42
GI-8	0.00	0.06	0.03	0.00	0.06	0.00	0.30	0.45	0.57	0.38	0.38	0.57	0.38	0.42	0.36	0.38	0.42	0.38	0.36	0.36	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.06	0.06	0.06	0.42	0.42	0.42	0.00	0.42	0.36	0.36	0.36	0.36	0.36	0.36	0.48	0.48	0.42	0.42	0.48	0.48	0.36	0.42	0.42
BA-42	0.00	0.06	0.03	0.00	0.06	0.00	0.30	0.45	0.57	0.38	0.38	0.57	0.38	0.42	0.36	0.38	0.42	0.38	0.36	0.36	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.06	0.06	0.06	0.42	0.42	0.42	0.00	0.42	0.36	0.36	0.36	0.36	0.36	0.36	0.48	0.48	0.42	0.42	0.48	0.48	0.36	0.42	0.42
NE2-1	0.00	0.06	0.03	0.00	0.06	0.00	0.30	0.45	0.57	0.38	0.38	0.57	0.38	0.42	0.36	0.38	0.42	0.38	0.36	0.36	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.06	0.06	0.06	0.42	0.42	0.42	0.00	0.42	0.36	0.36	0.36	0.36	0.36	0.36	0.48	0.48	0.42	0.42	0.48	0.48	0.36	0.42	0.42
NR-2	0.00	0.06	0.03	0.00	0.06	0.00	0.30	0.45	0.57	0.38	0.38	0.57	0.38	0.42	0.36	0.38	0.42	0.38	0.36	0.36	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.06	0.06	0.06	0.42	0.42	0.42	0.00	0.42	0.36	0.36	0.36	0.36	0.36	0.36	0.48	0.48	0.42	0.42	0.48	0.48	0.36	0.42	0.42
BO-4	0.00	0.06	0.03	0.00	0.06	0.00	0.30	0.45	0.57	0.38	0.38	0.57	0.38	0.42	0.36	0.38	0.42	0.38	0.36	0.36	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.06	0.06	0.06	0.42	0.42	0.42	0.00	0.42	0.36	0.36	0.36	0.36	0.36	0.36	0.48	0.48	0.42	0.42	0.48	0.48	0.36	0.42	0.42
SC-49	0.00	0.06	0.03	0.00	0.06	0.00	0.30	0.45	0.57	0.38	0.38	0.57	0.38	0.42	0.36	0.38	0.42	0.38	0.36	0.36	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.06	0.06	0.06	0.42	0.42	0.42	0.00	0.42	0.36	0.36	0.36	0.36	0.36	0.36	0.48	0.48	0.42	0.42	0.48	0.48	0.36	0.42	0.42
SK-1	0.00	0.00	0.03	0.00	0.00	0.00	0.30	0.45	0.57	0.38	0.38	0.57	0.38	0.42	0.31	0.38	0.42	0.38	0.36	0.36	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.12	0.12	0.12	0.41	0.41	0.41	0.06	0.41	0.41	0.41	0.41	0.41	0.41	0.41	0.52	0.52	0.46	0.46	0.52	0.52	0.31	0.41	0.41
NE1-6	0.06	0.13	0.10	0.06	0.13	0.06	0.43	0.52	0.57	0.44	0.44	0.57	0.44	0.48	0.48	0.44	0.48	0.44	0.41	0.41	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.12	0.00	0.00	0.07	0.39	0.39	0.39	0.03	0.39	0.39	0.39	0.39	0.39	0.39	0.39	0.52	0.52	0.45	0.45	0.52	0.52	0.45	0.39	0.39
LT-36	0.06	0.13	0.10	0.06	0.13	0.06	0.43	0.52	0.57	0.44	0.44	0.57	0.44	0.48	0.48	0.44	0.48	0.44	0.41	0.41	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.12	0.00	0.00	0.07	0.39	0.39	0.39	0.03	0.39	0.39	0.39	0.39	0.39	0.39	0.39	0.52	0.52	0.45	0.45	0.52	0.52	0.45	0.39	0.39
SK-2	0.06	0.09	0.06	0.06	0.09	0.06	0.36	0.45	0.57	0.33	0.33	0.57	0.33	0.42	0.42	0.33	0.42	0.33	0.36	0.36	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.12	0.07	0.07	0.00	0.46	0.46	0.46	0.12	0.41	0.36	0.36	0.36	0.36	0.36	0.41	0.52	0.52	0.46	0.46	0.52	0.52	0.46	0.46	0.46
GI-4	0.42	0.38	0.42	0.42	0.38	0.42	0.25	0.33	0.38	0.44	0.44	0.38	0.44	0.42	0.17	0.44	0.42	0.44	0.36	0.36	0.42	0.42	0.42	0.42	0.42	0.42	0.42	0.41	0.39	0.39	0.46	0.00	0.00	0.00	0.46	0.27	0.41	0.41	0.46	0.46	0.46	0.27	0.41	0.41	0.31	0.31	0.41	0.36	0.31	0.15	0.00
NE2-3	0.42	0.38	0.42	0.42	0.38	0.42	0.25	0.33	0.38	0.44	0.44	0.38	0.44	0.42	0.17	0.44	0.42	0.44	0.36	0.36	0.42	0.42	0.42	0.42	0.42	0.42	0.42	0.41	0.39	0.39	0.46	0.00	0.00	0.00	0.46	0.27	0.41	0.41	0.46	0.46	0.46	0.27	0.41	0.41	0.31	0.31	0.41	0.36	0.31	0.15	0.00
BO-52	0.42	0.38	0.42	0.42	0.38	0.42	0.25	0.33	0.38	0.44	0.44	0.38	0.44	0.42	0.17	0.44	0.42	0.44	0.36	0.36	0.42	0.42	0.42	0.42	0.42	0.42	0.42	0.41	0.39	0.39	0.46	0.00	0.00	0.00	0.46	0.27	0.41	0.41	0.46	0.46	0.46	0.27	0.41	0.41	0.31	0.31	0.41	0.36	0.31	0.15	0.00
NR-60	0.00	0.06	0.03	0.00	0.06	0.00	0.30	0.45	0.57	0.38	0.38	0.57	0.38	0.42	0.36	0.38	0.42	0.38	0.36	0.36	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.06	0.03	0.03	0.12	0.46	0.46	0.46	0.00	0.42	0.36	0.36	0.36	0.36	0.36	0.36	0.48	0.48	0.42	0.42	0.48	0.48	0.36	0.42	0.42
NE1-65	0.42	0.38	0.42	0.42	0.38	0.42	0.20	0.28	0.33	0.09	0.09	0.33	0.09	0.26	0.36	0.09	0.26	0.09	0.41	0.41	0.42	0.42	0.42	0.42	0.42	0.42	0.42	0.41	0.39	0.39	0.41	0.27	0.27	0.27	0.42	0.00	0.09	0.09	0.12	0.12	0.12	0.36	0.36	0.36	0.36	0.41	0.36	0.41	0.46	0.19	0.27
SO-1	0.36	0.38	0.36	0.36	0.38	0.36	0.11	0.19	0.29	0.00	0.00	0.29	0.00	0.17	0.36	0.00	0.17	0.00	0.31	0.31	0.36	0.36	0.36	0.36	0.36	0.36	0.36	0.41	0.39	0.39	0.36	0.41	0.41	0.41	0.36	0.09	0.00	0.00	0.03	0.03	0.03	0.27	0.27	0.27	0.27	0.31	0.27	0.31	0.46	0.31	0.41
LT-3	0.36	0.38	0.36	0.36	0.38	0.36	0.11	0.19	0.29	0.00	0.00	0.29	0.00	0.17	0.36	0.00	0.17	0.00	0.31	0.31	0.36	0.36	0.36	0.36	0.36	0.36	0.36	0.41	0.39	0.39	0.36	0.41	0.41	0.41	0.36	0.09	0.00	0.00	0.03	0.03	0.03	0.27	0.27	0.27	0.27	0.31	0.27	0.31	0.46	0.31	0.41
NR-48	0.36	0.38	0.36	0.36	0.38	0.36	0.15	0.23	0.33	0.00	0.00	0.33	0.00	0.21	0.42	0.00	0.21	0.00	0.36	0.36	0.36	0.36	0.36	0.36	0.36	0.36	0.36	0.41	0.39	0.39	0.36	0.46	0.46	0.46	0.36	0.12	0.03	0.03	0.00	0.00	0.00	0.29	0.29	0.29	0.29	0.33	0.29	0.33	0.50	0.33	0.44
BO-15	0.36	0.38	0.36	0.36	0.38	0.36	0.15	0.23	0.33	0.00	0.00	0.33	0.00	0.21	0.42	0.00	0.21	0.00	0.36	0.36	0.36	0.36	0.36	0.36	0.36	0.36	0.36	0.41	0.39	0.39	0.36	0.46	0.46	0.46	0.36	0.12	0.03	0.03	0.00	0.00	0.00	0.29	0.29	0.29	0.29	0.33	0.29	0.33	0.50	0.33	0.44
SK-5	0.36	0.38	0.36	0.36	0.38	0.36	0.15	0.23	0.33	0.00	0.00	0.33	0.00	0.21	0.42	0.00	0.21	0.00	0.36	0.36	0.36	0.36	0.36	0.36	0.36	0.36	0.36	0.41	0.39	0.39	0.36	0.46	0.46	0.46	0.36	0.12	0.03	0.03	0.00	0.00	0.00	0.29	0.29	0.29	0.29	0.33	0.29	0.33	0.50	0.33	0.44
NE1-34	0.36	0.38	0.36	0.36	0.38	0.36	0.11	0.10	0.24	0.29	0.29	0.24	0.29	0.17	0.17	0.29	0.17	0.29	0.23	0.23	0.36	0.36	0.36	0.36	0.36	0.36	0.36	0.41	0.39	0.39	0.41	0.27	0.27	0.27	0.36	0.36	0.27	0.27	0.29	0.29	0.29	0.00	0.10	0.10	0.07	0.07	0.10	0.10	0.19	0.10	0.19
BA-24	0.48	0.50	0.48	0.48	0.50	0.48	0.11	0.00	0.13	0.29	0.29	0.13	0.29	0.06	0.17	0.29	0.06	0.29	0.36	0.36	0.48	0.48	0.48	0.48	0.48	0.48	0.48	0.52	0.52	0.52	0.52	0.41	0.41	0.41	0.48	0.36	0.27	0.27	0.29	0.29	0.29	0.10	0.00	0.00	0.07	0.07	0.00	0.10	0.19	0.23	0.33
NR-1	0.48	0.50	0.48	0.48	0.50	0.48	0.11	0.00	0.13	0.29	0.29	0.13	0.29	0.06	0.17	0.29	0.06	0.29	0.36	0.36	0.48	0.48	0.48	0.48	0.48	0.48	0.48	0.52	0.52	0.52	0.52	0.41	0.41	0.41	0.48	0.36	0.27	0.27	0.29	0.29	0.29	0.10	0.00	0.00	0.07	0.07	0.00	0.10	0.19	0.23	0.33
SK-12	0.42	0.44	0.42	0.42	0.44	0.42	0.11	0.07	0.20	0.29	0.29	0.20	0.29	0.13	0.10	0.29	0.13	0.29	0.27	0.27	0.42	0.42	0.42	0.42	0.42	0.42	0.42	0.46	0.45	0.45	0.46	0.31	0.31	0.31	0.42	0.36	0.27	0.27	0.29	0.29	0.29	0.07	0.07	0.07	0.00	0.03	0.10	0.03	0.13	0.13	0.26
BA-41	0.42	0.44	0.42	0.42	0.44	0.42	0.15	0.07	0.20	0.33	0.33	0.20	0.33	0.13	0.10	0.33	0.13	0.33	0.27	0.27	0.42	0.42	0.42	0.42	0.42	0.42	0.42	0.46	0.45	0.45	0.46	0.31	0.31	0.31	0.42	0.41	0.31	0.31	0.33	0.33	0.33	0.07	0.07	0.07	0.03	0.00	0.10	0.06	0.13	0.17	0.26
SC-3	0.48	0.50	0.48	0.48	0.50	0.48	0.11	0.00	0.13	0.29	0.29	0.13	0.29	0.06	0.17	0.29	0.06	0.29	0.36	0.36	0.48	0.48	0.48	0.48	0.48	0.48	0.48	0.52	0.52	0.52	0.52	0.41	0.41	0.41	0.48	0.36	0.27	0.27	0.29	0.29	0.29	0.10	0.00	0.00	0.10	0.10	0.00	0.10	0.19	0.23	0.33
NE2-66	0.48	0.50	0.48	0.48	0.50	0.48	0.15	0.10	0.24	0.33	0.33	0.24	0.33	0.17	0.13	0.33	0.17	0.33	0.31	0.31	0.48	0.48	0.48	0.48	0.48	0.48	0.48	0.52	0.52	0.52	0.52	0.36	0.36	0.36	0.48	0.41	0.31	0.31	0.33	0.33	0.33	0.10	0.10	0.10	0.03	0.06	0.10	0.00	0.14	0.14	0.28
NR-40	0.36	0.29	0.36	0.36	0.29	0.36	0.20	0.19	0.33	0.50	0.50	0.33	0.50	0.26	0.06	0.50	0.26	0.50	0.31	0.31	0.36	0.36	0.36	0.36	0.36	0.36	0.36	0.31	0.45	0.45	0.46	0.31	0.31	0.31	0.36	0.46	0.46	0.46	0.50	0.50	0.50	0.19	0.19	0.19	0.13	0.13	0.19	0.14	0.00	0.24	0.29
NE1-19	0.42	0.38	0.42	0.42	0.38	0.42	0.15	0.23	0.33	0.33	0.33	0.33	0.33	0.31	0.17	0.33	0.31	0.33	0.31	0.31	0.42	0.42	0.42	0.42	0.42	0.42	0.42	0.41	0.39	0.39	0.46	0.15	0.15	0.15	0.42	0.19	0.31	0.31	0.33	0.33	0.33	0.10	0.23	0.23	0.13	0.17	0.23	0.14	0.24	0.00	0.10
NE2-37	0.42	0.38	0.42	0.42	0.38	0.42	0.25	0.33	0.38	0.44	0.44	0.38	0.44	0.42	0.17	0.44	0.42	0.44	0.36	0.36	0.42	0.42	0.42	0.42	0.42	0.42	0.42	0.41	0.39	0.39	0.46	0.00	0.00	0.00	0.42	0.27	0.41	0.41	0.44	0.44	0.44	0.19	0.33	0.33	0.26	0.26	0.33	0.28	0.29	0.10	0.00

Table 3.6. Table matrix of the genetic distance detected from the 31 representative indigenous soybean-nodulating bradyrhizobia in the Philippines through the Restriction Fragment Length Polymorphism (RFLP) treatment of the *rpoB* housekeeping gene.

Strain	31	46	61	76	94	130	4	6	38	62	110	115	122	123	124	125	127	129	135	3622	GI-4	NE2-3	BO-52	NE1-65	NR-48	NE2-37	NE1-34	NR-40	SK-12	BA-24	NR-1	SC-3	NE2-66	BA-41	IS-2	GI-8	BA-42	NE1-6	NE2-1	NR-2	BO-4	SC-49	NR-60	LT-36	SK-1	SK-2	SO-1	LT-3	BO-15	SK-5	NE1-19
USDA31	0.00	0.05	0.05	0.00	0.11	0.00	0.50	0.56	0.50	0.45	0.45	0.50	0.40	0.40	0.50	0.45	0.40	0.45	0.68	0.68	0.83	0.83	0.83	0.83	0.83	0.68	0.68	0.68	0.56	0.62	0.62	0.62	0.56	0.40	0.24	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.20	0.08	0.05	0.20	0.50	0.50	0.50	0.50	0.83
USDA46	0.05	0.00	0.00	0.05	0.14	0.05	0.62	0.62	0.56	0.56	0.56	0.56	0.50	0.50	0.56	0.56	0.50	0.56	0.68	0.68	0.75	0.75	0.75	0.75	0.75	0.62	0.68	0.75	0.56	0.68	0.68	0.68	0.68	0.50	0.17	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.27	0.02	0.11	0.27	0.62	0.62	0.62	0.62	0.75
USDA61	0.05	0.00	0.00	0.05	0.14	0.05	0.62	0.62	0.56	0.56	0.56	0.56	0.50	0.50	0.56	0.56	0.50	0.56	0.68	0.68	0.75	0.75	0.75	0.75	0.75	0.62	0.68	0.75	0.56	0.68	0.68	0.68	0.68	0.50	0.17	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.27	0.02	0.11	0.27	0.62	0.62	0.62	0.62	0.75
USDA76	0.00	0.05	0.05	0.00	0.11	0.00	0.50	0.56	0.50	0.45	0.45	0.50	0.40	0.40	0.50	0.45	0.40	0.45	0.68	0.68	0.83	0.83	0.83	0.83	0.83	0.68	0.68	0.68	0.56	0.62	0.62	0.62	0.56	0.40	0.24	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.20	0.08	0.05	0.20	0.50	0.50	0.50	0.50	0.83
USDA94	0.11	0.14	0.14	0.11	0.00	0.13	0.63	0.57	0.52	0.57	0.57	0.52	0.52	0.52	0.63	0.57	0.52	0.57	0.76	0.76	0.83	0.83	0.83	0.83	0.83	0.69	0.76	0.69	0.63	0.63	0.63	0.63	0.69	0.52	0.38	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.29	0.19	0.16	0.16	0.63	0.63	0.63	0.63	0.83
USDA130	0.00	0.05	0.05	0.00	0.13	0.00	0.50	0.56	0.50	0.45	0.45	0.50	0.40	0.40	0.50	0.45	0.40	0.45	0.68	0.68	0.83	0.83	0.83	0.83	0.83	0.68	0.68	0.68	0.56	0.62	0.62	0.62	0.56	0.40	0.24	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.20	0.08	0.05	0.20	0.50	0.50	0.50	0.50	0.83
USDA4	0.50	0.62	0.62	0.50	0.63	0.50	0.00	0.45	0.31	0.17	0.17	0.31	0.24	0.24	0.24	0.17	0.24	0.17	0.20	0.20	0.40	0.40	0.40	0.35	0.35	0.62	0.20	0.31	0.24	0.50	0.50	0.50	0.31	0.20	0.68	0.68	0.68	0.68	0.68	0.68	0.68	0.68	0.56	0.68	0.56	0.62	0.20	0.20	0.20	0.20	0.35
USDA6	0.56	0.62	0.62	0.56	0.57	0.56	0.45	0.00	0.25	0.43	0.43	0.25	0.38	0.25	0.38	0.43	0.25	0.43	0.54	0.54	0.60	0.60	0.60	0.54	0.54	0.54	0.48	0.43	0.54	0.08	0.05	0.05	0.48	0.54	0.90	0.67	0.67	0.67	0.67	0.67	0.67	0.67	0.54	0.67	0.60	0.67	0.48	0.48	0.48	0.48	0.54
USDA38	0.50	0.56	0.56	0.50	0.52	0.50	0.31	0.25	0.00	0.24	0.24	0.00	0.24	0.13	0.29	0.24	0.13	0.24	0.38	0.38	0.50	0.50	0.50	0.44	0.44	0.50	0.44	0.38	0.38	0.16	0.20	0.20	0.33	0.38	0.80	0.57	0.57	0.57	0.57	0.57	0.57	0.57	0.50	0.57	0.50	0.57	0.29	0.29	0.29	0.29	0.44
USDA62	0.45	0.56	0.56	0.45	0.57	0.45	0.17	0.43	0.24	0.00	0.00	0.29	0.05	0.21	0.18	0.00	0.21	0.00	0.33	0.33	0.48	0.48	0.48	0.38	0.38	0.54	0.38	0.29	0.29	0.48	0.48	0.48	0.21	0.21	0.67	0.60	0.60	0.60	0.60	0.60	0.60	0.60	0.48	0.60	0.48	0.60	0.03	0.03	0.03	0.03	0.38
USDA110	0.45	0.56	0.56	0.45	0.57	0.45	0.17	0.43	0.24	0.00	0.00	0.29	0.05	0.21	0.18	0.00	0.21	0.00	0.33	0.33	0.48	0.48	0.48	0.38	0.38	0.54	0.38	0.29	0.29	0.48	0.48	0.48	0.21	0.21	0.67	0.60	0.60	0.60	0.60	0.60	0.60	0.60	0.48	0.60	0.48	0.60	0.03	0.03	0.03	0.03	0.38
USDA115	0.50	0.56	0.56	0.50	0.52	0.50	0.31	0.25	0.00	0.29	0.29	0.00	0.24	0.13	0.29	0.24	0.13	0.24	0.38	0.38	0.50	0.50	0.50	0.44	0.44	0.50	0.44	0.38	0.38	0.16	0.20	0.20	0.33	0.38	0.80	0.57	0.57	0.57	0.57	0.57	0.57	0.57	0.50	0.57	0.50	0.57	0.29	0.29	0.29	0.29	0.44
USDA122	0.40	0.50	0.50	0.40	0.52	0.40	0.24	0.38	0.24	0.05	0.05	0.24	0.00	0.15	0.23	0.03	0.15	0.03	0.41	0.41	0.52	0.52	0.52	0.41	0.41	0.58	0.46	0.36	0.36	0.41	0.41	0.41	0.19	0.27	0.65	0.52	0.52	0.52	0.52	0.52	0.52	0.52	0.41	0.52	0.41	0.58	0.06	0.06	0.06	0.06	0.41
USDA123	0.40	0.50	0.50	0.40	0.52	0.40	0.24	0.25	0.13	0.21	0.21	0.13	0.15	0.00	0.19	0.26	0.00	0.26	0.38	0.38	0.52	0.52	0.52	0.47	0.47	0.63	0.47	0.57	0.47	0.29	0.33	0.33	0.38	0.42	0.76	0.57	0.57	0.57	0.57	0.57	0.57	0.57	0.42	0.57	0.47	0.52	0.29	0.29	0.29	0.29	0.47
USDA124	0.50	0.56	0.56	0.50	0.63	0.50	0.24	0.38	0.29	0.18	0.18	0.29	0.23	0.19	0.00	0.22	0.19	0.22	0.29	0.29	0.38	0.38	0.38	0.29	0.29	0.47	0.33	0.42	0.29	0.47	0.47	0.47	0.22	0.29	0.69	0.63	0.63	0.63	0.63	0.63	0.63	0.63	0.52	0.57	0.57	0.57	0.26	0.26	0.26	0.26	0.29
USDA125	0.45	0.56	0.56	0.45	0.57	0.45	0.17	0.43	0.24	0.00	0.00	0.24	0.03	0.26	0.22	0.00	0.21	0.00	0.33	0.33	0.48	0.48	0.48	0.38	0.38	0.54	0.38	0.29	0.29	0.48	0.48	0.48	0.21	0.21	0.67	0.60	0.60	0.60	0.60	0.60	0.60	0.60	0.48	0.60	0.48	0.60	0.03	0.03	0.03	0.03	0.38
USDA127	0.40	0.50	0.50	0.40	0.52	0.40	0.24	0.25	0.13	0.21	0.21	0.13	0.15	0.00	0.19	0.21	0.00	0.26	0.38	0.38	0.52	0.52	0.52	0.47	0.47	0.63	0.47	0.57	0.47	0.29	0.33	0.33	0.38	0.42	0.76	0.57	0.57	0.57	0.57	0.57	0.57	0.57	0.42	0.57	0.47	0.52	0.29	0.29	0.29	0.29	0.47
USDA129	0.45	0.56	0.56	0.45	0.57	0.45	0.17	0.43	0.24	0.00	0.00	0.24	0.03	0.26	0.22	0.00	0.26	0.00	0.33	0.33	0.48	0.48	0.48	0.38	0.38	0.54	0.38	0.29	0.29	0.48	0.48	0.48	0.21	0.21	0.67	0.60	0.60	0.60	0.60	0.60	0.60	0.60	0.48	0.60	0.48	0.60	0.03	0.03	0.03	0.03	0.38
USDA135	0.68	0.68	0.68	0.68	0.76	0.68	0.20	0.54	0.38	0.33	0.33	0.38	0.41	0.38	0.29	0.33	0.38	0.33	0.00	0.00	0.31	0.31	0.31	0.31	0.31	0.52	0.15	0.31	0.15	0.52	0.52	0.52	0.41	0.31	0.65	0.65	0.65	0.65	0.65	0.65	0.65	0.65	0.58	0.65	0.65	0.65	0.31	0.31	0.31	0.31	0.31
USDA3622	0.68	0.68	0.68	0.68	0.76	0.68	0.20	0.54	0.38	0.33	0.33	0.38	0.41	0.38	0.29	0.33	0.38	0.33	0.00	0.00	0.31	0.31	0.31	0.31	0.31	0.52	0.15	0.31	0.15	0.52	0.52	0.52	0.41	0.31	0.65	0.65	0.65	0.65	0.65	0.65	0.65	0.65	0.58	0.65	0.65	0.65	0.31	0.31	0.31	0.31	0.31
GI-4	0.83	0.75	0.75	0.83	0.83	0.83	0.40	0.60	0.50	0.48	0.48	0.50	0.52	0.52	0.38	0.48	0.52	0.48	0.31	0.31	0.00	0.00	0.00	0.03	0.03	0.17	0.21	0.26	0.26	0.48	0.48	0.48	0.36	0.26	0.79	0.62	0.62	0.62	0.62	0.62	0.62	0.62	0.62	0.55	0.70	0.70	0.36	0.36	0.36	0.42	0.03
NE2-3	0.83	0.75	0.75	0.83	0.83	0.83	0.40	0.60	0.50	0.48	0.48	0.50	0.52	0.52	0.38	0.48	0.52	0.48	0.31	0.31	0.00	0.00	0.00	0.03	0.03	0.17	0.21	0.26	0.26	0.48	0.48	0.48	0.36	0.26	0.79	0.62	0.62	0.62	0.62	0.62	0.62	0.62	0.62	0.55	0.70	0.70	0.36	0.36	0.36	0.42	0.03
BO-52	0.83	0.75	0.75	0.83	0.83	0.83	0.40	0.60	0.50	0.48	0.48	0.50	0.52	0.52	0.38	0.48	0.52	0.48	0.31	0.31	0.00	0.00	0.00	0.03	0.03	0.17	0.21	0.26	0.26	0.48	0.48	0.48	0.36	0.26	0.79	0.62	0.62	0.62	0.62	0.62	0.62	0.62	0.62	0.55	0.70	0.70	0.36	0.36	0.36	0.42	0.03
NE1-65	0.83	0.75	0.75	0.83	0.83	0.83	0.35	0.54	0.44	0.38	0.38	0.44	0.41	0.47	0.29	0.38	0.47	0.38	0.31	0.31	0.03	0.03	0.03	0.00	0.00	0.24	0.24	0.29	0.29	0.44	0.44	0.44	0.29	0.24	0.80	0.64	0.64	0.64	0.64	0.64	0.64	0.64	0.64	0.57	0.71	0.71	0.29	0.29	0.29	0.33	0.00
NR-48	0.83	0.75	0.75	0.83	0.83	0.83	0.35	0.54	0.44	0.38	0.38	0.44	0.41	0.47	0.29	0.38	0.47	0.38	0.31	0.31	0.03	0.03	0.03	0.00	0.00	0.24	0.24	0.29	0.29	0.44	0.44	0.44	0.29	0.24	0.80	0.64	0.64	0.64	0.64	0.64	0.64	0.64	0.64	0.57	0.71	0.71	0.29	0.29	0.29	0.33	0.00
NE2-37	0.68	0.62	0.62	0.68	0.69	0.68	0.62	0.54	0.50	0.54	0.54	0.50	0.58	0.63	0.47	0.54	0.63	0.54	0.52	0.52	0.17	0.17	0.17	0.24	0.24	0.00	0.36	0.42	0.36	0.42	0.42	0.42	0.48	0.42	0.79	0.48	0.48	0.48	0.48	0.48	0.48	0.48	0.42	0.42	0.55	0.62	0.42	0.42	0.42	0.48	0.24
NE1-34	0.68	0.68	0.68	0.68	0.76	0.68	0.20	0.48	0.44	0.38	0.38	0.44	0.46	0.47	0.33	0.38	0.47	0.38	0.15	0.15	0.21	0.21	0.21	0.24	0.24	0.36	0.00	0.24	0.20	0.45	0.45	0.45	0.40	0.27	0.68	0.62	0.62	0.62	0.62	0.62	0.62	0.62	0.56	0.62	0.62	0.56	0.35	0.35	0.35	0.40	0.24
NR-40	0.68	0.75	0.75	0.68	0.69	0.68	0.31	0.43	0.38	0.29	0.29	0.38	0.36	0.57	0.42	0.29	0.57	0.29	0.31	0.31	0.26	0.26	0.26	0.29	0.29	0.42	0.24	0.00	0.25	0.38	0.38	0.38	0.38	0.21	0.82	0.67	0.67	0.67	0.67	0.67	0.67	0.67	0.60	0.67	0.60	0.74	0.25	0.25	0.25	0.29	0.29
SK-12	0.56	0.56	0.56	0.56	0.63	0.56	0.24	0.54	0.38	0.29	0.29	0.38	0.36	0.47	0.29	0.29	0.47	0.29	0.15	0.15	0.26	0.26	0.26	0.29	0.29	0.36	0.20	0.25	0.00	0.48	0.48	0.48	0.29	0.25	0.54	0.48	0.48	0.48	0.48	0.48	0.48	0.48	0.48	0.48	0.48	0.54	0.25	0.25	0.25	0.29	0.29
BA-24	0.62	0.68	0.68	0.62	0.63	0.62	0.50	0.08	0.16	0.48	0.48	0.16	0.41	0.29	0.47	0.48	0.29	0.48	0.52	0.52	0.48	0.48	0.48	0.44	0.44	0.42	0.45	0.38	0.48	0.00	0.03	0.03	0.41	0.46	0.90	0.58	0.58	0.58	0.58	0.58	0.58	0.58	0.46	0.58	0.52	0.58	0.41	0.41	0.41	0.46	0.44
NR-1	0.62	0.68	0.68	0.62	0.63	0.62	0.50	0.05	0.20	0.48	0.48	0.20	0.41	0.33	0.47	0.48	0.33	0.48	0.52	0.52	0.48	0.48	0.48	0.44	0.44	0.42	0.45	0.38	0.48	0.03	0.00	0.00	0.41	0.46	0.90	0.58	0.58	0.58	0.58	0.58	0.58	0.58	0.46	0.58	0.52	0.58	0.41	0.41	0.41	0.46	0.44
SC-3	0.62	0.68	0.68	0.62	0.63	0.62	0.50	0.05	0.20	0.48	0.48	0.20	0.41	0.33	0.47	0.48	0.33	0.48	0.52	0.52	0.48	0.48	0.48	0.44	0.44	0.42	0.45	0.38	0.48	0.03	0.00	0.00	0.41	0.46	0.90	0.58	0.58	0.58	0.58	0.58	0.58	0.58	0.46	0.58	0.52	0.58	0.41	0.41	0.41	0.46	0.44
NE2-66	0.56	0.68	0.68	0.56	0.69	0.56	0.31	0.48	0.33	0.21	0.21	0.33	0.19	0.38	0.22	0.21	0.38	0.21	0.41	0.41	0.36	0.36	0.36	0.29	0.29	0.48	0.40	0.38	0.29	0.41	0.41	0.41	0.00	0.20	0.71	0.57	0.57	0.57	0.57	0.57	0.57	0.57	0.44	0.50	0.44	0.50	0.13	0.13	0.13	0.16	0.29
BA-41	0.40	0.50	0.50	0.40	0.52	0.40	0.20	0.54	0.38	0.21	0.21	0.38	0.27	0.42	0.29	0.21	0.42	0.21	0.31	0.31	0.26	0.26	0.26	0.24	0.24	0.42	0.27	0.21	0.25	0.46	0.46	0.46	0.20	0.00	0.57	0.47	0.47	0.47	0.47	0.47	0.47	0.47	0.52	0.47	0.38	0.47	0.22	0.22	0.22	0.26	0.24
IS-2	0.24	0.17	0.17	0.24	0.38	0.24	0.68	0.90	0.80	0.67	0.67	0.80	0.65	0.76	0.69	0.67	0.76	0.67	0.65	0.65	0.79	0.79	0.79	0.80	0.80	0.79	0.68	0.82	0.54	0.90	0.90	0.90	0.71	0.57	0.00	0.17	0.17	0.17	0.17	0.17	0.17	0.17	0.50	0.14	0.27	0.45	0.68	0.68	0.68	0.68	0.80
GI-8	0.08	0.02	0.02	0.08	0.19	0.08	0.68	0.67	0.57	0.60	0.60	0.57	0.52	0.57	0.63	0.60	0.57	0.60	0.65	0.65	0.62	0.62	0.62	0.64	0.64	0.48	0.62	0.67	0.48	0.58	0.58	0.58	0.57	0.47	0.17	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.24	0.00	0.08	0.24	0.56	0.56	0.56	0.62	0.64
BA-42	0.08	0.02	0.02	0.08	0.19	0.08	0.68	0.67	0.57	0.60	0.60	0.57	0.52	0.57	0.63	0.60	0.57	0.60	0.65	0.65	0.62	0.62	0.62	0.64	0.64	0.48	0.62	0.67	0.48	0.58	0.58	0.58	0.57	0.47	0.17	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.24	0.00	0.08	0.24	0.56	0.56	0.56	0.62	0.64
NE1-6	0.08	0.02	0.02	0.08	0.19	0.08	0.68	0.67	0.57	0.60	0.60	0.57	0.52	0.57	0.63	0.60	0.57	0.60	0.65	0.65	0.62	0.62	0.62	0.64	0.64	0.48	0.62	0.67	0.48	0.58	0.58	0.58	0.57	0.47	0.17	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.24	0.00	0.08	0.24	0.56	0.56	0.56	0.62	0.64
NE2-1	0.08	0.02	0.02	0.08	0.19	0.08	0.68	0.67	0.57	0.60	0.60	0.57	0.52	0.57	0.63	0.60	0.57	0.60	0.65	0.65	0.62	0.62	0.62	0.64	0.64	0.48	0.62	0.67	0.48	0.58	0.58	0.58	0.57	0.47	0.17	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.24	0.00	0.08	0.24	0.56	0.56	0.56	0.62	0.64
NR-2	0.08	0.02	0.02	0.08	0.19	0.08	0.68	0.67	0.57	0.60	0.60	0.57	0.52	0.57	0.63	0.60	0.57	0.60	0.65	0.65	0.62	0.62	0.62	0.64	0.64	0.48	0.62	0.67	0.48	0.58	0.58	0.58	0.57	0.47	0.17	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.24	0.00	0.08	0.24	0.56	0.56	0.56	0.62	0.64
BO-4	0.08	0.02	0.02	0.08	0.19	0.08	0.68	0.67	0.57	0.60	0.60	0.57	0.52	0.57	0.63	0.60	0.57	0.60	0.65	0.65	0.62	0.62	0.62	0.64	0.64	0.48	0.62	0.67	0.48	0.58	0.58	0.58	0.57	0.47	0.17	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.24	0.00	0.08	0.24	0.56	0.56	0.56	0.62	0.64
SC-49	0.08	0.02	0.02	0.08	0.19	0.08	0.68	0.67	0.57	0.60	0.60	0.57	0.52	0.57	0.63	0.60	0.57	0.60	0.65	0.65	0.62	0.62	0.62	0.64	0.64	0.48	0.62	0.67	0.48	0.58	0.58	0.58	0.57	0.47	0.17	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.24	0.00	0.08	0.24	0.56	0.56	0.56	0.62	0.64
NR-60	0.20	0.27	0.27	0.20	0.29	0.20	0.56	0.54	0.50	0.48	0.48	0.50	0.41	0.42	0.52	0.48	0.42	0.48	0.58	0.58	0.62	0.62	0.62	0.64	0.64	0.42	0.56	0.60	0.48	0.46	0.46	0.46	0.44	0.52	0.50	0.24	0.24	0.24	0.24	0.24	0.24	0.24	0.00	0.21	0.18	0.25	0.43	0.43	0.43	0.48	0.64
LT-36	0.08	0.02	0.02	0.08	0.19	0.08	0.68	0.67	0.57	0.60	0.60	0.57	0.52	0.57	0.57	0.60	0.57	0.60	0.65	0.65	0.55	0.55	0.55	0.57	0.57	0.42	0.62	0.67	0.48	0.58	0.58	0.58	0.50	0.47	0.14	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.21	0.00	0.14	0.30	0.60	0.60	0.60	0.66	0.57
SK-1	0.05	0.11	0.11	0.05	0.16	0.05	0.56	0.60	0.50	0.48	0.48	0.50	0.41	0.47	0.57	0.48	0.47	0.48	0.65	0.65	0.70	0.70	0.70	0.71	0.71	0.55	0.62	0.60	0.48	0.52	0.52	0.52	0.44	0.38	0.27	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.18	0.14	0.00	0.19	0.47	0.47	0.47	0.52	0.71
SK-2	0.20	0.27	0.27	0.20	0.16	0.20	0.62	0.67	0.57	0.60	0.60	0.57	0.58	0.52	0.57	0.60	0.52	0.60	0.65	0.65	0.70	0.70	0.70	0.71	0.71	0.62	0.56	0.74	0.54	0.58	0.58	0.58	0.50	0.47	0.45	0.24	0.24	0.24	0.24	0.24	0.24	0.24	0.25	0.30	0.19	0.00	0.56	0.56	0.56	0.62	0.71
SO-1	0.50	0.62	0.62	0.50	0.63	0.50	0.20	0.48	0.29	0.03	0.03	0.29	0.06	0.29	0.26	0.03	0.29	0.03	0.31	0.31	0.36	0.36	0.36	0.29	0.29	0.42	0.35	0.25	0.25	0.41	0.41	0.41	0.13	0.22	0.68	0.56	0.56	0.56	0.56	0.56	0.56	0.56	0.43	0.60	0.47	0.56	0.00	0.00	0.00	0.03	0.29
LT-3	0.50	0.62	0.62	0.50	0.63	0.50	0.20	0.48	0.29	0.03	0.03	0.29	0.06	0.29	0.26	0.03	0.29	0.03	0.31	0.31	0.36	0.36	0.36	0.29	0.29	0.42	0.35	0.25	0.25	0.41	0.41	0.41	0.13	0.22	0.68	0.56	0.56	0.56	0.56	0.56	0.56	0.56	0.43	0.60	0.47	0.56	0.00	0.00	0.00	0.03	0.29
BO-15	0.50	0.62	0.62	0.50	0.63	0.50	0.20	0.48	0.29	0.03	0.03	0.29	0.06	0.29	0.26	0.03	0.29	0.03	0.31	0.31	0.36	0.36	0.36	0.29	0.29	0.42	0.35	0.25	0.25	0.41	0.41	0.41	0.13	0.22	0.68	0.56	0.56	0.56	0.56	0.56	0.56	0.56	0.43	0.60	0.47	0.56	0.00	0.00	0.00	0.03	0.29
SK-5	0.50	0.62	0.62	0.50	0.63	0.50	0.20	0.48	0.29	0.03	0.03	0.29	0.06	0.29	0.26	0.03	0.29	0.03	0.31	0.31	0.42	0.42	0.42	0.33	0.33	0.48	0.40	0.29	0.29	0.46	0.46	0.46	0.16	0.26	0.68	0.62	0.62	0.62	0.62	0.62	0.62	0.62	0.48	0.66	0.52	0.62	0.03	0.03	0.03	0.00	0.33
NE1-19	0.83	0.75	0.75	0.83	0.83	0.83	0.35	0.54	0.44	0.38	0.38	0.44	0.41	0.47	0.29	0.38	0.47	0.38	0.31	0.31	0.03	0.03	0.03	0.00	0.00	0.24	0.24	0.29	0.29	0.44	0.44	0.44	0.29	0.24	0.80	0.64	0.64	0.64	0.64	0.64	0.64	0.64	0.64	0.57	0.71	0.71	0.29	0.29	0.29	0.33	0.00

