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Phylogenetic diversity of indigenous soya bean bradyrhizobia from different agro-climatic regions in Myanmar

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ABSTRACT: Soya bean (*Glycine max* Merr.) is the most important legume crop in the world and an important cash crop in Myanmar. In the present study, we characterized 43 isolates of indigenous soya bean rhizobia from Myanmar based on the sequence analysis of the bacterial 16S-23S rRNA internal transcribed spacer region. The sequence analysis confirmed that all isolates were categorized and identified as the genus *Bradyrhizobium* and they were conspecific with *B. japonicum*, *B. elkanii*, *B. yuanmingense*, and *Bradyrhizobium* sp. The highest genetic diversity of *Bradyrhizobium* strains was observed in the Shan State soya bean growing area. *B. elkanii* was distributed throughout the cultivation areas and was recorded as the dominant soya bean-nodulating strain in Myanmar. The present study revealed that there were two *Rj*-genotypes as Rj_4- and non-*Rj*-genotypes among the collected soya bean cultivars from Myanmar. These findings indicate a high compatibility between Rj_4-genotypes soya beans and nodulation type B strains in Shan State and Yangon region. In Mandalay region, the compatibility between non-*Rj*-genotype soya beans and nodulation type A strains was clearly investigated. This is the first report describing *B. yuanmingense* from nodules of soya bean and the geographical distribution of indigenous bradyrhizobia of soya bean from different regions in Myanmar.

KEYWORDS: diversity index, nodulation type, *Rj*-genotype, root nodule bacteria

INTRODUCTION

Soya bean (*Glycine max* L. Merr.), the most important grain legume crop in the world, maintains soil fertility since it can assimilate nitrogen from the atmosphere through symbiotic biological N_2 fixation (BNF) with bradyrhizobia[1]. As biological nitrogen fixation reduces the need for chemical nitrogen fertilizer for agriculturally-important crops such as soya bean and alfalfa[2], nitrogen fixation in the root nodule is given considerable emphasis. The symbiosis between soya bean and bradyrhizobia results from a complex process involving many genes of both partners that leads to the formation of N_2-fixing nodules on roots[3].

Soya bean-nodulating bacteria in the genus *Bradyrhizobium* are gram-negative bacteria that are slow-growing and produce alkaline substances on a yeast extract-mannitol (YM)[4] medium. They are found worldwide, and their genetic diversity may reflect geographical and climatic differences as well as host diversity[5]. Efficient strains of soya bean rhizobia have been selected for commercial production of soya bean rhizobium biofertilizers worldwide[1,6,7].

Some soya bean cultivars possess nodulation regulatory genes known as *Rj* genes, and the genotypes non-*Rj*, Rj_1, Rj_2, Rj_3, and Rj_4 have been confirmed to exist in nature[7]. The *Rj* genes play a role in controlling the compatibility between the plant and specific rhizobial strains, and the preference for indigenous soya bean-nodulating rhizobia[8,9]. *Rj*-genotype soya bean cultivars might affect not only the compatibility with particular bradyrhizobia, but also the nodulation[10]. Hence to determine indigenous soya bean-nodulating rhizobia, it is important to use several kinds of *Rj*-soya bean cultivars. The compatibility and nodule preference of bradyrhizobial strains for *Rj*-genotype soya bean cultivars has been tested[8]. Type A strains formed effective nodules with all *Rj*-genotype cultivars and were preferred by the non-*Rj*-genotype cultivars for nodulation. Type B strains that were

incompatible with the Rj_2Rj_3-genotype were preferred by Rj_4-cultivars, whereas type C strains that were incompatible with the Rj_4-genotype were preferred by Rj_2Rj_3-cultivars.

In Myanmar, peat-based rhizobial inoculants using *Bradyrhizobium japonicum* TAL 377, TAL 379, and TAL 102 from NifTAL Project are produced and distributed throughout the country by the Ministry of Agriculture and Irrigation for seven leguminous crops. Recently, the effectiveness of native *Bradyrhizobium* strains isolated from soya bean growing areas of Myanmar was studied[11,12]. These authors demonstrated the effectiveness of native bradyrhizobial isolates from Myanmar in symbiosis with soya bean cultivars[13]. However, research about native root nodule bacteria compatible with soya bean is still limited in Myanmar. Moreover, it is necessary to characterize and identify the nitrogen-fixing bacteria in order to eventually avoid the use of urea fertilizer in food legume crops. For this reason, we aimed to isolate and identify the native Myanmar bradyrhizobial isolates, to determine their distribution in the soya bean growing areas, and to evaluate their compatibility with *Rj* genotypic host soya beans for the nodulation type. In the present study, phylogenetic analysis was carried out to investigate the diversity of indigenous soya bean-nodulating rhizobia in Myanmar based on sequence analysis of the 16S-23S rRNA internal transcribed spacer (ITS) region of the isolates.

