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REGULAR ARTICLE

Genetic diversity of indigenous soybean-nodulating *Bradyrhizobium elkanii* from southern Japan and Nueva Ecija, Philippines

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Abstract
Aims Understanding the factors that influence the diversity of soybean-nodulating rhizobia is important before doing inoculation. Since studies about this topic in tropical regions are limited, this could lay the groundwork for related research particularly on *Bradyrhizobium elkanii*.
Methods To determine the genetic diversity of *B. elkanii* in different regions, we conducted Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) and sequence analysis of 16S rRNA gene, internal transcribed spacer (ITS) region and *rpo*B gene. Also, sequence analysis of symbiotic *nif*D and *nod*D1 genes was conducted.
Results Analysis of the *rpo*B gene revealed a higher genetic diversity than the ITS region, and possible endemic *B. elkanii* strains were observed. Meanwhile, no variation was detected among the strains in both *nif*D and *nod*D1 phylogenies. Through *rpo*B gene analysis, variations in the ITS-*rpo*B type of *B. elkanii* strains were distinguished and differentiated with that of the closest reference strains. We identified potential soybean inoculants which possess symbiotic efficiency regardless of the *Rj* genotypes used, suggesting broad host-range of the strains.
Conclusions We show how the genetic diversity of soybean-nodulating *B. elkanii* strains in subtropical and tropical regions might be influenced by temperature and soil pH and, provided some insights between the symbiotic genes and *Rj* genotypes.

Keywords *Bradyrhizobium elkanii* · *rpo*B gene · ITS region · Philippines · Symbiotic genes

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Introduction

Soybean (*Glycine max* [L.] Merrill.) is a high protein legume (40%) and can be used as food, animal feed and as an industrial raw material. This legume can establish a symbiotic relationship with the nitrogen-fixing bacteria, known as rhizobia. Recent literatures reported that 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*, *Bradyrhizobium diazoefficiens, Bradyrhizobium elkanii,* and *Sinorhizobium/Ensifer fredii* (Jordan 1982; Scholla and Elkan 1984; Kuykendall et al. 1992; Young 2003; Delamuta et al. 2013). In soybean, some cultivars possess nodulation regulatory genes known as *Rj* genes, and the genotypes which have been confirmed to exist in nature are non-*Rj*, rj_1, Rj_2, Rj_3, and Rj_4 (Devine and Kuykendall 1996).

Unlike 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, soybean production in both countries cannot supply its local demand as reflected in the amount of soybean importation (Manuel et al. 1986; Wang 2016). One way to increase soybean yield per unit area is through inoculation. Inoculating useful rhizobia may lead to an increase in the yield of soybean as revealed by several studies (Alves et al. 2003; Njira et al. 2013; Alam et al. 2015; Sanz-Sáez et al. 2015). However, soybean inoculation does not always succeed due to competition between the inoculated and indigenous rhizobia in the soil (Yamakawa et al. 2003). Thus, there is a need to first understand the ecology of indigenous rhizobia in the soil before inoculation should be conducted.

Investigation of diversity and distribution of indigenous soybean rhizobia in Japan identified that *B. japonicum* strains were dominant in the northern part whereas *B. elkanii* strains were dominant in the southern part (Suzuki et al. 2008; Saeki et al. 2006, 2008, 2010, 2013) and temperature was the most influential factor in its dominance (Saeki and Shiro 2014). However, no studies about indigenous soybean rhizobia have been done in the Philippines as of this time.

Diversity analyses of soybean rhizobia in Japan were conducted mainly by analyzing the 16S rRNA gene and the ITS region between the 16S and 23S rRNA gene (Saeki et al. 2006, 2008, 2013; Suzuki et al. 2008; Minami et al. 2009; Saeki and Shiro 2014). But, a major disadvantage of 16S rRNA gene in taxonomic studies is that it is often multiple-copy (Vos et al. 2012) and has little resolution below the species level (Germano et al. 2006; Martens et al. 2008). Meanwhile, ITS region and *rpo*B gene provided better discriminatory power up to 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). Therefore, it is better to analyze other genes in addition to ITS region to provide better identification of rhizobial strains. However, taxonomical studies of rhizobia do not necessarily reflect their symbiotic features, particularly their host range, which is an important character of a suitable inoculant. Thus, sequence analysis of symbiotic genes *nif*D (encoded the α subunit of dinitrogenase) and *nod*D1 (nodulation regulation protein) was also conducted. Several studies reported that genes located in symbiosis island might not show diversity even among related species in rhizobial genera commonly due to 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). But the role of NodD regulator proteins (including *nod*D1) 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 evaluation of potential inoculant strains. Meanwhile, the role of *nif*D in partner quality for *Rhizobium* was investigated and reported that there might be a causal relationship between the locus and measures of partner quality (Gordon et al. 2016) which in turn, could influence mutualism between macro and microsymbionts for effective N fixation.