Presented in Figure 3.3 is the dendrogram of the representative isolates from the unique RFLP patterns which were obtained from the 16S rRNA gene. From this cluster, it is evident that some isolates indeed have different fragment patterns with each other but almost all were grouped under the different species of bradyrhizobia.

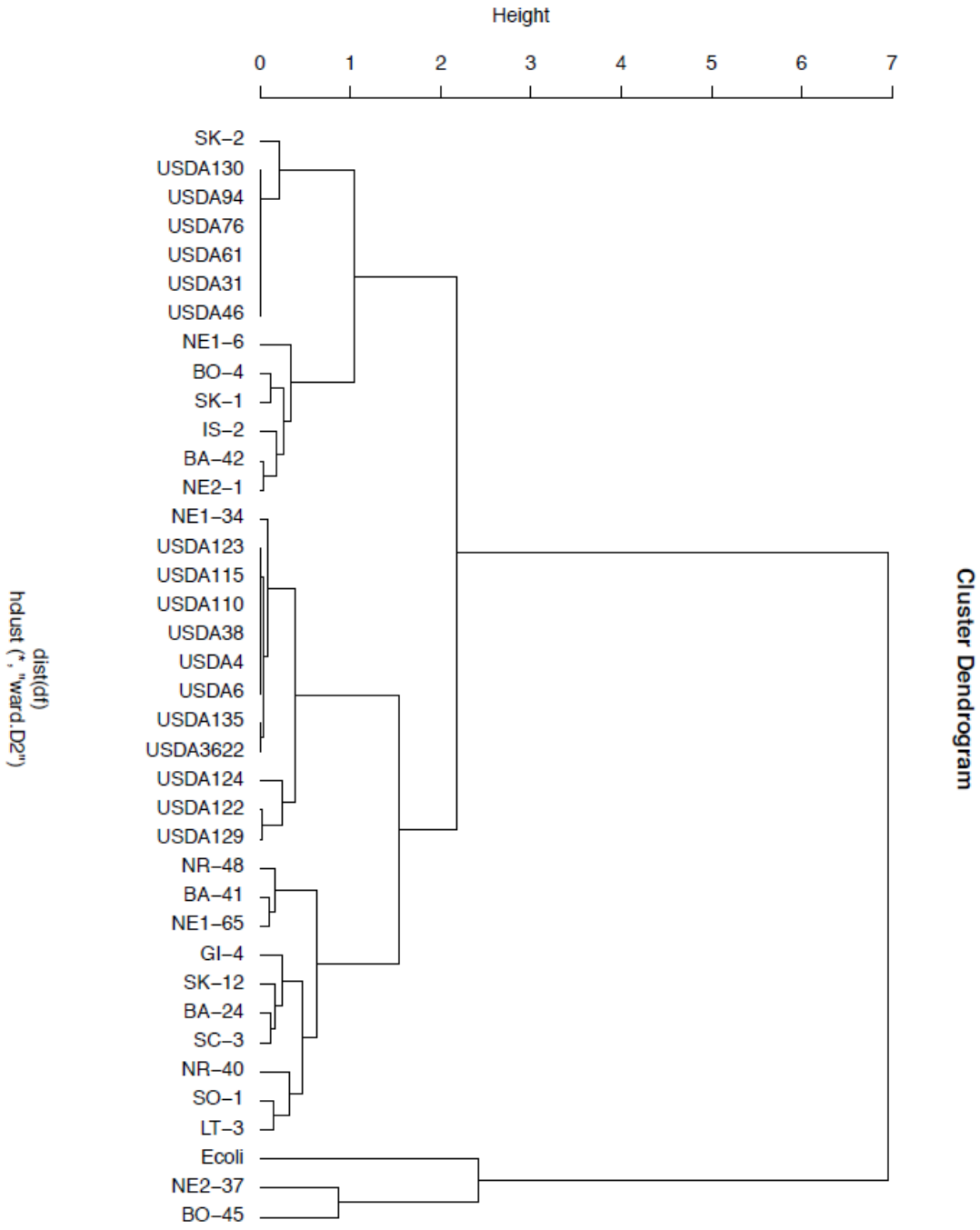

Figure 3.3. Dendrogram of the unique band patterns obtained from the RFLP analysis of the 16S rRNA gene for the representative isolates which can nodulate soybean. The names starting with USDA are the *Bradyrhizobium* reference strains used in this study. The figure was constructed with the ward.D2 method of the R software v.3.4.0.

Then, the unique RFLP patterns obtained from the 16S-23S rRNA gene ITS region of the representative isolates is shown in Figure 3.4. In this dendrogram, the three species which are *B. japonicum*, *B. diazoefficiens* and *B. elkanii* are clearly separated.

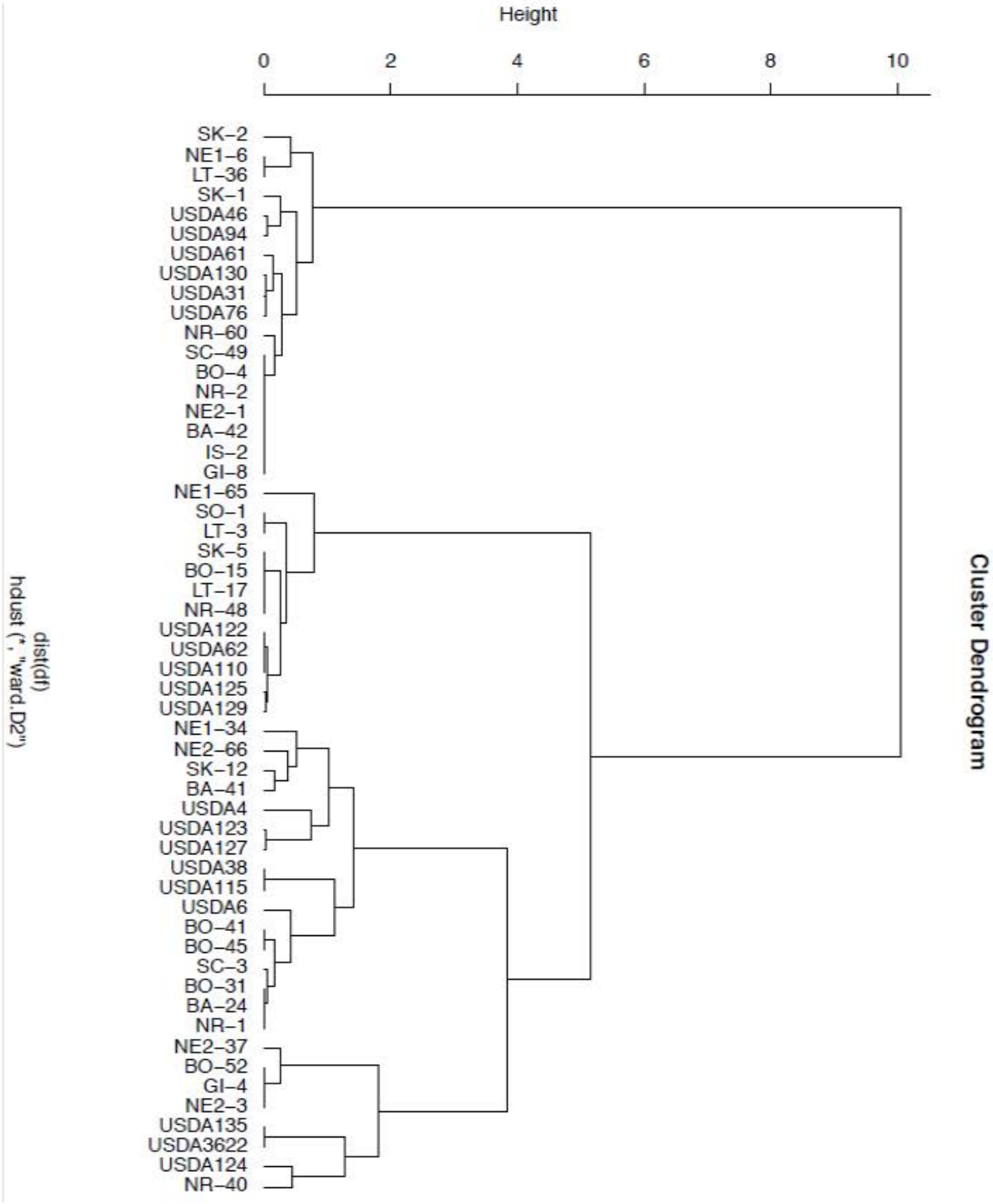

Figure 3.4. Dendrogram of the unique band patterns obtained from the RFLP analysis of the 16S-23S rRNA gene ITS region for the representative isolates which can nodulate

soybean. The names starting with USDA are the *Bradyrhizobium* reference strains used in this study. The figure was constructed with the ward.D2 method of the R software v.3.4.0.

Meanwhile, presented in the figure below (Fig. 3.5) is the cluster dendrogram that was constructed from the distinct RFLP patterns from the *rpoB* housekeeping gene of the soybean-nodulating bradyrhizobia. In this figure, more distinctions between the strains were seen such between the USDA76 and USDA 46.

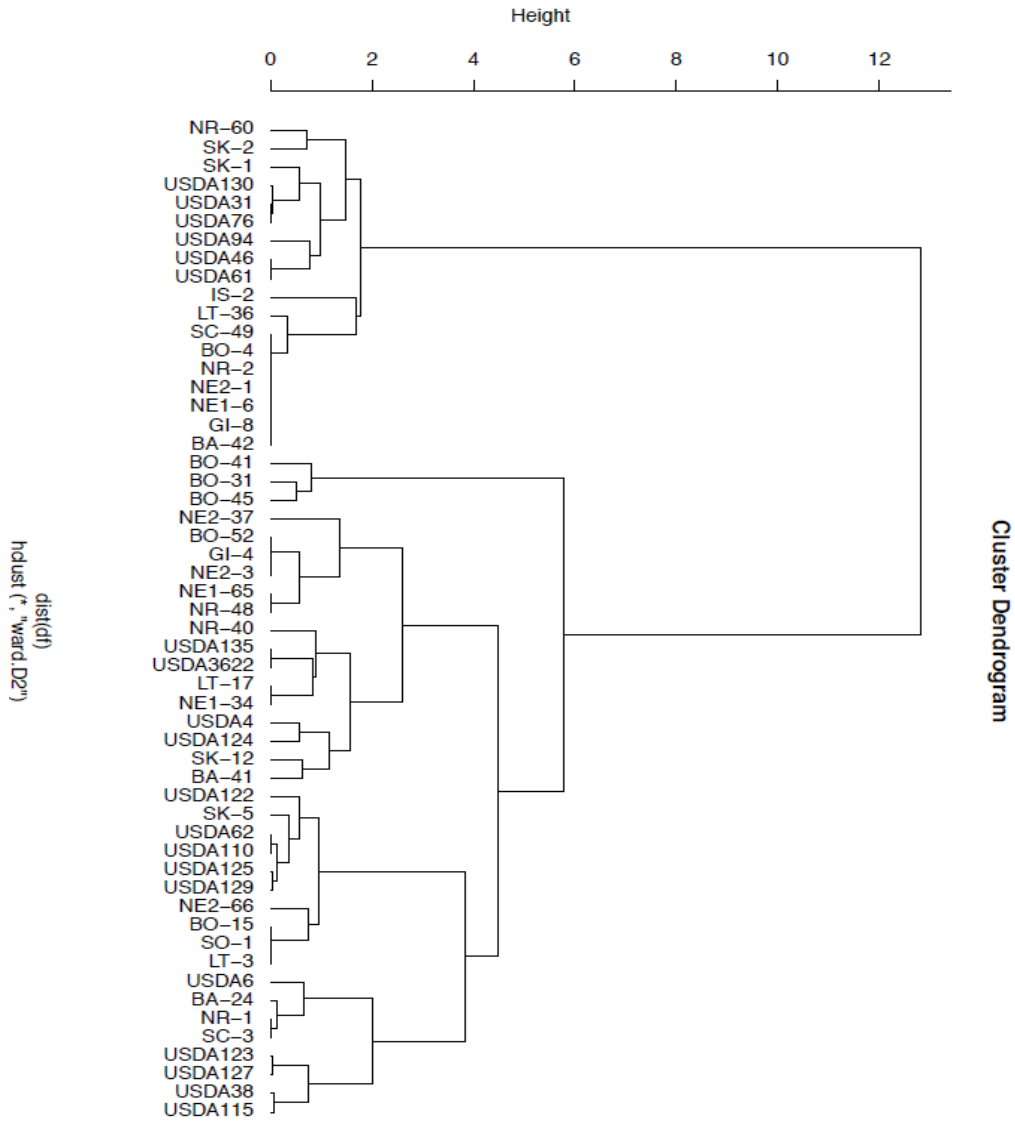

Figure 3.5. Dendrogram of the unique band patterns obtained from the RFLP analysis of the *rpoB* housekeeping gene for the representative isolates which can nodulate soybean. The names starting with USDA are the *Bradyrhizobium* reference strains used in this study. The figure was constructed with the ward.D2 method of the R software v.3.4.0.

Then, on figures 3.9, 3.10 and 3.11, the distinct fragment sizes obtained from the digestion with restriction enzymes of the 16S rRNA gene, 16S-23S rRNA ITS region and *rpoB* gene are presented. It is noticeable that some isolates have unique patterns that are indication of their nucleotide divergence.

3. Sequence analysis of the 16S rRNA, ITS region and *rpoB* gene

Presented in Figure 3.6 is the phylogenetic tree from the sequence analysis of the ITS region showing the genetic diversity of soybean bradyrhizobia across the country. The isolates were predominantly grouped under four bradyrhizobia species which are *B. elkanii* (37.74%), *B. diazoefficiens* (29.48%), *B. japonicum* (16.51%), and *B. yuanmingense* (0.94%). A *Bradyrhizobium* sp. clade that makes up about 15.09% of the population was also observed including two independent single isolates NE1-34 and NE2-66. On the other hand, almost similar clusters were observed from the *rpoB* gene phylogenetic tree (Fig. 3.3) wherein eight delineations were also distinguished similarly with the ITS region. Notably, some genetic variations were detected only on specific isolates between the two gene loci. For example, SK-1 belonged to Be46 cluster in the ITS region but it was reclassified into Be130 cluster in the *rpoB* gene. This observation mainly occurred with the isolates clustered under *B. elkanii* strains except for those clustered with the Be94. On the other hand, no remarkable genetic diversity was observed for the other clusters particularly for those under *B. diazoefficiens* and *B. yuanmingense*.

Meanwhile, the phylogenetic tree from the sequence analysis of the 16S rRNA gene is presented in Fig. 3.4. It is clear that all the 424 isolates considered in this study belong to the different species of *Bradyrhizobium* and were delineated with that of

Sinorhizobium fredii. For clarity, the 16S rRNA gene nucleotide sequence of the *Escherichia coli* was used as the outgroup. The sequence alignment of the indigenous bradyrhizobia are presented in the appendix.

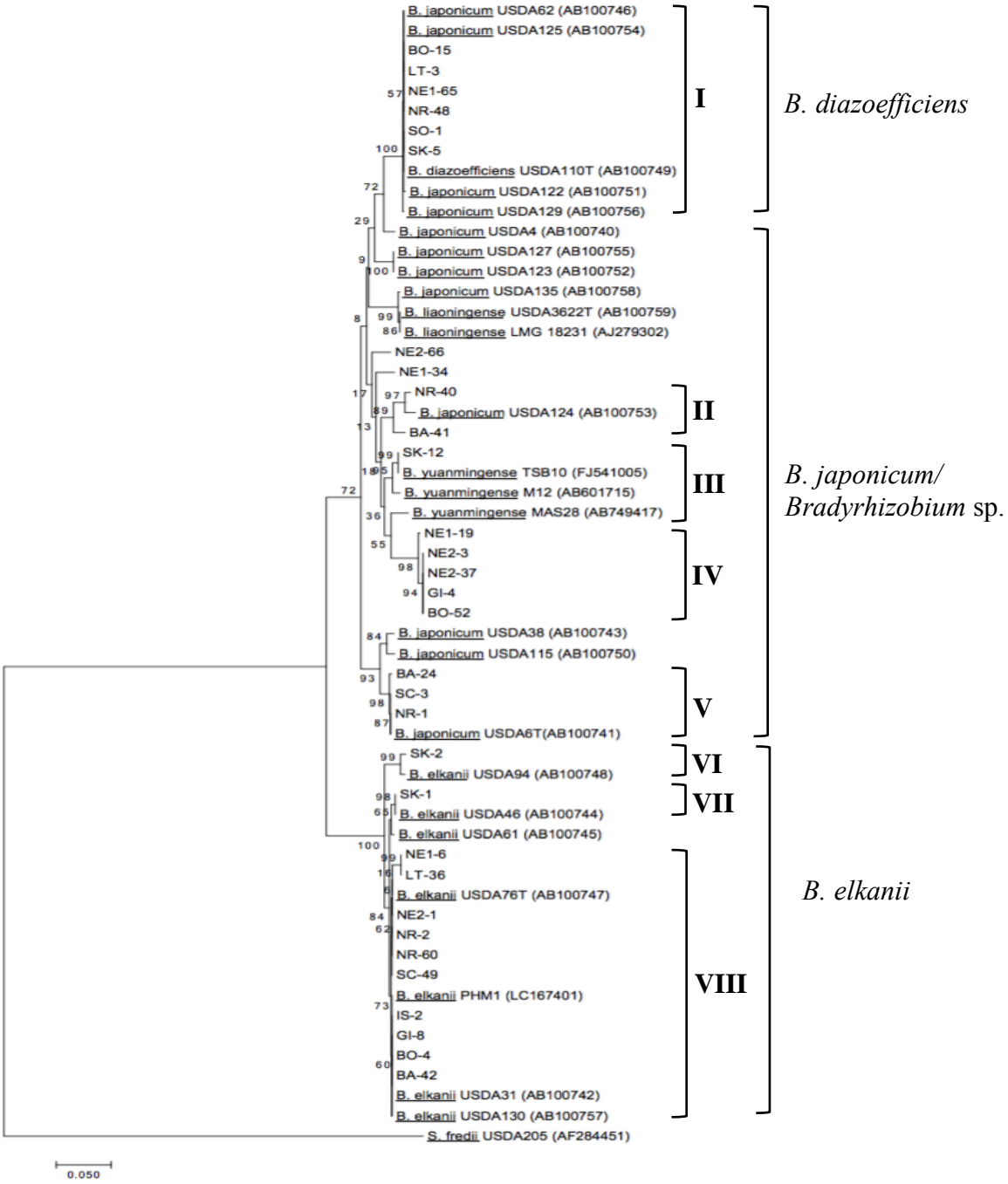

Figure 3.6. Phylogenetic tree based on sequence analysis of the 16S – 23S rRNA gene ITS region. The tree was constructed using the Neighbor-Joining method with the Kimura 2-parameter distance correlation model and 1000 bootstrap replications in MEGA v.7 software. The accession numbers are indicated only for sequences obtained from BLAST. The isolates in this study are indicated with letters and number combinations, for example: **IS-2** – isolate no. 2 collected from Ilagan, Isabela.

