

Phylogenetic Diversity and Evaluation the Effectiveness of Indigenous *Bradyrhizobium* Strains for Myanmar Black Gram (*Vigna mungo* L. Hepper) Cultivars

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Abstract

Black gram (*Vigna mungo* L. Hepper) is one of the main leguminous crops that provide chief source of food. Several *Bradyrhizobium* species are able to induce effective nodules in black gram cultivars. In the present study, we characterized forty isolates of indigenous black gram bradyrhizobia from Myanmar based on the sequence analysis of the bacterial 16S rRNA gene. The sequence analysis confirmed that all isolates were categorized and identified as the genus *Bradyrhizobium* and they were conspecific with *B. elkanii*, *B. sp.*, *B. liaoningense*, *B. japonicum* and *B. yunamingense*. Almost all the collected isolates from major black gram growing regions of Nyaunglebin Bago Regio, Chaungzon Mon State, Sittwe Rakhine State, Danubyu Ayeyarwady Region and Launglon Tanintharyi Region were identified as *B. liaoningense*. At Danubyu Ayeyarwady Region and Pyinmanar Nay Pyi Taw Region, most of the strains were identified as *B. japonicum*. On the other hand, more or less all the isolates from Launglon Tanintharyi Region and Hpa-an Kayin State were related to *B. elkanii*. However, all *B. sp.* strains were found in Salingyi Sagaing Region black gram growing region. This is the first report describing *Bradyrhizobium* strains that were isolated from soil samples of major black gram growing areas in Myanmar. Evaluation of the effectiveness of Myanmar *Bradyrhizobium* strains isolated from soil samples of major black gram growing

areas of Myanmar for plant growth and nitrogen fixation was studied in pot experiments with completely randomized design and three replicates. The nodule dry weight, shoot dry weight and acetylene reduction activity of the plant inoculated with *Bradyrhizobium elkanii* LauBG38 were significantly higher in ARA per plant, nodule and shoot dry weights than the other tested isolates in both Yezin-4 and Yezin-7 black gram varieties. We expect that Myanmar *Bradyrhizobium elkanii* LauBG38 will be able to use as Biofertilizer for black gram cultivars.

Keywords

Black Gram, Myanmar, 16S rRNA Gene, *Bradyrhizobium*, Nitrogen Fixation

1. Introduction

Grain legumes play an important nutritional role in the diet of millions of people in the developing countries [1]. Black gram (*Vigna mungo* L. Hepper) is a short duration crop that belongs to grain legumes family and rich in protein [2]. It is an important legume crop in Asia [3]. In Myanmar, it is one of the major exportable crops and it is the second largest cultivated Legume crop as well [4]. During 2017-2018, black gram growing areas in Myanmar was about 9.77 million hectares with a production of 1.37 metric tons, and the average yield was 1.41 metric ton·ha⁻¹ [5].

Nitrogen (N) fixation through legume-*Rhizobium* symbiosis is important for enhancing agricultural productivity and is therefore of great economic interest [6]. Leguminous crops have a reputation for maintaining soil fertility since it can assimilate nitrogen from the atmosphere through symbiotic biological N₂ fixation (BNF) with Rhizobia [7]. Biological nitrogen fixation (BNF) is an important component of sustainable agriculture [8], and rhizobial inoculants have been applied frequently as biofertilizers.

Rhizobial inoculant can be used to substitute the nitrogenous fertilizers in food legume crops. Recently, peat-based root nodule bacterial inoculants containing TAL strains are using as biofertilizer in seven legumes distributed by Ministry of Agriculture and Irrigation, Myanmar [9]. However, the effectiveness of symbiotic N₂ fixation may be an important factor to take into consideration through successful management of symbiosis between black gram cultivars and effective bradyrhizobial strains.

Commercial production of *Rhizobium* inoculant has been developed for several decades. The main objective of using *Rhizobium* inoculant is to substitute the nitrogenous fertilizers in food legume production. It is cheaper and lighter in weight than urea and easier to use for the farmers [10]. To improve high quality inoculant production, the evaluation of highly effective rhizobial strains for specific legume is one of the principle obligations [11] [12]. Indigenous rhizobial strains also play an important role since they have adapted to local environmen-

tal conditions. Therefore, the investigation of effective indigenous rhizobial strains should be considered for current inoculant production in Myanmar.

Myanmar farmers have used, and continue to use, rhizobial inoculants when sowing legumes, but the practice is currently not extensive. The Department of Agricultural Research, Ministry of Agriculture, Livestock and Irrigation is responsible for producing inoculants in Myanmar for their distribution to farmers. Production by DAR peaked during the 1980s at 600 - 700,000 packets annually. Current production is < 100,000 packets, due to limitations in the whole supply chain from production and quality assurance to distribution to demand. Myanmar farmers use nitrogenous (N) fertilizers sparingly, particularly on legume crops. Thus, low-nodulation induced N deficiencies of the legumes are not remedied by inputs of fertilizer N and the value of lost production could exceed \$100 million annually [13].