MATERIALS AND METHODS

Soya bean nodule collection sites

Nodules of soya bean were collected from the soya bean growing fields in three locations in Myanmar (Fig. 1): Yangon Region, pH 5.0–5.5, lateritic soils; Mandalay Region, pH 6.0–8.0, meadow and meadow alluvial soil; and Shan State, pH 5.0–5.5, red earths and yellow earths[14]. Yangon Region (17° 0′ N 96° 10′ E) is located in Lower Myanmar and has a tropical monsoon climate. Mandalay Region (21° 0′ N 95° 45′ E) is located in the central dry zone of Myanmar and features a tropical wet and dry climate. Shan State (21° 30′ N 98° 0′ E) is a hilly plateau and possesses a humid equatorial climate with dry winters.

Collection of nodule samples and isolation of *Bradyrhizobium*

Forty-eight indigenous root nodules bacteria (MAS1–MAS48, where MAS means Myanmar Agriculture Service) were collected from different agro-climatic regions of Myanmar in order to evaluate their nitrogen fixing ability for soya bean production. After purification, forty-three isolates gave pure colonies and were authenticated for nodule formation on host soya beans[15]. The forty-three native *Bradyrhizobium* strains were isolated from soya bean root nodules of local varieties such as; Yezin-3, Yezin-6, Yezin-8, Yezin-11, Yezin-14, Shan Sein, Southern Shan Local, Shan Wha, and Northern Shan Local collected from different agro-climatic regions of Myanmar. The host variety and location of collection site as well as the collection of the nodules samples were recorded (Fig. 1 and Table 1). Isolation of *Bradyrhizobium* was done at *Rhizobium* Section, Department of Agricultural Research, Ministry of Agriculture and Irrigation, Myanmar. Isolation of the bacteria from nodule samples was done after surface sterilizing the nodules, and culturing on yeast extract mannitol agar (YMA) medium[4] containing Congo red (25 µg/ml). The nodules were crushed in small bottles containing 1 ml of sterilized water. For every sample, a loopful of the suspension was streaked on YMA Congo red plate and single colonies were selected after incubation at 30 °C for 5–7 days. The isolates were nominated as MAS (Myanmar Agriculture Service).

Identification of bradyrhizobial isolates

DNA extraction: The purified bacterial isolates were conserved in a glycerol-stock culture at −85 °C. Prior to DNA extraction, isolates from the glycerol stock were streaked on A1E[16] agar plates and incubated at 30 °C to allow colony development. Total DNA as the PCR template was then extracted from an A1E liquid culture grown to the exponential phase ($0.4 < \mathrm{OD}_{600\,\mathrm{nm}} < 0.6$), using an ISOPLANT kit (Nippon Gene, Tokyo) following the instructions of the manufacturer.

PCR analysis of 16S-23S ITS rRNA region: The PCR reactions were performed as described[17]. The primer sets ITS1512F (5′-GTCGTAACAAGGTAGCCGT-3′) and ITSLS23R (5′-TGCCCAAGGCATCCACC-3′) were used to amplify the 16S-23S rRNA ITS genes. The PCR cycle was as described[18], except that the annealing temperature was raised from 55 °C to 60 °C and the total number of cycles was increased from 30–33 for a higher DNA yield. PCR products were then purified using the Wizard Gel and PCR Clean-up System (Promega, Madison, WI, USA), and the corresponding concentrations were estimated by NIH image 1.62 (National Institute of Health, Bethesda, MD, USA) after agarose gel electrophoresis (2% agarose gel in 1× TAE buffer) and staining with ethidium bromide (Toyobo, Tokyo).

