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Diversity and distribution of soybean-nodulating bradyrhizobia isolated from major soybean-growing regions in Myanmar

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Rhizobia play an important role in plant nutrition by fixing nitrogen that is subsequently available for uptake by plants. In Myanmar, it has been difficult to characterize the nitrogen-fixing indigenous bradyrhizobia strains. In this study, 120 strains were isolated from five major soybean-growing regions of Myanmar. The strains were characterized based on sequence analysis of the 16S-23S rRNA internal transcribed spacer (ITS) region. Determination of nodulation types of native isolates was based upon their compatibility between strain and soybean cultivars by inoculation test. Our goal was to describe the polygenetic diversity among indigenous bradyrhizobial strains isolated from Myanmar and to identify their nodulation types. Sequence analysis indicated that all isolates belonged to the *Bradyrhizobium* genus and were conspecific with *B. liaoningense*, *B. elkanii*, *Bradyrhizobium* spp., *B. japonicum* and *B. yuanmingense*. A phylogenetic tree showed that 40, 33.3, 19.2, 5 and 2.5% of the isolates were related to *B. liaoningense*, *B. elkanii*, *Bradyrhizobium* spp., *B. japonicum* and *B. yuanmingense*, respectively. These results suggested that *B. liaoningense* and *B. elkanii* were the predominant *Bradyrhizobium* species present in Myanmar soils. *B. elkanii*, *B. liaoningense* and *Bradyrhizobium* spp. strains were dominant in acid soils, slightly acid to slightly alkaline soils and alkaline soils, respectively. Among tested isolates, Type A strains accounted for 74% of the isolates, while Type B and C strains accounted for 22 and 4%, respectively. It can be concluded that the diversity and distribution of indigenous bradyrhizobia differed markedly among sampling sites and among sites within the same region, and were affected by soil pH and climate. Determination of nodulation types of indigenous bradyrhizobia provides useful information for selection of strains compatible with different soybean cultivars. This is the first report describing *B. liaoningense* strains isolated from soybean in Myanmar.

Key words: Indigenous bradyrhizobia, internal transcribed spacer (ITS), Myanmar, phylogenetic diversity, soybean, nodulation type.
INTRODUCTION

Soybean (Glycine max L.) plays an important role in plant nutrition by fixing nitrogen that is subsequently available for uptake by plants. In the world, soybean is the major grain legume crop, representing about 50% of global legume acreage and 68% of global legume production (Herridge et al., 2008). In Myanmar, soybean has become the second largest cultivated crop, due to the increasing domestic consumption and increasing export demand (CSO, 2006). Soybean also plays an important role in the economy of Myanmar, due to its high nitrogen fixing ability through symbiosis with rhizobia.

Rhizobia are soil bacteria, differentiated by their unique ability to infect root hairs of leguminous plants and induce nodule formation on roots. Most Bradyrhizobium strains are associated with soybeans. Soybean-nodulating bacteria belong to the genus Bradyrhizobium and are gram-negative, slow-growing and alkali-producing when cultured on yeast extract mannitol agar (YMA) medium (Vincent, 1970). Rhizobialae are highly diverse and classified into several genera and species. The slow-growing, soybean-nodulating rhizobia are B. japonicum (Jordan, 1982), B. elkanii (Kuykendall et al., 1992), B. liaoningense (Xu et al., 1995), B. huanghuaihuiense (Zhang et al., 2012), B. daqingense (Wang et al., 2013), B. diazoefficiens (Delamuta et al., 2013) and B. ottawaense (Yu et al., 2014). The fast-growing bradyrhizobia are classified into two Sinorhizobium (Ensifer) species: S. fredii (Chen et al., 1998) and S. sojae (Li et al., 2011b). The diversity of soybean-nodulating bradyrhizobia depends on climate and soil properties (Adhikari et al., 2012). The community structure of bradyrhizobia varies according to the soybean cultivar, the host soybean Rj-genotype, and cultivation temperature (Minami et al., 2009; Shiro et al., 2012). Howieson and Ballard (2004) stated that the soybean-nodulating bradyrhizobia community might not depend only on the host cultivar and cultivation temperature even in the same field but also on geographical, soil texture, soil pH, salinity, and other differences among fields. The abundant diversity of rhizobia in the soil provides a large source of natural germplasm to select strains with desired characteristics.

Saeki et al. (2005) stated that Rj genotypes of soybean cultivars have the ability to affect both preference and compatibility for nodulation between the host cultivar and soybean rhizobia. Identifying the Rj genotypes of cultivars and nodulation types of bradyrhizobia is critical to select the best cultivar and strain to boost soybean yield through enhanced biological nitrogen fixation. Recently, Soe et al. (2013) mentioned that Myanmar soybean cultivars harbor non-Rj and Rj genes. Therefore, it is necessary to identify nodulation types of isolated strains. Ishizuka et al. (1991a) and Ishizuka et al. (1991b) tested compatibility and preference of Rj-genotype soybean cultivars with specific Bradyrhizobium strains. The Bradyrhizobium strains are classified into nodulation types A, B, and C, based on their compatibility with Rj cultivars. Type A strains are preferred by the non-Rj-genotype cultivars and nodulate with all Rj genotype cultivars. Type B strains are preferred by Rj4 cultivars and inhibit nodulation with the Rj2-Rj5-gene harboring cultivars. Type C strains are preferred by Rj6-Rj5 cultivars and restrict effective nodule formation with the Rj4 genotype cultivars.