Majority of the studies about diversity and distribution of soybean rhizobia were conducted in temperate and subtropical 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) but there is limited research about this topic in tropical regions (Loureiro et al. 2007; Sharma et al. 2010; Ansari et al. 2013). 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, was thought to be originated from 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*, the diversity of soybean rhizobia has not yet been clearly described (Man et al. 2008). Therefore, this study would be a helpful foundation for future research and studies about the diversity and endemism of soybean rhizobia in subtropical and tropical regions. It aimed to determine the possible endemism and genetic diversity of soybean-nodulating *B. elkanii* species between three different

geographical regions and provide the first report in the Philippines and in Kumamoto, Japan.

Materials and methods

Soil collection

Soil samples were collected from 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) previously planted with soybean and/or other legumes. The surface was cleared with litters before obtaining a bar of soil with dimension of 20 cm depth and 2 to 3 cm thickness and weighed approximately 1 kg. Half of the 1 kg soil sample was air dried and pulverized for soil pH and electrical conductivity (EC) analyses by water extraction method (1:2.5 soil: water for pH and 1:5 soil: water for EC) whereas the remaining 0.5 kg was freshly used for soybean cultivation. Data of 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 temperature data from Kumamoto and Okinawa, Japan were obtained from Japan Meteorological Agency website at http://www.data.jma.go.jp/. All data were averages from this last decade.

Isolation of indigenous soybean rhizobia

Three soybean cultivars of three *Rj* genotypes, 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 1-l culture pots ($n = 3$). 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. Soil sample (2 to 3 g) was placed on the vermiculite at a depth of 2 to 3 cm, the seeds were then sown on the soil, and the pot was weighed. Plants were grown for 4 weeks in a growth chamber (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 nodules were randomly collected from the soybean roots per *Rj* genotype and sterilized by soaking them in 70% ethanol for 3 min and in a diluted sodium hypochlorite solution (0.25% available chlorine) for 30 min; then washed with sterile distilled water. Each nodule was homogenized in sterile distilled water, streaked onto a yeast extract mannitol agar (YMA; Vincent 1970) plate medium, and incubated for about 1 week in the dark at 28 °C. A 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.

Inoculation test

From the primary 16S rRNA gene and ITS region RFLP analysis of all collected isolates, representative isolates were selected and tested for their capability to form nodules on host soybean by inoculation test with the three *Rj* genotypes of soybean cultivars used in this study. Each isolate was cultured in YM broth (Vincent 1970) 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 soybean seeds were sown as described above but without soil and inoculated with 1 ml aliquot of each isolate per seed, replicated thrice. Nodule formation was assessed after 4 weeks in growth chamber under similar conditions mentioned above. Control pots (un-inoculated) for both Japanese (AK, BO, FK) and 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 obtained for symbiotic analysis. Oven drying was done at 70 °C for 48 h. Shoot was finely ground into 2 mm size prior to Nitrogen analysis. Total N was analyzed by automatic high sensitive NC Analyzer Sumigraph NC-220F (Sumika Chemical Analysis Service. Ltd., Tokyo, Japan). 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 obtained by the following formula: (mg N fixed/mg dry nodule) × 100 (Risal et al. 2010). Statistical analysis was conducted employing R software

(v. 3.3.2) and means of three replicates were compared by 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.

DNA extraction

Each isolate was cultured in HEPES-MES (HM) broth culture (Cole and Elkan 1973; Sameshima et al. 2003) for 6 days at 28 °C with continuous agitation at 120 rpm, and the bacteria cells cultured in the HM medium were collected by centrifugation and washed with sterile distilled water. Extraction of DNA was done by using BL buffer as described (Minami et al. 2009) from the method reported by Hiraishi et al. (1995). Similar method was done for the DNA extraction of reference strains.

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

Amplification of target genes were conducted using *Ex Taq* DNA polymerase (TaKaRa Bio, Otsu, Shiga, Japan) and previously designed primers (Table S1). The PCR cycle consisted of a pre run at 94 °C for 5 min, 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 1 min, with a final post run extension at 72 °C for 10 min. This cycle was used for all the five (5) target genes except that: for 16S rRNA gene, denaturation was done for 1 min while for *rpo*B gene, annealing was done at 60 °C for 1 min; then annealing was conducted at 57 °C for 1 min for both *nif*D and *nod*D1 genes.

RFLP analysis

The RFLP analysis of the ITS region was performed using the restriction enzymes *Hae*III, *Hha*I, *Msp*I and *Xsp*I (TaKaRa Bio) whereas for *rpo*B gene, *Hae*III, *Msp*I and *Alu*I (TaKaRa Bio) were used. *Bradyrhizobium* USDA strains *B. japonicum* 4, 6^{T}, 38, 122, 123, 124, 129, 135, *B. diazoefficiens* 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 16S rRNA gene, ITS region and *rpo*B 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 TBE buffer by means of electrophoresis and visualized with ethidium bromide.

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 (data not shown). Since almost all isolates collected from Nueva Ecija, Philippines belonged to Be 76 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 *rpo*B 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, *rpo*B, *nif*D and *nod*D1 genes.