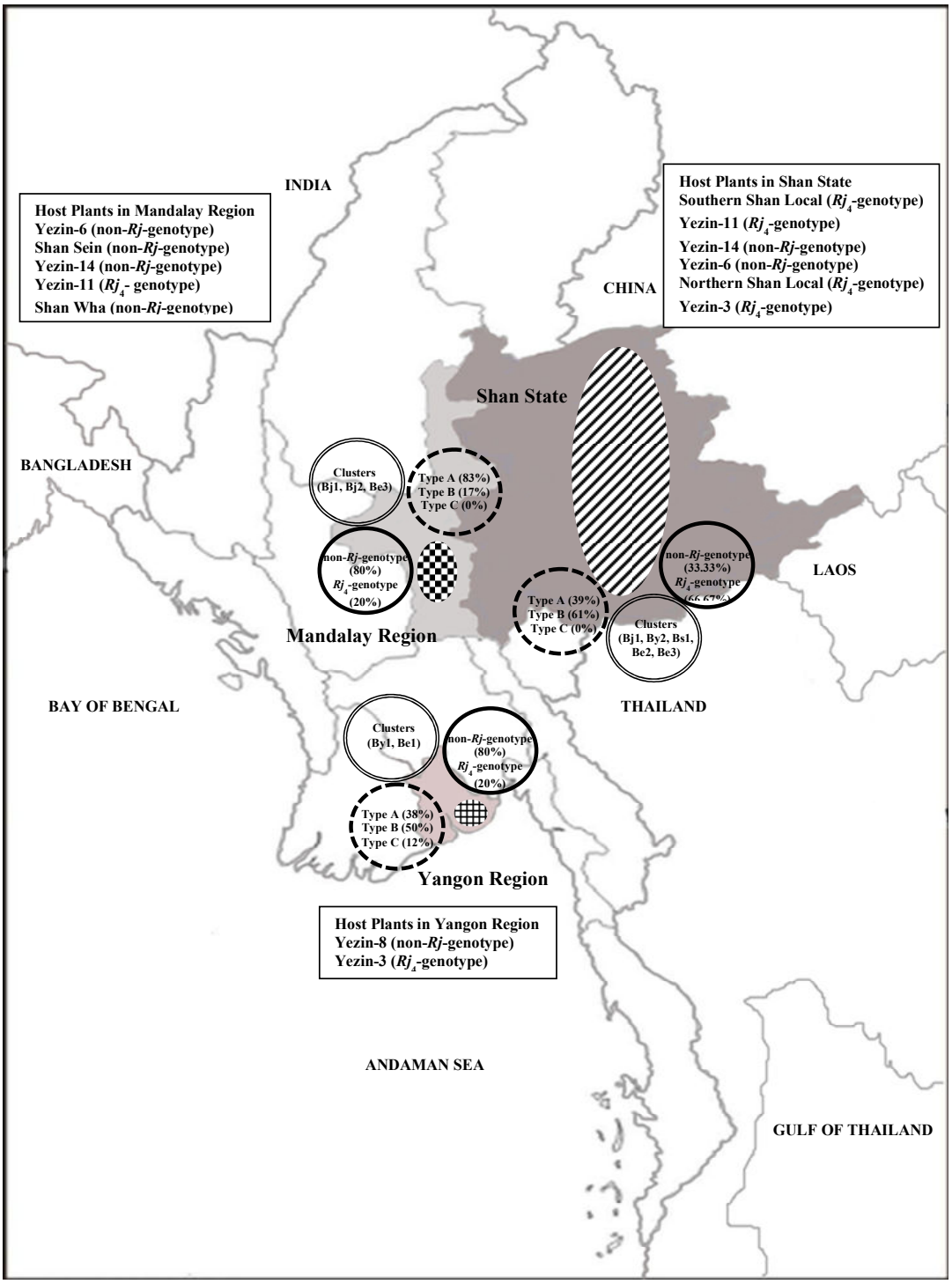

Fig. 1 Locations of nodule sampling sites in Myanmar.

Table 1 The origin and nodulation type of soya bean bradyrhizobial isolates from Myanmar.