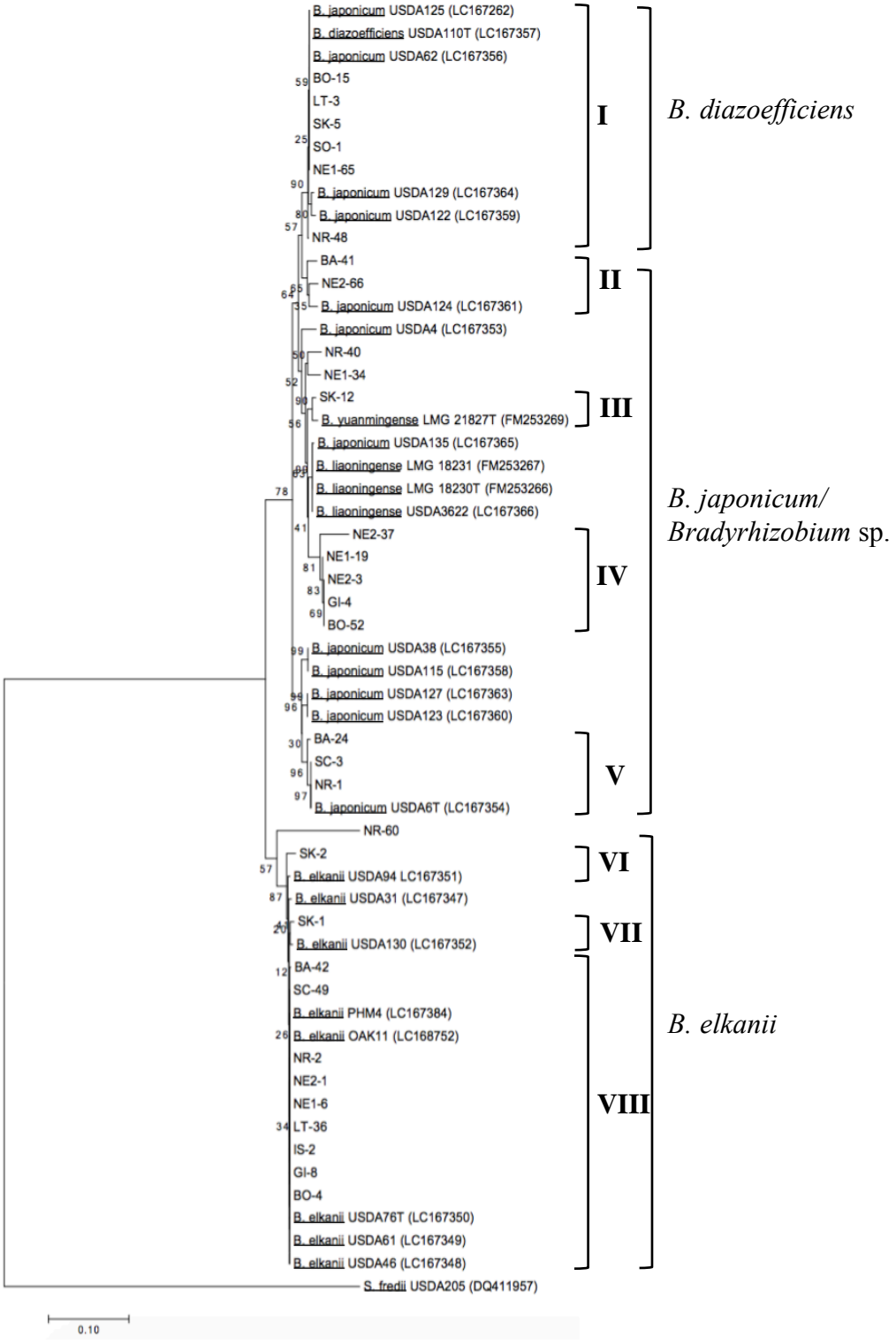

Figure 3.7. Phylogenetic tree based on sequence analysis of the *rpoB* housekeeping gene. The tree was constructed using the Neighbor-Joining method with the Kimura 2-parameter distance correlation model and 1000 bootstrap replications in MEGA v.7 software. The accession numbers are indicated only for sequences obtained from BLAST. The isolates in this study are indicated with letters and number combinations, for example: **IS-2** – isolate no. 2 collected from Ilagan, Isabela.

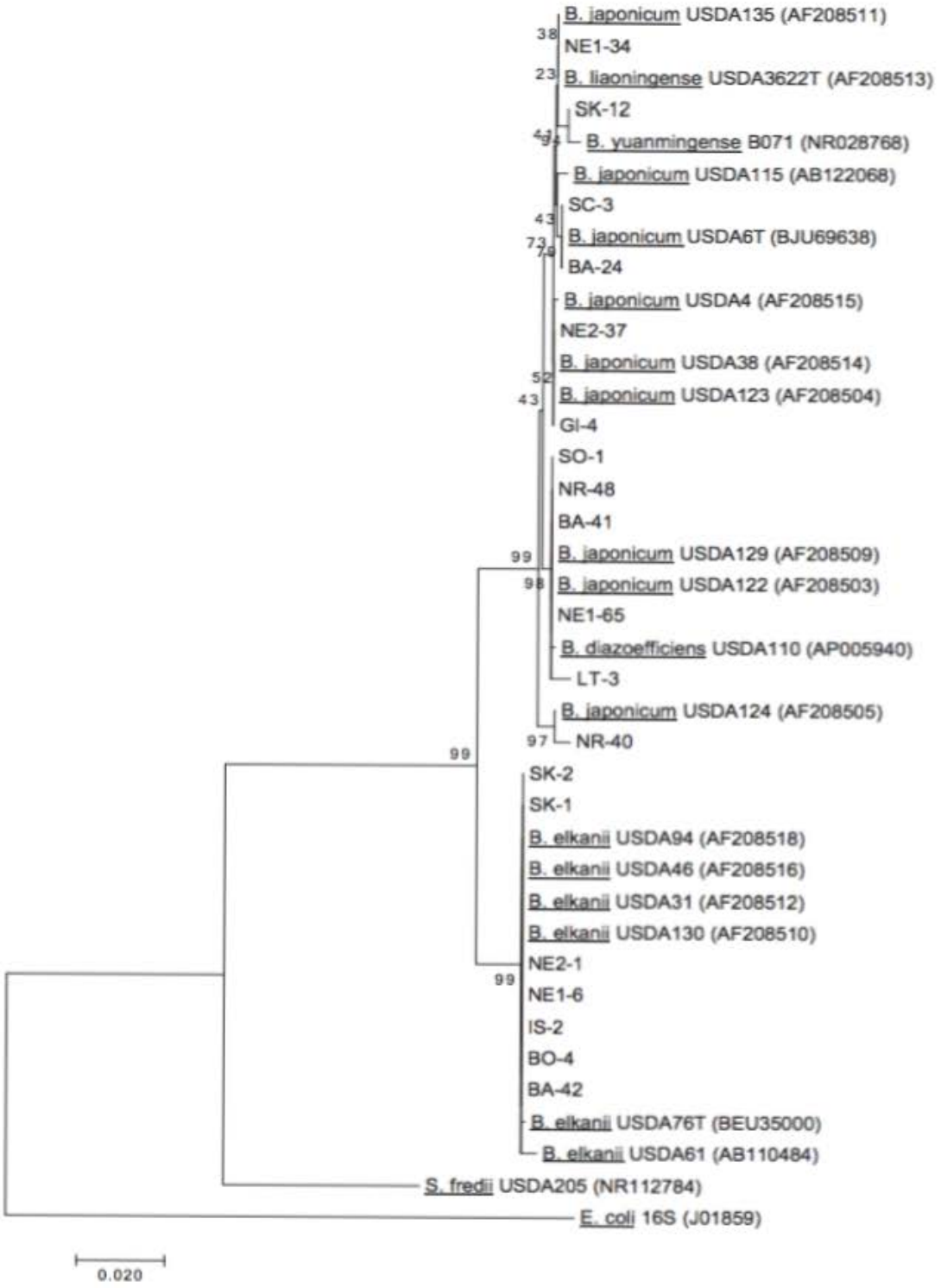

Figure 3.8. Phylogenetic tree based on sequence analysis of 16S rRNA gene. The tree was constructed using the Neighbor-Joining method with the Kimura 2-parameter (K2P) distance correlation model and 1000 bootstrap replications in MEGA v.7 software. The accession numbers are indicated only for sequences obtained from BLAST. The isolates in this study are indicated with letters and number combinations, for example: IS-2 – isolate no. 2 collected from Ilagan, Isabela.

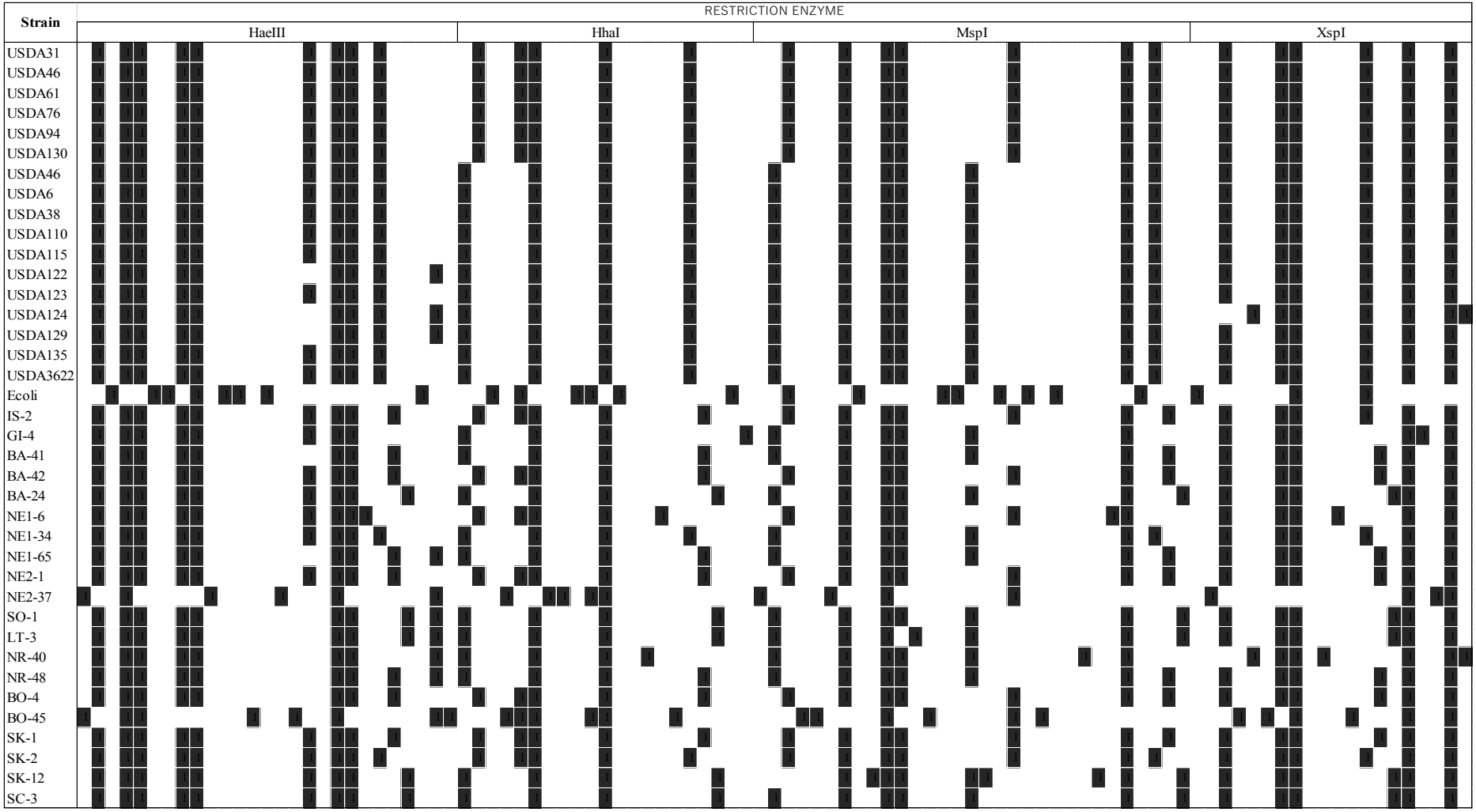

Figure 3.9. Schematic representation of the difference in the fragment sizes from the four restriction enzymes. This was obtained from the 16S rRNA gene sequence analysis and was determined by using the software Genetyx for mac v. 16.

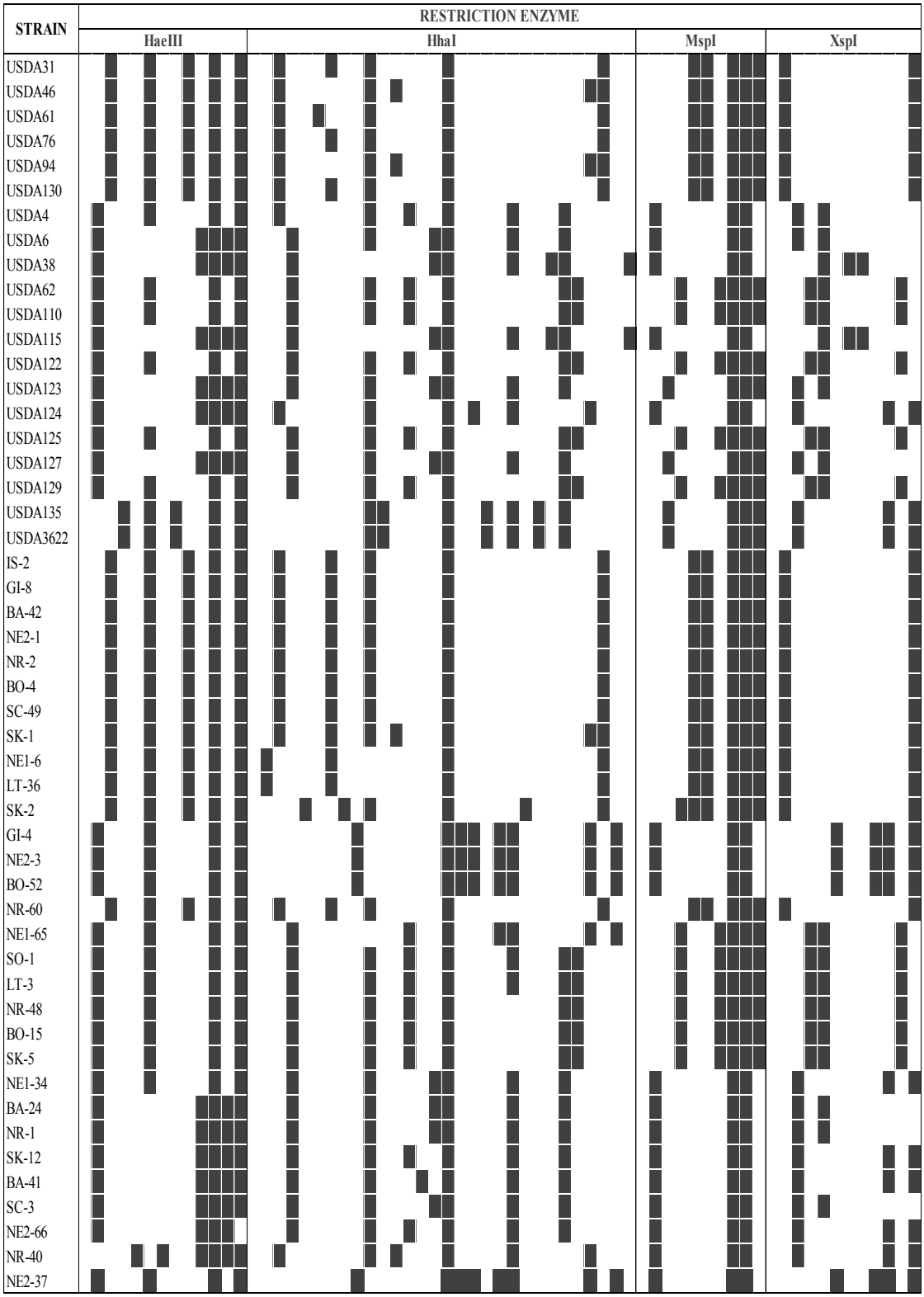

Figure 3.10. Schematic representation of the difference in the fragment sizes from the four restriction enzymes. This was obtained from the 16S-23S rRNA gene ITS region sequence analysis and was determined by using the software Genetyx for mac v. 16.

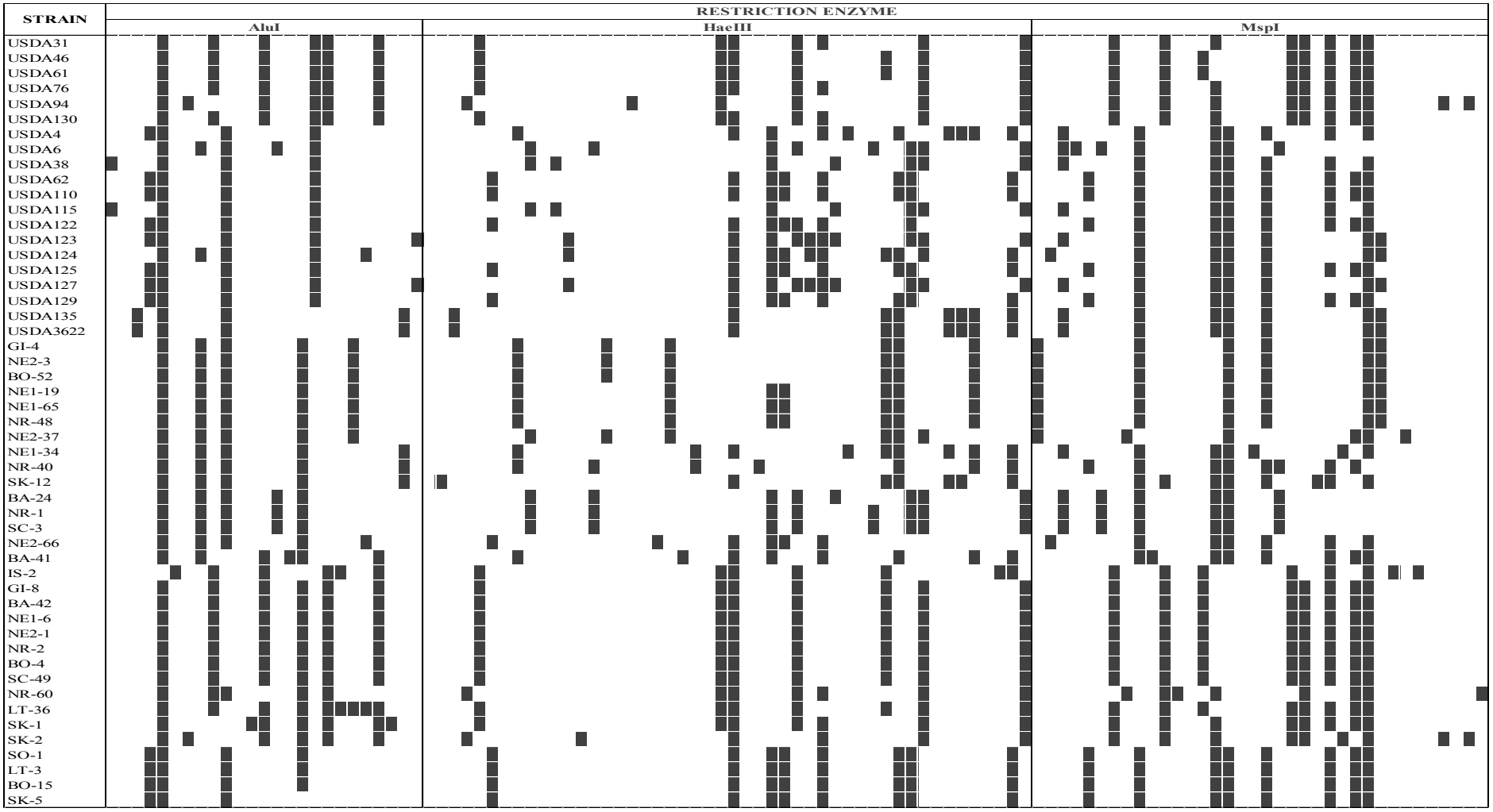

Figure 3.11. Schematic representation of the difference in the fragment sizes from the four restriction enzymes. This was obtained from the *rpoB* housekeeping gene sequence analysis and was determined by using the software Genetyx for mac v. 16.

4. Distribution of isolates and diversity analysis

The distribution of the isolates in the locations and the diversity indices of soybean bradyrhizobia in the Philippines are presented in Table 3.1 and are graphically seen in Figure 3.12. Those isolates that were clustered under the *B. elkanii* strains were present in almost all the locations, suggesting a prevalence of this species in the regions with high temperature. The highest diversity and equitability indices were obtained from Sultan Kudarat (SK) (*H'*=0.98, *Eh*=0.71), followed by Negros Occidental (NR) (*H'*=0.82, *Eh*=0.59). On the other hand, the lowest indices (*H'*=0.00, *Eh*=0.00) were obtained from Isabela (IS) and Sorsogon (SO) where all isolates belonged to *B. elkanii* and *B. diazoefficiens*, respectively.

Table 3.10. Population distribution and diversity indices of the indigenous bradyrhizobia in the Philippines. The Shannon's diversity (*H'*) and equitability (*Eh*) indices were computed with the formulae ($H' = -\sum Pi \ln Pi$; $Eh = H' / \ln S$).

Cluster/ Location	IS	GI	BA	NE1	NE2	SO	LT	NR	BO	SK	SC
Be31	40	6	1	0	0	0	0	0	24	0	0
Be46	0	0	0	0	0	0	0	0	0	4	0
Be76	0	0	0	49	3	0	1	23	0	0	3
Be94	0	0	0	0	0	0	0	0	0	6	0
Bj6	0	0	31	0	0	0	0	4	0	0	31
Bj124	0	0	1	0	0	0	0	3	0	0	0
Bd110	0	0	0	5	0	44	42	1	4	29	0
By	0	0	0	0	0	0	0	0	0	4	0
Bradyrhizobium sp.	0	30	0	1	31	0	0	0	1	0	0
Independent bradyrhizobia	0	0	0	1	1	0	0	0	0	0	0
Total	**40**	**36**	**33**	**56**	**35**	**44**	**43**	**31**	**29**	**43**	**34**
H'	**0.00**	**0.45**	**0.27**	**0.48**	**0.42**	**0.00**	**0.11**	**0.82**	**0.55**	**0.98**	**0.30**
Eh	**0.00**	**0.65**	**0.25**	**0.34**	**0.38**	**0.00**	**0.16**	**0.59**	**0.50**	**0.71**	**0.43**

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

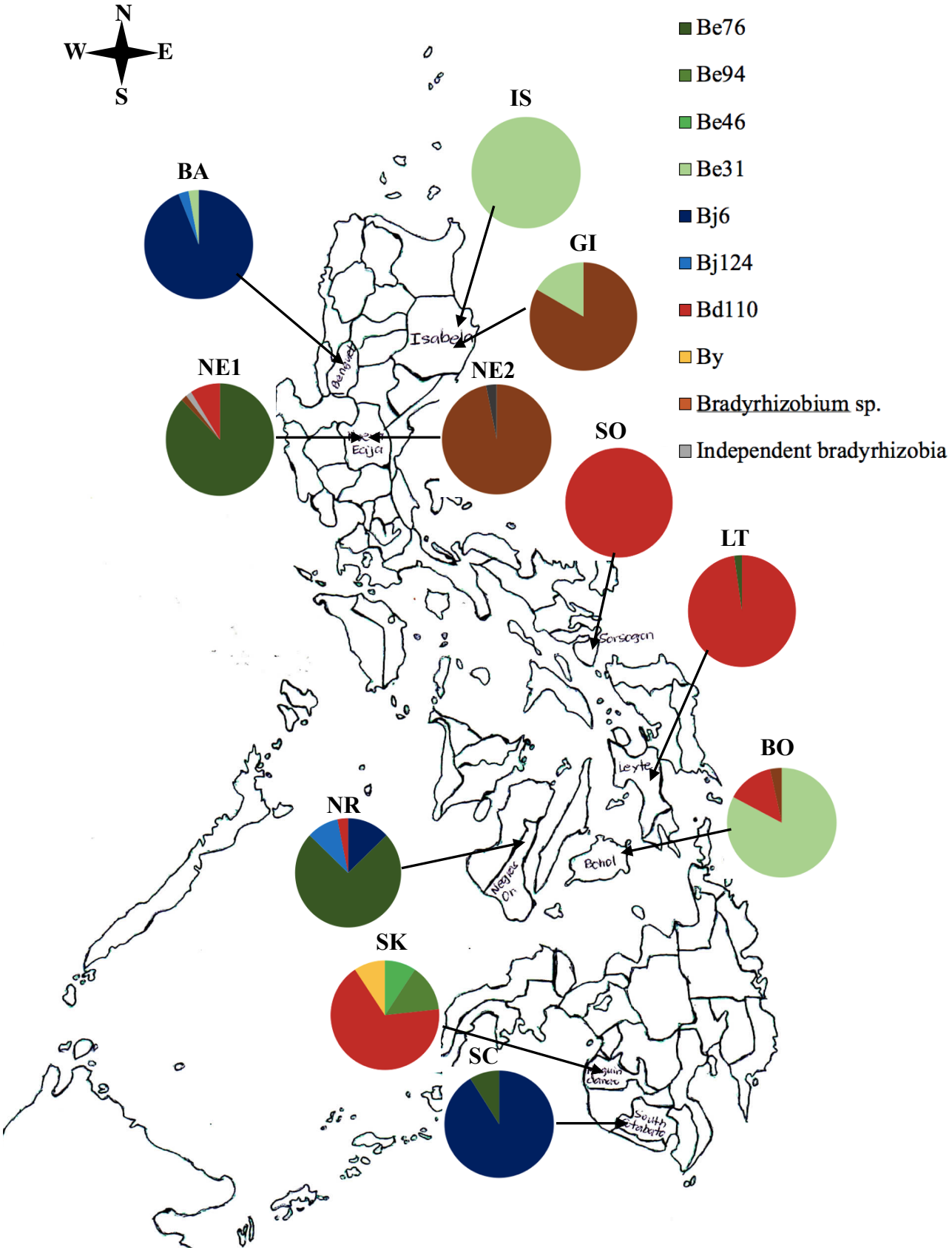

Figure 3.12. Geographic distribution of the soybean-nodulating bradyrhizobia in the Philippines from the result of Restriction Fragment Length Polymorphism (RFLP) and sequence analysis of the 16S-23S rRNA gene ITS region. The detailed name of each location is listed in Table 3.1.

Meanwhile, the MDS plots clearly indicate the community structure and population dominance of each *Bradyrhizobium* species in accordance with their clusters and respective location (Fig. 3.13 and 3.14). The different clusters of *Bradyrhizobium* sp. obtained from in the phylogenetic trees of the ITS region and *rpo*B gene are clustered together in the plots (GI, NE2). Conversely, SO, LT and SK were grouped together because these locations are dominated by those clustered to *B. diazoefficiens*. Similar observations were obtained for the locations dominated by *B. elkanii* (IS, BO, NR, NE1) and *B. japonicum* USDA6 (BA, SC).

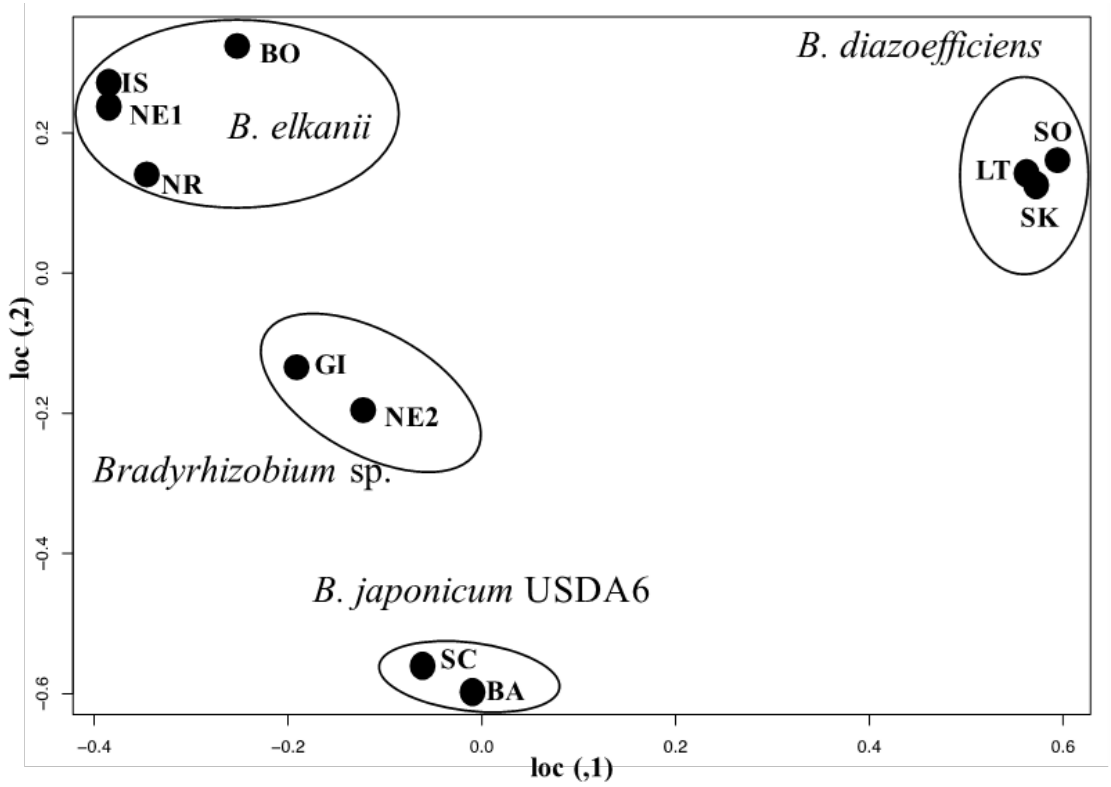

Figure 3.13. Community structure of indigenous soybean-nodulating bradyrhizobia from the 16S-23S internal transcribed spacer (ITS) region in the Philippines showing the dominance of each *Bradyrhizobium* species at respective location. The figure was constructed using Bray-Curtis index through R software v.3.4.0.