Soe et al. (2013) studied the phylogenetic diversity of indigenous soybean bradyrhizobia, which were isolated from root nodules in three ecological regions. They isolated 43 indigenous strains. They were 23, 12 and 8 strains from Shan State, Mandalay and Yangon regions, respectively. Among 12 isolates from Madalay region, 10 and 2 were collected from Yezin and Bagan site, respectively. Among 23 isolates from Shan State, 3, 4, 5 and 11 were collected from Namlatt, Taungyi, Aungban and Kyaukme areas, respectively. In Yangon region, they collected 8 isolates from Insein area. We thought that unequal sample number among sampling sites within the same region or among regions cannot represent the diversity and distribution of each sampling site. Moreover, a similar study focusing on genetic diversity in Ayeyawaddy, Bago and Sagaing, which are the major soybean growing regions, is needed. Major soybean growing areas of Myanmar are in the Shan State, followed by the Ayeyarwady, Bago, Sagaing, and Mandalay regions (FAO, 2009). They are located in different ecological zones. Therefore, the present study was conducted to investigate the polygenetic diversity and distribution of indigenous bradyrhizobia isolated from five agro-climatic regions in Myanmar and to identify the nodulation types of indigenous isolates to estimate their compatibility with different soybean cultivars.

MATERIALS AND METHODS

Sampling sites

Soil samples were collected from five major soybean-growing regions in Myanmar. The collection sites were Heho and Aungban (Shan State), Hinthada (Ayeyawaddy Region), Letpandan (Bago Region), Myaung (Sagaing Region) and Madaya (Mandalay Region). Heho and Aungban sites are located on a hilly plateau in a humid temperate area, Hinthada and Letpandan sites are located in the lower part of Myanmar, in a humid tropical area. Mandalay and Sagaing regions are located in the middle and upper parts of Myanmar, respectively and both are tropical areas. The sampling...
site locations are shown in Figure 1. The soil samples were collected from fields with a long history of soybean cultivation and with no history of rhizobial inoculation. The soil pH (1:2.5 soil: H$_2$O) of sampling sites was measured using a pH meter (Beckman ϕ 360 pH/Temp/mV Meter; Beckmann Coulter, Brea, CA). Location, climate, soil type, and pH of sampling sites are presented in Table 1.

Estimation of indigenous rhizobia population in the soil

The most probable number count method (MPN) was used to estimate indigenous rhizobial populations in soil samples by inoculating a diluted soil suspension to Yezin-3 (R$_{j4}$) and Yezin-6 (non-R$_{j}$) (Vincent, 1970).

Isolation of soybean-nodulating indigenous bradyrhizobia

One gram of each composite soil sample was diluted with 99 ml of sterilized one-half strength modified Hoagland nutrient solution (MHN) in a 200-ml conical flask. The flasks were shaken on a rotary shaker at 120 rpm for 1 h to prepare a well-mixed soil suspension. The culture pots (1-L volume) were filled with 1 L of vermiculite and 0.6 L of MHN nutrient solution. The pots were covered with aluminum foil and autoclaved at 120°C for 20 min. For surface sterilization, the seeds were soaked in a 2.5% sodium hypochlorite solution for 5 min, rinsed five times with 10 ml of 99.5% ethanol and washed five times with sterilized MHN nutrient solution to remove traces of sodium hypochlorite and ethanol. Yezin-6 (non-R$_{j}$) and Yezin-3 (R$_{j4}$) varieties were used as trap hosts for all soil samples. Soe et al. (2013) reported that Myanmar soybean cultivars were non-R$_{j}$- and R$_{j4}$-genes harboring cultivars. Devine and Briethaupt (1981) also stated that 71.2% of cultivars from Myanmar were R$_{j4}$ genes harboring cultivars. Therefore, we used both Yezin-6 (non-R$_{j}$) and Yezin-3 (R$_{j4}$) varieties to isolate the native bradyrhizobia.

A total of 13 culture pots, corresponding to the two varieties grown with six soil suspensions and one control pot, were prepared. Five surface-sterilized seeds for each variety were planted in the sterilized vermiculite pots. A 5-ml aliquot of soil suspension was inoculated per seed. The control was soybean plants without inoculation to assess the possibility of contamination by non-relevant rhizobia. The plants were cultivated in the control room.
(25°C and 75% relative humidity) for four weeks. Autoclaved deionized water was poured when the original weight of the pots decreased by ~300 g.

After uprooting, about 15 nodules (≥ 2 mm) were collected per pot from Yeizin-6 (non-Rj) and Yeizin-3 (Rj), respectively. A total of 30 nodules from each sampling site were used to extract bacteria. Nodules were arranged from the largest to the smallest size to designate the name of isolates numerically. For surface sterilization, the nodules were immersed in 70% ethanol for 3 min, soaked in 2.5% sodium hypochlorite (NaClO) solution for 15 min, and washed five times with 0.9% autoclaved sodium chloride (NaCl) solution. The surface-sterilized nodules were transferred separately into the autoclaved small test tubes and crushed. The milky bacterial juice was streaked onto yeast extract mannitol agar (YMA) plates (Vincent, 1970) containing 25 µg Congo red (Somasegaran and Hoben 1984), using sterilized glass rods. The plates were incubated at 30°C for 7 days to promote colony development. Some small nodules did not form colony and some were contaminated.

Therefore, we chose 20 pure isolates from each sampling site for phylogenetic analysis (10 isolates from each cultivar). For storage of the isolates, a single pure colony of each pure isolate was cultured in A1E broth medium and incubated on a rotary shaker at 100 rpm at 30°C for 7 days. One milliliter of each liquid bacterial culture was mixed with 0.45 ml of Glycerol Liquid Medium (GLM) in an autoclaved Eppendorf tube. GLM was made by mixing 50% of HM salts and 50% glycerol. The glycerol stock isolates were stored at -85°C for later use.