Isolate	Species name	Nodulation type	Soya bean growing location	Soil type[†]	pH[†]	Host soya bean	Climate (Temp., RF)[‡]
MAS1	*B. elkanii*	Type *A*	Yezin, MR	MMa	6.0–8.0	Yezin-6	21–32 °C, 1028 mm
MAS4	*B. japonicum*	Type *B*	Yezin, MR	MMa	6.0–8.0	Shan Sein	21–32 °C, 1028 mm
MAS5	*B. japonicum*	Type *B*	Yezin, MR	MMa	6.0–8.0	Yezin-6	21–32 °C, 1028 mm
MAS6	*B. elkanii*	Type *A*	Yezin, MR	MMa	6.0–8.0	Yezin-6	21–32 °C, 1028 mm
MAS7	*B. elkanii*	Type *A*	Yezin, MR	MMa	6.0–8.0	Yezin-14	21–32 °C, 1028 mm
MAS8	*B. elkanii*	Type *A*	Yezin, MR	MMa	6.0–8.0	Yezin-11	21–32 °C, 1028 mm
MAS9	*B. elkanii*	Type *A*	Yezin, MR	MMa	6.0–8.0	Yezin-6	21–32 °C, 1028 mm
MAS10	*B. elkanii*	Type *A*	Yezin, MR	MMa	6.0–8.0	Yezin-14	21–32 °C, 1028 mm
MAS11	*B. elkanii*	Type *A*	Yezin, MR	MMa	6.0–8.0	Yezin-11	21–32 °C, 1028 mm
MAS12	*B. elkanii*	Type *A*	Yezin, MR	MMa	6.0–8.0	Yezin-11	21–32 °C, 1028 mm
MAS13	*B. yuanmingense*	Type *B*	Taunggyi, SS	MMa	5.0–5.5	Shan Wha	15–29 °C, 1022 mm
MAS14	*B. japonicum*	Type *B*	Aungban, SS	MMa	5.0–5.5	Yezin-11	15–29 °C, 1022 mm
MAS15	*B.* sp.	Type *B*	Taunggyi, SS	MMa	5.0–5.5	Shan Wha	15–29 °C, 1022 mm
MAS17	*B. elkanii*	Type *A*	Aungban, SS	MMa	5.0–5.5	Yezin-6	15–29 °C, 1022 mm
MAS18	*B. elkanii*	Type *B*	Aungban, SS	MMa	5.0–5.5	Yezin-6	15–29 °C, 1022 mm
MAS19	*B. elkanii*	Type *A*	Taunggyi, SS	Re&Ye	5.0–5.5	Shan Wha	15–29 °C, 1022 mm
MAS20	*B. elkanii*	Type *A*	Taunggyi, SS	Re&Ye	5.0–5.5	Shan Wha	15–29 °C, 1022 mm
MAS21	*B. elkanii*	Type *A*	Aungban, SS	Re&Ye	5.0–5.5	Yezin-11	15–29 °C, 1022 mm
MAS22	*B. elkanii*	Type *A*	Aungban, SS	Re&Ye	5.0–5.5	Yezin-14	15–29 °C, 1022 mm
MAS23	*B. elkanii*	Type *B*	Insein, YR	Ls	5.0–5.5	Yezin-8	23–32 °C, 2757 mm
MAS25	*B. elkanii*	Type *A*	Insein, YR	Ls	5.0–5.5	Yezin-3	23–32 °C, 2757 mm
MAS26	*B. elkanii*	Type *B*	Insein, YR	Ls	5.0–5.5	Yezin-3	23–32 °C, 2757 mm
MAS27	*B. elkanii*	Type *A*	Insein, YR	Ls	5.0–5.5	Yezin-3	23–32 °C, 2757 mm
MAS28	*B.yuanmingense*	Type *C*	Insein, YR	Ls	5.0–5.5	Yezin-8	23–32 °C, 2757 mm
MAS29	*B. elkanii*	Type *A*	Insein, YR	Ls	5.0–5.5	Yezin-3	23–32 °C, 2757 mm
MAS30	*B. elkanii*	Type *B*	Insein, YR	Ls	5.0–5.5	Yezin-8	23–32 °C, 2757 mm
MAS31	*B. elkanii*	Type *B*	Insein, YR	Ls	5.0–5.5	Yezin-3	23–32 °C, 2757 mm
MAS32	*B. japonicum*	Type *A*	Bagan, MR	Pcss	5.0–7.0	Shan Wha	21–32 °C, 1028 mm
MAS33	*B. japonicum*	Type *A*	Bagan, MR	Pcss	5.0–7.0	Shan Wha	21–32 °C, 1028 mm
MAS34	*B.yunamingense*	Type *A*	KyautMae, SS	Re&Ye	5.0–5.5	NSL	15–29 °C, 1022 mm
MAS35	*B. japonicum*	Type *B*	KyautMae, SS	Re&Ye	5.0–5.5	Yezin-3	15–29 °C, 1022 mm
MAS36	*B. japonicum*	Type *B*	KyautMae, SS	Re&Ye	5.0–5.5	Yezin-6	15–29 °C, 1022 mm
MAS37	*B. japonicum*	Type *B*	KyautMae, SS	Re&Ye	5.0–5.5	NSL	15–29 °C, 1022 mm
MAS38	*B. japonicum*	Type *B*	KyautMae, SS	Re&Ye	5.0–5.5	NSL	15–29 °C, 1022 mm
MAS39	*B. japonicum*	Type *B*	KyautMae, SS	Re&Ye	5.0–5.5	NSL	15–29 °C, 1022 mm
MAS40	*B. elkanii*	Type *B*	KyautMae,SS	Re&Ye	5.0–5.5	Yezin-3	15–29 °C, 1022 mm
MAS42	*B. elkanii*	Type *A*	Nam Latt, SS	Re&Ye	5.0–5.5	NSL	15–29 °C, 1022 mm
MAS43	*B. japonicum*	Type *B*	Kyaut Mae,SS	Re&Ye	5.0–5.5	Yezin-6	15–29 °C, 1022 mm
MAS44	*B. japonicum*	Type *B*	Nam Latt, SS	Re&Ye	5.0–5.5	NSL	15–29 °C, 1022 mm
MAS45	*B. japonicum*	Type *B*	Nam Latt, SS	Re&Ye	5.0–5.5	NSL	15–29 °C, 1022 mm
MAS46	*B.yuanmingense*	Type *A*	Kyaut Mae, SS	Re&Ye	5.0–5.5	NSL	15–29 °C, 1022 mm
MAS47	*B.yuanmingense*	Type *A*	Kyaut Mae, SS	Re&Ye	5.0–5.5	NSL	15–29 °C, 1022 mm
MAS48	*B. japonicum*	Type *B*	Kyaut Mae, SS	Re&Ye	5.0–5.5	Yezin-6	15–29 °C, 1022 mm

MR: Mandalay Region, SS: Shan State, YR: Yangon Region, NSL: Northern Shan Local, MMa: meadow and meadow alluvial soil, Re&Ye: red earths and yellow earths, Ls: lateritic soils, Pcss: primitive crushed stone soils, Temp: average minimum to maximum temperature, RF: average annual rainfall per year

[†] Soil classification estimated based on the book *Soil Types and Characteristics of Myanmar* published by the Ministry of Agriculture and Irrigation (2004).

[‡] Records of the Meteorological Stations and Rainfall Stations of the Department of Meteorology and Hydrology, Myanmar.

Sequencing and phylogenetic analysis: The purified PCR products ($\geqslant$ 50 ng/µl) were directly sequenced by Macrogen (Tokyo) using the primer set described above. Raw sequence results were edited by DNASIS Mac ver. 2.0 (Hitachi, San Bruno, CA, USA) to create ITS sequence fragments. The sequencing of each strain was replicated twice and bidirectional sequences were aligned using GENETYX-MAC ver.10.1 (Software Development, Tokyo) to obtain the consensus sequence of the strain.