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 NanoDrop 2000 Spectrophotometer (Thermo Scientific, U.S.A.).

Preparation of samples for sequence analysis from purified DNA followed the protocol for the premixed template and primer of the company (EUROFINS GENOMICS). After preparation, samples were sent to the company for sequence analysis. The sequence primers (Table S1) used were all designed for this study and calculated using OligoEvaluator™ by Sigma-Aldrich® Co. LLC.

Sequence alignment and construction of phylogenetic trees

To search the homology of sequences, Basic Local Alignment Search Tool (BLAST) program in DNA Databank of Japan (DDBJ) was used. Those sequences of type strains having 100% similarity with our isolates were retrieved from BLAST database. The alignment also included sequences of *Bradyrhizobium* genospecies for 16S rRNA gene and ITS region which were previously determined (Saeki et al. 2004; van Berkum and Fuhrmann 2000). Alignment of sequences obtained were performed using ClustalW. Phylogeny was determined by the Neighbor-Joining (Saitou and Nei 1987)

method for the 16S rRNA, ITS region, *rpo*B, *nif*D and *nod*D1 genes. Genetic distances were calculated using Kimura 2-parameter model (Kimura 1980) in the Molecular Evolutionary Genetic Analysis (MEGA v7) software (Kumar et al. 2016). Phylogenetic trees were bootstrapped with 1000 replications of each sequence to evaluate the reliability of the tree topology. 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 the supplementary information (Table S3).

Results

Soil pH and EC

The soils collected from Kumamoto, Japan and Nueva Ecija, Philippines were both slightly acidic (6.23 and 6.21, 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.

Isolation of soybean rhizobia and selection of representative isolates

A total of 216 isolates were obtained from the three locations (72 isolates per location) and their nodulation capability were confirmed through inoculation test. The number of isolates which belonged to Be76 cluster collected from 216 samples were 21, 42, and 71 from Kumamoto, Okinawa, and Nueva Ecija, respectively. The rest of the isolates belonged to *B. japonicum* USDA6^T and *B. diazoefficiens* USDA110^T and other minor *B. elkanii* strains. Therefore, 20 isolates which belonged to Be76 cluster only from each location were selected for final RFLP analysis of ITS region and *rpo*B gene which totaled to 60 isolates. Afterwards, 6 isolates from each location where randomly selected based from the different *rpo*B gene type that was observed from RFLP analysis. However, since some Okinawa samples showed similar ITS-*rpo*B type from RFLP analysis with both Kumamoto and Nueva Ecija, we selected 7 isolates from this location to represent the differences. Thus, 19 isolates were used for sequence analysis of ITS, *rpo*B, *nif*D and *nod*D1 whereas 12 isolates were used for 16S rRNA gene.

Nodulation and symbiotic analysis

The oven dry weight of shoot and nodules were obtained as well as nodule number for the three *Rj* genotypes of soybean cultivars that we used (Table S2). All the isolates were able to form nodules on both non-*Rj* and Rj_4 genotypes. Four (4) Kumamoto isolates (HFK2, HFK10, HFK12, HBO21) and 2 out 7 Okinawa isolates (OAK10, OFK6) did not form nodules with Rj_2Rj_3. In contrast, all isolates from Nueva Ecija formed nodules with all the *Rj* genotypes used in this study. Accordingly, control plants did not produce any nodule. The highest number of nodules produced were obtained from OBO4, OFK8, PBM1 and PHM4 isolates regardless of the *Rj* genotpyes which were significantly different than the other isolates. HFK2, HFK10 and HBO21 showed comparably high number of nodules for both non-*Rj* and Rj_4 genotypes.

The amount of N fixed showed significant differences among the isolates (Fig. 1a). For non-*Rj* genotype, HFK2, OBO4 and PHM1 fixed the highest N; HBO14 and PHM have the highest fixed N for Rj_2Rj_3; and for Rj_4, HFK2 fixed the highest amount of N. Some isolates did not form nodules with soybean cultivars harboring Rj_2Rj_3 (HFK2, HFK10, HFK12, HBO21, OAK10, OFK6) which indicated the absence of symbiosis (Fig. 1b). In general, higher amount of N fixed and symbiotic efficiency were observed with plants that produced higher number of nodules for particular *Rj* genotype.

RFLP analysis of ITS region and rpoB gene

Figure 2 showed the dendrogram of the preliminary RFLP analysis of 60 *B. elkanii* isolates from the three locations that elucidated the clusters formed from ITS region (Fig. 2a) and *rpo*B gene (Fig. 2b). All the 60 *B. elkanii* isolates belonged to Be76 cluster for the ITS region-RFLP analysis but were divided into two clusters (Be76 and Be46) for the *rpo*B gene-RFLP analysis. The RFLP band patterns of all the 60 isolates for ITS region were similar to each other on all four restriction enzymes (*Hae*III, *Hha*I, *Msp*I and *Xsp*I) indicating 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. 3a). Meanwhile, the RFLP band patterns of the 60 isolates for *rpo*B gene digested with 3 restriction enzymes (*Hae*III, *Msp*I and *Alu*I) showed two distinct band patterns similar to clusters Be76 and Be46 (data not shown). All Kumamoto isolates and three Okinawa isolates (represented by OAK11) have identical band patterns with Be76 cluster whereas, all Nueva Ecija isolates and the remaining Okinawa isolates have identical band patterns with Be46 cluster (data not shown). Therefore, one isolate per location which represented the band patterns clearly were chosen and plotted against *B. elkanii* reference strains (Fig. 3b) indicating the two distinct band patterns for *rpo*B gene.