### Table 1. Locations, soil type and pH of sampling sites.

| Sampling site | Soil Type | Soil pH | Location | Climate (Temp; RF) |
|---------------|-----------|---------|----------|-------------------|
| Shan State    |           |         |          |                   |
| Heho          | Red Earth and Yellow Earth | 8.0 | 20° 72' N 96° 77' E | 15.4°C-27.1°C, 1187 mm |
| Aungban       | Red Earth and Yellow Earth | 5.1 | 20° 67' N 96° 63' E | 15.4°C-27.1°C, 1187 mm |
| Ayeyarwady Region |         |         |          |                   |
| Hinthada      | Alluvial Soil | 6.7 | 17° 38'N 95° 30' E | 23.3°C-33.6°C, 2386 mm |
| Bago Region   | Meadow Alluvial soil | 5.9 | 17° 46' 95° 45' E | 20.6°C-33.1°C, 2531 mm |
| Sagaing Region | Meadow and Meadow Alluvial soil | 7.1 | 21° 25' 95° 58' E | 21.6°C-35.2°C, 1152 mm |
| Mandalay Region | Compact Soil | 7.2 | 22° 13' 96° 7' E | 23.0°C-35.1°C, 1018 mm |

Source. 1 MOAI (2004); 2 MOAI (2010); Temp: average minimum to maximum temperature; RF, average annual rainfall.

### PCR analysis and sequencing of 16S-23S rRNA internal transcribed spacer regions

The primers ITS1512F (5’-GTCGTAACAA GGTAGCCGT-3’) and ITSLS23R (5’-TGCCCAA GCCATCCACC-3’) were used to amplify the 16S-23S rRNA ITS region of bradyrhizobia. The PCR reaction consisted of a pre-run at 94°C for 5 min, denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 1 min. The cycle was repeated for 33 cycles, followed by a final extension at 72°C for 10 min (Sarr et al., 2011). PCR products were purified using the Wizard Gel and PCR Clean-up System (Promega, Madison, WI, USA). Purified PCR products (≥50 ng µL-1) were subjected to direct sequencing by Macrogen (Tokyo, Japan), using the primer set described above. Raw sequence results were edited using DNASIS Mac ver. 2.0 (Hitachi, San Bruno, CA, USA) to create ITS sequence fragments.

### Construction of phylogenetic trees

For homology searches, sequences were compared with the DNA Data Bank of Japan (DDBJ) using the Basic Local Alignment Search Tool (BLAST) program (Altschul et al., 1997). To construct the phylogenetic tree, sequences of type strains and closely related strains of *Bradyrhizobium* genospecies were retrieved from the BLAST database. Two isolates from each group, which have the 100% sequence similarity, were selected. All selected sequences including type strains and closet strains were aligned using the CLUSTALW function of the MEGA version 6 software (Tamura et al., 2013). After alignment, a phylogenetic tree was constructed according to the neighbor-joining method (Saitou and Nei, 1987). The phylogenetic tree was bootstrapped with 1,000 replications of each sequence to evaluate the tree topology for reliability. Genetic distances were calculated using the Kimura two-parameter model (Kimura, 1980).

### Nucleotide sequence accession numbers

The nucleotide sequences of 16-23S rRNA genes of 120 isolates were deposited in the DDBJ under the accession numbers (LC037234-LC037353).
**Table 2. The population of indigenous rhizobia at each soil sampling site.**

| Sampling sites | Number of rhizobia per gram dry soil |
|---------------|-------------------------------------|
|               | Yezin-3 (R<sub>lj</sub>)           | Yezin-6 (non-Rj) |
| Hinthada      | 0.85 × 10<sup>6</sup>              | 1.55 × 10<sup>8</sup> |
| Myaung        | 0.85 × 10<sup>6</sup>              | 0.85 × 10<sup>6</sup> |
| Madaya        | 2.9 × 10<sup>5</sup>               | 0.5 × 10<sup>5</sup> |
| Letpandan     | 0.5 × 10<sup>5</sup>               | 0.85 × 10<sup>5</sup> |
| Heho          | 2.9 × 10<sup>4</sup>               | 0.85 × 10<sup>5</sup> |
| Aungban       | 0.85 × 10<sup>4</sup>              | 2.9 × 10<sup>4</sup> |

**Diversity index of rhizobia at each sampling site**

Diversity of the native bradyrhizobial isolates at each sampling site was calculated using Shannon’s diversity index (H’), based on clusters in the phylogenetic tree (Pielou, 1969). The index was calculated using the following equation:

\[ H' = -\sum (P_i \ln P_i) \]

Where, \( P_i \) is the dominance of the isolates expressed as \( n_i/N \), and \( N \) and \( n_i \) are the total number of isolates tested at a site and the number of isolates belonging to a particular cluster at the site, respectively.

**Determination of nodulation types**

Nodulation types for the 120 isolated indigenous bradyrhizobial strains (including the three reference strains \( B. japonicum \) USDA 110 (Type A), \( B. japonicum \) Is 1 (Type B) and \( B. japonicum \) Is 34 (Type C) were assessed to estimate their cultivar preference (Ishizuka et al., 1991b). Soybean cultivars Yeziin-6 (non-Rj), CNS (R<sub>lj</sub>lR<sub>lj</sub>) and Hill (R<sub>lj</sub>) were used as trap hosts. Before inoculation, these three reference strains and 120 indigenous strains were cultured in A1E liquid medium (Kuykendall, 1979) and incubated on a rotary shaker at 30°C for 7 days. One milliliter of liquid culture of each isolate was diluted with 99 ml of sterilized MHN solution. The surface-sterilized seeds were sown in culture pots prepared as described above, and inoculated with a 5-ml aliquot of one bacterial suspension per seed. Sterilization and cultivation of seeds were performed as described above. After one month, the presence or absence of nodules was checked to identify nodulation types of all tested isolates. Unclear results were confirmed using the same procedure.