Sequences were compared with the DNA Data Bank of Japan (DDBJ) using the Basic Local Alignment Search Tool (BLAST) program[19]. For the construction of the phylogenetic tree, type strains of *Bradyrhizobium* species were chosen from BLAST search and aligned with the CLUSTALW function of the MEGA version 4 computer program[20]. After alignment, MEGA version 4 was used to construct the phylogenetic tree by the neighbour-joining method[5]. The topologies of the trees were evaluated in bootstrap analyses of 1000 replicates, and the Kimura 2-parameter model[21] was used to calculate the genetic distances.

Nucleotide sequence accession numbers

The nucleotide sequences of the ITS region between the 16S and 23S rRNA genes of 43 isolates were deposited in the DDBJ under the set of accession numbers AB749394-AB749436.

Diversity index of rhizobia at each collected site

The diversity of the isolates at each collection field site was investigated. For this, Shannon's diversity index (H') was calculated for each site in terms of the clusters in the phylogenetic tree. The index was calculated using the equation[22]

$$H' = -\sum_i P_i \ln P_i,$$

where P_i is the dominance of the isolates expressed as n_i/N, with N and n_i being the total number of isolates tested in a site and the number of isolates belonging to a particular cluster in the site, respectively.

Nodulation test for *Rj* gene determination

To estimate compatibility of soya bean cultivars collected from Myanmar with reference strains to judge the *Rj*-genotype, 14 soya bean cultivars (Yezin-3, Yezin-6, Yezin-8, Yezin-11, Yezin-14, Shan Sein, Hinthada, Shan Wha, Northern Shan Local, Southern Shan Local, Daewoncong, Daepung, Cheongga-3, Zhongpin-661) including three reference cultivars D51 (Rj_3), CNS (Rj_2Rj_3) and Hill (Rj_4) were inoculated with *Bradyrhizobium* strains Is 1, USDA 33 and Is 34, respectively[23]. Prior to inoculation, these *Bradyrhizobium* strains were cultured on A1E liquid medium[16] and diluted with Hoagland solution to give 10^6 cells/ml. Sterilized seeds were sown into 1 l prepared culture pots containing sterilized vermiculite in the control room (25 °C, 70% RH). After one month, the formation of effective nodules was checked on the soya bean roots.

Determination of nodulation type

The nodulation types of the collected 43 *Bradyrhizobium* isolates from Myanmar were investigated. There compatibility for effective nodule formation on two *Rj*-genotype soya bean cultivars, CNS (Rj_2Rj_3) and Hill (Rj_4) was assessed using the reference strains such as Is 1 (B type), Is 34 (C type) and USDA 110 (A type)[8]. The experiment was performed using the same conditions as described above.

RESULTS

The forty three bacteria were isolated from root nodules of different soya bean hosts from three different agro-climatic regions of Myanmar. According to Somasegaran and Hoben[15], these strains were proved as pure *Bradyrhizobium* strains. In YMA plates, the bradyrhizobial colonies reached 1–3 mm diameter with undulated pulvinate, entirely pulvinate, and entirely capitate shapes after 7 days incubation (Table 1).

The results of the phylogenetic analysis based on the ITS sequence, indicated that all the 43 isolates belonged to the genus *Bradyrhizobium* (Table 1). The results of phylogenetic analysis of these sequences are shown in Fig. 2.

The closest reference strain with at least 97% sequence similarity was used as the criterion for cluster separation of the isolates. Eight clusters were identified in the phylogenetic tree including 2 clusters which belong to *B. japonicum*. *Bj1* was highly related to *B. japonicum* strains USDA 452 and 456 with 100% sequence similarity while *Bj2* cluster was 100% sequence similar to *B. japonicum* strains USDA110, 122, 125, and 129. Among the last six clusters, three belonged to *B. elkanii*, with *Be1* showing between 99% and 100% sequence similarity with *B. elkani* USDA 46. *Be2* groups were related to *B. elkanii* USDA 26, 61, and 83 with at least 99% sequence similarity. The last cluster, *Be3* had 99% sequence similarity with closest *B. elkanii* strains USDA 29, 39, 40, 67, 116, and 120. In the rest of the three clusters, two clusters belonged to *B. yuanmingense* and shared 97% and 99% ITS sequence similarity