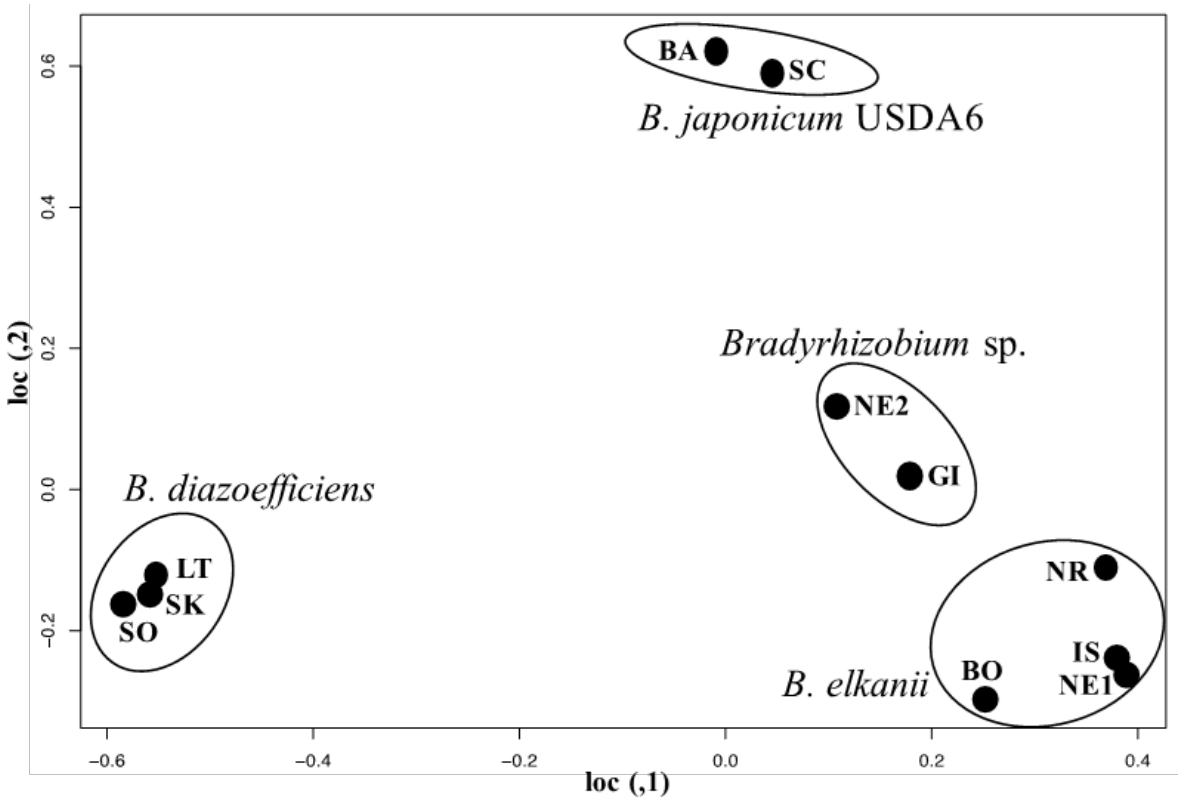

Figure 3.14. Community structure of indigenous soybean-nodulating bradyrhizobia from the *rpoB* housekeeping gene in the Philippines showing the dominance of each *Bradyrhizobium* species at respective location. The figure was constructed using Bray-Curtis index through R software v.3.4.0.

5. Correlation analysis among the factors that affect the diversity and distribution of bradyrhizobia

In Figure 3.15, the PCA plot showed the relationship between the agro-environmental factors and the dominance of each indigenous bradyrhizobia species in the country. The PC1 generated 47.74% proportion that is accounted for most of the variance and indicated the correlation between the parameters considered and the distribution of bradyrhizobia in the specific locations.

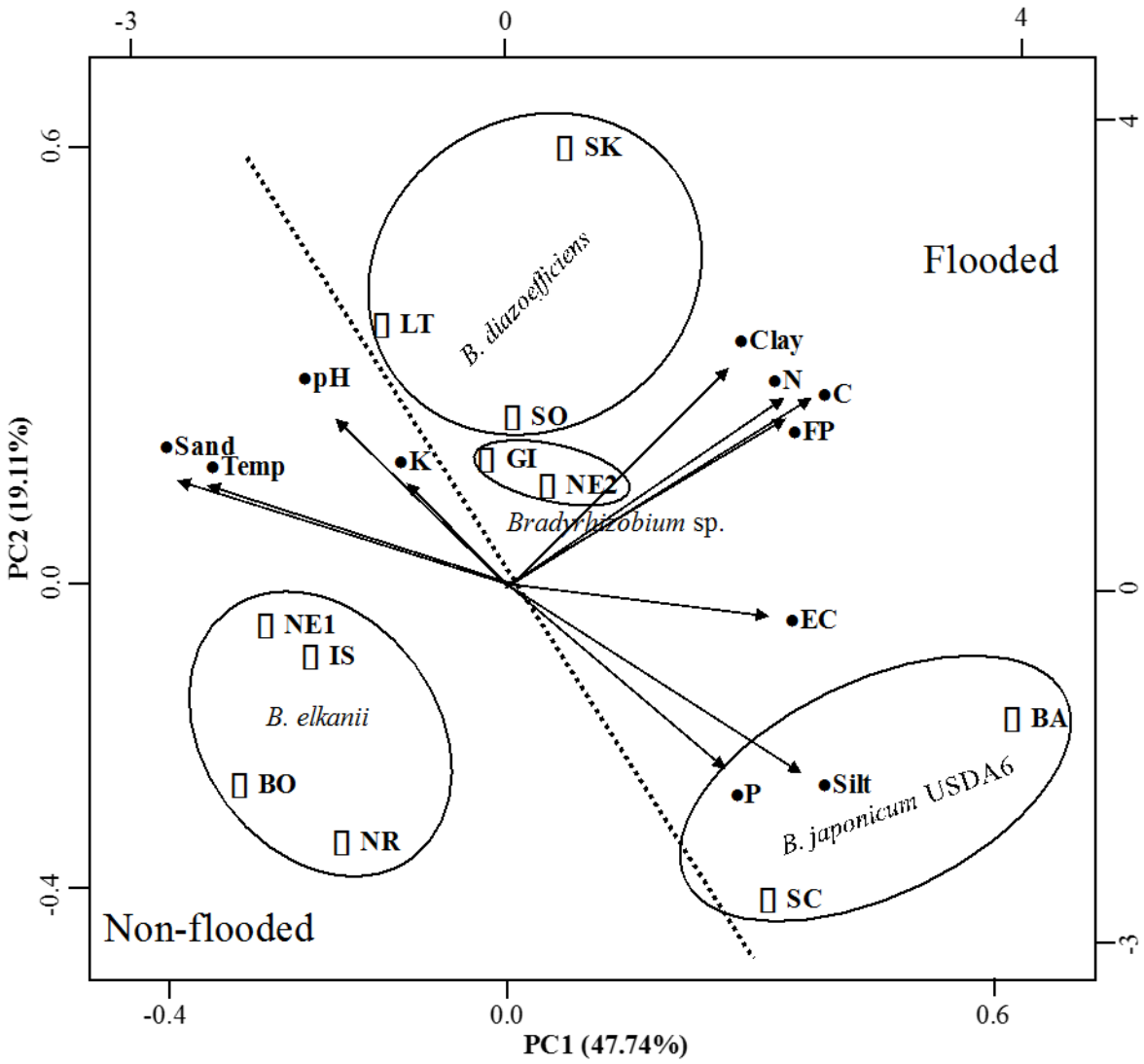

Figure 3.15. Principal Component Analysis (PCA) plot depicting the relationship between the dominance of *Bradyrhizobium* species in the respective locations and the agro-environmental factors considered in this study. • Blackened circle indicates the agro-environmental factors. □Hollow rectangle indicates the location of the soil sampling collection. FP-period of flooding. Dotted straight line indicates the separation between the flooded and non-flooded condition.

The isolates that are clustered under the *Bradyrhizobium* sp. and Bd110 seemed to be dominant in specific locations and can be attributed to the influence of flooding period (FP), N, C, pH and clay content as shown by the arrows whereas the dominance of the isolates that are clustered under the Bj6 can be attributed to the influence of high silt and P content in the soil. Meanwhile, the abundance and distribution of the isolates

under the *B. elkanii* clusters is generally correlated with the high temperature in the country and possibly with the higher amount of sand. Since there were only a few isolates which were identified to be related with *B. yuanmingense*, a definite correlation between these isolates and the agro-environmental parameters could not be made generally.

Discussion

1. Genetic diversity of indigenous bradyrhizobia

The high species diversity of the indigenous bradyrhizobia as revealed from the Shannon's index (*H'* – 0.98) was observed and additionally, the genomic variations between the ITS region and the *rpoB* gene showed a high genetic diversity. This is not a new observation as a similar result was reported previously (Mason et al., 2017) where intra-genomic variations in the clusters of Be76 and Be46 were detected and were attributed to the temperature and pH gradient. Although our results alone could not properly explain the specific reason for this phenomenon, we hypothesize that a gene transfer or gene shuffling might have occurred for the isolates which are classified under the Be76, Be46, and Be31 clusters. From the complete genome map of the *B. elkanii* USDA76^{T} with a genome size of 9,484,767bp, the *rpoB* gene (6,539,500bp) is located on almost opposite locus with the ITS region (411,600 – 410,900bp) as reported previously (Reeve et al., 2017) so it may easily reflect genetic changes that occurred within the species, or even within the strain.

At large, the genomic variations between the two genetic loci on *B. elkanii* clusters are helpful for the identification of tropical bradyrhizobia and merit further research to explain this event.

2. Factors that influence the diversity and distribution of bradyrhizobia

The several agro-environmental factors were considered in order to investigate which parameter/s could impact the distribution and genetic diversity of the soybean-nodulating bradyrhizobia in the Philippines. The positive influence of high Phosphorus content on the abundance of *B. japonicum* sp., which were phylogenetically clustered with Bj6 was also observed by another report (Yan et al., 2014). Another study affirmed that the Andisols in Japan were dominated by the isolates that are clustered to Bj6 (Shiina et al., 2014). This is similar to the soils of South Cotabato (SC) which are classified as Andisols with mixed alluvium and sedimentary deposits where dominant isolates of the Bj6 cluster were observed herein. Since the *B. japonicum* USDA6^T was reported to release N_2O (Sameshima-Saito et al., 2006), which is one of the greenhouse gases, its significance for both agriculture and environment merits more attention for better and thorough understanding.

For the distribution of Bd110 cluster in flooded soils, this result is supported by our previous reports where the dominance of the *B. diazoefficiens* USDA110^T was enhanced by flooding condition in the soil (Saeki et al., 2017) and that the USDA110 cluster is dominant on fine-textured soil that are affected by water status and oxidation-reduction potential in the soil (Saeki and Shiro, 2014). Moreover, it was reported that the anaerobic condition in the flooded soils of an alluvial origin resulted in the dominance of Bd110 cluster (Shiina et al., 2014). The isolates which are clustered under Bd110 were found in areas which are usually planted with rice during the wet season then, planted with rice and/or legume during the dry season. In both seasons, the rice plant is always cultivated under a waterlogged status. This provided an interesting vision especially for the Philippines' agriculture since the strain *B. diazoefficiens* USDA110^T was proven to be a highly effective and an efficient inoculant for soybean

(Siqueira et al., 2014; Liu et al., 2017) with a complete denitrification ability (Itakura et al., 2013; Shiina et al., 2014; Akiyama et al., 2016) that is beneficial for mitigating the effects of climate change.

A different species of bradyrhizobia dominated each location in Nueva Ecija (NE), wherein the locations were almost similar in all aspect except for the soil water status. The NE1 isolates which were dominated by *B. elkanii* were collected from the non-flooded condition while the NE2 isolates which were dominated by the *Bradyrhizobium* sp. were collected from the flooded condition. These results implied that soil management could alter the population dominance of certain species of bradyrhizobia. As stated by an earlier report, the diversity and abundance of *Bradyrhizobium* species were altered by cultural management and other soil-related properties (Yan et al., 2014). These strains might be novel species in the Philippines since the stated locations have no history of rhizobia inoculation and we did not obtain any highly similar sequences from the BLAST engine for its identification.

The abundance and widespread distribution of the isolates that belong to the *B. elkanii* clusters in the Philippines is consistent with some previous findings wherein this species was distributed in areas with a slight to moderate acidity and sub-tropical to tropical region (Saeki et al., 2006; Adhikari et al., 2012; Shiro et al., 2013; Mason et al., 2017). Upon consideration, it could be one of the reasons for the low soybean production across the country. It was reported that the *B. elkanii* provides lower N fixation and symbiotic efficiency in comparison with *B. diazoefficiens* USDA110^{T} (Risal et al., 2010). Also, the *B. elkanii* species produce NO_2^-, and it is known that the interaction of nitrites to some soil components are related to some environmental concerns. Thus, these species of bradyrhizobia are important research specimens for its role in agriculture and environment.

With regards to our objectives, the following ideas are suggested: (a) the major micro-symbionts of soybean in the Philippines are bradyrhizobia under the clusters of *B. elkanii*, Bd110, Bj6 and *Bradyrhizobium* sp., (b) the prevalence of bradyrhizobia in the country are classified accordingly: *B. elkanii* clusters for non-flooded soil with high temperature, Bd110 cluster for fine-textured flooded soils, and Bj6 cluster for flooded soils that are high in phosphorus and silt content, (c) the isolated *Bradyrhizobium* sp. strains are endemic and might be potential novel species, and (d) the distribution and genetic diversity of bradyrhizobia in the Philippines was mainly influenced by the period of flooding and other soil properties such pH, soil type and nutrient content.

Lastly, this study was able to identify the indigenous and potentially new endemic strains of bradyrhizobia, and understood the agro-environment conditions wherein they are abundant and dominant. This information would be helpful for crafting inoculation strategies that are location-specific. Further studies on the characteristics, possession of denitrification genes, and symbiotic relationship of the indigenous bradyrhizobia with the different soybean cultivars that are already adapted to the local conditions will be helpful to test its potential as a useful soybean inoculant. It is also recommended that the results generated from this research would be conducted in field trials as to test its efficacy in natural soil and environment conditions. It is understood in through this study that the ecology of bradyrhizobia is complicated, particularly for a tropical region; and it depends on several abiotic and biotic factors.

Summary

Inoculation is one of the strategies that is commonly used in the Philippines to improve the production of soybean. However, this technique often fails mainly due to the lack of information about the indigenous soybean rhizobia in the Philippines soil.

In this report, the diversity of indigenous bradyrhizobia collected from the non-flooded and flooded soil conditions at 11 locations in the country was investigated using a local soybean cultivar as the host plant. The Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) treatment and sequence analysis for the 16S rRNA gene, 16S-23S rRNA internal transcribed spacer (ITS) region and *rpoB* housekeeping gene of the 424 isolates detected the intra-genomic variations on specific species of bradyrhizobia. A majority of the isolates were classified under the four species of *Bradyrhizobium* namely: *B. elkanii*, *B. diazoefficiens*, *B. japonicum*, *B. yuanmingense* and a considerable percentage of the isolates were clustered under *Bradyrhizobium* sp. The isolates which were classified under the *Bradyrhizobium* sp. are considered endemic to Philippines soil as evidenced by their nucleotide divergence against the known rhizobia and the historical absence of rhizobia inoculation at the study areas. The distribution and diversity of soybean bradyrhizobia across the country is mainly attributed to the difference in the flooding period, followed by other soil properties such as pH, soil type, and nutrient content. So, it is suggested that the major micro-symbiont of soybean in the Philippines are *B. elkanii* for non-flooded soils, then *B. diazoefficiens* and *B. japonicum* for flooded soils.

Chapter 4

Symbiotic performance of indigenous soybean bradyrhizobia from the Philippines with various soybean (*Glycine max* [L.] Merill) cultivars harboring different *Rj* genotypes

Introduction

The Philippines is a tropical archipelago located in the Southeast Asia with a land area of about 298 km^2. From the approximately 7M hectares used for agriculture, only about 1,000 ha. are allocated to soybean. This is due to the low yield (≤1.0 ton/ha) of soybean and also low government support to soybean planters. Although the Government's program entitled "Philippine Soybean Development Program" launched in 2011 was able to increase the production area from ≈1,000 ha. to a record high of 5,280 has., soybean yield is still low at about 1.0 ton/ha. Thus, projects and studies on how to increase soybean yield in a sustainable manner prompted researchers to venture into development of plant varieties with high-yielding ability. However, breeding of high-yielding soybean varieties alone does not guarantee that it would attain its optimal yield. Other factors such as soil management and cultural practices has to be improved too.

Soybean (*Gycine max* [L.] Merill) can establish a symbiotic relationship with rhizobia, a general term for diazotrophic bacteria in the soil that are able to convert the atmospheric N into ammonia and renders it available for the plant's growth and development. In turn, the plant provides food for the rhizobia from the product of photosynthesis. Nitrogen fixation takes place inside the root nodule, a specialized organ that was formed from the infection of the rhizobia. An efficient and effective symbiotic relationship between the plant (soybean) and the bacteria (rhizobia) could lessen the chemical inputs, particularly the nitrogenous fertilizers that are applied to the crop or to the succeeding crop. It is a proven knowledge that the soils with low N content respond better with rhizobia inoculation so, this technique is very helpful for soil restoration activities. If this technique is properly utilized, farmers will have better

profit from buying less chemical fertilizers and lesser soil degradation from using chemicals.

Previous studies reported that aside from various agro-environmental factors, competition with the native rhizobia is a hindrance for successful inoculation (Yamakawa and Saeki, 2013; Grönemeyer et al., 2014). Therefore, it is essential to select and evaluate the symbiotic competitiveness of the indigenous strains which are native and existing in high density.

In soybean, there are genetic loci which are known as *Rj*(s) or *rj*(s) that were reported to restrict the nodulation between a certain rhizobia strains and legume plants. A non-nodulating line of soybean was first identified by Williams and Lynch (1954) and reported that it was due to a single recessive gene in the host plant, rj_1. Thereafter, the discovery of dominant *Rj* genes allowed the nodulation between certain rhizobia as summarized by Hayashi et al. (2012). The recessive *rj* genes (rj_1, rj_5, rj_6, rj_7) restrict the nodulation with all strains while the dominant *Rj* genes (Rfg_1, Rj_2, Rj_3, Rj_4) restrict the nodulation only with certain strains. In one study about soybean preference for nodulation using the *B. diazoefficiens* USDA110, it was observed that the $Rj_2Rj_3Rj_4$ line of soybean were superior than the other *Rj* lines in relation to the inoculation with a type A strain (Yamakawa et al., 2003).

In the Philippines, the first study about the diversity of soybean bradyrhizobia was reported by Mason et al. (2017) where the author compared the dominance of *B. elkanii* strains found among Kumamoto and Okinawa in Southern Japan and in Nueva Ecija, Philippines. It was then stated that the difference in temperature and similarity in soil pH were the driving force for the presence of the particular strains in those three locations. Additionally, Mason et al. (2018) reported the pioneer work about the diversity and distribution of indigenous bradyrhizobia collected from the northern to

the southern areas in the Philippines. This report was able to identify the strains of bradyrhizobia that are dominant and abundant in specific locations and at the same time, identified the agro-environment conditions that influenced the prevalence of certain species. In the latter report, indigenous strains clustered under the *B. elkanii* were identified to be dominant in the non-flooded soils whereas *B. japonicum* and *B. diazoefficiens* were prevalent in flooded soils of the country. Many strains of *B. elkanii* were reported to be relatively inefficient microsymbionts of soybean and can induce chlorosis in soybean plants (Devine et al. 1988), and they lack *nos*Z gene (Sameshima-Saito et al., 2006) that encodes the N_2O reductase responsible for reducing N_2O into N_2. Meanwhile, the strain *B. diazoeffic*iens USDA110 is globally used in soybean research as it has a high symbiotic efficiency and nitrogen fixation ability (Soe et al., 2012; Chibeba et al., 2017), as well as it possess a complete denitrification ability that allows the release of N_2 in the atmosphere as the end product (Sameshima-Saito et al., 2006; Itakura et al., 2013; Shiina et al., 2014; Akiyama et al., 2016), rather than N_2O which is the end product of certain *B. japonicum* strains (Sameshima-Saito et al., 2006). This led us to hypothesize that the *B. diazoefficiens* indigenous strains from the Philippines might be useful as potential inoculant in the country to increase the yield of soybean rather than the other *Bradyrhizobium* species. Consequently, the present study aimed to confirm this by conducting a single-strain inoculation test on tropical and temperate soybean cultivars harboring various *Rj* genotypes using the most dominant strains in specific locations against the *B. diazoefficiens* USDA110 as the positive control.

Materials and Methods

1. Selection and preparation of inoculant strains

In our previous experiment, we were able to identify the most dominant bradyrhizobia at a particular location in the Philippines which are listed in Table 4.1. The identification of the strains was based on the Restriction Fragment Length Polymorphism (RFLP) treatment and sequence analysis of the 16S rRNA gene, internal transcribed spacer (ITS) region of the 16S-23S rRNA gene and the *rpoB* housekeeping gene (Mason et al., 2018).

Table 4.1. List of indigenous bradyrhizobia strains isolated from the Philippines and used as single-strain inoculant in this study and the site of isolation.

Location	Coordinate	Strain for inoculation	No. of isolates	Species
Ilagan, Isabela (**IS**)	17.30°N,122.01°E	IS-2	40	*B. elkanii*
Gamu, Isabela (**GI**)	17.08°N,121.79°E	GI-4	30	*Bradyrhizobium* sp.
Baguio, Benguet (**BA**)	16.40°N,120.60°E	BA-24	31	*B. japonicum*
Nueva Ecija 1 (**NE1**)	15.74°N,120.93°E	NE1-6	49	*B. elkanii*
Nueva Ecija 2 (**NE2**)	15.74°N,120.93°E	NE2-37	26	*Bradyrhizobium* sp.
Irosin, Sorsogon (**SO**)	12.72°N,124.04°E	SO-1	44	*B. diazoefficiens*
Abuyog, Leyte (**LT**)	10.67°N,125.04°E	LT-3	42	*B. diazoefficiens*
La Carlota, Negros Occidental (**NR**)	10.24°N,122.59°E	NR-2	22	*B. elkanii*
Ubay, Bohol (**BO**)	9.99°N,124.45°E	BO-4	24	*B. elkanii*
Sultan Kudarat, Maguindanao (**SK**)	6.51°N,124.42°E	SK-5	29	*B. diazoefficiens*
Tupi, South Cotabato (**SC**)	6.34°N,124.97°E	SK-12	4	*B. yuanmingense*
		SC-3	31	*B. japonicum*
Total		**12**	**372**	

The selection process was based on the most abundant strain in the total population of bradyrhizobia at a specific location. For example, in Ilagan, Isabela, 80 isolates were collected from which 50% of the population (40 out of 80) belonged to *B. elkanii* (Mason et al., 2018), then IS-2 was selected to represent the 40 isolates. The

most prevalent strain in the specific location was selected in the present study for a single-strain inoculation test, with an exemption for Sultan Kudarat (SK) where two strains were selected, SK-5 and SK-12. Sultan Kudarat is the only location where *B. yuanmingense* was isolated of which SK-12 was phylogenetically similar.

Each strain was grown in yeast-extract mannitol agar (YMA, Vincent 1970) with Congo Red until a single colony appears then, the single colony was cultured in YM broth (Vincent, 1970) for about 1 week at 28°C in a dark shaker with continuous agitation at 120 rpm. The culture was diluted with sterile distilled water at a rate of 1 x 10^6 ml^{-1} as an inoculant.

2. Cultivation in culture pots, maintenance and harvesting

For the soybean plant, two cultivars from the Philippines, PSB-SY2 (Rj_4) and Collection 1 (non-*Rj*), and three cultivars from Japan, Orihime (Rj_3), IAC-2 (Rj_2Rj_3), and Akisengoku (Rj_4), were used in cultivation. For easier referral in this manuscript, the two cultivars from the Philippines are referred to as tropical cultivars while the three cultivars from Japan are referred to as temperate cultivars from henceforth. Soybean seeds were surface-sterilized by soaking in 70% ethanol and sodium hypochlorite solution as formerly described (Saeki et al., 2000) and planted in 1-liter culture pots (n = 4). Next, the culture pots were filled with vermiculite then, N-free nutrient solution was added at 40% (vol/vol) distilled water content and were autoclaved for 20 min at 121°C. The seeds were then sown on the vermiculite, and the pot was weighed. Afterwards, the plants were grown for 4 weeks inside the growth chamber (33°C for 16 h, day; 28°C for 8h, night), and were supplied weekly with sterile distilled water until the initial weight of the pot was reached.

For reference, *B. diazoefficiens* USDA110 was inoculated as a positive control and a pot without inoculated strain served as the negative control. All treatments were conducted with four replications.

3. Data collection and analysis

After 4 weeks, data gathering was conducted as follows: 1) the chlorophyll content or the greenness of the plant was taken with the use of SPAD 502 Plus Chlorophyll Meter (Minolta Camera Co., Japan) at three points in the youngest fully expanded leaf of each plant and the average was recorded; 2) plants were gently uprooted and were washed with distilled water until the roots were free from vermiculite; 3) plant height was measured from the base of the plant to the tip of the longest and youngest leaf; 4) clean roots containing the nodules were immediately separated and placed in 100 mL Erlenmeyer flask with a rubber cork then were used for the Acetylene Reduction Assay (ARA) using gas-chromatography (gas chromatograph GC-8A, Shimadzu, Japan); 5) then, nodules were collected from the roots, counted and the fresh biomass was recorded along with the shoot and root fresh weight; 6) shoot, root, and nodules were then placed in an oven to dry at 70°C for 48 hours; then finally, the dry biomass of the shoot, root, and nodules were read and recorded. The oven-dried shoot were ground up to about 2 mm and were used for Total N analysis using an Automatic NC Analyzer Sumigraph NC-220F (Sumika Chemical Analysis Service. Ltd., Tokyo, Japan).

The symbiotic efficiency was computed with the formula adapted from Risal et al. (2010) which is: (amount of N fixed / dry weight of nodule) x 100. The amount of N fixed was obtained from the difference between the N content of the inoculated treatments against the uninoculated pots. The nitrogenase activity was expressed as the

concentration of ethylene produced and computed with the following formulae: (C_E x S_E) / (K_E x $_E$) where C_E and K_E are the ethylene concentration (µl/L) in the sample and standard, respectively, and E (sample) and S_E (standard) are the respective areas obtained after the chromatographic analyses (Unkovich et al., 2008).

Statistical analysis was conducted using R software v.3.4.0 and the comparison among means was analyzed by Tukey's Honest Significant Difference (HSD) test. For the nitrogenase activity, the analysis was performed only with 2 replications so statistical test was not applied.

Results

1. Nodule number, oven dry weight of shoot and nodules

Shown in Table 4.2 is the influence of the single-strain inoculation test on the tropical and temperate soybean cultivars in terms of the number of nodules, and the dry biomass of the shoot and nodules. All the indigenous strains from the Philippines were able to form nodules on all the soybean cultivars, regardless of the *Rj* genotype, except for strain BO-4. USDA110 showed the highest number of nodules significantly in comparison with all the indigenous strains, except with Akishirome (*Rj*$_4$), wherein IS-2 showed the highest nodule number. For the tropical cultivars, strain BO-4 has the least nodulation ability, regardless of the *Rj* genotype. The PSB-SY2 (*Rj*$_4$) showed similar preference with strains IS-2, GI-4, BA-23, NE1-6, SO-1 and LT-3 while Collection 1 (non-*Rj*) seemed to prefer the strains GI-4, NR-2, and SK-5. For the temperate cultivars, the strain BO-4 did not form any nodule with all cultivars, regardless of the *Rj* genotype. For Orihime (*Rj*$_3$), both the strains NR-2 and SK-5 showed the highest nodulation ability among the rest. In the case of Akisengoku (*Rj*$_4$), the strain IS-2 showed the highest nodule number, followed similarly by the strains GI-

4, BA-24, NE1-6, NE2-37, SO-1, LT-3, NR-2, and SK-5. Meanwhile, IAC-2 (Rj_2Rj_3) seemed to have an almost similar preference with all the indigenous strains, with the lowest nodule numbers obtained from the strains LT-3 and SC-3.