**RESULTS**

**Indigenous rhizobia in sampling soils**

Before bacterial isolation, the population of indigenous rhizobia was estimated by the most probable number method. The populations of indigenous rhizobia in Hinthada, Myaung, Madaya, Letpandan, Heho and Aungban soils, nodulated to the Yeziin-3 (R<sub>lj</sub>) and to Yeziin-6 (non-Rj) cultivars are presented in Table 2.

**Isolation and growth properties of isolates**

Twenty isolates were extracted from root nodules of two different soybean cultivars, Yeziin-3 (R<sub>lj</sub>) and Yeziin-6 (non-Rj). A total of 120 strains were obtained from six sampling sites in five major soybean growing regions of Myanmar. The isolates were named SHY3 1-10 and SHY6 1-10 for Heho soil from the Shan State, SAY3 1-10 and SAY6 1-10 for Aungban soil from the Shan State, AHY3 1-10 and AHY6 1-10 for Hinthada soil from the Ayeyarwady Region, BLY3 1-10 and BLY6 1-10 for Letpandan soil from the Bago Region, SMY3 1-10 and SMY6 1-10 for Myaung soil from the Sagaing Region, and MMY3 1-10 and MMY6 1-10 for Madaya soil from the Mandalay Region, for the Yeziin-3 and Yeziin-6 varieties, respectively. After incubation for seven days on YMA plates, the colonies of \( B. liaoningense \) and \( B. japonicum \) isolates, which had entire pulvinate shapes were up to 0.5-2 mm in diameter. \( B. elkanii \), \( B. yuanmingense \) and \( Bradyrhizobium \) spp. isolates showed the same undulating flat colony shape with different diameters, ranging from 0.5 to 3 mm. Colony characteristics of each isolate are shown in Table 3.

**Sequence analysis of 16S-23S rRNA ITS genes**

PCR products of 16S-23S rRNA from each isolate showed a single band of approximately 0.85 kb, which were sequenced. BLAST search results of ITS sequences indicated that all isolates belonged to the genus \( Bradyrhizobium \). Phylogenetic analysis results, based on ITS sequences of indigenous soybean nodulating bradyrhizobia, are shown in Figure 2. The closest reference strain, with at least 97% sequence similarity, was used as the criterion for cluster separation of the isolates. Thirteen clusters were identified in the phylogenetic tree. Among them, five clusters belonged to \( B. elkanii \). \( B. elkanii \) strains were clearly separated into five distinct clusters. Be1 and Be5 shared 99% sequence similarity with \( B. elkanii \) strains USDA 90 and USDA 94, respectively. Be2, Be3, and Be4 had 100% sequence similarity with \( B. elkanii \) strains USDA 86 and USDA 76, respectively. \( Bradyrhizobium \) liaoningense represented clusters BI1 and BI2. Clusters BI1 and BI2 showed 100% sequence similarity with \( B. liaoningense \) strains SEMIA 5003 and SEMIA 5062, respectively. \( B. japonicum \)
Table 3. Colony characteristics of isolates on YMA plates after 7 day incubations at 30°C.