Several studies also reported significant increase growth parameters and yield due to the inoculation of rhizobial isolates in chickpea [14] in mung bean and in soybean [15] [16] [17] [18] in Myanmar to identify the strains, plant growth and nitrogen fixation. However, there was nobody investigated the native bradyrhizobial isolates from Myanmar black gram cultivars on diversity, plant growth and N₂ fixation. So, this research is very first report for phylogenetic diversity and evaluation the effectiveness of indigenous *Bradyrhizobium* strains for Myanmar black gram (*Vigna mungo* L.) cultivars and this research finding is needed in order to guarantee the potential of these indigenous *Bradyrhizobium* strains for black gram production. Not only bradyrhizobial inoculation is important for black gram cultivation by poor Myanmar farmers as valuable cheap source of nitrogen enough N₂ fixation by biological means are also necessary and attractive topic for investigation.

For this reason, we aimed to isolate indigenous root nodule bacteria from collected soil samples of major black gram growing areas of Myanmar, to identify the phylogenetic diversity of indigenous black gram-nodulating bradyrhizobia in Myanmar based on sequence analysis of the 16S rRNA region of the isolates, and to evaluate the effectiveness of indigenous Myanmar *Bradyrhizobium* strains for plant growth and nitrogen fixation of Myanmar black gram varieties are required for investigation.

2. Materials and Methods

2.1. Origin of Soil Sample Collection Sites to Obtain Black Gram Bradyrhizobial Isolates

Soil samples were collected from eight major black gram growing regions of Myanmar (Figure 1). Meadow soil occurs in Delta Plains Region of Nyaunglebin Bago Region (17°57'N 96°43'E) with a pH of 5.04, Coastal Strips Region of Chaungzon Mon State (16°23'N 97°32'E) with a pH of 4.69 and Sittwe Rakhine State (20°30'N 93°20'E) with a pH of 5.91. The Coastal Strips Region of Launglon Tanintharyi Region (14°05'N 98°12'E) with a pH of 4.79 and Mountain and Hill