These results showed that although the RFLP analysis of ITS region indicated that all the isolates have Be76 ITS type, the analysis of *rpo*B gene indicated that Okinawa (except for three isolates represented by OAK11) and Nueva Ecija samples have different *rpo*B type than their ITS type.

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

The phylogenetic tree for 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. 4a). 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 *B. elkanii* LMG 6135 and *B. elkanii* MAS8 showed 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.

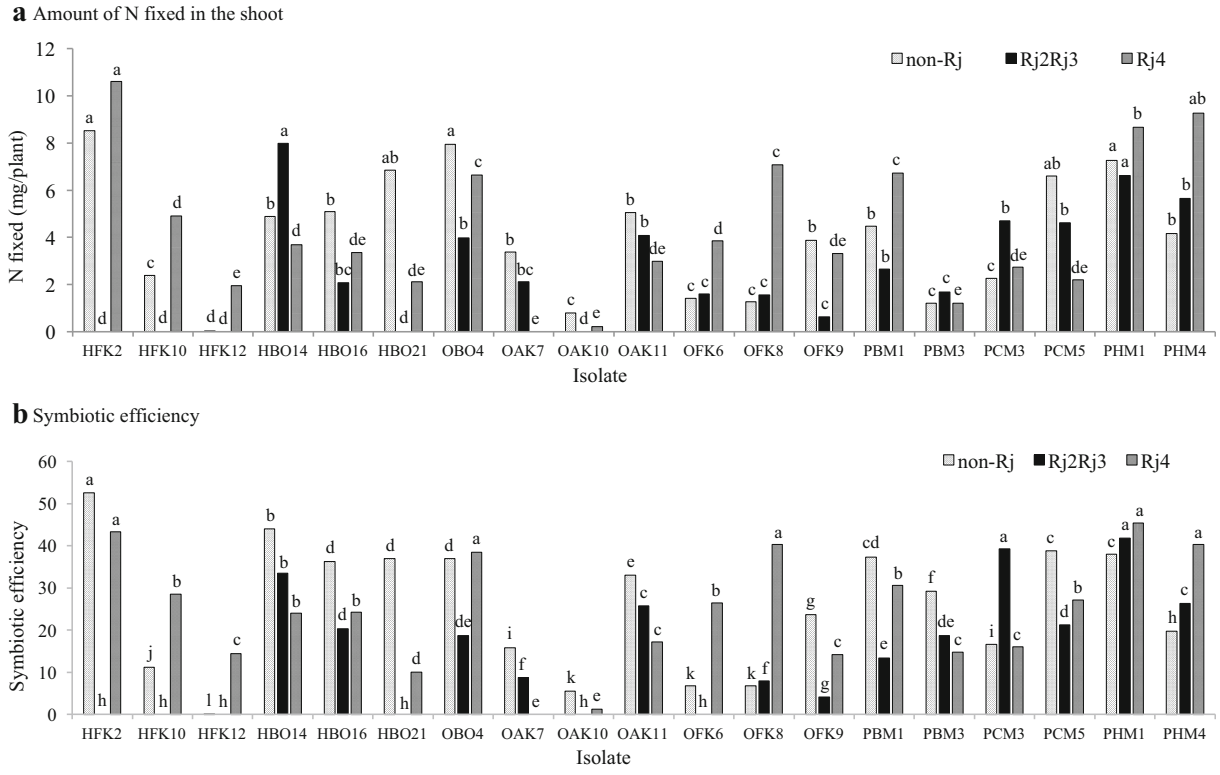

Fig. 1 N fixation in shoot **a** and symbiotic efficiency **b** of the 19 representative *B. elkanii* isolates employing three *Rj* genotypes. Mean comparison was conducted in triplicates only between isolates within the same *Rj* genotype