| Isolates | Species name        | Shape | Size (mm) | Isolates | Species name        | Shape | Size (mm) |
|----------|---------------------|-------|-----------|----------|---------------------|-------|-----------|
| SAY3-1   | B. elkanii          | UF    | 1         | SHY3-1   | B sp.               | UF    | 1         |
| SAY3-2   | B. elkanii          | UF    | 0.5       | SHY3-2   | B sp.               | UF    | 3         |
| SAY3-3   | B. japonicum        | UF    | 1         | SHY3-3   | B sp.               | UF    | 1         |
| SAY3-4   | B. elkanii          | UF    | 1         | SHY3-4   | B sp.               | UF    | 1         |
| SAY3-5   | B. elkanii          | UF    | 0.5       | SHY3-5   | B sp.               | UF    | 1         |
| SAY3-6   | B. elkanii          | UF    | 3         | SHY3-6   | B sp.               | UF    | 1         |
| SAY3-7   | B. japonicum        | EP    | 1         | SHY3-7   | B sp.               | UF    | 1         |
| SAY3-8   | B. elkanii          | UF    | 2         | SHY3-8   | B sp.               | UF    | 1         |
| SAY3-9   | B. elkanii          | UF    | 2         | SHY3-9   | B sp.               | UF    | 1         |
| SAY3-10  | B. japonicum        | EP    | 3         | SHY3-10  | B. japonicum        | EP    | 1         |
| SAY6-1   | B. japonicum        | EP    | 1         | SHY6-1   | B sp.               | UF    | 3         |
| SAY6-2   | B. elkanii          | UF    | 1         | SHY6-2   | B sp.               | UF    | 1         |
| SAY6-3   | B. elkanii          | UF    | 3         | SHY6-3   | B sp.               | UF    | 3         |
| SAY6-4   | B. japonicum        | EP    | 3         | SHY6-4   | B sp.               | UF    | 3         |
| SAY6-5   | B. elkanii          | UF    | 1         | SHY6-5   | B sp.               | UF    | 1         |
| SAY6-6   | B. elkanii          | UF    | 1         | SHY6-6   | B sp.               | UF    | 3         |
| SAY6-7   | B. elkanii          | UF    | 1         | SHY6-7   | B sp.               | UF    | 3         |
| SAY6-8   | B. elkanii          | UF    | 1         | SHY6-8   | B sp.               | UF    | 3         |
| SAY6-9   | B. elkanii          | UF    | 1         | SHY6-9   | B sp.               | UF    | 3         |
| SAY6-10  | B. elkanii          | UF    | 3         | SHY6-10  | B sp.               | UF    | 3         |
| AHY3-1   | B. elkanii          | UF    | 3         | BLY3-1   | B. elkanii          | UF    | 2         |
| AHY3-2   | B. liaoningense     | EP    | 1         | BLY3-2   | B. elkanii          | UF    | 1         |
| AHY3-3   | B. liaoningense     | EP    | 1         | BLY3-3   | B. elkanii          | UF    | 1         |
| AHY3-4   | B. liaoningense     | EP    | 2         | BLY3-4   | B. liaoningense     | EP    | 2         |
| AHY3-5   | B. liaoningense     | EP    | 2         | BLY3-5   | B. liaoningense     | UF    | 0.5       |
| AHY3-6   | B. spp.             | EP    | 3         | BLY3-6   | B. liaoningense     | EP    | 1         |
| AHY3-7   | B. elkanii          | UF    | 3         | BLY3-7   | B. elkanii          | UF    | 2         |
| AHY3-8   | B. elkanii          | UF    | 3         | BLY3-8   | B. elkanii          | UF    | 2         |
| AHY3-9   | B. spp.             | EP    | 3         | BLY3-9   | B. elkanii          | UF    | 3         |
| AHY3-10  | B. liaoningense     | EP    | 1         | BLY3-10  | B. liaoningense     | EP    | 1         |
| AHY6-1   | B. liaoningense     | EP    | 1         | BLY6-1   | B. elkanii          | UF    | 3         |
| AHY6-2   | B. elkanii          | UF    | 1         | BLY6-2   | B. elkanii          | UF    | 3         |
| AHY6-3   | B. liaoningense     | EP    | 1         | BLY6-3   | B. elkanii          | UF    | 3         |
| AHY6-4   | B. liaoningense     | EP    | 1         | BLY6-4   | B. elkanii          | UF    | 3         |
| AHY6-5   | B. liaoningense     | UF    | 1         | BLY6-5   | B. liaoningense     | EP    | 1         |
| AHY6-6   | B. elkanii          | UF    | 1         | BLY6-6   | B. liaoningense     | EP    | 2         |
| AHY6-7   | B. elkanii          | UF    | 3         | BLY6-7   | B. elkanii          | UF    | 3         |
| AHY6-8   | B. elkanii          | UF    | 1         | BLY6-8   | B. liaoningense     | EP    | 1         |
| AHY6-9   | B. elkanii          | UF    | 1         | BLY6-9   | B. elkanii          | UF    | 1         |
| AHY6-10  | B. liaoningense     | EP    | 1         | BLY6-10  | B. liaoningense     | UF    | 0.5       |
| SMY3-1   | B. liaoningense     | EP    | 1         | MMY3-1   | B. liaoningense     | EP    | 1         |
| SMY3-2   | B. liaoningense     | EP    | 1         | MMY3-2   | B. liaoningense     | EP    | 1         |
| SMY3-3   | B. liaoningense     | EP    | 1         | MMY3-3   | B. liaoningense     | EP    | 1         |
| SMY3-4   | B. liaoningense     | EP    | 1         | MMY3-4   | B. liaoningense     | EP    | 1         |
| SMY3-5   | B. elkanii          | UF    | 3         | MMY3-5   | B sp.               | UF    | 1         |
| SMY3-6   | B. liaoningense     | EP    | 1         | MMY3-6   | B. liaoningense     | EP    | 1         |
| SMY3-7   | B. liaoningense     | EP    | 1         | MMY3-7   | B sp.               | UF    | 1         |
| SMY3-8   | B. liaoningense     | EP    | 1         | MMY3-8   | B. liaoningense     | EP    | 1         |
| SMY3-9   | B. liaoningense     | EP    | 1         | MMY3-9   | B. liaoningense     | UF    | 1         |
| SMY3-10  | B. liaoningense     | EP    | 1         | MMY3-10  | B. liaoningense     | EP    | 1         |
| SMY6-1   | B. liaoningense     | EP    | 1         | MMY6-1   | B. elkanii          | UF    | 1         |
isolates were separated into two clusters. Bj1 cluster had 99% sequence similarity to the *B. japonicum* strain USDA 4 while Bj2 was highly related to *B. japonicum* 456, with 100% sequence similarity. *Bradyrhizobium yuanmingense* isolates comprised one cluster with 98% sequence similarity to the *B. yuanmingense* strain T15. Several strains were not assigned to a specific Bradyrhizobium species, so were identified as *Bradyrhizobium* spp. in the study. These were clearly separated into three distinct clusters. Bs1, Bs2 and Bs3 showed 100, 97 and 100% sequence similarity with *Bradyrhizobium* spp. strains TSA15y, KO13 and CCBAU 15774, respectively. Willems et al. (2003) suggested that strains belong to the same genospecies if their ITS regions have ≥95.5% sequence similarity. *Bradyrhizobium betae*, *B. canariense*, *B. iriomotense* and *Sinorhizobium fredii* were not represented among the tested isolates.