Table 4.2. Effect of the single-strain inoculation of indigenous Philippines bradyrhizobia and *B. diazoefficiens* USDA110 on the number of nodules, and dry weight of shoot and nodules of the two tropical and three temperate soybean cultivars.

Strain	Ph-Col1 (non-*Rj*)			Ph-SY2 (*Rj*$_4$)			Ja-Ori (*Rj*$_3$)			Ja-Aki (*Rj*$_4$)			Ja-IAC2 (*Rj*$_2$*Rj*$_3$)		
	NN	SDW	NDW	NN	SDW	NDW	NN	SDW	NDW	NN	SDW	NDW	NN	SDW	NDW
IS-2	22^{b}	247.2^{b}	21.5^{cd}	26^{b}	203.2^{c}	30.3^{bc}	21^{b}	268.4^{b}	25.5^{b}	36^{a}	278.6^{a}	27.9^{b}	22^{b}	270.2^{a}	29.0^{a}
GI-4	30^{a}	248.4^{b}	31.3^{b}	27^{ab}	234.7^{b}	34.1^{b}	15^{b}	276.8^{a}	21.7^{d}	24^{ab}	272.6^{a}	25.8^{b}	18^{b}	280.1^{a}	23.9^{b}
BA-24	20^{b}	229.6^{d}	32.5^{b}	26^{b}	238.9^{b}	34.2^{b}	18^{b}	277.3^{a}	26.3^{ab}	24^{ab}	279.0^{a}	28.9^{b}	20^{b}	274.7^{a}	28.3^{a}
NE1-6	18^{b}	249.3^{b}	37.8^{a}	27^{ab}	209.8^{c}	31.7^{b}	18^{b}	269.3^{ab}	28.5^{a}	29^{ab}	257.4^{a}	37.0^{a}	20^{b}	260.7^{b}	28.9^{a}
NE2-37	18^{b}	202.8^{f}	31.8^{b}	21^{c}	249.1^{b}	40.7^{a}	14^{b}	274.9^{a}	21.8^{d}	32^{ab}	244.8^{b}	29.7^{b}	18^{b}	268.4^{ab}	23.7^{b}
SO-1	20^{b}	210.7^{e}	31.2^{b}	25^{b}	228.5^{b}	29.4^{bc}	20^{b}	279.4^{a}	27.2^{ab}	25^{ab}	289.5^{a}	24.2^{c}	17^{bc}	232.5^{bc}	23.1^{b}
LT-3	19^{b}	236.3^{c}	20.4^{cd}	25^{b}	176.7^{d}	29.7^{bc}	19^{b}	267.9^{b}	25.1^{b}	22^{ab}	256.4^{a}	21.2^{cd}	16^{c}	244.3^{b}	21.5^{b}
NR-2	27^{a}	214.9^{e}	31.1^{b}	20^{c}	237.4^{b}	31.6^{b}	27^{a}	261.4^{b}	25.4^{b}	25^{ab}	240.5^{b}	28.1^{b}	19^{b}	243.6^{b}	22.1^{b}
BO-4	15^{b}	241.1^{bc}	24.1^{c}	12^{d}	200.0^{c}	32.5^{b}	0^{c}	269.4^{ab}	0^{e}	0^{c}	279.3^{a}	0^{d}	0^{d}	208.7^{c}	0^{c}
SK-5	30^{a}	259.7^{a}	25.8^{c}	17^{c}	202.2^{c}	22.1^{c}	26^{a}	272.6^{a}	27.1^{ab}	29^{ab}	276.8^{a}	32.1^{b}	21^{b}	261.3^{b}	25.4^{b}
SK-12	24^{ab}	236.4^{c}	29.2^{bc}	16^{c}	234.1^{b}	24.2^{c}	21^{b}	265.7^{b}	25.9^{ab}	18^{b}	273.4^{a}	22.9^{c}	19^{b}	275.9^{a}	23.9^{b}
SC-3	21^{b}	215.4^{e}	25.6^{c}	19^{c}	227.3^{b}	25.2^{c}	18^{b}	238.5^{c}	23.6^{c}	15^{b}	272.7^{a}	22.2^{c}	15^{c}	277.3^{a}	26.8^{a}
USDA110	33^{a}	260.1^{a}	24.6^{c}	31^{a}	275.9^{a}	20.3^{d}	30^{a}	289.6^{a}	21.4^{d}	31^{ab}	272.0^{a}	27.9^{b}	30^{a}	290.5^{a}	25.1^{b}
Un-inoculated	1^{c}	256.8^{a}	0.68^{d}	0^{e}	200.5^{c}	0^{e}	0^{c}	222.6^{c}	0^{e}	0^{c}	265.0^{a}	0^{d}	0^{d}	189.2^{c}	0^{c}

Ph – Philippines; **Ja** – Japanese; **Col1** – Collection1; **SY2** – PSB-SY2; **Ori** – Orihime; **Aki** – Akisengoku; **NN –** nodule number; **SDW –** shoot dry weight; **NDW –** nodule dry weight.

Table 4.3. Nodulation ability of the indigenous bradyrhizobia from the Philippines and the *B. diazoefficiens* USDA110 on two soybean cultivars from the Philippines and three soybean cultivars from Japan that harbors different *Rj* genotypes.

Strain	Ph-Col1 (non-*Rj*)	Ph-SY2 (*Rj*$_4$)	Ja-Ori (*Rj*$_3$)	Ja-Aki (*Rj*$_4$)	Ja-IAC2 (*Rj*$_2$*Rj*$_3$)
IS-2	22^{b}	26^{b}	21^{b}	36^{a}	22^{b}
GI-4	30^{a}	27^{a}	15^{b}	24^{a}	18^{b}
BA-24	20^{ab}	26^{a}	18^{ab}	24^{a}	20^{ab}
NE1-6	18^{b}	27^{a}	18^{b}	29^{a}	20^{b}
NE2-37	18^{b}	21^{b}	14^{b}	32^{a}	18^{b}
SO-1	20^{ab}	25^{a}	20^{ab}	25^{a}	17^{ab}
LT-3	19^{ab}	25^{a}	19^{ab}	22^{a}	16^{b}
NR-2	27^{a}	20^{ab}	27^{a}	25^{a}	19^{ab}
BO-4	15^{a}	12^{a}	0^{b}	0^{b}	0^{b}
SK-5	30^{a}	17^{b}	26^{a}	29^{a}	21^{ab}
SK-12	24^{a}	16^{b}	21^{a}	18^{ab}	19^{ab}
SC-3	21^{a}	19^{a}	18^{a}	15^{a}	15^{a}
USDA110	33^{a}	31^{a}	30^{a}	31^{a}	30^{a}
Un-inoculated	1^{a}	0^{a}	0^{a}	0^{a}	0^{a}

Ph – Philippines; **Ja** – Japanese; **Col1** – Collection1; **SY2** – PSB-SY2; **Ori** – Orihime; **Aki** – Akisengoku; **N fixed** - amount of N fixed by the shoot; **S.E.** – symbiotic efficiency. The subscript letters showed a significant difference by Tukey's HSD test at $p > 0.05$, $n = 4$.

For the nodule dry weight, although USDA110 showed the highest number of nodules, it did not possess the highest dry nodule biomass, indicating that the indigenous strains were able to form larger nodules than the ones formed by the inoculation with USDA110. For the temperate cultivars, it is noticeable that the strains However, it is visible that in both the tropical and temperate cultivars, the indigenous strains have better nodulation compatibility with the cultivars that harbor the *Rj*$_4$ genotype, except for BO-4 (Table 4.3). This shows that BO-4 might not be compatible with the temperate cultivars as it failed to nodulate any of the three cultivars. Furthermore, it also has the least nodulation compatibility with the tropical cultivars. NE1-6 and SK-5 had the highest nodule biomass regardless of the *Rj* genotype, while

for the tropical cultivars, the strains NE1-6 and NE2-37 obtained the highest nodule biomass with Collection 1 (non-*Rj*) and PSB-SY2 (*Rj*$_4$), respectively.

In terms of the shoot dry weight, the tropical cultivars generally had lower biomass than the temperate cultivars, which could be attributed to the fact that the tropical cultivars were also generally smaller than the temperate cultivars. Although the data on plant height was not shown in this report, the plant height of the tropical cultivars generally ranged from 18 to 22 cm, whereas the temperate cultivars generally ranged from 23 to 27 cm.

2. Symbiotic performance and nitrogen fixation ability of bradyrhizobia from the Philippines

Presented in Figure 4.1 and Table 4.4 is the symbiotic efficiency of the indigenous bradyrhizobia strains isolated from the Philippines against that of the USDA110. It is evident that USDA110 had significantly the highest symbiotic efficiency, regardless of the origin of the cultivars or the *Rj* genotypes. For the tropical cultivars, it is noticeable that the most efficient indigenous strain was the IS-2 while the least efficient was LT-3, regardless of the *Rj* genotype. On the other hand, the highest symbiotic efficiency with the temperate cultivars from the indigenous strains was obtained with the inoculation of SK-5, while the least was obtained with BO-4. Additionally, the strain LT-3 showed a comparably low efficiency with BO-4 on the temperate cultivars harboring the *Rj*$_3$ and *Rj*$_4$ genotypes.

Meanwhile, the symbiotic performance of the possible novel species GI-4 and NE2-37 also merit further evaluation as these two, which were classified as *Bradyrhizobium* sp. were comparably efficient with both the tropical and temperate cultivars. Unlike the strains IS-2 ad SK-5, these two strains did not show any preference

with the origin of the cultivars. The two isolates BA-24 and SC-3 which were classified under *B. japonicum* USDA6 also showed better performance with the temperate cultivars in particular with the Rj_4 plants. In contrast to the strain IS-2, NE1-6, which is also a *B. elkanii* strain showed preference to the temperate cultivars than to the tropical cultivars.

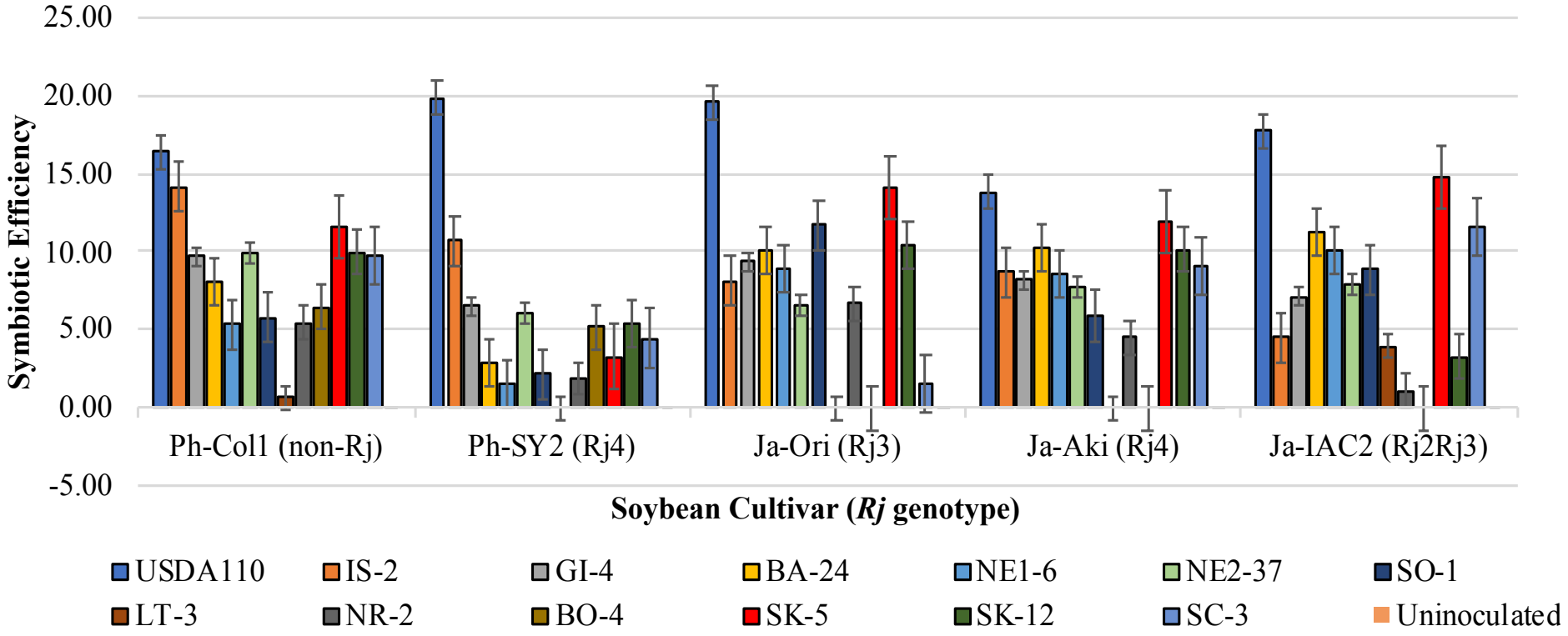

Figure 4.1. Symbiotic efficiency as influenced by the single-strain inoculation of the 12 indigenous bradyrhizobia from the Philippines and *B. diazoefficiens* USDA110 for the two tropical and three temperate soybean cultivars. **Ph** – Philippines; **Ja** – Japanese; **Col1** – Collection1; **SY2** – PSB-SY2; **Ori** – Orihime; **Aki** – Akisengoku.

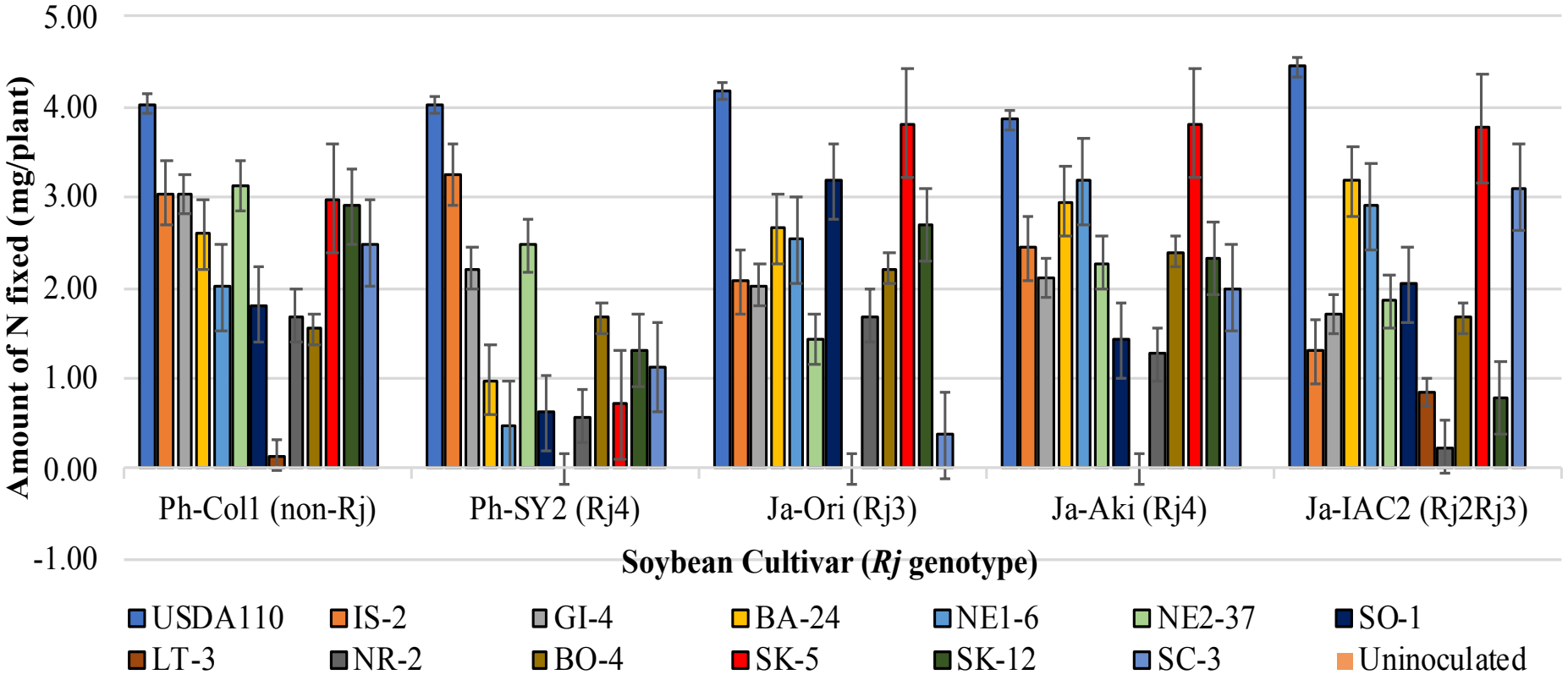

Figure 4.2. Amount of N fixed by the shoot as influenced by the single-strain inoculation of the 12 indigenous bradyrhizobia from the Philippines and *B. diazoefficiens* USDA110 for the two tropical and three temperate soybean cultivars. **Ph** – Philippines; **Ja** – Japanese; **Col1** – Collection1; **SY2** – PSB-SY2; **Ori** – Orihime; **Aki** – Akisengoku.

Table 4.4. Amount of N fixed by the shoot and symbiotic efficiency as influenced by the single-strain inoculation of the 12 indigenous bradyrhizobia from the Philippines and *B. diazoefficiens* USDA110 for the two tropical and three temperate soybean cultivars.

Strain	**Ph-Col1 (non-*Rj*)**		**Ph-SY2 (*Rj*$_4$)**		**Ja-Ori (*Rj*$_3$)**		**Ja-Aki (*Rj*$_4$)**		**Ja-IAC2 (*Rj*$_2$*Rj*$_3$)**	
	N fixed mg^{-plant}	S.E.	N fixed mg^{-plant}	S.E.	N fixed mg^{-plant}	S.E.	N fixed mg^{-plant}	S.E.	N fixed mg^{-plant}	S.E.
IS-2	4.03^{a}	16.41^{a}	4.02^{a}	19.82^{a}	4.18^{a}	19.56^{a}	3.86^{a}	13.82^{a}	4.45^{a}	17.69^{a}
GI-4	3.04^{b}	14.15^{b}	3.25^{b}	10.72^{b}	2.07^{bc}	8.14^{c}	2.43^{bc}	8.72^{d}	1.29^{e}	4.46^{e}
BA-24	3.03^{b}	9.67^{d}	2.21^{c}	6.49^{c}	2.03^{bc}	9.36^{c}	2.11^{bc}	8.17^{d}	1.70^{de}	7.09^{d}
NE1-6	2.59^{c}	7.98^{e}	0.97^{f}	2.83^{d}	2.65^{bc}	10.08^{c}	2.95^{b}	10.18^{c}	3.18^{c}	11.24^{c}
NE2-37	2.01^{d}	5.30^{fg}	0.47^{g}	1.49^{de}	2.53^{bc}	8.87^{c}	3.18^{b}	8.58^{d}	2.91^{c}	10.03^{c}
SO-1	3.13^{b}	9.85^{d}	2.47^{c}	6.06^{c}	1.44^{c}	6.59^{c}	2.28^{bc}	7.68^{d}	1.85^{d}	7.83^{d}
LT-3	1.81^{d}	5.79^{fg}	0.62^{fg}	2.13^{de}	3.18^{b}	11.71^{bc}	1.43^{d}	5.90^{e}	2.04^{d}	8.84^{d}
NR-2	0.14^{e}	0.67^{g}	<0^{h}	<0.01^{e}	<0^{e}	<0.01^{e}	<0^{e}	<0.01^{g}	0.84^{f}	3.92^{e}
BO-4	1.68^{de}	5.42^{fg}	0.58^{g}	1.83^{de}	1.69^{c}	6.67^{c}	1.26^{d}	4.49^{f}	0.24^{g}	1.10^{f}
SK-5	1.54^{de}	6.41^{f}	1.67^{d}	5.14^{cd}	2.21^{bc}	0.00^{e}	2.40^{bc}	0.00^{g}	1.67^{de}	0.00^{f}
SK-12	2.98^{b}	11.55^{c}	0.71^{f}	3.28^{d}	3.81^{a}	14.06^{b}	3.82^{a}	11.91^{b}	3.76^{b}	14.79^{b}
SC-3	2.90^{b}	9.95^{d}	1.31^{e}	5.40^{c}	2.70^{b}	10.42^{c}	2.32^{bc}	10.14^{c}	0.78^{f}	3.27^{e}
USDA110	2.49^{c}	9.74^{d}	1.12^{e}	4.45^{cd}	0.37^{d}	1.55^{d}	2.00^{c}	9.03^{cd}	3.11^{c}	11.61^{c}
Un-inoculated										

Ph – Philippines; **Ja** – Japanese; **Col1** – Collection1; **SY2** – PSB-SY2; **Ori** – Orihime; **Aki** – Akisengoku; **N fixed** - amount of N fixed by the shoot; **S.E.** – symbiotic efficiency. The subscript letters showed a significant difference by Tukey's HSD test at p>0.05, n = 4.

With regards to the amount of Nitrogen fixed in the shoot, it is again the USDA110 that showed the highest performance (Fig. 4.2, table 4.4). For the indigenous strains, IS-2, GI-4, NE2-37, SK-5, and SK-12 have comparable N-fixing ability with Collection 1 (non-*Rj*) while the lowest was obtained with the inoculation of LT-3. Similarly, LT-3 had the lowest amount of fixed N with PSB-SY2 (Rj_4) while the highest was obtained with the inoculation of IS-2. Meanwhile, SK-5 showed the highest N-fixation ability with the temperate cultivars while the lowest was obtained again with the inoculation of LT-3 for the Rj_3 and Rj_4-harboring cultivars, and NR-2 had the least amount of N fixed with the Rj_2Rj_3-harboring cultivar.

3. Chlorophyll content and nitrogenase activity of indigenous bradyrhizobia from the Philippines

The chlorophyll content or the greenness of the leaves is presented in Figure 4.3. The plants inoculated with USDA110 have the greenest leaves regardless of the cultivars and the *Rj* genotypes. However, it was not significantly different with some of the indigenous strains. For tropical cultivars, the plants inoculated with the strains IS-2 and NE2-37 have the greenest leaves or relatively higher chlorophyll content similar with the USDA110. The tropical plants inoculated with LT-3 showed the lowest chlorophyll content. For the temperate cultivars, the plants that were inoculated with SK-5 had the highest chlorophyll content similar with the USDA110, regardless of the *Rj* genotypes. The lowest chlorophyll content was observed on the Rj_3 and Rj_4 plants inoculated with LT-3 while for the Rj_2Rj_3 plants, both the strains NR-2 and SK-12 provided the lowest chlorophyll content. Most of the readings obtained from the SPAD-502 meter showed a consistent result with the amount of N fixed in the shoot of the plants.

Meanwhile, the nitrogenase activity as obtained from the ARA (Fig. 4.4) showed an almost similar result with the N fixation ability (Fig. 4.2) on most of the strains. It is noticeable that USDA110 obtained the highest activity for both the tropical and the temperate cultivars. For the indigenous strains, IS-2 had the highest nitrogenase activity with the tropical cultivars whereas SK-5 was the highest with the temperate cultivars. Again, the strains LT-3 and BO-4 showed the least activity with the tropical cultivars and temperate cultivars, respectively.

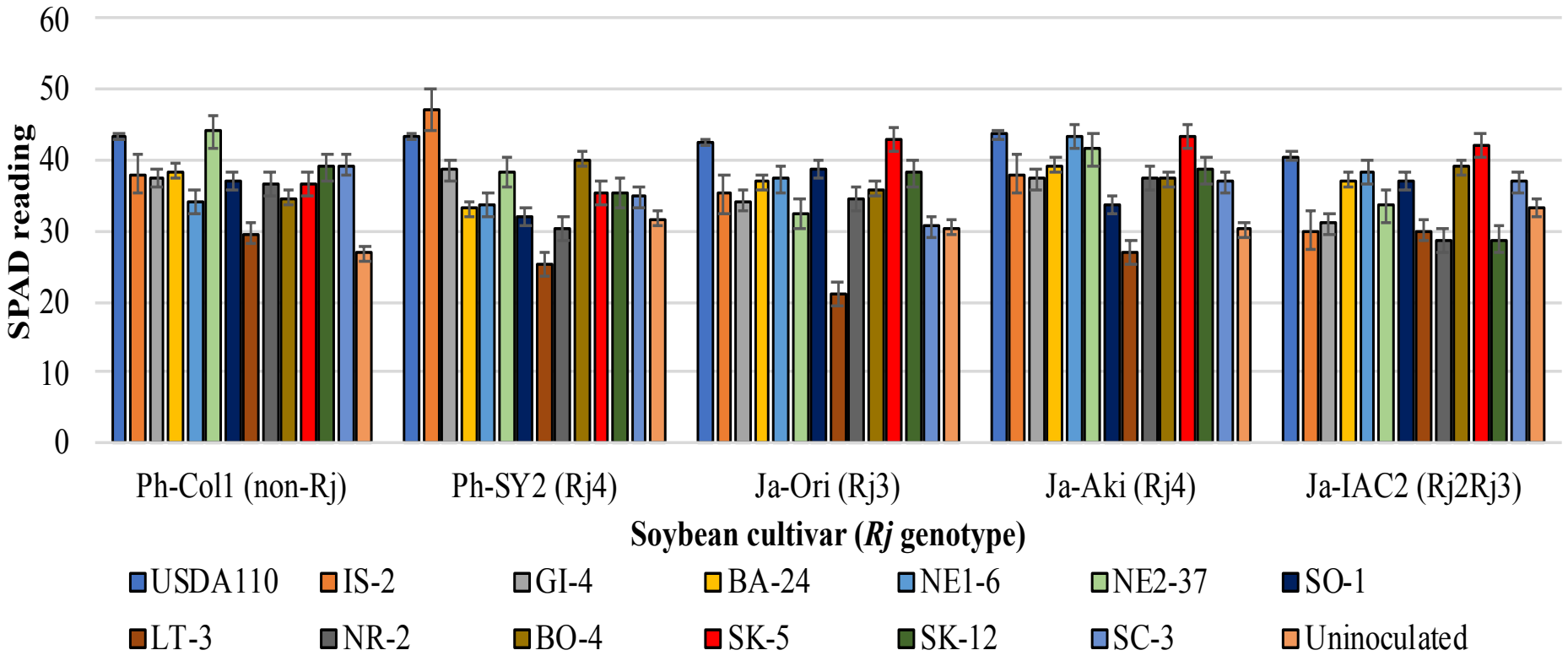

Figure 4.3. Greenness of the shoot or chlorophyll content as influenced by the single-strain inoculation of the 12 indigenous bradyrhizobia from the Philippines and *B. diazoefficiens* USDA110 for the two tropical and three temperate soybean cultivars. **Ph** – Philippines; **Ja** – Japanese; **Col1** – Collection1; **SY2** – PSB-SY2; **Ori** – Orihime; **Aki** – Akisengoku.

Table 4.5. Greenness of the shoot or chlorophyll content as influenced by the single-strain inoculation of the 12 indigenous bradyrhizobia from the Philippines and *B. diazoefficiens* USDA110 for the two tropical and three temperate soybean cultivars.

Strain	**SPAD Reading**				
	Ph-Col1 (non-*Rj*)	**Ph-SY2 (*Rj*$_4$)**	**Ja-Ori (*Rj*$_3$)**	**Ja-Aki (*Rj*$_4$)**	**Ja-IAC2 (*Rj*$_2$*Rj*$_3$)**
IS-2	43.4^{a}	43.3^{a}	42.6^{a}	43.6^{a}	40.6^{a}
GI-4	38.0^{a}	47.2^{a}	35.2^{b}	38.1^{ab}	30.1^{b}
BA-24	37.5^{ab}	38.6^{a}	34.3^{b}	37.3^{ab}	31.0^{b}
NE1-6	38.4^{a}	33.1^{b}	37.0^{b}	39.3^{ab}	37.1^{a}
NE2-37	34.1^{a}	33.7^{b}	37.3^{b}	43.5^{a}	38.4^{a}
SO-1	44.1^{a}	38.3^{a}	32.5^{b}	41.5^{a}	33.5^{b}
LT-3	37.2^{ab}	32.0^{b}	38.7^{b}	33.6^{c}	36.9^{ab}
NR-2	29.7^{b}	25.2^{c}	21.1^{c}	27.0^{e}	30.1^{b}
BO-4	36.5^{ab}	30.5^{b}	34.4^{b}	37.4^{ab}	28.7^{c}
SK-5	34.6^{ab}	40.1^{a}	35.9^{b}	37.3^{ab}	39.1^{a}
SK-12	36.7^{ab}	35.5^{ab}	43.1^{a}	43.4^{a}	42.2^{a}
SC-3	39.1^{a}	35.4^{ab}	38.2^{b}	38.7^{ab}	28.8^{c}
USDA110	39.3^{a}	34.8^{b}	30.6^{bc}	36.9^{b}	37.0^{ab}
Un-inoculated	26.8^{b}	31.8^{b}	30.5^{bc}	30.2^{d}	33.3^{b}

Note: Ph – Philippines; **Ja** – Japanese; **Col1** – Collection1; **SY2** – PSB-SY2; **Ori** – Orihime; **Aki** – Akisengoku. The subscript letters showed a significant difference by Tukey's HSD test at $p>0.05$, $n = 4$.