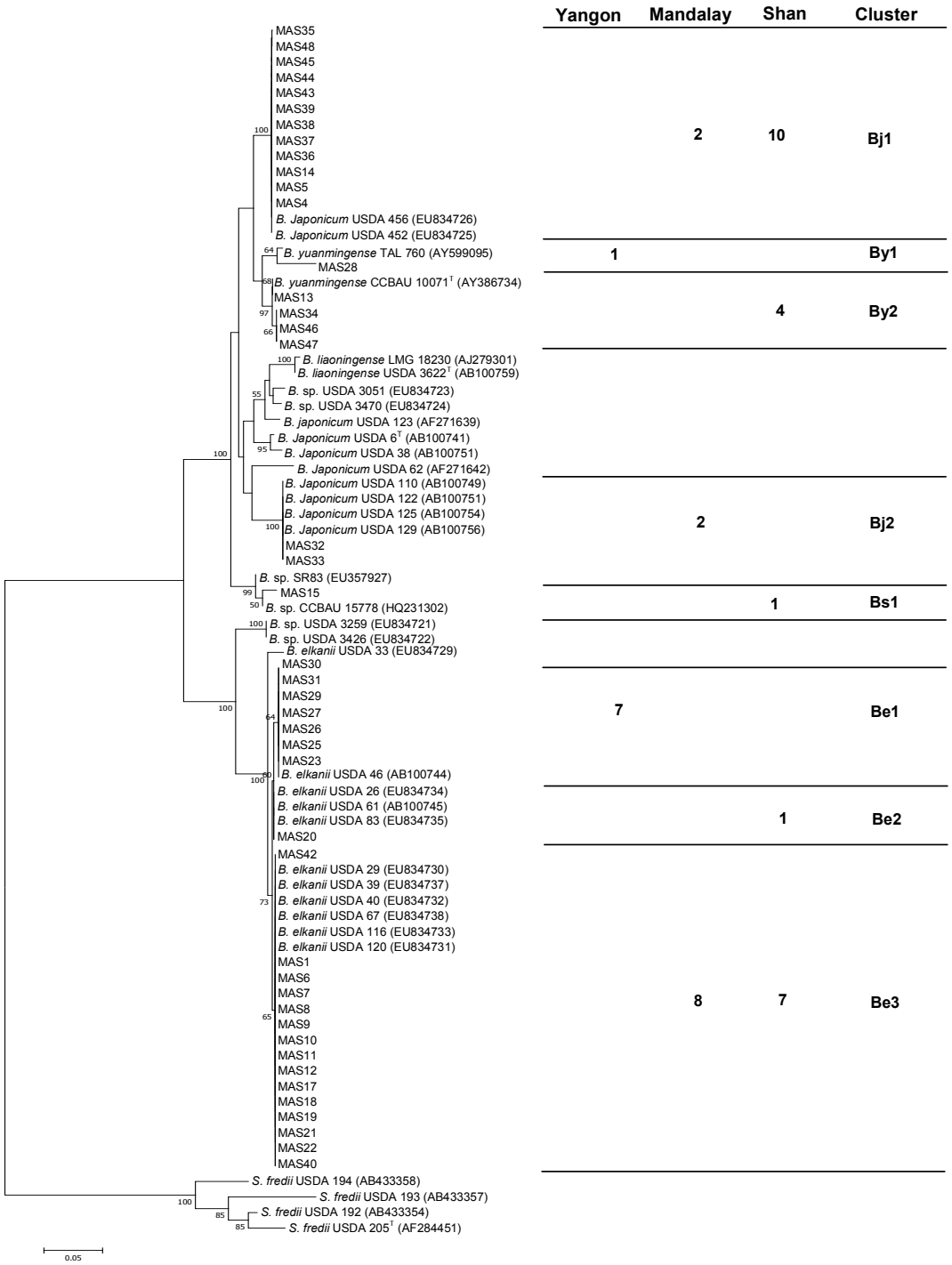

Fig. 2 Position of the 43 strains in the phylogenetic tree based on the ITS (16S-23S rRNA) sequences of related *Bradyrhizobium* strains (in italics) retrieved from GenBank. The tree was constructed by the neighbour-joining method with the Kimura 2-parameter (K2P) distance correlation model and 1000 bootstrap replications. Bootstrap values above 50% are indicated at the nodes. Bar indicates 0.02 K_{nuc} in nucleotide sequences. Accession numbers of the reference strains, including all type strains of *Bradyrhizobium*, are shown in parentheses. *B: Bradyrhizobium* and *S: Sinorhizobium*. The clustering of isolates and their distribution throughout the total studied area is noted in the tree: presence of clusters at the Yangon region: presence of clusters at the Mandalay region: presence of clusters at the Shan State: this excludes *B. liaoningense* from being grouped with *B. japonicum* in the tree.

Table 2 Percentage distribution (%) of isolates and diversity index (H') of clusters at each field site.

Field sites	*B. japonicum*		*B. yuanmingense*		*B. elkanii*		*B.* sp.		Diversity
	%	No. of clusters	%	No. of clusters	%	No. of clusters	%	No. of clusters	(H')
Shan State	44	1	17	1	35	2	4	1	1.30
Mandalay Region	33	2	–	–	67	1	–	–	0.87
Yangon Region	–	–	12	1	88	1	–	–	0.38
Total	33		12		53		2		

The total is the percentage distribution of isolates in the total studied area.

with *B. yuanmingense* TAL760 and *B. yuanmingense* CCBAU 10071^{T}, respectively. *Bradyrhizobium* sp. was found in the last cluster *Bs1* which showed 100% sequence similarity with *B.* sp. CCBAU 15778.

Among the collected strains, almost all from Yangon Region soya bean were identified as *B. elkanii* except MAS28 which belonged to *B. yuanmingense*. At Mandalay Region, most of the strains were related to *B. elkanii* and only a few were *B. japonicum* (Table 1 and Fig. 1). However, almost all the soya bean-nodulating bacteria: *B. japonicum*, *B. elkanii*, *B. yuanmingense*, and *Bradyrhizobium* sp. were found in Shan State soya bean growing region.