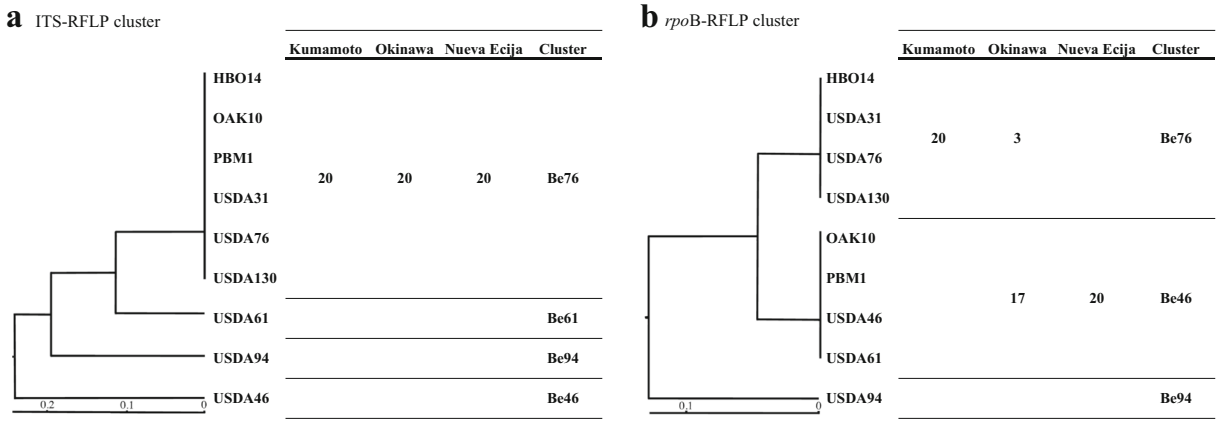

Fig. 2 Dendrogram of 60 *B. elkanii* isolates and *B. elkanii* reference strains based on primary PCR-RFLP analysis of **a** ITS region and **b** *rpo*B gene showing clusters of *B. elkanii*

Meanwhile, sequence analysis of the *rpo*B gene revealed three (3) distinct groups under Be46 and Be76 clusters (Fig. 4b). 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. Additionally, Fig. 4 showed the first phylogeny of *Bradyrhizobium* USDA strains with specific serogroups from *rpo*B gene sequence analysis. This phylogeny is similar with the band patterns of the *rpo*B gene obtained from RFLP treatment which indicated its usefulness (Fig. S1). 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. S2).

a 16S-23S rRNA gene ITS region-RFLP pattern

	HBO14	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				

b *rpo*B housekeeping gene-RFLP pattern

	HBO14	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

Fig. 3 Schematic representation of gel electrophoresis patterns based on **a** ITS region and **b** *rpo*B gene 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

Sequence analysis of symbiotic genes nifD and nodD1

The 19 representative isolates which were used for the ITS region and *rpo*B gene were classified phylogenetically based from the DNA fragments of *nif*D and *nod*D1 genes. The results are shown as supplementary material (Fig. S3) as we did not detect diversity among the isolates. All the isolates from the three locations have homogenous nucleotide (nt) sequences for both *nif*D (785 nt) and *nod*D1 (717 nt). It is evident that all the isolates were grouped under *B. elkanii* in *nif*D wherein separation of *B. elkanii* and *B. japonicum* strains was distinguished. For *nod*D1, all the isolates showed 100% similarity with *B. elkanii* USDA94 and *B. elkanii* M13 and was differentiated from *B. japonicum* and *B. diazoefficiens* strains. We were not able to find other *B. elkanii* strains in DDBJ database for the *nod*D1 which had at least 97% similarity with any of our isolates. In relation, we used *B. elkanii* USDA94 *nod*D2 nucleotide sequence as the outgroup.

Summary of RFLP and sequence analysis

The results from RFLP and sequence analysis were summarized (Table 1) and showed that from the 19 representative isolates used in this study, ITS-*rpo*B types 31′-46′ and 31–46 were found to be possibly endemic in Okinawa, Japan and Nueva Ecija, Philippines, respectively. We also observed in this study that some isolates have ITS type which was different from its *rpo*B type and these isolates were found in Okinawa, Japan and Nueva Ecija, Philippines.

We were able to observe the genetic diversity of the isolates through sequence analysis better than by just RFLP analysis. Two ITS-*rpo*B types were observed from Nueva Ecija which were Be31-Be46 and