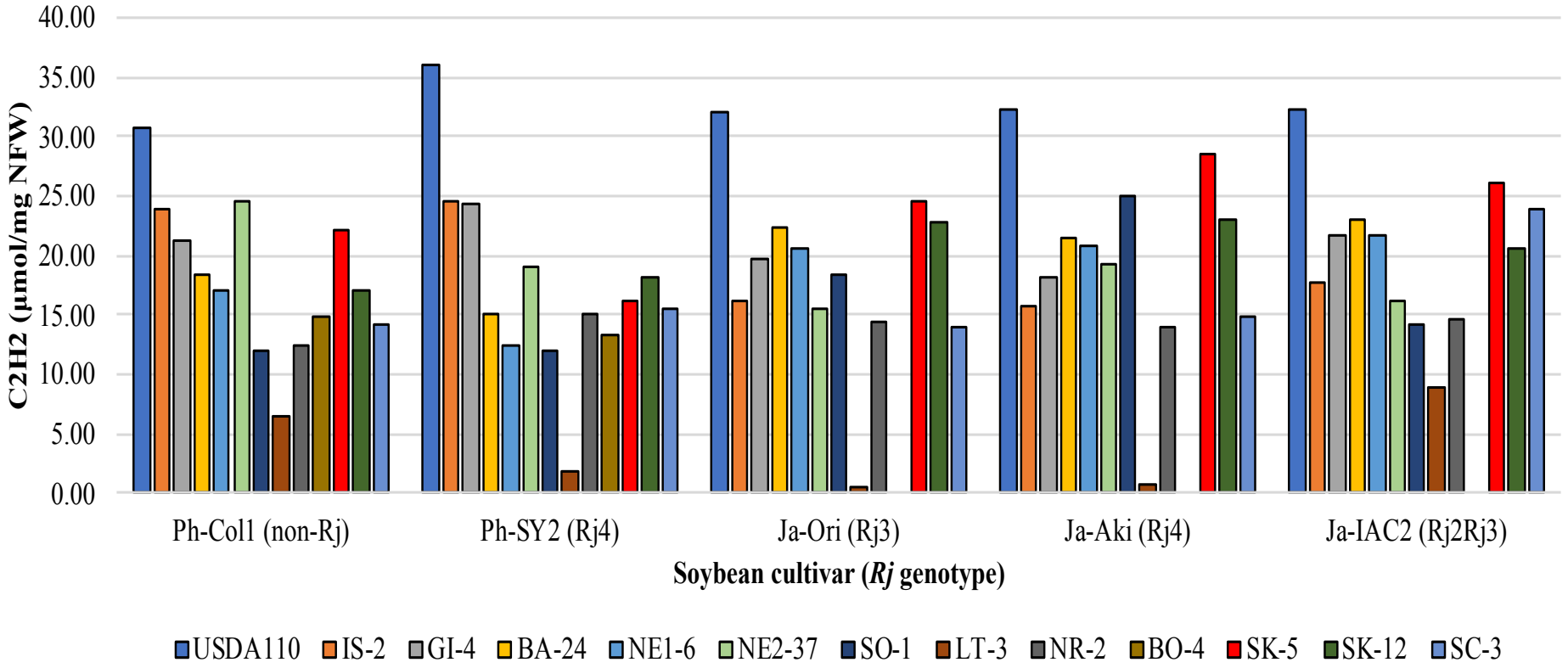

Figure 4.4. Nitrogenase activity as influenced by the single-strain inoculation of the 12 indigenous bradyrhizobia from the Philippines and *B. diazoefficiens* USDA110 for the two tropical and three temperate soybean cultivars. **Ph** – Philippines; **Ja** – Japanese; **Col1** – Collection1; **SY2** – PSB-SY2; **Ori** – Orihime; **Aki** – Akisengoku.

Discussion

1. Nodulation and N-fixation ability of the indigenous bradyrhizobia

Although the number of nodules did not differ significantly with each cultivar, it is fairly noticeable that most of the strains slightly favored the cultivars that harbor the Rj_4 genotype regardless whether the cultivar was from the tropical or the temperate region. Yet, all the indigenous strains from the Philippines was able to form nodules on all the cultivars that harbor a different *Rj* genotypes, except BO-4, so it is proposed that these indigenous and dominant strains isolated from the Philippines be considered as type A strains. This observation is first obtained from this report and will provide the foundation for future research in the country.

In relation to the ability of the indigenous strains to fix N, it is an interesting finding that the strains IS-2 and SK-5 showed a certain pattern in their performance. The strain SK-5, which is classified as *B. diazoefficiens* (Mason et al., 2018), seemed to perform better along with the temperate cultivars than with the tropical cultivars. On the other hand, IS-2, which was classified under the *B. elkanii* species, showed the highest N fixation ability with the tropical cultivars, although it has a comparably similar performance with the other strains for the non-*Rj* plants.

2. Symbiotic performance of the indigenous bradyrhizobia

All the indigenous bradyrhizobia isolated from the Philippines showed lesser performance in comparison to the *B. diazoefficiens* USDA110 but better performance than the uninoculated treatment. Although using USDA 110 as a reference strain for symbiotic association with soybean has been established in many reports, a study noted that the effect of inoculating USDA110 in the presence of native rhizobia was not significantly higher under ambient and elevated CO_2 especially in field condition (Sanz-

Saez et al., 2015). Thus, our result might change if the indigenous strains would be inoculated under natural conditions were they exist in higher population than the USDA110.

In tropical regions like Brazil, it was also reported that some novel *Bradyrhizobium* species even showed a better symbiotic performance than the commercial inoculant *B. elkanii* SEMIA 5019 which was approved by their Ministry of Agriculture, Livestock and Supply (Ribeiro et al., 2015). Additionally, similar observation was obtained from another report conducted at Mozambique where the relative efficiency of native bradyrhizobia were higher than the *B. diazoefficiens* USDA110 (Chibeba et al., 2017). Hence, it is better to harness and use the locally-adapted strains for inoculation.

This is the first time that the USDA110 was used to compare with the indigenous bradyrhizobia isolated from the Philippines and we were able to show that the selected strains IS-2 and SK-5 could be further studied as potential inoculants. Henceforth, it was shown in this report that the symbiotic efficiency of indigenous bradyrhizobia from the Philippines have different preference and performance according to the cultivar to be used. For the tropical cultivars from the Philippines, it is evident that the strains with the highest potential to become inoculant would be IS-2, followed by the GI-4 and NE2-37.

We have elucidated in our two previous reports (Mason et al., 2017, 2018) that *B. elkanii* was the most dominant and have the widest distribution in Philippines soil so it is suggested that among all the indigenous strains used in this study, IS-2 have the highest potential to be used as a candidate inoculant. Although we have hypothesized that the indigenous strains which were classified under the Bd110 cluster would be

good inoculant in the Philippines, their symbiosis with the local soybean cultivars proved to be lower than IS-2, which is a strain of *B. elkanii* species.

In summary, this study was able to provide the following observations: (1) the use of indigenous and locally-adapted bradyrhizobia for soybean production might be comparably efficient in symbiosis and N-fixation with the known model strain of bradyrhizobia, which is the *B. diazoefficiens* USDA110; (2) all the indigenous bradyrhizobia used in this study are classified as type A strains based on its nodulation ability with various *Rj* genotypes; and (3) the symbiotic performance and N-fixation ability of the indigenous strains are in this order for tropical cultivars, *B. elkanii* > *Bradyrhizobium* sp. > *B. japonicum* > *B. diazoefficiens* but is reversed for the temperate cultivars. Lastly, we recommend that for further research, if soybean yield is to be considered, we propose the use of IS-2 as a potential inoculant to increase soybean yield but if the objective is for climate change mitigation, SK-5 or LT-3 should be used.

For future prospect, it would be helpful if field trials using IS-2 in comparison or in combination with the commercially available inoculants would be conducted under different agro-environmental gradient in the country. Further on, evaluation of different carrier materials that would be suitable for the said strain should be conducted for mass production if IS-2 was proven in field trials to be competitive.

Summary

This report aimed to evaluate the ability of the dominant indigenous strains of bradyrhizobia obtained from different locations in the Philippines in terms of their symbiosis, N-fixation and nodulation with both the Philippines and Japanese soybean cultivars harboring different *Rj* genotypes. This was done to select the most efficient and effective indigenous strain that can be used as an inoculant under the Philippines'

local condition. Two soybean cultivars from the Philippines and three cultivars from Japan harboring different *Rj* genotypes were planted in culture pots and inoculated with 12 indigenous bradyrhizobia isolated from the Philippines and the reference strain *Bradyrhizobium diazoefficiens* USDA110. The pots were grown inside the growth chamber for four weeks with 33°C for 16 h as daytime and 28°C for 8 h as night time. At harvest, fresh and dry biomass of the shoot, root, and nodules, number of nodules, and chlorophyll content via SPAD meter were obtained. The nitrogenase activity by Acetylene Reduction Assay (ARA) was performed immediately at harvest while Total N analysis was conducted after oven drying. All the indigenous bradyrhizobia strains were able to form nodules on both the Philippines and Japanese soybean cultivars, except for *B. elkan*ii BO-4, which did not form nodules on all the Japanese cultivars. As expected, *B. diazoefficiens* USDA110 obtained the highest symbiotic efficiency and nitrogen fixation against the indigenous bradyrhizobia isolated form the Philippines. However, upon comparing only the performance of the indigenous strains, *B. elkanii* IS-2 is significantly the most efficient and effective N-fixer than the other strains for the Philippines' cultivars. In contrast, *B. diazoefficiens* SK-5 was found to be the most efficient and effective N-fixer for Japanese' cultivars. The chlorophyll content at harvest matched with the amount of N fixed in the shoot. Meanwhile, the nitrogenase activity did not differ among all the strains but the highest activity was obtained from the plants inoculated with the USDA110, followed by SK-5 and IS-2. Although an efficient and effective strain of *B. diazoefficiens* was isolated from the Philippines, it seemed that this strain performed better in synergy with the temperate soybean cultivars, regardless of the soybean's *Rj* genotype. Meanwhile, *B. elkanii* IS-2 was the most compatible with the tropical soybean cultivars. These results showed that while *B. diazoefficiens* can exist abundantly in tropical soils, the symbiosis with a tropical

soybean crop will not be as efficient and effective with that of the temperate soybean cultivars. Thus, we were able to identify the most promising indigenous bradyrhizobia that could be used to increase the soybean yield in the Philippines.

Chapter 5

Species diversity of nodulating and non-nodulating soybean rhizobia from different soil management practices in the Philippines

Introduction

Soybean (*Glycine max* [L.] Merill), a member of Fabacea family, is an important food legume planted in about 120M hectares in the world wherein Philippines only share 495 ha. with an average yield of 1.1 tons/ha (Food and Agriculture Organization FAO 2016). Nutritionally, soybean is very valuable as it contains 40% protein (highest among cereals and legumes) and 20% oil (second highest among food legumes), among its other nutritional composition (Liu, 1997). In agriculture, its importance lies in its symbiosis with rhizobia, a general term for root-nodule bacteria that are capable of fixing atmospheric N and renders it available for plant's use. In return, the plant provides the "food" for the rhizobia in the form of carbon substrates from photosynthesis. However, symbiosis is a highly specific and mutual relationship between the micro-symbionts and their host guided by an interaction between the flavonoids released by the legume and the *nod* genes in rhizobia. For soybean, the major nodulating rhizobia are reportedly *Bradyrhizobium japonicum*, *B. elkanii*, *B. diazoefficiens* and *Sinorhizobium/Ensifer fredii* (). In case of the Philippines, we reported that the above stated three species of bradyrhizobia were the most abundant and dominant, with the prevalence of *B. elkanii* over the other two species in non-flooded soils with moderate to slight acidic condition (Mason et al., 2017, 2018). However, those studies were only focused on soybean bradyrhizobia. It is necessary to take into consideration the other possible symbiovars of soybean that also exist in the soil. In this manner, more information can be gained as to the species diversity and richness in a particular location.

The ecological niche of soybean rhizobia has been well documented decades ago particularly for temperate and sub-tropical regions such as Japan, USA, and China. In Japan and USA, *B. japonicum* and *B. diazoefficiens* were predominant in cooler

temperature whereas *B. elkanii* was prevalent in warmer temperature, with soil acidity ranging from acidic to neutral (Saeki et al., 2006, Shiro et al., 2013). In terms of soil acidity and alkalinity, *S./E. fredii* and *B. liaoningense* were dominant in alkaline soils of Vietnam and Nepal, respectively (Saeki et al., 2004; Adhikari et al., 2012). In China, it was reported that *Bradyrhizobium* sp. were affected by rich organic C, available P and soil pH, and that the abundance of rhizobia was impacted by land use and cultural management (Yan et al., 2014). The same author (Yan et al., 2017) also reported that under a long-term fertilization study, the diversity of rhizobial species was significantly decreased and that the community structure was influenced by organic C, available N and soil pH. Another study stated that under a cereal-based cropping system in India, the symbiotically efficient slow-growers dominated the proportion of rhizobia whereas, the less symbiotically efficient fast-growers were dominant in soybean-based cropping system (Kumar et al., 2017). Under the Philippine condition, it was indicated that the period of flooding and some soil properties such as soil type, pH, and nutrients affected the community structure and genetic diversity of soybean bradyrhizobia (Mason et al., 2018). In summary, these reports signify that the ecological niche of soybean rhizobia inhabiting the soil has such high variation across geographical location. Understanding the elements of diversity in rhizobia might lead to an improved and sustainable soybean production in a wide variety of agricultural and environmental gradients.

In this present report, a local soybean cultivar PSB-SY2 was used to isolate the rhizobia from soils collected at eleven locations in the Philippines. Our aim is to determine the richness and abundance of rhizobia species in Philippine soils, whether nodulating or non-nodulating, and understand the stimuli of diversity in their community structure under different soil management practices. This is an important

aspect to devise strategies on how to manipulate the population of efficient and effective rhizobia to increase soybean yield and its influence on soil fertility restoration.

Materials and Methods

1. Cropping patterns, soil collection and preparation

Eleven soil samples were collected from the eleven locations in the Philippines with the following cropping patterns: rice-based (R) – planted with rice twice in a year but also planted with other legumes or soybean during a 2 month period in between rice cropping season; legume-based (L) – usually planted with different legumes such as soybean, mungbean, cowpea, stringbeans, and other legumes during dry season then planted with rice during the wet season; soybean-based (S) – only planted with soybean and sometimes intercropped with other legumes or leafy vegetables). The rate of chemical fertilizers applied are generally 120-60-60 kg, 90-60-60 kg, and 20-30-30 kg NPK per ha per cropping season for dry season rice, wet season rice, and legumes, respectively. Ten soil sub-samples were collected per site from a 20 cm depth then mixed and quartered until a 1 kg composite soil was obtained as described previously (Mason et al., 2017). Data of the annual average temperature and rainfall were obtained from Philippine Atmospheric Geophysical and Astronomical Services Administration (PAGASA) website (www.pagasa.com.ph/) which were averages from the last two decades. Information on the study sites are listed in Table 5.1.

2. Isolation of indigenous soybean rhizobia

A commonly available soybean cultivar in the Philippines, PSB-SY2, which harbor *Rj*$_4$ genotype (Mason et al., 2018), was used to isolate the soybean rhizobia.

Table 5.1. Information on the physical and some chemical properties of the soil and the cropping pattern on the study sites in the Philippines.

Location	Coordinate	Cropping pattern	pH	EC (dS/m)	C (%)	Sand (%)	Silt (%)	Clay (%)
Ilagan, Isabela **(IS)**	17.30°N,122.01°E	Soybean-based **(S)**	5.90	0.08	1.34	28.0	34.5	37.5
Gamu, Isabela **(GI)**	17.08°N,121.79°E	Legume-based **(L)**	5.52	0.15	1.85	28.2	33.8	38.0
Baguio, Benguet **(BA)**	16.40°N,120.60°E	Soybean-based **(S)**	5.22	0.20	3.10	19.0	41.4	39.6
Nueva Ecija1 **(NE1)**	15.74°N,120.93°E	Legume-based **(L)**	6.21	0.05	1.37	28.7	34.6	36.7
Nueva Ecija2 **(NE2)**	15.74°N,120.93°E	Rice-based **(R)**	5.81	0.12	2.36	27.4	34.7	37.9
Irosin, Sorsogon **(SO)**	12.72°N,124.04°E	Legume-based **(L)**	5.26	0.15	1.92	28.9	33.6	37.5
Abuyog, Leyte **(LT)**	10.67°N,125.04°E	Soybean-based **(S)**	5.80	0.12	1.50	29.2	32.8	38.0
La Carlota, Negros Occidental **(NR)**	10.24°N,122.59°E	Legume-based **(S)**	5.62	0.15	0.63	28.0	34.2	37.8
Ubay, Bohol **(BO)**	9.99°N,124.45°E	Soybean-based **(S)**	5.82	0.11	0.63	29.7	33.5	36.8
Sultan Kudarat, Maguindanao **(SK)**	6.51°N,124.42°E	Legume-based **(L)**	6.64	0.14	2.48	24.0	34.1	41.9
Tupi, South Cotabato **(SC)**	6.34°N,124.97°E	Rice-based **(R)**	5.52	0.15	1.36	19.5	42.5	38.0

Cropping pattern: S – main crop is soybean, with some intercrop of leafy vegetables and other legumes; **R** – main crop is rice, planted twice in a year; **L** – usually planted with different legumes such as soybean, mung bean, cowpea, string beans, and other legumes during dry season then planted with rice during the wet season.

Surface-sterilized soybean seeds were planted in 1-liter culture pots (n = 4). Prior to planting, soybean seeds were surface-sterilized by soaking in ethanol and sodium hypochlorite solution (Saeki et al., 2006). The culture pots were filled with vermiculite and applied with N-free nutrient solution (Saeki et al., 2004) at 40% (vol/vol) water content then, were autoclaved at 121°C for 20 min. The planting of sterilized soybean seeds to the culture pots until the isolation of soybean rhizobia from the root nodules followed the similar procedure as previously described (Mason et al., 2017).

3. DNA extraction

A pure single colony obtained from the YMA plate was then cultured in HEPES-MES (HM) broth (Cole and Elkan, 1973; Sameshima et al., 2003) for 3 to 4 days at 28°C with continuous agitation at 120 rpm. Afterwards, the cultured bacteria cells were collected by centrifugation and washed with sterile distilled water. DNA extraction was done by using BL buffer as described by Minami et al. (2009) from the method reported by Hiraishi et al. (1995).

4. PCR amplification of 16S rRNA and ITS region

The amplification of 16S rRNA gene was conducted using *Ex Taq* DNA polymerase (TaKaRa Bio, Otsu, Shiga, Japan). A universal primer set for the 16S rRNA was used which were listed in the Materials and Methods section of Chapter 2. Then, the specific primer sets for the *Bradyrhizobium* (Bra-ITS-F: 5'-GACTGGGGTGAAGTCGTAAC-3' and Bra-ITS-R1: 5'-ACGTCCTTCATCGCCTC-3') from the report of Saeki et al. (2006) and *Sinorhizobium* (ITS1512F: 5'-GTCGTAACAAGGTAGCCGT-3' and ITSLS23R: 5'-TGCCAAGGCATCCACC-3') from the report of Hiraishi et al. (1997) were used for the ITS region.

5. Nodulation test for symbiosis

An inoculation test was performed to determine the nodulation capability of each individual isolate. Each isolate was cultured in YM broth (Vincent, 1970) for about 1 week at 28°C in the dark at 120 rpm, and the cultures were diluted with sterile distilled water to approximately 10^6 cells ml^{-1}. The soybean seeds were sown as described above in the Materials and Methods section of Chapter 2 but without soil, and inoculated with a 1^{-ml} aliquot of each isolate per seed with three replication. The nodule formation was evaluated after 4 weeks in a growth chamber under similar conditions described above. As control, a culture pot was prepared similarly without any inoculation.

6. RFLP treatment of 16S rRNA gene and ITS region

The RFLP analyses of the 16S rRNA and the ITS region were performed using the restriction enzymes *HaeIII, HhaI, MspI* and *XspI* (TaKaRa Bio). For reference, we used the *Bradyrhizobium* strains *B. japonicum* USDA 4, 6^T, 38, 122, 123, 124, 129, 135, *B. diazoefficiens* USDA110 ^{T}, *B. elkanii* USDA 31, 46, 76^T, 94, and 130 and *B. liaoningense* USDA 3622^T (Saeki et al., 2004). A 5.0 µl aliquot of the PCR product was digested with the restriction enzymes for 16 h at 37°C in a 20 µl reaction mixture. A 3 or 4% agarose gels in TBE buffer was used for the submerged gel electrophoresis to separate the restriction fragments then, stained with ethidium bromide and visualized with Luminiscent Image Analyzer (LAS-4000, Tokyo, Japan).

9. Species diversity

The diversity analysis was performed by Shannon-Wiener' index and Simpson's index as described previously (MacArthur 1965; Pielou 1969; Saeki et al., 2008) as well as the Simpson's index (Simpson, 1949).

Results

1. Soil fertility status of the sampling sites

In this report, all the soil samples were slightly to moderately acidic with non-saline condition. This is ideal for rice, as well as legumes. In terms of the nutrient status, all the soils have typically low fertility as can be seen from low N content, low to medium P, low to moderate K and low cation exchange capacity (CEC) as seen in Table 5.2. This result is typical from agricultural soils that are continuously used for cropping all throughout the year. The soil texture was mostly silty clay loam and clay loam which is also appropriate for the crops being planted in each location (Table 5.1).

2. Isolation of soybean rhizobia and nodulation test

We were able to obtain a total of 880 nodules (>2 mm size) of which only 771 grew and formed single colonies after streaking onto YMA plates containing Congo Red CR. A majority of the isolates (65.11%) were slow-growers (5-7 days) and produced alkaline substance on YMA plates containing BTB. The remaining 34.89% (269 out of 771) of the isolates were fast-growers (2-3 days) which produced acid substances on the BTB-containing media. The data of the nodule collection are presented in Table 5.3.

Table 5.2. Chemical properties of the soil samples used in this study.

Location	**N (%)**	**P (ppm)**	**K (ppm)**	**Ca (ppm)**	**Mg (ppm)**	**Na (ppm)**	**CEC (meq/100g)**
Ilagan, Isabela **(IS)**	0.13	1.86	51.80	205.5	39.3	51.4	1.71
Gamu, Isabela **(GI)**	0.17	2.30	58.60	242.1	115.9	98.0	2.74
Baguio, Benguet **(BA)**	0.24	22.22	51.00	470.6	30.5	45.4	2.93
Nueva Ecija1 **(NE1)**	0.13	6.74	73.90	389.5	98.0	17.1	3.01
Nueva Ecija2 **(NE2)**	0.22	21.63	49.40	344.9	93.2	36.0	2.77
Irosin, Sorsogon **(SO)**	0.22	2.57	55.80	215.8	52.2	86.8	2.03
Abuyog, Leyte **(LT)**	0.15	6.39	174.20	443.2	156.3	26.8	4.06
La Carlota, Negros Occidental **(NR)**	0.07	20.44	74.10	197.5	14.1	26.3	1.41
Ubay, Bohol **(BO)**	0.06	2.80	47.80	118.7	6.0	47.1	0.97
Sultan Kudarat, Maguindanao **(SK)**	0.19	4.53	59.60	370.0	241.1	15.3	4.05
Tupi, South Cotabato **(SC)**	0.14	31.18	47.20	148.4	9.7	6.8	0.97

Table 5.3. List of sampling sites and the number of nodules collected from using the Philippines soybean cultivar PSB-SY2.

No.	Location	Nodules collected (no.)
1	Ilagan, Isabela **(IS)**	79 (80)
2	Gamu, Isabela **(GI)**	67 (80)
3	Baguio, Benguet **(BA)**	70 (80)
4	Nueva Ecija1 **(NE1)**	72 (80)
5	Nueva Ecija2 **(NE2)**	70 (80)
6	Irosin, Sorsogon **(SO)**	77 (80)
7	Abuyog, Leyte **(LT)**	70 (80)
8	La Carlota, Negros Occidental **(NR)**	64 (80)
9	Ubay, Bohol **(BO)**	64 (80)
10	Sultan Kudarat, Maguindanao **(SK)**	63 (80)
11	Tupi, South Cotabato **(SC)**	75 (80)
	Total	**771 (880)**

Note: The numbers written inside the parenthesis refers to the total number of nodules collected per location and the numbers outside the parenthesis refers to the isolates from all the nodules per location which were able to grow on YMA media with Congo Red.

The single-strain nodulation test showed that the 550 isolates (71.34% of the 771 isolates) were able to form nodules on soybean while the remaining 221 isolates (28.66%) did not form nodules. The control pots (un-inoculated) also did not form any nodules with soybean which eliminated the possibility of contamination in this test. The data on the nodule formation by the single strain and the co-inoculation test is presented in the Table 5.4.

3. Cluster analysis and species diversity of nodulating soybean rhizobia

The distribution of the different clusters of bradyrhizobia that nodulate soybean in the Philippines is presented in Figure 5.1 and it is clear that all throughout the country, the species of *Bradyrhizobium* genus were the most dominant. In the country, 73.64% of the soybean-nodulating rhizobia (405 out of 550 isolates) were classified under the three species of bradyrhizobia, which were *B. japonicum*, *B. diazoefficiens*, and *B. elkanii*. The isolates which were classified under *S./Ensifer* species comprised only 8.73% of the total population (48 out of 550) while the remaining 18.36% (97 out of 550) of the isolates did not belong to a particular genus according to the RFLP patterns from the 16S rRNA gene.

Meanwhile, the phylogenetic tree of the soybean-nodulating rhizobia based from the RFLP patterns of the 16S rRNA gene is presented in Figure 5.2. In this dendrogram, 39 representative isolates from the 11 locations showed the prevalence of the *Bradyrhizobium* species and were distinguished from the *S./Ensifer* species and those isolates which were not classified in either of the two genera of rhizobia which is referred to in this manuscript as Not in Reference (NR). The species diversity of the soybean-nodulating rhizobia is shown in Table 5.4 and it indicated that the species diversity as calculated from two diversity indices (Shannon's and Simpson's) is highest in rice-based (R) cropping systems, followed by soybean-based (S) then the least diversity of rhizobia species was observed at the locations which were planted with different legumes (L).

Based on location, Nueva Ecija2 (NE2), which is a rice-based system obtained the highest indices which are $H' - 0.87, Eh - 0.79, D - 0.55$ while the lowest was found in two locations which are Ilagan, Isabela (IS) and Baguio, Benguet (BA) wherein only *Bradyrhizobium* species were found.

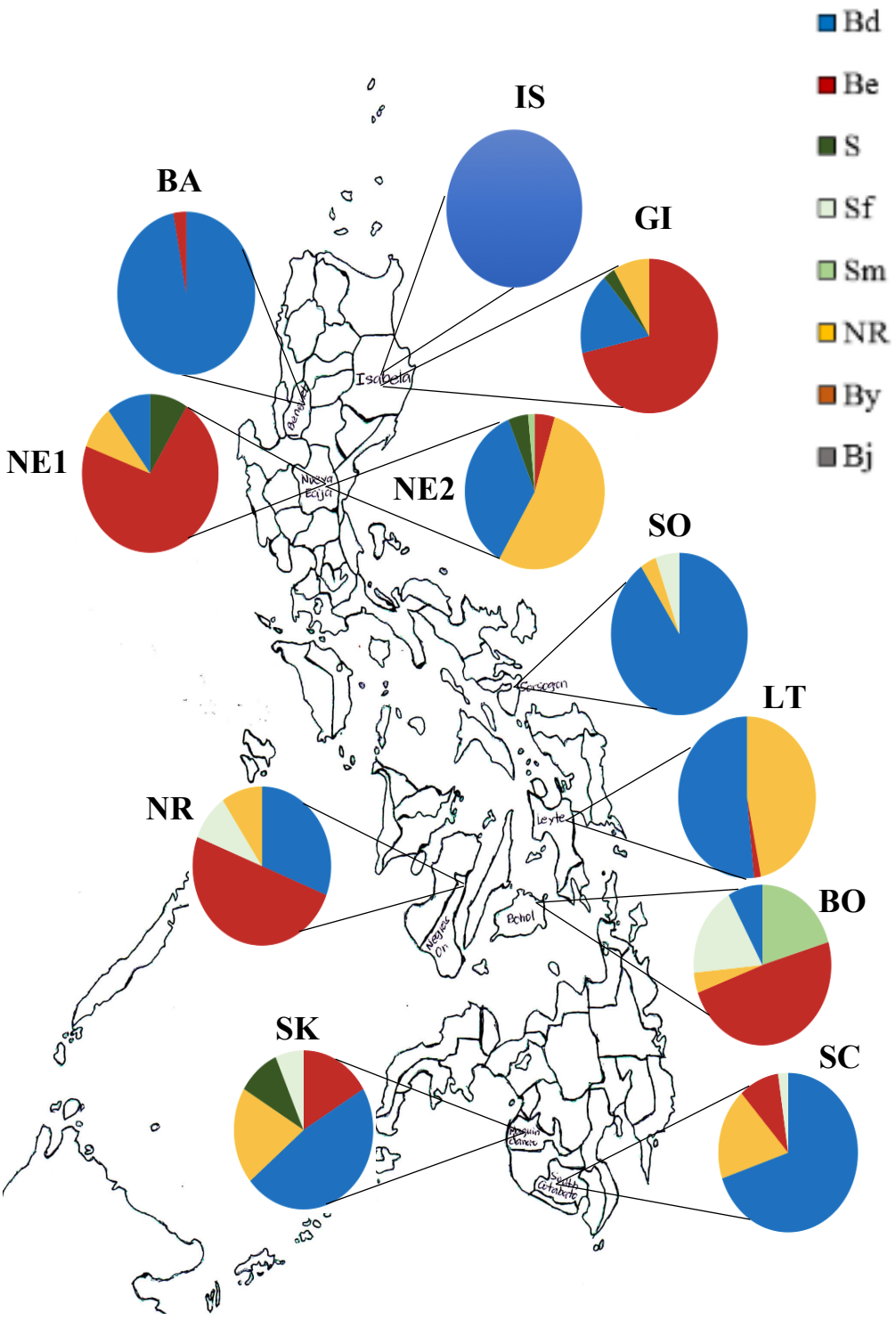

Figure 5.1. Geographic distribution of the soybean-nodulating bradyrhizobia in the Philippines from the result of Restriction Fragment Length Polymorphism (RFLP) and sequence analysis of the 16S rRNA gene.