In this study, cluster *Be1* and *By1* were observed only in Yangon Region soya bean growing area. However, *Bj1*, and *Be3* clusters were distributed in both Mandalay Region and Shan State soya bean growing sites. The percentage distribution of isolates in the different sites is shown in Fig. 2 and Table 2.

The diversity index of the clusters present at each field site is shown in Table 2. The differences in diversity index (H') were obtained among the studied regions. The high diversity was found at the Shan State where *B. japonicum*, *B. yuanmingense* and *Bradyrhizobium* sp. were present. This area also showed a higher variance in *B. elkanii* with a higher diversity index (H') value of 1.30. However, the diversity index (H') at the Mandalay Region soya bean growing areas showed high variance in *B. japonicum*. In Yangon soya bean growing regions, there was a low variance in *B. elkanii* and *B. yuanmingense*. The results of this study revealed that *B. elkanii* is distributed throughout the soya bean cultivation areas and appeared as the dominant strain in Myanmar.

The use of different types of *Rj*-genotype soya beans is important for the isolation and analysis of indigenous soya bean-nodulating bacteria. Among the checked soya bean cultivars for nodulating genotypes, six cultivars such as Hinthada, Southern Shan local, Northern Shan local, Yezin-3, Yezin-11, and Daewoncong harboured the Rj_{4} gene, but the remaining eight cultivars (Shan Sein, Shan Wha, Yezin-6, Yezin-8, Yezin-14, Daepung, Cheongga-3, and Zhongpin-661) were non-*Rj*. The nodule occupancy of the isolated *Bradyrhizobium* strains with host soya bean genotypes and their level of compatibility are shown in Fig. 1. From these results, the isolated native *Bradyrhizobium* strains were derived from the non-*Rj* genotype and Rj_4 genotype.

When the nodulation types of the collected native bradyrhizobia were determined, 22 bradyrhizobia showed type A, 20 bradyrhizobia were type B and only MAS28 showed nodulation type C (Table 1). It was found that *Bradyrhizobium* sp. and all *B. japonicum* strain *Bj1* were type B. However, almost all of the bacterial strains in rest clusters showed nodulation type A.

DISCUSSION

Forty three soya bean root nodule bacterial isolates from three agro-climatic regions of Myanmar were successfully collected and proved as pure *Bradyrhizobium* strains [15]. Among them, 8 isolates were obtained from Yangon Region, 12 isolates were from Mandalay Region, and 23 isolates were collected from soya bean cultivation regions of Shan State. The bacterial strains collected from three different agro-climatic regions of Myanmar were higher in number at Shan State followed by Mandalay soya bean growing site and Yangon site (Table 1).

Based on the results of 16S-23S rRNA gene ITS sequence analyses, these isolates were related to *B. japonicum*, *B. elkanii*, *B. yuanmingense*, and *Bradyrhizobium* sp. The ITS and maintenance genes are currently used as markers for molecular systematics and for estimations of phylogenetic relationships among bradyrhizobia [24–28], with the 16S rRNA gene having few polymorphisms within the *Bradyrhizobium* genus [25,27,28]. The ITS genes of Myanmar bradyrhizobia were classified by using the ITS sequences of *Bradyrhizobium* USDA serotype strains: six *B. japonicum* (USDA 452, 456, 110, 122, 125, and 129) and ten *B. elkanii* (USDA 46, 26, 61, 83, 116, 120, 67, 40, 39, and 29), indicating a higher

diversity of soya bean bradyrhizobia among *B. elkanii* than *B. japonicum*. Recently, the isolation and characterization of *B. yuanmingense* strains nodulate mung bean and soya bean were reported[29–31]. In the present study, *B. yuanmingense* strains were isolated from the root nodule of soya bean from Yangon Region and Shan State. Additionally, this strain was also isolated from soya bean in China[32], Nepal[33], and Thailand[29].

The phylogenetic tree showed that the indigenous bradyrhizobial strains were distributed throughout the soya bean growing regions of Myanmar. Our results showed that most of the strains were observed in the clusters Bj1 and Be3. Among these genotypic strains, *B. elkanii* was widely distributed in the all soya bean growing areas of Myanmar. Hence according to our findings, *B. japonicum* and *B. elkanii* were the dominant strains in Myanmar where three soya bean-growing areas were considered for bacterial nodule identification. Moreover, bradyrhizobia distribution in Japan[34,35], similar to our finding, that bradyrhizobia might diversify in individual fields depending on the associated host plants and local soil conditions. The soya bean-nodulating rhizobial community might change depending on the host cultivar and cultivation temperature even in the same field as well as in different fields with geographical, soil texture, soil pH, salinity, and other differences[36]. From our finding, the genotypic analysis of soya bean bradyrhizobia from Myanmar implies a relationship between the field distribution of indigenous root nodule bacteria, latitude and soil pH. From which, it was noticed that *B. yuanmingense* cluster 1 and *B. elkanii* cluster 1 were distributed in tropical monsoon, Yangon region with soil pH 5.0–5.5. The native strains belong to the cluster *Bj1* and *Be3* were found in central dry zone of Myanmar, Mandalay region, and hilly plateau area, Shan State, of soya bean growing regions with soil pH ranging between 5.0 and 8.0. However, strains in cluster *Bj2* were observed only in soya bean growing area of central Myanmar, Mandalay. Although a geographical distribution was observed in our study, further studies are required to examine the many environmental factors affecting the indigenous bradyrhizobia. This is in accordance to the suggestion that bradyrhizobia might diversify in individual fields depending on the associated host plants and local soil conditions[35].