At the Heho site, most of the isolates were classified as *Bradyrhizobium* spp. strains and only one isolate belonged to *B. japonicum*. At the Aungban site, most of the isolates were identified as *B. elkanii* and a few strains as *B. japonicum*. At the Letpandan site, 60% of the isolates belonged to *B. elkanii* and 40% were classified as *B. liaoningense*. At the Hinthada site, *B. elkanii* and *B. liaoningense* were equally represented and only two isolates were identified as *Bradyrhizobium* spp. At the Myaung site, *B. liaoningense* strains were dominant, in addition to a single *Bradyrhizobium* spp. and a few *B. yuanmingense* isolates. At the Madaya sites, most of the isolates were *B. liaoningense* strains and only a few isolates were classified as *B. elkanii* or *Bradyrhizobium* spp. In this study, *B. japonicum* strains were found only at the Shan State (Aungban and Heho sites). *Bradyrhizobium yuanmingense* was detected only at the Sagaing Region. Among all isolates from the various sampling sites, the dominant strains were *B. liaoningense*, followed by *B. elkanii*, *Bradyrhizobium* spp., *B. japonicum* and *B. yuanmingense*. *Bradyrhizobium* liaoningense was widely distributed throughout the Myaung, Madaya, Letpandan and Hinthada sites. However, *B. liaoningense* strains, the most abundant strains in Myanmar, were not found in the Shan State. *Bradyrhizobium elkanii* strains were the second most dominant and were widely distributed throughout all soybean-growing regions of Myanmar.

### Diversity index and percent distribution of isolates

Diversity indices are shown in (Table 4). The Aungban site had the highest diversity index (1.57), followed by Hinthada (1.11), Madaya (1.03) and Letpandan (0.94). The lowest diversity index (0.20) was at the Heho site, followed by the Myaung site (0.61). The highest diversity index was at the Aungban site, where *B. elkanii* and *B. japonicum* strains were diverse. The high diversity indices at the Hithada and Letpandan sites were related to the high variability of *B. elkanii* strains, although *B. liaoningense* and *Bradyrhizobium* spp. isolates were less variable. The high diversity index at the Madaya site was due to the high variability of *B. liaoningense*, with a few strains of *Bradyrhizobium* spp. and *B. elkanii*. The lowest diversity index was at the Heho area, where *Bradyrhizobium* spp. strains were more abundant, but grouped in a single cluster; followed by Myaung, where *B. liaoningense* strains were highly abundant. This result revealed that the diversity indices differed markedly among the regions and among sites within the same region.

Distribution percentages of isolates are shown in Figure 3. At the Aungban site, 75 and 25% of the isolates were identified as *B. elkanii* and *B. japonicum*, respectively. At the Heho site, 95 and 5% of the isolates were identified as *Bradyrhizobium* spp. and *B. japonicum* strains, respectively. At the Letpandan site, 60 and 40% of the isolates were identified as *B. elkanii* and *B. liaoningense*, respectively. *Bradyrhizobium* species identified at the Hinthada site were *B. liaoningense* (45%), *B. elkanii* (45%) and *Bradyrhizobium* spp. (10%). At the Madaya and Myaung sites, 75% of the isolates were *B. liaoningense*, followed by *B. elkanii* (15%), *Bradyrhizobium* spp. (10%), *B. liaoningense* (80%), *B. liaoningense*
yuanmingense (15%) and B. elkanii (5%). At all sites, the isolates identified were B. liaoningense (40% of total isolates), followed by B. elkanii (33.3%), Bradyrhizobium spp. (19.2%), B. japonicum (5%), and B. yuanmingense (2.5%). These results indicate that the distribution of rhizobia varies, depending on the soybean cultivation area both between and within the various regions.

Nodulation types of indigenous bradyrhizobia

In this study, all B. liaoningense and B. yuanmingense...
Table 4. Numbers of isolates and diversity index (H’) of clusters at each field site.

| Sampling site | B. liaoningense | B. elkanii | B. japonicum | B. yuanmingense | Bradyrhizobium spp. | Diversity (H’) |
|---------------|-----------------|-------------|--------------|-----------------|---------------------|----------------|
|               | No. of isolate  | No. of cluster | No. of isolate | No. of cluster | No. of isolate | No. of cluster | No. of isolate | No. of cluster | No. of isolate | No. of cluster | No. of isolate | No. of cluster |
| Heho          | 19              | 1            | 1            | 1               | 19                | 1              | 0.20          |
| Aungban       | 15              | 4            | 5            | 2               | 19                | 1              | 1.57          |
| Hinthada      | 9               | 1            | 9            | 2               | 2                  | 1              | 1.11          |
| Letpandan     | 8               | 1            | 12           | 2               | 2                  | 1              | 0.94          |
| Madaya        | 15              | 2            | 3            | 1               | 2                  | 1              | 1.03          |
| Myaung        | 16              | 1            | 1            | 1               | 1                  | 1              | 0.61          |
| Total         | 48              | 40           | 6            | 3               | 23                 |                |               |

The diversity index (H’) was calculated using the following equation: 

\[ H’ = - \sum (P_i \ln P_i) \]

where \( P_i \) is the dominance of the isolates expressed as \( \frac{n_i}{N} \), where \( N \) and \( n_i \) are the total number of isolates tested in a site and the number of isolates belonging to a particular cluster in the site, respectively.