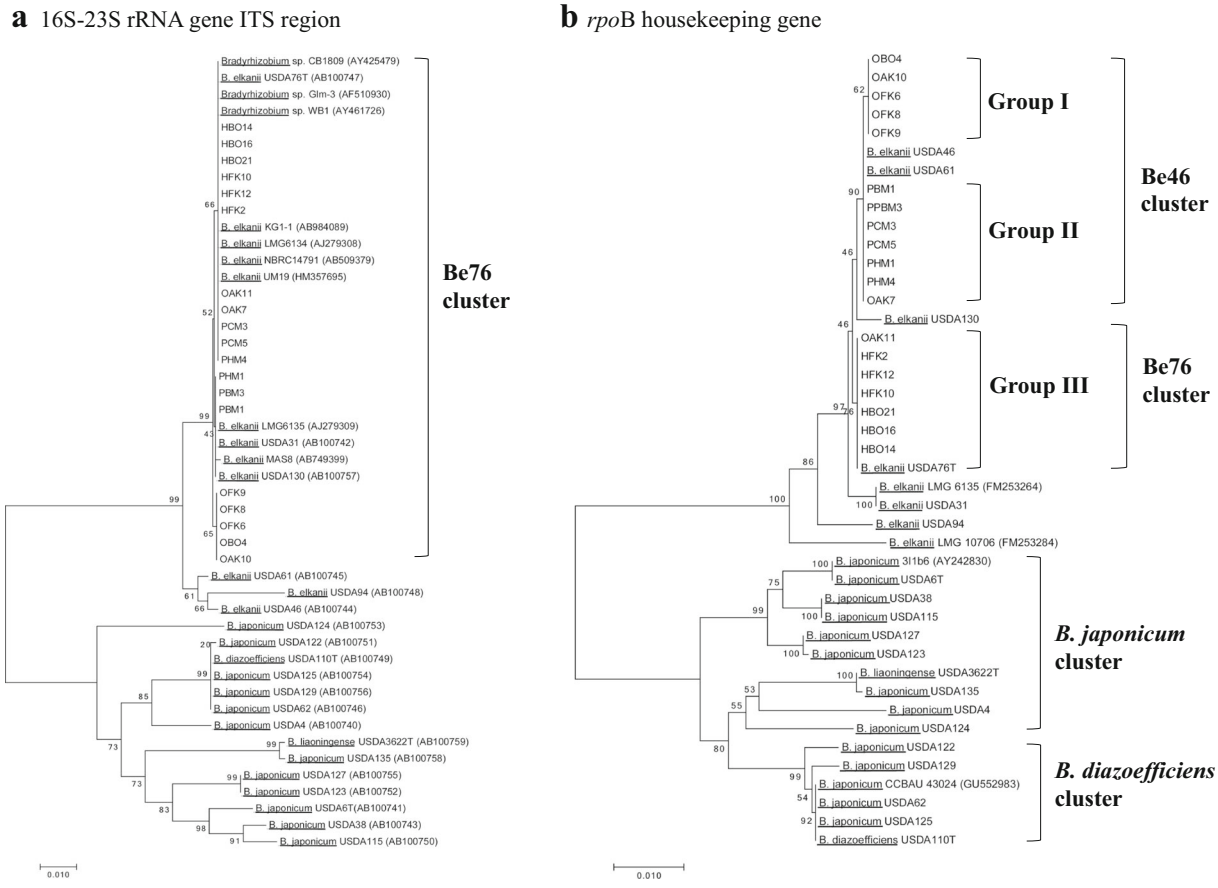

Fig. 4 Phylogenetic tree based on sequence analysis of **a** 16S–23S rRNA gene ITS region and **b** *rpo*B housekeeping gene. The tree was constructed using the Neighbor-Joining method with the Kimura 2-parameter (K2P) distance correlation model and 1000 bootstrap replications in MEGA v.7 software. The first letter of isolates' name indicates the location as follows: **H** - Kumamoto; **O** - Okinawa; **P** - C. Luzon, Philippines

Be76-Be46 whereas, three ITS-*rpo*B types were observed from Okinawa as follows: Be76-Be76', Be76-Be46, and Be31′-Be46'. All Kumamoto isolates have the same ITS-*rpo*B types which was Be76-Be76'. Highest diversity among the isolates was observed in Okinawa, followed by Nueva Ecija then, Kumamoto. Meanwhile, no diversity was observed for both *nif*D and *nod*D1 genes but both have similar results with 16S rRNA gene showing high homogeneity with *B. elkanii* USDA strains and *B. elkanii* M13 strain, which was isolated from *Vigna radiata* plant in Nepal.

Discussion

*Genetic diversity of indigenous soybean rhizobia by RFLP analy*sis

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

As previously reported, 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, the most influential factors in our study might as well be temperature and soil pH. Probably, this is not the case for salinity level since it was almost similar for all the study sites. It is observed that in the RFLP analysis of Okinawa isolates, where the soil was strongly acidic, all isolates with Be76 ITS type were divided into Be76 and Be46 type in *rpo*B gene. In case of Nueva Ecija isolates, all Be76 ITS type became Be46 type in *rpo*B gene. Additionally, Okinawa, Japan is considered as a subtropical region whereas Nueva Ecija,

Table 1 Difference in ITS-*rpo*B type of the isolates against the two closest *B. elkanii* reference strains as detected by sequence analysis