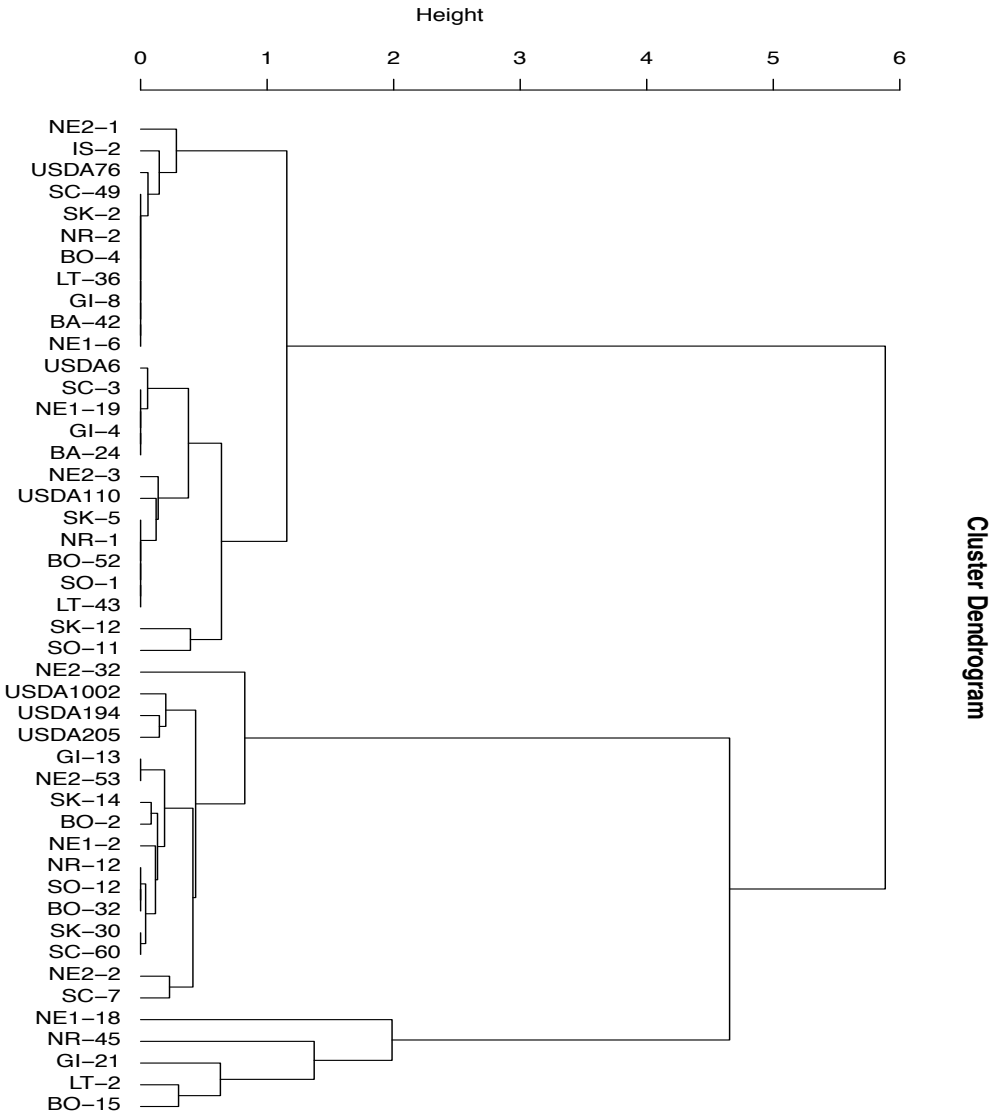

Figure 5.2. Phylogenetic tree based on the Restriction Fragment Length Polymorphisms (RFLP) band patterns digested with four restriction enzymes *HaeIII*, *HhaI*, *XspI* and *MspI*. The tree was constructed with the ward.D2 method in R software v. 3.5.0.

Table 5.4. Diversity and equitability of nodulating soybean rhizobia in the Philippines as calculated with Shannon-Wiener and Simpson's Indices. The Shannon's diversity (*H'*) and equitability (*Eh*) indices were computed with the formulae ($H' = -\sum Pi \ln Pi$; $Eh = H' / \ln S$). The Simpson's (*D*) index was calculated with the formula ($D = 1-(\sum n(n-1))/ N(N-1)$).

Cropping pattern	**Soybean – based (S)**					**Legume – based (L)**				**Rice – based (R)**	
Location	**IS**	**BA**	**LT**	**NR**	**BO**	**GI**	**NE1**	**SO**	**SK**	**NE2**	**SC**
Bradyrhizobium	40	33	33	34	28	31	55	48	43	26	34
Sinorhizobium	0	0	0	4	19	1	6	3	10	4	1
Not in reference (NR)	0	0	29	4	2	3	6	2	8	35	8
Total	40	33	62	42	49	35	67	53	61	65	43
H'	0.00	0.00	0.69	0.62	0.82	0.42	0.59	0.38	0.81	0.87	0.59
E_H	0.00	0.00	1.00	0.56	0.74	0.38	0.54	0.34	0.74	0.79	0.53
D	0.00	0.00	0.51	0.33	0.53	0.21	0.31	0.18	0.47	0.55	0.35

Cropping pattern: S – main crop is soybean, with some intercrop of leafy vegetables and other legumes; **R** – main crop is rice, planted twice in a year; **L** – usually planted with different legumes such as soybean, mung bean, cowpea, string beans, and other legumes during dry season then planted with rice during the wet season.

Note: The *Pi* is the dominance of the isolate, expressed as (*ni/N*), where *N* and *ni* are the total number of isolates tested and the number of isolates belonging to a particular cluster, respectively. *S* is the total number of clusters, indicating the taxonomic group, at each field site. The *n* is the number of individuals displaying one trait while *N* is the total number of individuals.

4. Species diversity of the non-nodulating rhizobia

For the 221 isolates which were not able to form nodules on soybean at single inoculation, a phylogenetic tree was not constructed. Hence, it is only presented in this manuscript the diversity of the species of the non-nodulating rhizobia according to the RFLP patterns of the 16S rRNA gene (Fig. 5.5).

It can be noticed that for the non-nodulating rhizobia, most isolates were deemed to be classified under the *S.*/*Ensifer* genus which comprised 65.15% (144 out of 221) isolates while those that were classified under the *Bradyrhizobium* genus only comprised 3.17% (7 out of 221). Some isolates which comprised 31.67% (70 out of 221) did not belong to either of the two genera of rhizobia.

In terms of the location, the legume-based (L) system which was located in Irosin, Sorsogon (SO) obtained the highest diversity of non-nodulating rhizobia at *H'* – 0.69, *Eh* – 1.00, *D* – 0.50 while the four locations which are: Abuyog, Leyte (LT); Ubay, Bohol (BO); Nueva Ecija1 (NE1) and Sultan Kudarat, Maguindanao (SK) showed a similarly low diversity at *H'* – 0.00, *Eh* – 0.00, *D* – 0.00. Regarding the population of the non-nodulating rhizobia, the highest was found on soybean-based (S) cropping system (53.39%), followed by legume-based (L) which comprised 31.22% and the lowest was at rice-based (R) cropping system with 15.39%.

Table 5.5. Diversity and equitability of non-nodulating soybean rhizobia in the Philippines as calculated with Shannon-Wiener and Simpson's Indices. The Shannon's diversity (*H'*) and equitability (*Eh*) indices were computed with the formulae ($H' = -\sum Pi \ln Pi$; $Eh = H' / \ln S$). The Simpson's (*D*) index was calculated with the formula ($D = 1-(\sum n(n-1))/ N(N-1)$).

Cropping pattern	**Soybean – based (S)**					**Legume – based (L)**				**Rice – based (R)**	
Location	**IS**	**BA**	**LT**	**NR**	**BO**	**GI**	**NE1**	**SO**	**SK**	**NE2**	**SC**
Bradyrhizobium	0	0	0	5	0	1	0	0	0	1	0
Sinorhizobium	38	36	0	4	0	29	5	13	0	0	19
Not in Reference (NR)	1	1	8	13	15	2	0	11	2	4	13
Total	39	37	8	22	15	32	5	24	2	5	32
H'	0.12	0.10	0.00	0.50	0.00	0.12	0.00	0.69	0.00	0.50	0.56
E_H	0.17	0.09	0.00	0.36	0.00	0.11	0.00	1.00	0.00	0.46	0.51
D	0.05	0.11	0.00	0.59	0.00	0.18	0.00	0.50	0.00	0.38	0.66

Cropping pattern: S – main crop is soybean, with some intercrop of leafy vegetables and other legumes; **R** – main crop is rice, planted twice in a year; **L** – usually planted with different legumes such as soybean, mung bean, cowpea, string beans, and other legumes during dry season then planted with rice during the wet season.

Note: The *Pi* is the dominance of the isolate, expressed as (*ni/N*), where *N* and *ni* are the total number of isolates tested and the number of isolates belonging to a particular cluster, respectively. *S* is the total number of clusters, indicating the taxonomic group, at each field site. The *n* is the number of individuals displaying one trait while *N* is the total number of individuals.

Discussion

1. Diversity of soybean-nodulating rhizobia as impacted by the cropping patterns

As shown in the results above, the diversity of rhizobia species which can nodulate soybean in the Philippines as obtained from the 16S rRNA gene RFLP analysis might be considered as moderately high. Although the 16S rRNA gene is highly conserved especially in *Bradyrhizobium* species (Vos et al., 2012), it was not enough to accurately separate the different species so the computation of diversity was calculated based only on whether the species were clustered under *Bradyrhizobium*, *S./Ensifer*, or neither of the two major genera of rhizobia.

Interestingly, the rice-based (R) cropping system showed the highest diversity (*H'* – 0.87) which could be attributed in the soil management practices. Previously, we have reported that the Philippines is dominated by the *B. elkanii* species which was basically due to the slight to moderate soil pH, non-saline condition, high temperature, and non-flooded condition (Mason et al., 2017, 2018). This result was also observed in several studies where *B. elkanii* dominated the areas with the same agro-environmental conditions (Bizarro et al., 2011; Shiro et al., 2013,).

In a report by Ferreira et al. (2000), the diversity of bradyrhizobia which were evaluated according to the tillage methods and crop rotation system showed that no-tillage and crop rotation systems provided a higher population and diversity of the species. In contrast, the area which underwent a conventional tillage method showed the least diversity. Another study about different soil managements in Brazil was able to obtain a very high genetic diversity of bradyrhizobia through serological typing of 75 isolates at *H'* – 5.46, and through the 16S rRNA gene, they were able to identify the dominance of *Bradyrhizobium* species which are *B. japonicum* and *B. elkanii* (Bizarro et al., 2011).

In another report which investigated the diversity of rhizobia in an agricultural-forestry ecosystem, it showed a variety of rhizobia which belonged to both the *Bradyrhizobium* and *Rhizobium* genera and the diversity varied dramatically according to the species and hosts of origin (Wang et al., 2009). Meanwhile, fields from South Brazil which were used for the commercial production of soybean and with frequent rhizobia inoculation, a high diversity of bradyrhizobia (*H'* – 6.17) was obtained owing to the influence of soil pH, followed by the clay and organic matter (Giongo et al., 2008). Additionally, it was reported that in the different agro-climatic regions of Ethiopia, the soils also contained a high diversity of bradyrhizobia in some locations which was attributed to the high available P content and the lower diversity on some locations was attributed to the high CEC in the soil (Jaiswal et al., 2016).

In a study that compared the diversity of *Rhizobium leguminosarum* biovar *viciae* from arable soils and grass soils, it was noted that the high genetic diversity was indeed influenced by the soil management practices (Palmer and Young, 2000). Their study showed that the arable lands which were subjected to repeated cultivation provided a higher diversity of rhizobia than the less cultivated soils, which could be the same case in our study such that the rice-based system were more frequently cultivated than the legume-based and the soybean-based system.

Meanwhile in Kenya, it was observed that the different cropping patterns which included a maize and soybean in intercropping, rotation or monocropping with different fertilization strategies obtained a very low diversity of rhizobia which were identified to be *B. elkanii* and that the rhizobial diversity was not affected by the cropping systems nor the nitrogen fertilization (Herrmann et al., 2014). This is an indication that the driving force for the diversity of rhizobia differs across a wide variety of regions.

2. Diversity of non-nodulating rhizobia in the Philippines

In this report, we obtained a considerable number of rhizobia that were not able to form nodules on soybean upon single-inoculation (28.66% of the total rhizobial population). Since most research on soybean rhizobia were conducted on isolates that can have a good symbiotic relationship with the host plant, diversity studies of rhizobia which do not possess this quality were seldom performed. However, these non-nodulating rhizobia, which lack the key loci (nodulation genes) to induce the process of nodulation, also exist naturally in soils. In some regions in America, nonnodulating rhizobia were even observed to be dominant in the soil populations (Laguerre et al., 1993; Pongsilp et al., 2002; VanInsberghe et al., 2015; Hollowell et al., 2016).

The importance of the nonnodulating rhizobia lies in the fact that their presence in the nodule may decrease the occupancy of the nodulating rhizobia (Winarno and Lie, 1979; Singh and Ahmad, 1991). In a study by Gano-Cohen et al. (2016), the nonnodulating rhizobia did not affect the growth of the legume plant when inoculated in isolation. However, co-inoculating the symbiotic strains with the nonnodulating strains reduced the host performance in comparison to the inoculation of only the symbiotic strains. Their results indicated that the nonnodulating rhizobia may play an important role in modulating the symbiosis between the legume host and the rhizobia. This inter-strain competition within the root nodule might have a specific mechanism that has to be investigated further because of the high density of the nonnodulating rhizobia in the soil.

Summary

This report aimed to determine the diversity of the nodulating and nonnodulating rhizobia in the Philippines under different cropping patterns. This was done through the

PCR-RFLP analysis of the 16S rRNA gene and the 16S-23S rRNA gene ITS region. The species diversity on the rice-based system was the highest for the nodulating rhizobia while the lowest diversity was found on soybean-based system. Meanwhile, the nonnodulating rhizobia showed the highest species diversity for legume-based system while the lowest was found on soybean-based system. Our results indicated that the presence of nonnodulating rhizobia is highest for legume-based system that have the least cultivation management while the nodulating rhizobia was highest in rice-based system which are frequently cultivated. The lowest presence of both the nodulating and nonnodulating rhizobia in the soybean-based system indicated that monocropping could lead to lesser diversity of rhizobia.

In this result, we found that a majority of the soybean-nodulating rhizobia belong to *Bradyrhizobium* genus while the non-nodulating rhizobia belong to the *Sinorhizobium* genus. For both the genera of rhizobia, the least diversity was found on soybean-based system which was basically a monocrop system.

Chapter 6

General Discussion

Rhizobia are an interesting group of soil microorganisms. Their presence in the soil is not only important to maintain soil health and biodiversity, but also essential for their role in crop production for food security and sustainability. Although these bacteria has been well studied for a long time, their evolution and adaptation to a wide variety of geographical, agricultural, and environmental gradients continuously fascinates the scientific community.

As illustrated in Figure 6.1, the symbiotic relationship between rhizobia and the host legume provides an efficient mutual interaction wherein the bacteria supplies the N needed for the plant's growth and development by N fixation and in return, the photosynthetic products from the plant such as carbohydrates are being supplied as food for the bacteria. Also, it can be noted that a rhizobium is not only important for its ability to establish a symbiotic relationship with the host legume. It has also a vital role for its function in the release of nitrous oxide (N_2O), a potent greenhouse gas to the atmosphere. Human activities, particularly agricultural activities, is reported to be the single largest source of anthropogenic N_2O emission (Reay et al., 2012). Recently, a study by Akiyama et al. (2016) showed that inoculating the soil with an indigenous *nosZ*+ strains has high potential to mitigate N_2O emission from soybean ecosystems. The *nosZ* gene is a nitrous oxide reductase which is responsible for the conversion of N_2O to N_2 and is known to be present in some strains of *B. diazoefficiens* species. (Breitenbeck and Bremner, 1989; Zumft, 1997; Sameshima-Saito et al., 2004, 2006). Thus, rhizobia is not only necessary in food production and soil restoration, but also in climate change mitigation.

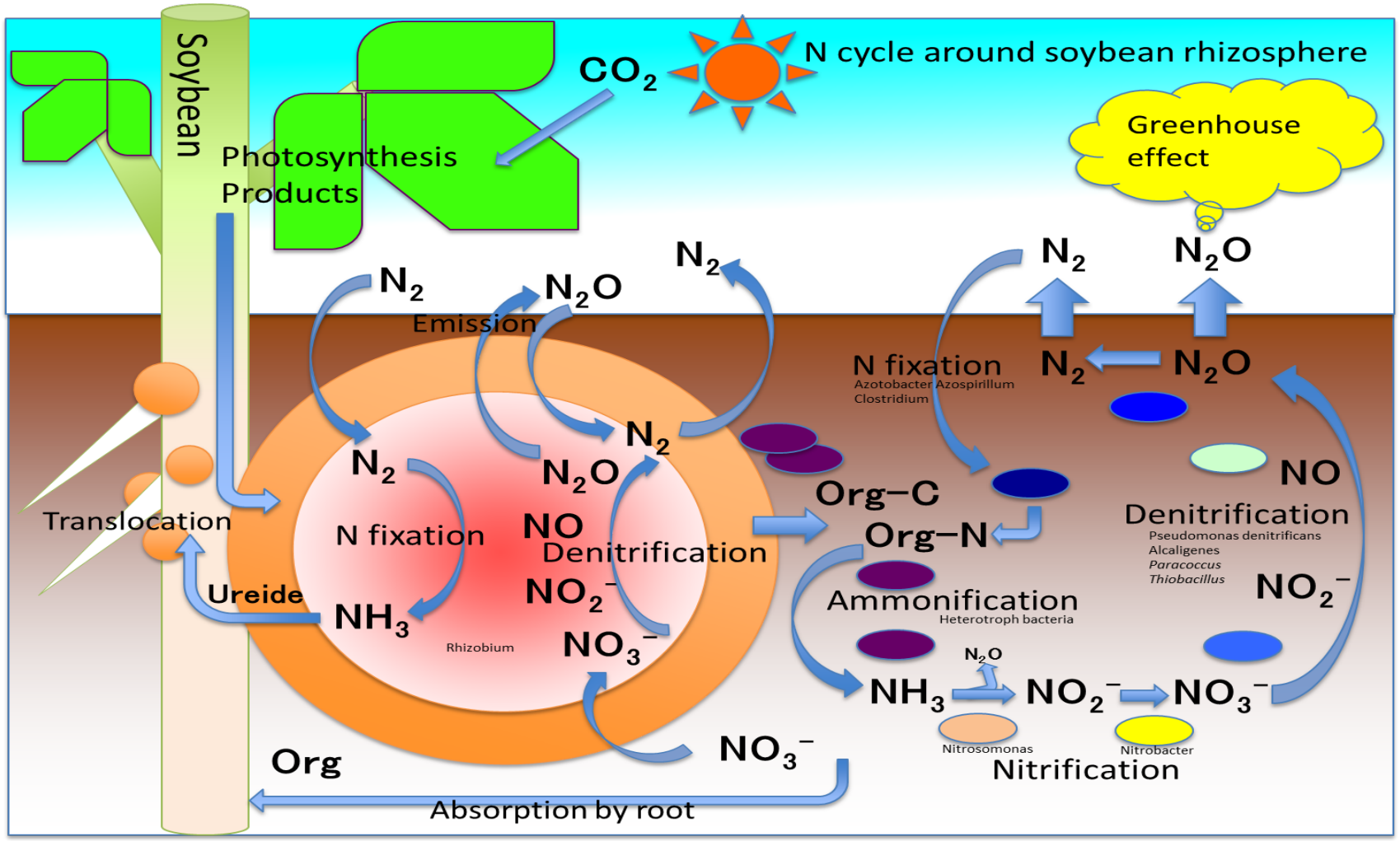

Figure 6.1. The soybean rhizosphere showing the interactions between the various microorganisms in the soil and the N cycle. (Figure drawn by Yuichi Saeki, Faculty of Agriculture, University of Miyazaki).

In this study, which was composed of four experimental chapters, we were able to elucidate that the endemism and diversity of soybean rhizobia in a tropical region is different form the well-studied temperate regions. First, we obtained this information firsthand on the 2nd chapter of this manuscript, where the change in temperature among the three sampling locations provided an insight that *B. elkanii* species tend to adapt differently in higher temperatures than in lower temperatures. Although the presence of this species could be found in a wide variety of regions with acidic to moderately acid soils, its intra-strain diversity tends to become higher with high temperature regions (Mason et al., 2017).

Then, in order to further verify this observation, more locations in the Philippines were included for the second experiment wherein 11 locations from the northern to the southern regions in the country were obtained and used to isolate the indigenous rhizobia. In here, it was further elucidated that the diversity and distribution of bradyrhizobia in a tropical region like the Philippines is indeed different from the temperate regions like Japan and USA. The previous reports which studied the diversity of soybean rhizobia in USA (Shiro et al., 2013) showed that the *B. japonicum* species are dominant in the north while *B. elkanii* species are dominant in the south and in Japan (Saeki et al., 2006) which showed a similar result. Additionally, the prevalence of *B. diazoefficiens* were observed on the middle regions of Japan. However, we found that in case of the Philippines, there was no such clear pattern of distribution that were observed from north to south. One reason is that the temperature in the country was almost similar everywhere (ave. - 27°C) except for on location, which is Baguio City (ave. - 18°C). The similarity in temperature, soil pH and EC seemed to be the most influential factors for the abundance of *B. elkanii* in Philippines soil. Yet, an interesting observation was that we detected a high correlation between the relationship of flooding

condition in the soil and the distribution of different species of bradyrhizobia. The dominance of *B. japonicum* and *B. diazoefficiens* strains on specific locations correlated with the longer periods that the soil was in flooded condition while the dominance of *B. elkanii* in some locations was attributed to the non-flooded condition of the soil (Mason et al., 2018). Additionally, it was noted that upon closer investigation between the dominance of the *B. japonicum* and *B. diazoefficiens*, we found that the *B. japonicum* strains similar to the Bj6 cluster are prevalent on soils with high amount of silt and phosphorus. Moreover, the prevalence of the *B. diazoefficiens* was higher at flooded soils with higher clay content. These observations were similar with other reports (Shiina et al., 2014; Saeki et al., 2017; Siquiera et al., 2017) wherein it was reported that the *B. diazoefficiens* tends to be prioritized in a more anaerobic condition than the *B. japonicum* due to the presence of the low efficiency of NO_3- reduction.

Thus, we were able to collect and identify some indigenous strains which are similar to the symbiotically efficient *B. diazoefficiens* USDA110 as well as the other species. In order to test the hypothesis that the Bd110 cluster strains would have the same high symbiotic efficiency in the Philippines, we conducted a single strain inoculation test of selected indigenous strains against the USDA110 reference strain. Unfortunately, we found that the indigenous strains of Bd110 cluster were not the most efficient for the Philippines soybean cultivars that harbor the non-*Rj* and *Rj*$_4$ genotypes. Instead, we observed that the indigenous *B. elkanii* strain and some potentially novel *Bradyrhizobium* sp. strains were more efficient for the tropical soybean plants. However, it is interesting to note that for the temperate soybean cultivars from Japan, the indigenous *B. diazoefficiens* strain was the most efficient. It seemed that for the tropical regions, *B. elkanii* would provide a better symbiosis with the tropical soybeans. It might be because *B. elkanii* species are more abundant in tropical regions and more

adaptive to higher temperatures than the *B. diazoefficiens*. It was reported by Hungria and Vargas (2000) from their previous research that at temperature above 39°C, a decrease in the release of *nod*-gene inducers from soybean and common bean roots was observed.

In relation to the diversity of rhizobia species, we were able to gather interesting data for both the nodulating and non-nodulating soybean rhizobia in the Philippines. As reported in the discussion part of Chapter 5, more than 26% of the total rhizobial population (771) did not possess the ability to nodulate the soybean upon single inoculation. When the diversity of the different species where considered, it was found that the highest diversity of soybean-nodulating rhizobia were found on rice-based system, followed by the legume system, then the least was on the soybean-system. On the other hand, the non-nodulating rhizobia have the highest diversity in the legume-based system and the lowest was found on the soybean-based system. These indicate that for the nodulating rhizobia, continuous and frequent cultivation could be a factor in their diversification, while for the non-nodulating rhizobia, least cultivation provided for the highest diversity. This result has been similarly observed in some studies (Ferreira et al., 2000; Palmer and Young, 2000; Bizarro et al., 2011; Herrmann et al., 2014).

Adhikari et al. (2012) reported that in Nepal, the following dominance of bradyrhizobia was observed in sub-tropical soils; *B. elkanii* for acid soils, *B. yuanmingense* for moderate acidity and *B. liaoningense* for slightly alkaline condition and it was noted that *B. japonicum* was the dominant species in temperate region. However, in the case of the Philippines' Baguio City, which is classified as a sub-tropical highland climate under the Köppen climate classification, the most abundant species was the *B. japonicum* with a minor population that belonged to *B. elkanii*. Since

the pH of the soil in the said location was only slightly acid, it is thought in case of the Philippines, temperature played a major role for the presence of *B. japonicum*.

It was previously noted that soils which contain higher amounts of clay and silt also harbor a greater diversity of rhizobia (Sessitsch et al., 2002). A report also showed that soils with high CEC will have lower biodiversity and in this particular study, a positive relationship between the amount of P in the soil and rhizobia diversity was found (Jaiswal et al., 2016), which is also similar with our results that is reported in Chapter 3. However, a result by Palmer and Young (2000) did not indicate a significant influence of nutrient contents on the rhizobia diversity from the ITS region analysis.

In summary, this study was able to provide some similar and new insights on the diversity, distribution, and endemism of soybean rhizobia in a tropical region, with emphasis on a tropical archipelago like the Philippines. Our future prospects will include the utilization of the isolated indigenous strains for their practical application in the field as potential inoculants in Philippines' local conditions and their compatibility with the different soybean varieties in the country. This study is the first report that focused on the genetic characterization and symbiotic efficiency evaluation of the indigenous soybean rhizobia along the 11 locations in the Philippines and would provide a valuable information for future related research.

Abstract

The ultimate goal of this study is to help improve the soybean production in the Philippines without the use of chemical fertilizers. This could be done through the utilization of the indigenous rhizobia in the soil which can establish a symbiotic relationship with the leguminous plants, particularly for soybean. This complex and specific relationship allows the biological nitrogen fixation to take place inside a nodule

on the soybean root wherein the rhizobia provides the nitrogen requirement of the plant and in return, the plant provides the energy needed by the rhizobia. However, inoculation does not always succeed due to several reasons such as the plant-rhizobia incompatibility, factors that affect the diversity, abundance, and distribution of the effective and efficient rhizobia, as well as the diverse agro-environmental conditions that affect both the rhizobia and the plant. These kind of studies about soybean rhizobia in the Philippines is still limited and accurate information is scarce. So, this study was conceptualized to answer these limitations.

In this study, one location in the Philippines was first used in comparison to the southern regions of Japan. According to the result, it was necessary to obtain more information from different location in the country to accurately represent the diversity of rhizobia. Through the Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) and sequence analysis of chromosomal and symbiotic genes, the diversity and endemism of soybean rhizobia were elucidated. Furthermore, the factors that influence the diversity and distribution of soybean rhizobia in specific locations were also understood.

In general, this study was able to identify the most abundant and widely-distributed rhizobia in the Philippines, obtained some potential novel species, as well as understood the factors that influence the diversity and endemism of these indigenous rhizobia in the country. In addition, the strains' symbiotic efficiency and N-fixation ability were quantified that would be useful in field application. Overall, the specific goals of each individual study in this research manuscript were all achieved.

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To my father, who died before I was able to make him proud, this is for you.