Figure 3. Percent distribution of isolates at each sampling site.

strains were classified as nodulation Type A strains. The Bj1 strain, which was similar to USDA 4, was identified as Type A. The Bj2 strain, which was similar to B. japonicum USDA 456, was classified as Type B. Among all Bradyrhizobium spp. isolates, Bs1 isolates was similar to
Bradyrhizobium spp. TSA15y were classified as a Type C strains. Bs2 isolates similar to Bradyrhizobium spp.KO13 were classified as Type A strains. Bs3 strains similar to Bradyrhizobium spp. CCBAU 15574 were classified as Type B strains. Be1, Be2 and Be5 strains similar to the B. elkanii strains USDA 90, USDA 86 and USDA 94, respectively, were classified as Type A strains.However, Be3 and Be4 isolates similar to B. elkanii strains CCBAU 51010 and USDA 23, respectively, were classified into different nodulation types, although the isolates belonged to the same cluster. The Be3 isolates from the Letpandan, Bago Region such as BLY3-8 and BLY6-1 were classified in nodulation Type B. The Be3 isolates from the Hinthada, Ayeyawaddy Region were identified as Type A. Similarly, Be4 isolates from Madaya such as MMY6-1, MMY6-2 and MMY6-5 were Type C. Be4 isolates from Aungban and Letpandan were classified as Type A. Overall, Type A, B and C strains represented 74, 22 and 4%, respectively, of the total. These results indicate that the nodulation type might be different, even if the isolates belong to the same species and cluster. The nodulation types of isolates are shown in Table 5.

**DISCUSSION**

The isolation and characterization of rhizobia provides the natural biological resources to select the strains that have adapted to local environmental conditions to boost agricultural production through enhancing symbiotic nitrogen fixation. Therefore, in this present study, soil samples were collected from soybean fields with no
history of inoculation treatment and long history of soybean cultivation for isolation of native rhizobia. The population of rhizobia in sampling soils ranged from $1.55 \times 10^3$ to $0.85 \times 10^4$. Therefore, 120 strains were successfully isolated from five major soybean-growing regions of Myanmar, where soil pH ranged from 5.1 to 8.0.

Soybean plays the essential role in plant nutrition by supporting fixed nitrogen to the plant. In Myanmar, the isolated strains were characterized based on the colony morphology of the isolates according to their size, shape and color on YMA plate (Vincent, 1970). In this study, the colony shapes of *B. liaoningense* and *B. japonicum* isolates were very similar. Besides, the *Bradyrhizobium elkanii*, *Bradyrhizobium yuenmingense* and *Bradyrhizobium* spp. isolates showed the same colony shape. Therefore, characterization of isolates based on morphology and molecular biology technique is needed. However, characterization of nitrogen fixing indigenous bradyrhizobia using molecular biology technique is still limited in Myanmar. In this study, sequence analysis of 16S-23S rRNA ITS region was done because PCR analysis of the 16S-23S rRNA ITS region is a useful technique to group soybean-nodulating bradyrhizobia (Saeki et al., 2004).

In this study, all isolates were in the genus *Bradyrhizobium*, based upon their ability to form nodules on roots of Yezin-6 (*non-Rj*) soybean cultivars, alkali production on YMA plates, and ITS sequence analysis results. According to the results from PCR analysis of the 16S-23S rRNA ITS region, the isolates were classified into five *Bradyrhizobium* species: *B. japonicum*, *B. elkanii*, *B. liaoningense*, *B. yuenmingense*, and *Bradyrhizobium* spp. Some known species, such as *B. canariensis*, and fast-growing *Sinorhizobium (Ensifer)* species such as *S. xinjiangense* and *S. fredii*, were not detected in this study. The absence of fast-growing rhizobia could be related to the soil pH, as the abundance of these strains in alkaline soils has been reported (Saeki et al., 2005; Han et al., 2009; Zhang et al., 2011).

A phylogenetic tree showed that indigenous bradyrhizobial strains were distributed throughout the soybean-growing regions of Myanmar, with varying diversity indices. *B. liaoningense* strains were dominant in Myanmar. *B. liaoningense* strains were isolated from soybeans grown in alkaline soils in China (Xu et al., 1995; Han et al., 2009; Li et al., 2011a), Nepal (Adhikari et al., 2012) and India (Appunu et al., 2008). In Myanmar, *B. liaoningense* strains were isolated from four different ecological zones with a pH range of 5.9-7.2. These findings suggested that *B. liaoningense* strains can also be isolated from slightly acidic soils.

Assignment of a significant proportion of Myanmar isolates to *B. elkanii* was not surprising, as this species is widely distributed as soybean rhizobia in Asian countries (Vinuesa et al., 2008, Li et al., 2011a, Maruekaratjintpleng et al., 2012), the United States (Shiro et al., 2013), Brazil (Barcellos et al., 2007), Africa (Wasike et al., 2009) and Paraguay (Chen et al., 2000). Recently, Soe et al. (2013) reported that *B. elkanii* are dominant and widely distributed, in all studied regions of Myanmar (Yangon, Mandalay and Shan State). This is in agreement with our findings, in which *B. elkanii* strains were more diverse and more widely distributed throughout all regions of Myanmar.

In Myanmar, *B. japonicum* strains were detected only in the Shan State, which is one of the cooler regions of Myanmar. *B. japonicum* strains have been reported to be dominant, especially in the soils of cooler regions in Japan (Suzuki et al., 2008; Sarr et al., 2011) and Nepal (Vinuesa et al., 2008). In Myanmar, *B. japonicum* strains were found abundantly in Shan State, with fewer in the Mandalay region, and none in the Yangon region (Soe et al., 2013). This is in line with our finding.