Strain	Location	16S rRNA gene	*nif*D	*nod*D1	RFLP cluster		Sequence cluster		ITS-*rpo*B type
					ITS region	*rpo*B gene	ITS region	*rpo*B gene	
HBO 14	Kumamoto	*B. elkanii*	*B. elkanii*	*B. elkanii*	Be76	Be76	Be76	Be76'	76–76'
HBO 16	Kumamoto	*B. elkanii*	*B. elkanii*	*B. elkanii*	Be76	Be76	Be76	Be76'	76–76'
HBO 21	Kumamoto		*B. elkanii*	*B. elkanii*	Be76	Be76	Be76	Be76'	76–76'
HFK 2	Kumamoto		*B. elkanii*	*B. elkanii*	Be76	Be76	Be76	Be76'	76–76'
HFK 10	Kumamoto	*B. elkanii*	*B. elkanii*	*B. elkanii*	Be76	Be76	Be76	Be76'	76–76'
HFK 12	Kumamoto		*B. elkanii*	*B. elkanii*	Be76	Be46	Be76	Be76'	76–76'
OBO 4	Okinawa	*B. elkanii*	*B. elkanii*	*B. elkanii*	Be76	Be46	Be31'	Be46'	31′-46'
OAK 7	Okinawa		*B. elkanii*	*B. elkanii*	Be76	Be46	Be76	Be46	76–46
OAK 10	Okinawa	*B. elkanii*	*B. elkanii*	*B. elkanii*	Be76	Be46	Be31'	Be46'	31′-46'
OAK 11	Okinawa		*B. elkanii*	*B. elkanii*	Be76	Be76	Be76	Be76'	76–76'
OFK 6	Okinawa		*B. elkanii*	*B. elkanii*	Be76	Be46	Be31'	Be46'	31′-46'
OFK 8	Okinawa	*B. elkanii*	*B. elkanii*	*B. elkanii*	Be76	Be46	Be31'	Be46'	31′-46'
OFK 9	Okinawa	*B. elkanii*	*B. elkanii*	*B. elkanii*	Be76	Be46	Be31'	Be46'	31'-46'
PBM 1	Nueva Ecija	*B. elkanii*	*B. elkanii*	*B. elkanii*	Be76	Be46	Be31	Be46	31–46
PBM 3	Nueva Ecija	*B. elkanii*	*B. elkanii*	*B. elkanii*	Be76	Be46	Be31	Be46	31–46
PCM 3	Nueva Ecija		*B. elkanii*	*B. elkanii*	Be76	Be46	Be31	Be46	31–46
PCM 5	Nueva Ecija	*B. elkanii*	*B. elkanii*	*B. elkanii*	Be76	Be46	Be76	Be46	76–46
PHM 1	Nueva Ecija	*B. elkanii*	*B. elkanii*	*B. elkanii*	Be76	Be46	Be76	Be46	76–46
PHM 4	Nueva Ecija	*B. elkanii*	*B. elkanii*	*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

' - indicates a slight variation in the nucleotide sequence of the isolate from that of the reference strain

Philippines is a tropical region. On the other hand, Kumamoto isolates obtained from a temperate region, remained to have the same Be76 ITS type with *rpo*B gene type. 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 *rpo*B type. The effect of different temperature regimes on the changes of population occupancy of bradyrhizobia in Japan was evaluated and revealed that 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 subtropical and tropical climates of the three locations in this study by RFLP analysis of ITS region.

We observed that by analyzing the polymorphisms of *rpo*B gene, possible endemic strains of soybean rhizobia in Okinawa and Nueva Ecija isolates were detected which were not distinguished in the ITS region. Also, we observed the existence of strains with similar ITS type but completely different *rpo*B type as detected from Okinawa and Nueva Ecija isolates. This observation could be due to the influence of temperature and soil acidity, although more detailed analyses should be conducted to verify this. The observed inconsistency between the ITS and *rpo*B type within the same strains could be likely due to recombination events which is widespread within bacteria, more often to 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).

Genetic diversity of indigenous soybean rhizobia as revealed by sequence analysis of ITS and rpoB

We showed the usefulness of sequence analysis for ITS region to distinguish *Bradyrhizobium* species even at the strain level (Fig. 4a). However, the ITS region failed to detect a clear endemism and genetic diversity of soybean rhizobia in this study. Considering the previous studies on the diversity of soybean rhizobia in temperate regions as earlier cited, it is possible that the evolution rate of ITS region of soybean rhizobia in subtropical and tropical regions was not similarly affected by environmental conditions, particularly temperature. Thus, we have elucidated that for subtropical and tropical regions, the use of ITS region solely was not enough to detect diversity among *B. elkanii* strains.

On the other hand, sequence analysis of the *rpo*B gene (Fig. 4b) provided a better discrimination among the strains and revealed that the genetic diversity of indigenous soybean rhizobial isolates varied geographically and we believe that this variation could be due to temperature as well as soil pH. High discriminatory power of *rpo*B gene was already proven (Vinuesa et al. 2008; Rivas et al. 2009; Degefu et al. 2013; Yan et al. 2014; Guimarães et al. 2015). Also, the existence of Okinawa isolates (OAK7 and OAK11) in Nueva Ecija and Kumamoto groups indicated that distribution of soybean rhizobia could be affected by the change in temperature as well as soil pH. It was previously stated that physical proximity, genetic distance and environmental changes are some of the factors for the occurrence of recombination events (Didelot and Maiden 2010) and in our case, it might be the differences in temperature and soil pH. Thus, we demonstrated that *rpo*B gene is useful for the analysis of diversity and detection of potential endemic strains of soybean rhizobia in these three locations, particularly for *B. elkanii*.