This study detected a genetic diversity of indigenous Myanmar soya bean bacterial strains. Diverse *B. elkanii* clusters were recorded at the Yangon, Mandalay, and Shan State areas. These results suggested a high variance of *B. elkanii* which resulted in the higher diversity indexes (H') recorded at the soya bean growing regions of Myanmar. Due to its wide distribution, *B. elkanii* was considered as the distinctive strains in the studied soya bean cultivation area in Myanmar, and the cluster *Be3* was highly represented in middle Myanmar.

The distribution of soya bean nodulating bacteria in the USA and record of the indigenous rhizobia survival, demonstrated a high presence of serogroup 123 in the northern regions and a high presence of *B. elkanii* in the southern regions[37]. However, the indigenous soya bean nodulating rhizobia of Myanmar, serogroups USDA 452 and 456 were found abundant in upper hilly plateau Myanmar. The differences in dominance of bradyrhizobial clusters between the strains of the US collections and the current study collections from Myanmar might be affected by geographic condition such as latitude that differs greatly, including different in climate and soil type.

This study reports the first finding of *B. yuanmingense* from nodules of soya bean in Myanmar. There were some reports on the isolation and classification of *B. yuanmingense* strains that nodulated mung bean and soya bean[30,31] and *B. yuanmingense* strain that did not nodulate soya bean but nodulated legume species of the genus *Lespedeza*[32]. In this study, five *B. yuanmingense* strains; MAS28 from Yangon Region and MAS13, MAS34, MAS46, and MAS47 from Shan State were isolated.

Differences in compatibility of the native *Bradyrhizobium* isolates to *Rj*-soya bean genotypes were found in this experiment. From this finding, the nodulation results stated that the collected isolates were incompatible with Rj_2Rj_3 genotypic soya beans in nodule formation. However, they were compatible with Rj_4 and non-*Rj* genotype of soya beans; leading to nodule formation. A similar result[8] stated that rhizobial strains incompatible with Rj_2Rj_3-genotype soya beans prefer nodulation on Rj_4-genotype soya bean cultivars, strains incompatible with Rj_4-genotype soya beans prefer Rj_2Rj_3-genotype soya bean cultivars, and strains compatible with every *Rj*-genotype prefer for non-*Rj*-genotype soya bean cultivars.

Moreover, they classified *B. japonicum* into three nodulation-types, type A, type B, and type C based on the compatibility with the *Rj*-genotypes of the soya bean cultivars. Here, we determined the nodulation type of the collected root-nodule bacteria. It appeared that all of the isolates in cluster *Bj1* and *Bs1* belonged to the type B nodulation that prefer on Rj_4 and non-*Rj* genotypic soya beans. A bradyrhizobial strain in *By1* cluster was compatible with non-*Rj* genotypes soya bean for nodulation type C. Nevertheless, almost

all native bacterial isolates in the remaining clusters preferred to form both types A and B on non-*Rj* and *Rj*$_4$ soya beans. Most of the bacterial strains in Shan State and Yangon Region were produced nodulation type B, though in Mandalay type A nodules were formed in inoculated plants. These results suggest that inoculated native bradyrhizobial strains were compatible with *Rj*$_4$ genotypes for the preference of nodulation type B in Shan State and Yangon Region. However, the compatibility of indigenous *Bradyrhizobium* strains with non-*Rj* genes has been observed in Mandalay Region that preferred the formation of type A nodulation.

In conclusion, this is the first report on the identification and the biogeographic distribution of soya bean-nodulating *Bradyrhizobium* isolates from three different soya bean growing areas in Myanmar. The 43 indigenous bradyrhizobia were successfully isolated and their geographic distribution was determined based on the analysis of the 16S-23S rRNA internal transcribed spacer region. Our results indicated that *B. elkanii* strain was widely distributed in the soya bean growing regions of Myanmar whereas *B. japonicum* and *B. elkanii* were found more abundant in Mandalay Region and Shan State. When indigenous bacteria were evaluated in compatibility for *Rj*-genotypes in preference of nodule formation, two *Rj*-genotypes *Rj*$_4$ and non-*Rj* determined the nodulation types of strains. *Rj*$_4$ genotype soya beans in Shan State and Yangon Region were compatible with indigenous bacteria for the formation of nodulation type B, and non-*Rj* genotypic soya beans with bacterial strains were compatible for nodulation type A in Mandalay Region. Only type C was found in MAS28. Hence *Rj* genes might affect the nodulation compatibility between *Rj*-genotype soya beans and bradyrhizobia. However, potential efficacy of soya bean-nodulating bacteria in symbiosis interaction for nitrogen fixation should be investigated further for the selection of effective inoculants used as a sustainable approach in soya bean production.

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