*B. yuenmingense* strains have also been isolated as dominant strains for mungbean in Nepal (Risal et al., 2012) and cowpea in Japan (Sarr et al., 2009). Several authors have isolated *B. yuenmingense* strains from soybean as a minor group in China (Man et al., 2008), Japan (Sarr et al., 2011), Nepal (Risal et al., 2010; Adhikari et al., 2012), Thailand (Maruekaratjintpleng et al., 2012), and Myanmar and Vietnam (Vinuesa et al., 2008; Soe et al., 2013). This is in accordance with our findings. In the present study, *B. yuenmingense* isolates represented an only minor group at the Myaung site.

Soil pH also has a marked effect on diversity at the subspecies level (Adhikari et al., 2012). Our results indicated that soil pH affects the diversity of indigenous bradyrhizobia. *Bradyrhizobium elkanii* strains were dominant over *B. japonicum* and *B. liaoningense* strains in acid soils with a pH 5.1 and 5.9, respectively. When pH increased from 5.9 to pH 6.7, *B. liaoningense* and *B. elkanii* were equally distributed. When pH increased from 6.7 to 7.1 and 7.2, *B. liaoningense* strains were abundant, with a few *B. elkanii* strains. When pH increased to 8.0, *Bradyrhizobium* spp. strains were dominant. Suzuki et al. (2008) reported *B. elkanii* strains to be dominant not only in acidic soils (pH 4.6-6.1) but also in alkaline soils (pH 7.5). In Myanmar, *B. elkanii* strains predominated in acid soils with a pH of 5.1 and 6.7 as a major group and in slightly alkaline soils with a pH of 7.1 and 7.2 as a minor group. In Heho site, soil pH was optimal for *B. liaoningense*, but this *Bradyrhizobium* species was absent from that area. The absence of this species might be due to geographic factors, such as climate and latitude.

Shiro et al. (2013) stated that the indigenous soybean bradyrhizobial community structure varies with geography and it is also highly correlated with latitude. Adhikari et al. (2012) indicated that *B. liaoningense*, *B. elkanii* and *B. yuenmingense* were present in subtropical areas of Nepal but *B. japonicum* was not observed. This is consistent with our findings. In Myanmar also, *B. japonicum* were abundant in temperate areas. Similarly, *B. liaoningense*
strains were predominant in tropical areas but those strains were not found in temperate areas.

Diversity and distribution seems to be related to the cultivars grown in the regions. In the Madaya and Myaung sites, B. liaoningense strains were more abundant and accounted for 80 and 75%, respectively, of the Bradyrhizobium species. Although geographic location and soil type were different, the same soybean variety (Madaya) was planted at the Madaya and Myaung sites. Higher diversity index resulted from Aungban site. In Aungban sampling site, cultivars produced by Department of Agricultural Research such as Yezin-3, Yezn-6 cultivars were used as main cultivars. In Heho which had lowest diversity index, local cultivar (Shan Seine) was grown as a main cultivar. The higher diversity is related to high variance of Bradyrhizobium species. High variance of Bradyrhizobium species can obtain if many cultivars are planted in soils because of the compatibility and preference of rhizobia to specific host cultivars. Therefore, it seems that the relative proportion of Bradyrhizobium species is determined by the cultivars grown in those regions. Minamisawa et al. (1999) described that bradyrhizobial diversity might change in individual fields depending on the associated host plants and local soil conditions.

Determination of nodulation type is important for matching isolates with soybean cultivars and optimizing soybean productivity. Therefore, nodulation types of indigenous Bradyrhizobium strains were evaluated to estimate their compatibility with Myanmar soybean cultivars. In Myanmar, Type A strains appeared to be dominant, followed by Type B. A few Type C strains were found only at Madaya site. Soe et al. (2013) also reported that Type A and Type B strains were abundant in Myanmar.

Recently, Soe et al. (2013) firstly reported that diversity and distribution of indigenous bradyrhizobia from three different ecological zones. They pointed out that B. elkanii and B. japonicum were observed as the major dominant species, and B. yuanningense as the minor species. Our results showed that B. elkanii and B. liaoningense were found as the major dominant species, and Bradyrhizobium spp., B. japonicum and B. yuanningense as the minor species in Myanmar soils. Although Soe et al. (2013) did not find B. liaoningense strains, we abundantly found those strains in tropical regions of Myanmar. Our results supported the findings of Soe et al. (2013) that is, B. elkanii strains were widely distributed throughout the soybean growing regions.

Conclusion

Conclusively, this is the first report of B. liaoningense strains being dominant in soybean fields in Myanmar. B. elkanii strains were dominant in acid soils with a pH 5.1 and 6.7. B. liaoningense strains were found as dominant strains in soils with a pH range of 6.7-7.2.

Bradyrhizobium spp. strains were dominant in alkaline soil with pH 8.0. In Myanmar, the dominant strains were B. liaoningense, followed by B. elkanii, Bradyrhizobium spp., B. japonicum and B. yuanningense. B. liaoningense and B. elkanii were the abundant strains in Myanmar. However, B. yuanningense was not detected in the Shan State, temperate region of Myanmar. In contract, none of B. japonicum strains were observed in tropical regions of Myanmar. According to our results, it can be concluded that the diversity and distribution of indigenous bradyrhizobia are dependent on geographical location, soil pH, climate and associated host cultivar. Among tested isolates, Type A strains appeared to predominate, followed by Types B and C. This study on determination of nodulation types of native strains provides useful information for selection of strains compatible with soybean cultivars. Further study is needed on the effectiveness of indigenous bradyrhizobia on different Rf genotype soybean cultivars of Myanmar to improve soybean yield by enhancing symbiotic nitrogen fixation.

Conflict of interests

The authors have not declare any conflict of interest.

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