Genetic diversity of indigenous soybean rhizobia based from symbiotic genes

In contrast with the ITS region and *rpo*B gene, sequence analysis of *nif*D and *nod*D1 genes did not provide diversity among the isolates. The very high similarity (99–100%) observed between the isolates and *B. elkanii* strains for both *nif*D and *nod*D1 and its congruence with 16S rRNA gene phylogeny might be an indication that the evolution of symbiotic genes from the isolates of Kumamoto and Okinawa, Japan and Nueva Ecija, Philippines have progressed similarly with their conserved genes. Previous studies (Minamisawa et al. 2002; Barcellos et al. 2007; Ling et al. 2016) stated that horizontal gene transfer seldom occur for symbiotic genes in rhizobial genera that commonly causes the conformity in phylogenetic analyses. Although we cannot say that this is the same case with our study because we did not perform an analysis that will support this. However, our result is also similar with earlier report (Risal et al. 2010) stating the similarity of phylogenies obtained from conserved 16S rRNA gene region and symbiotic genes *nif*D and *nod*D1 for Nepalese isolates. Thus, we suggest that symbiotic genes may not be enough as indicators for genetic diversity observations,

particularly for *B. elkanii*. This idea is supported by a previous study stating that even distinct rhizobial species can share similar symbiotic genes and because they are located in easily interchangeable elements like the symbiosis island (Ramirez-Bahena et al. 2009). Another possible reason for the similar phylogenies we obtained from *nif*D and *nod*D1 genes might be due to 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 *nif*D and *nod*D1 with that of *rpo*B gene in our study is possibly due to 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 *nod*D sequence as a representative gene of common nod gene. The common nod genes including *nod*D, A, B, C are concerned with the construction of based structure of Nod factor. The Nod factor is related with host specificity between rhizobia and leguminous species, not with the compatibility between rhizobia and *Rj*-genotype varieties. The responsible gene in bradyrhizobia for incompatibility with *Rj*-genotype soybean is not clarified yet with some candidate genes (Tsurumaru et al. 2008; Yasuda et al. 2016). For incompatibility with Rj_2-genotype soybean, Tsurumaru et al. (2008) reported 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 yet, the gene may be important not only for compatibility but also for genomic diversity.

Symbiotic efficiency of the isolates

One very important feature of an inoculant is its efficiency in symbiosis with the host. Here, we observed that most of the isolates have broad host-range, which is a positive characteristic for a potential inoculant, particularly for Nueva Ecija isolates where 100% possessed this quality. The fact that the phylogeny of *nod*D1 did not detect any differences among the isolates, it might be 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. Not a single isolate in this study was host-specific which could be generally due to the role of *nod*D1 as a nodulation regulator. These isolates also showed varied symbiotic efficiency, which were significantly different between isolates within the same *Rj* genotype. Although there was no indication of this result in the phylogeny of *nif*D gene, it might be a possibility that *nif*D gene had no direct or little relationship with the *Rj* genotypes. Nevertheless, it is worthy to note that some isolates from 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.

Genetic diversity and detection of some endemic soybean rhizobia

Our research group already established that for diversity investigation of soybean rhizobia in temperate regions, ITS region provided high diversity (Saeki et al. 2006, 2008, 2010; Shiro et al. 2013). However, for subtropical and tropical regions, we suggest that *rpo*B gene should at least be included in addition to ITS region as one of the target genes. We proposed the use of the following *Bradyrhizobium* USDA reference strains (*B. japonicum* 4, 6^T, 38, 122, 123, 124, 129, 135, *B. diazoefficiens* 110^T, *B. elkanii* 31, 46, 76^T, 94, and 130 and *B. liaoningense* 3622^T) in the analysis of *rpo*B gene for soybean rhizobia. This study was able to distinguish that the ITS-*rpo*B type of *B. elkanii* isolates from Kumamoto and Okinawa, Japan and Nueva Ecija, Philippines were not 100% identical to the closest reference strains, which were USDA 76^T and USDA46. Thus, we reported the presence of possible endemic strains of *B. elkanii* that nodulate soybean in Okinawa, Japan and Nueva Ecija, Philippines and that genetic diversity of the isolates studied might have varied with temperature and soil pH as revealed by sequence analysis of *rpo*B gene. We also proposed that the symbiotic genes *nif*D and *nod*D1 were possibly not correlated with the compatibility of the *Rj* genotypes used in this study, although more detailed analyses are recommended to confirm this statement.

The significant results of this study were: first, the production of the first phylogenetic tree of *Bradyrhizobium* USDA reference strains for *rpo*B gene with specific serogroups; second, first study that reported the existence of different *rpo*B 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. Additionally, the strains that have possibly broad host-range compatibility and could be efficient microsymbionts of soybean were identified in the three locations that could be further studied for their efficiency and effectiveness as suitable inoculants. The information obtained in this research might help inoculation strategy to be more successful specifically in Nueva Ecija, Philippines and Kumamoto, Japan since the indigenous soybean rhizobia have been identified. But of course, more locations should be considered particularly in the Philippines that could represent the whole country.

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