Maria Luisa Tabing Mason

University of Miyazaki

March, 2019

Appendix

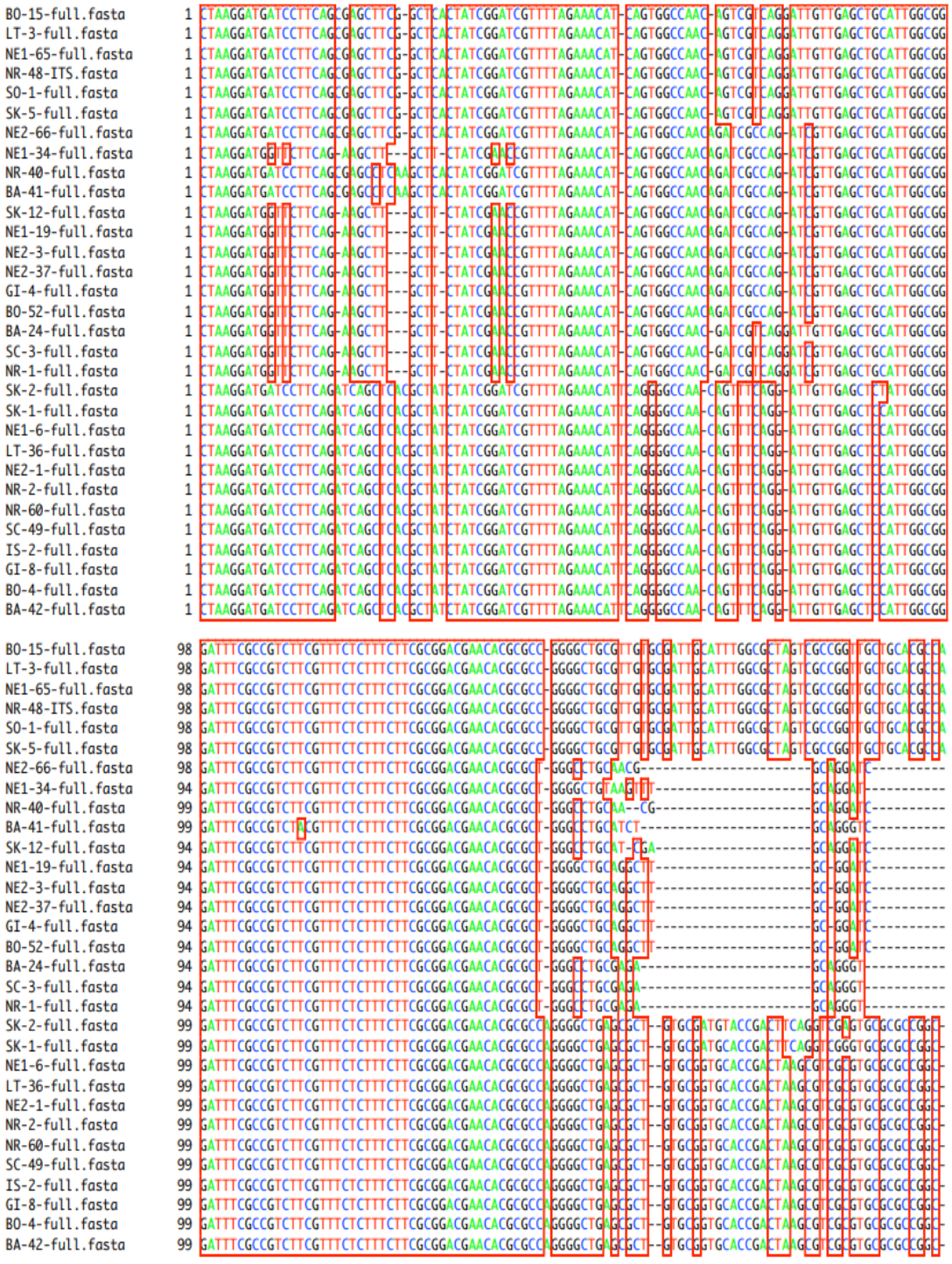
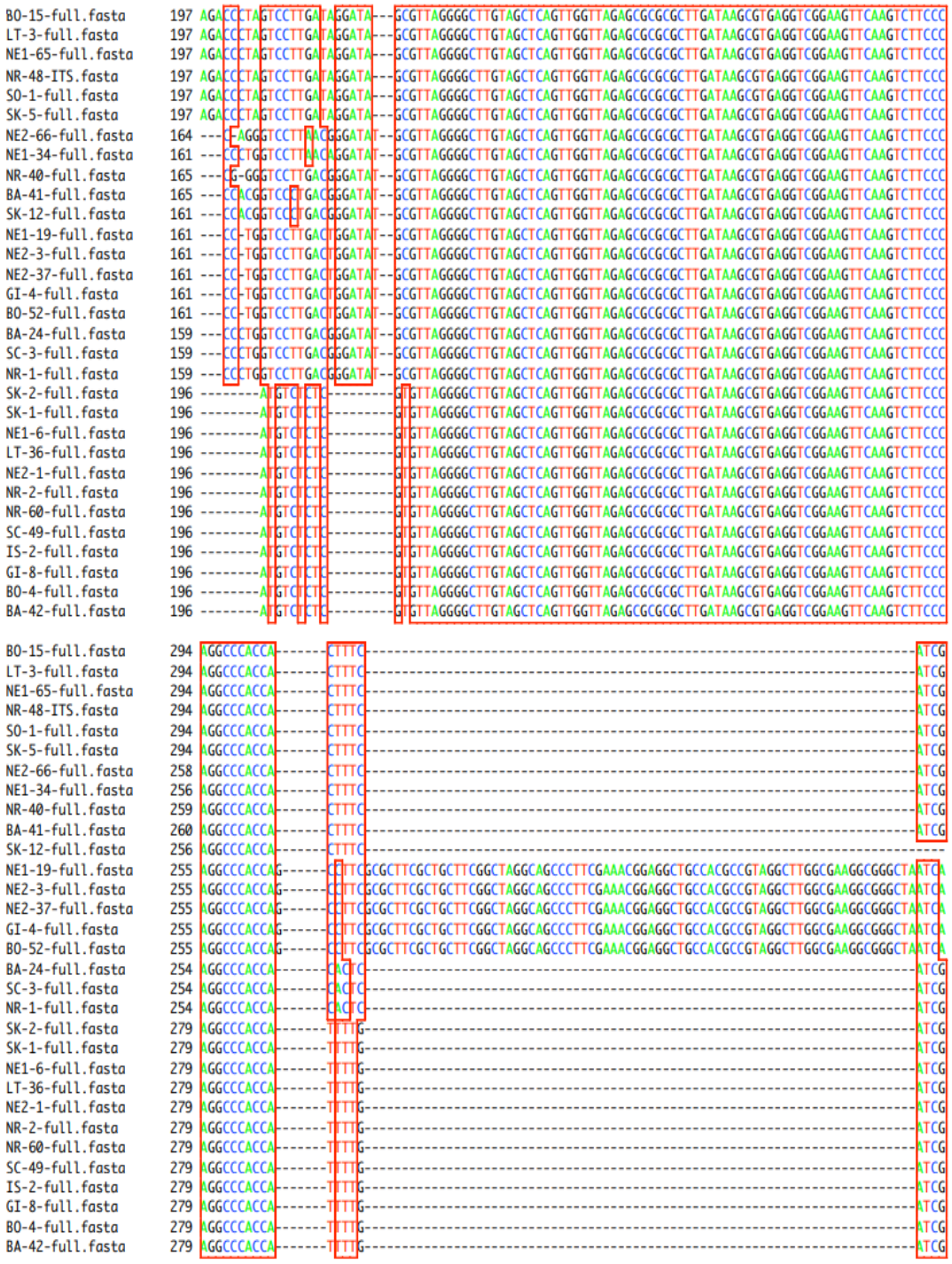


Fig. S1. Alignment of the indigenous soybean-nodulating bradyrhizobia in the Philippines from the 16S-23S rRNA ITS region.



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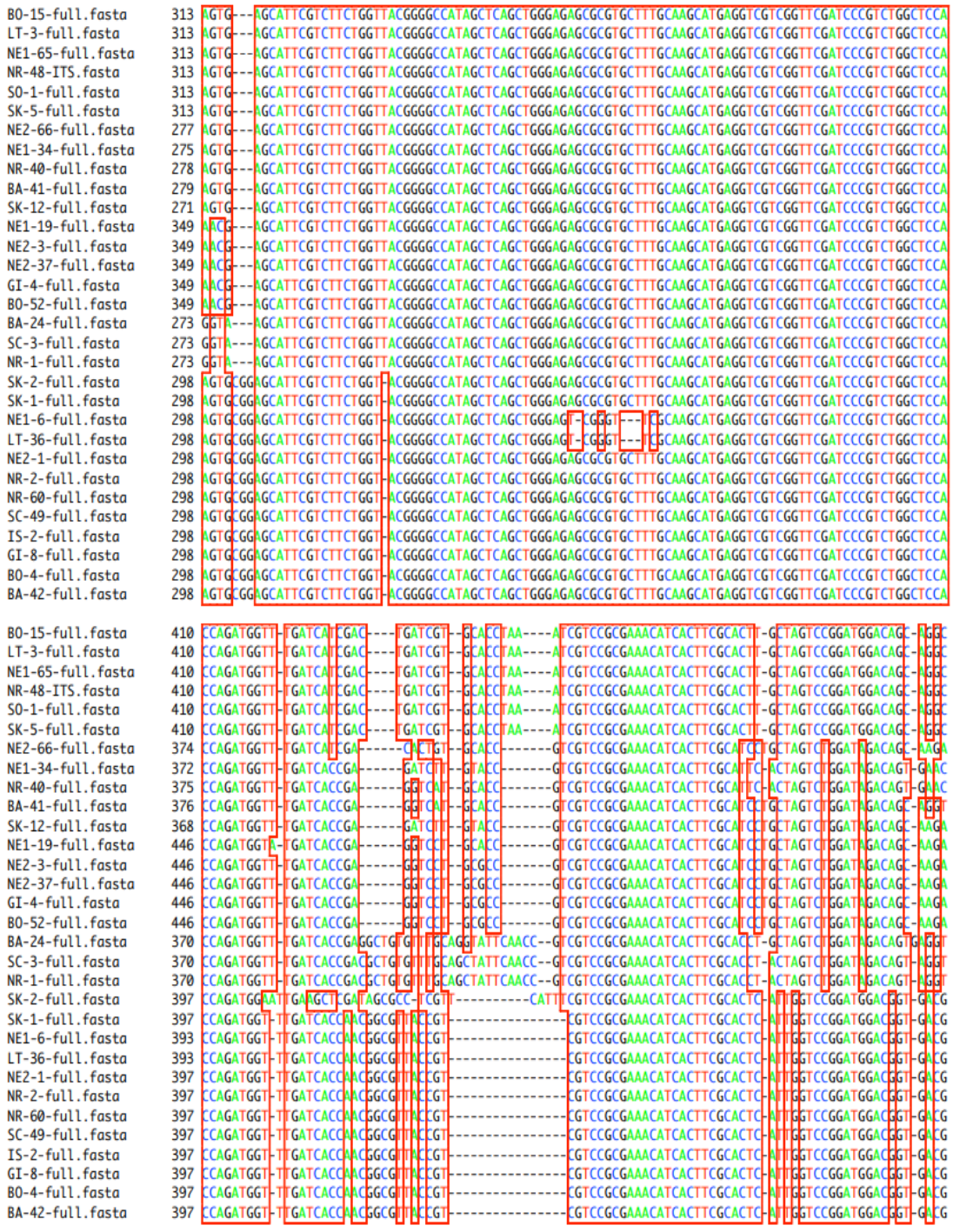

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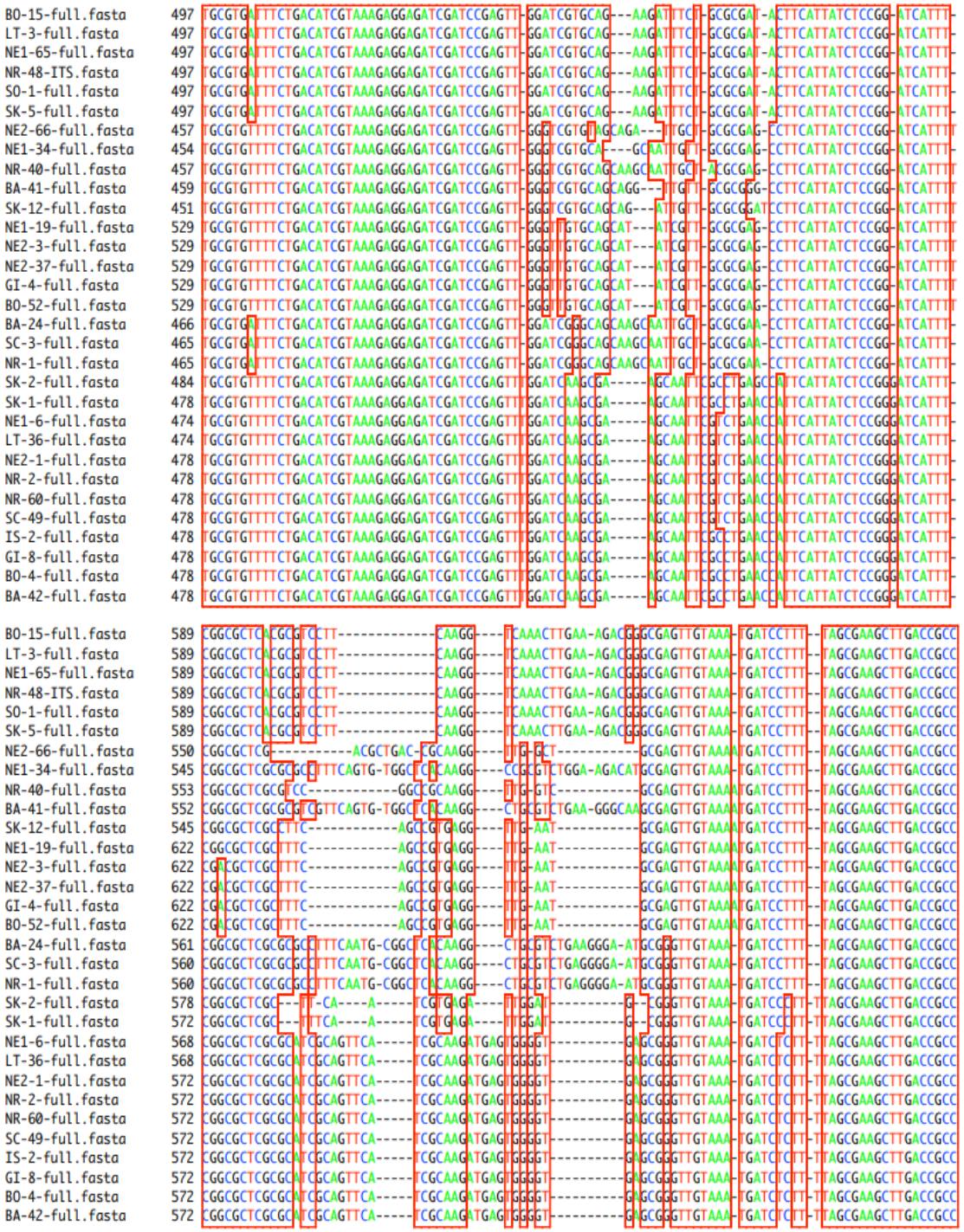

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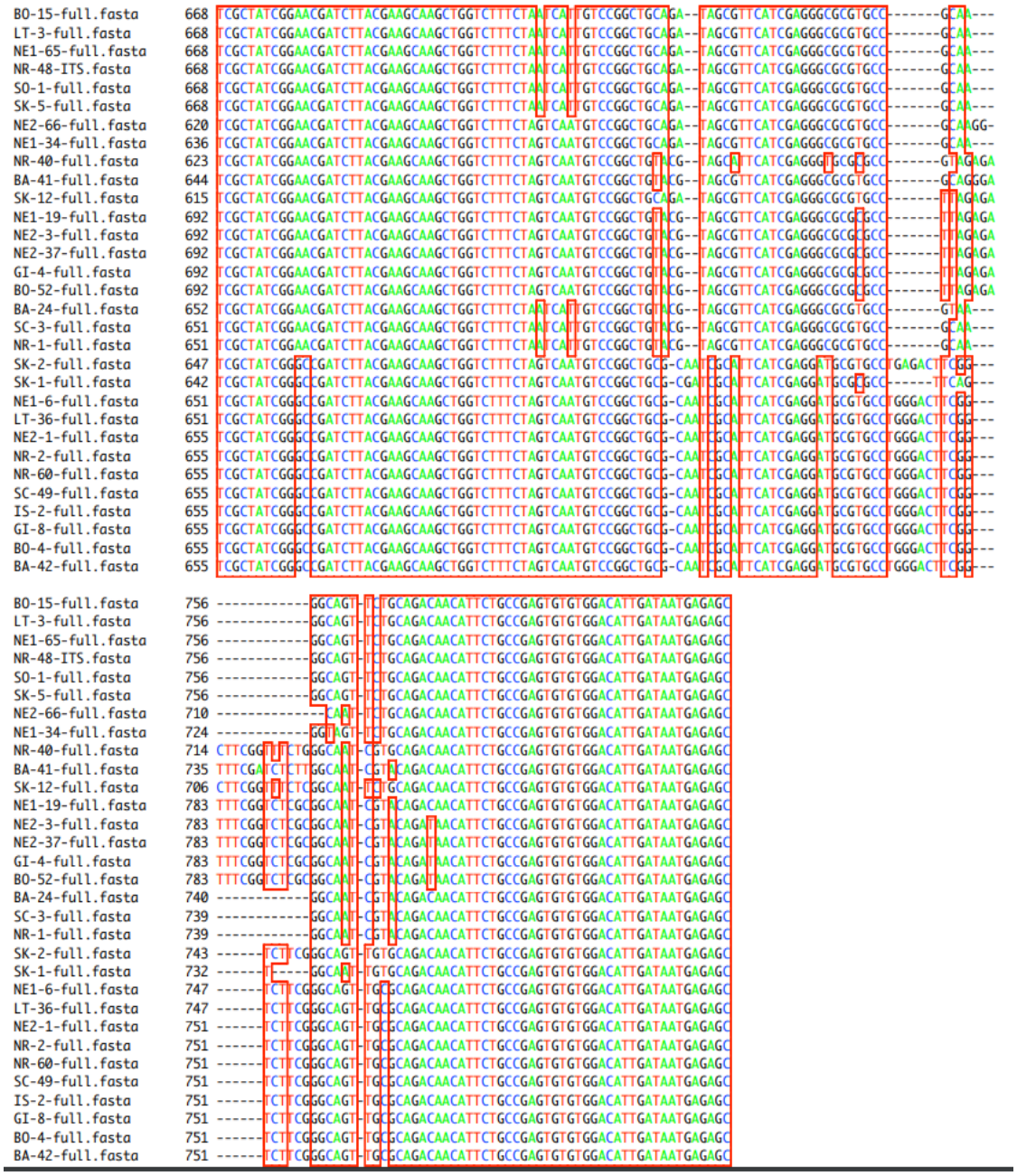

Fig. S1 (continued). Alignment of the indigenous soybean-nodulating bradyrhizobia in the Philippines from the 16S-23S rRNA ITS region.

Fig. S2. Alignment of the indigenous soybean-nodulating bradyrhizobia in the Philippines from the *rpoB* housekeeping gene.

Fig. S2 (continued). Alignment of the indigenous soybean-nodulating bradyrhizobia in the Philippines from the *rpoB* housekeeping gene.

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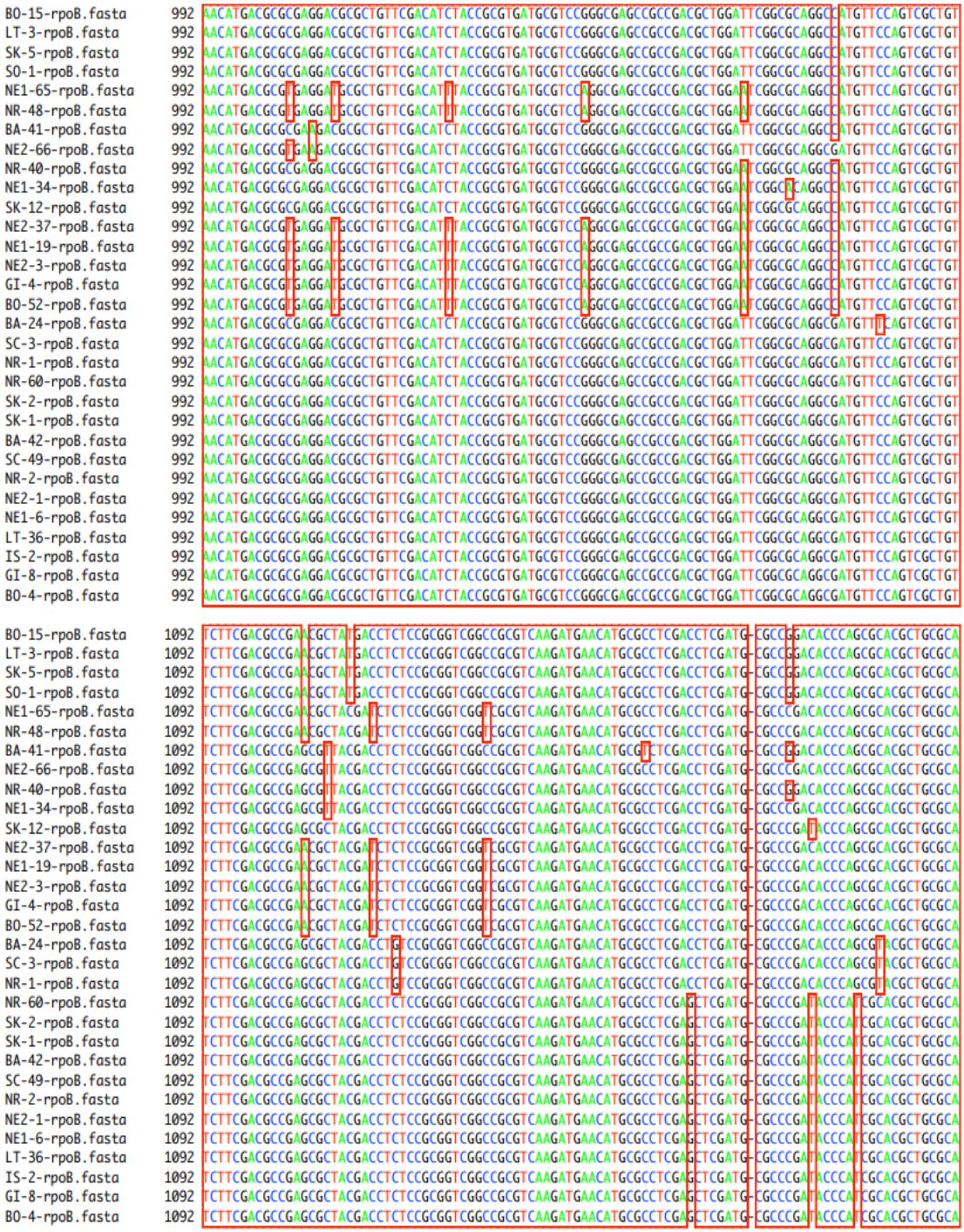

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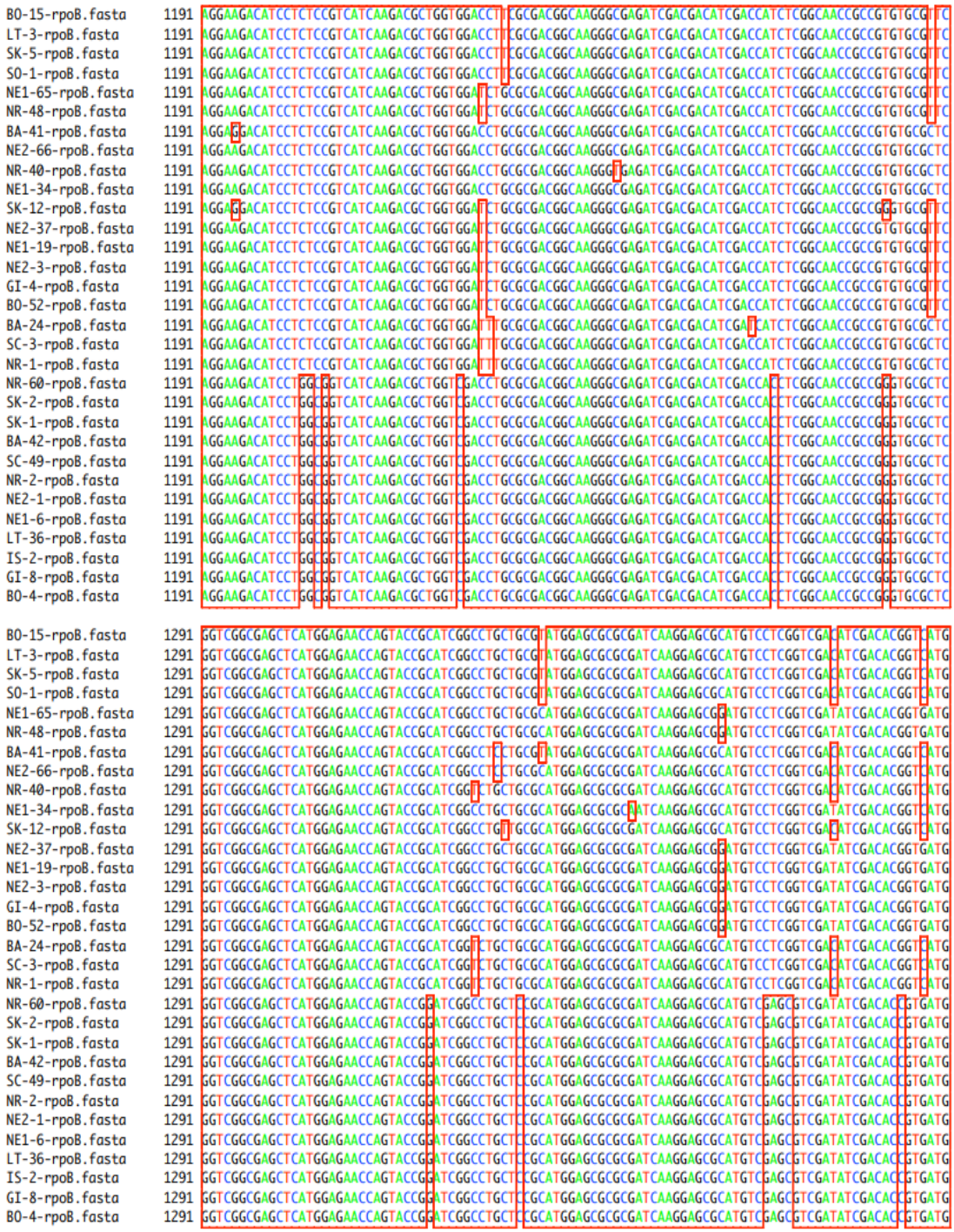

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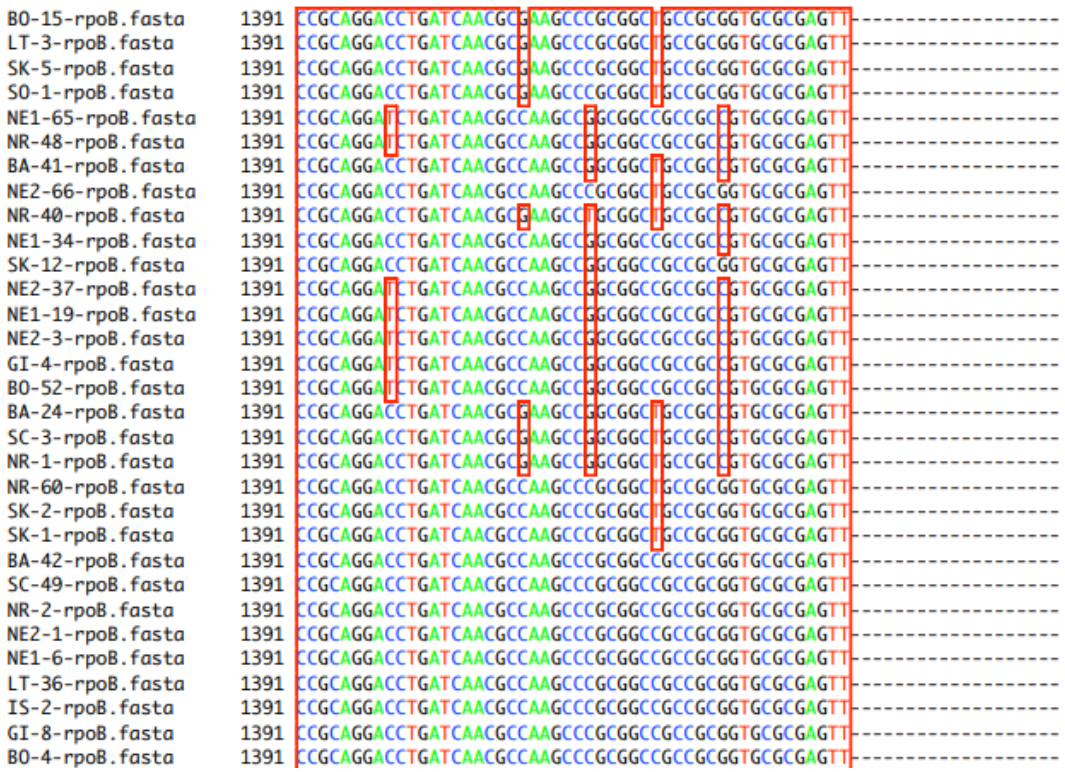

Fig. S2 (continued). Alignment of the indigenous soybean-nodulating bradyrhizobia in the Philippines from the *rpoB* housekeeping gene.