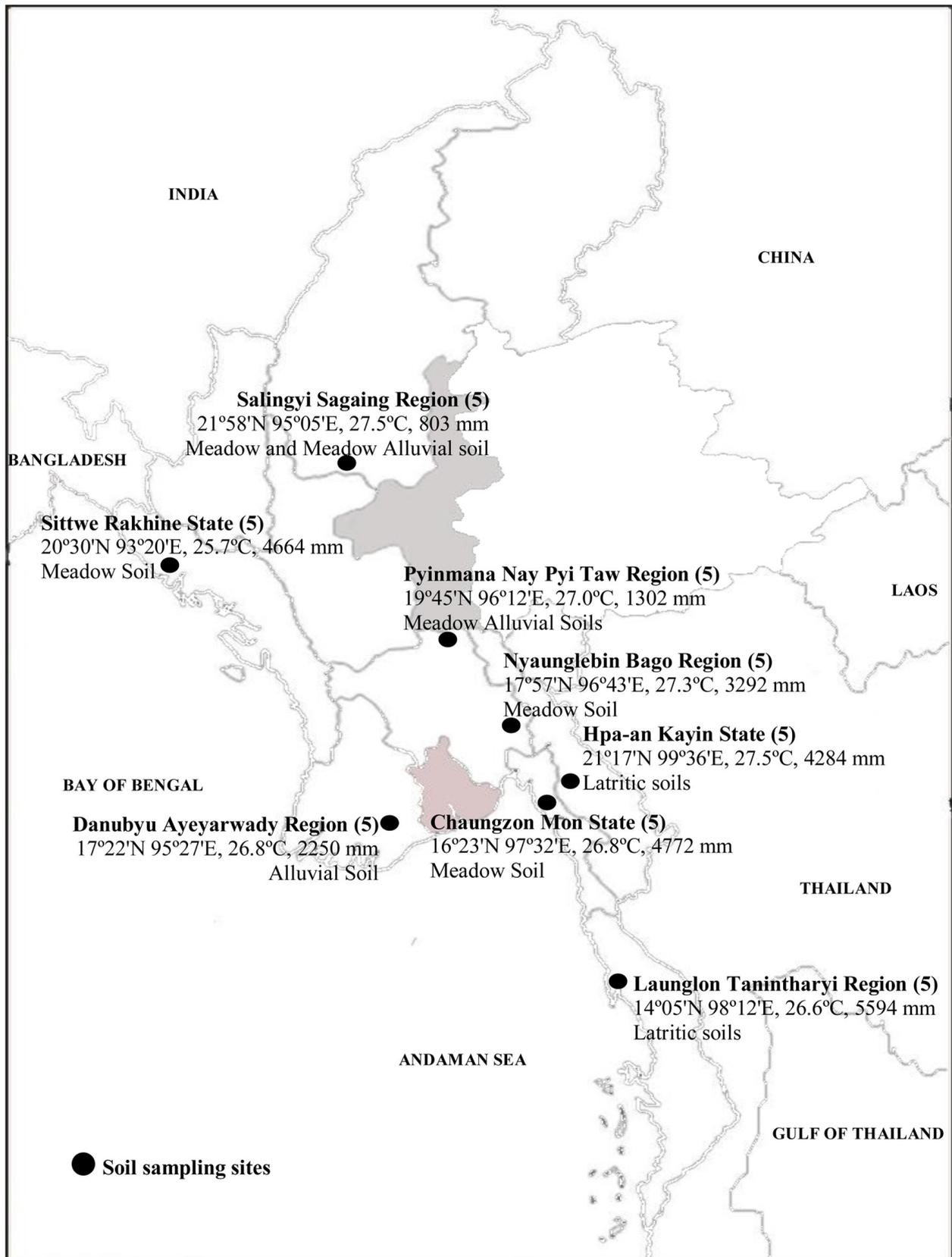


Figure 1. Map of Myanmar showing soil sampling sites from black gram major growing areas in Myanmar. Numbers in parentheses represent the total number of isolates collected from each site.

Region of Hpa-an Kayin State (16°53'N 97°38'E) with a pH 5.13 was Latritic Soils [19]. The Central Regions of Pyinmanar Nay Pyi Taw Region (19°45'N 96°12'E) was a Meadow Alluvial Soil with a pH of 6.01 and Salingyi Sagaing Region (21°58'N 95°05'E) with a pH 6.97 was Meadow and Meadow Alluvial Soil are performing a tropical wet and dry climate [20]. Alluvial Soil occurs in Delta Plains Region of Danubyu Ayeyarwady Region (17°22'N 95°27'E) with a pH of 6.70 (Table 1, Table 2 and Figure 1).

Table 1. Soil classification, location and climate of soil samples collected from major black gram growing areas of Myanmar.

Soil sampling site	Soil Classification ¹⁹⁾	Location	Climate ²⁰⁾ (Avg. Temp; RF)
Nyaunglebin Bago Region	Meadow Soil	17°57'N 96°43'E	27.3°C, 3292 mm
Danubyu Ayeyarwady Region	Alluvial Soil	17°22'N 95°27'E	26.8°C, 2250 mm
Chaungzon Mon State	Meadow Soil	16°23'N 97°32'E	26.8°C, 4772 mm
Launglon Tanintharyi Region	Latritic soils	14°05'N 98°12'E	26.6°C, 5594 mm
Sittwe Rakhine State	Meadow Soil	20°30'N 93°20'E	25.7°C, 4664 mm
Pyinmanar Nay Pyi Taw Region	Meadow Alluvial Soil	19°45'N 96°12'E	27.0°C, 1302 mm
Salingyi Sagaing Region	Meadow and Meadow Alluvial Soil	21°58'N 95°05'E	27.5°C, 803 mm
Hpa-an Kayin State	Latritic soils	16°53'N 97°38'E	27.5°C, 4284 mm

Sources: ¹⁹⁾Shein, H.A. The soil types and characteristics of Myanmar. Department of Agriculture, Ministry of Agriculture, Livestocks and Irrigation: Nay Pyi Taw, Myanmar, 2015. ²⁰⁾Aung, L.L.; Zin, E.E.; Theingi, P.; Elvera, N.; Aung, P.P.; Han, T.T.; Oo, Y.; Skaland, R.G. Myanmar Climate Report published by Department of Meteorology and Hydrology Myanmar, Ministry of Transport and Communications, Government of the Republic of the Union of Myanmar, 2017.

Table 2. Characteristics of soils used to obtain black gram rhizobia isolate from major black gram growing regions of Myanmar.

Soil samples	Physicochemical property				
	Soil pH (Soil: H ₂ O; 1:2.5)	Total N (%)	Mineralizable N (g/kg)	Total P ₂ O ₅ (%)	Total K ₂ O (%)
Nyaunglebin Bago Region	5.04	0.31	1.31	0.23	1.14
Danubyu Ayeyarwady Region	6.70	0.12	0.91	0.06	0.73
Chaungzon Mon State	4.69	0.23	1.57	0.10	1.74
Launglon Tanintharyi Region	4.79	0.73	1.62	0.08	2.98
Sittwe Rakhine State	5.91	0.16	2.07	0.05	0.42
Pyinmanar Nay Pyi Taw Region	6.01	0.25	0.95	0.09	0.76
Salingyi Sagaing Region	6.97	0.05	0.42	0.03	0.16
Hpa-an Kayin State	5.13	0.16	0.75	0.07	0.75

Soil sample analyses were performed in the plant nutrition laboratory, faculty of agriculture, Kyushu University, Japan.

2.2. Analysis of Collected Soil Samples

The collected soil samples were analyzed at Plant Nutrition Laboratory, Faculty of Agriculture, Kyushu University, Fukuoka, Japan. For each collected soil sample, soil pH H₂O (1:2.5 soil: H₂O) was measured using a pH meter (HM-10P; DKK-TOA Corp., Tokyo, Japan). Soil samples were also digested using the salicylic acid-sulfuric acid-hydrogen peroxide method [21]; then, total N was examined using the indophenol method [22], and total P was tested using the ascorbic acid method [23]. Total K was analyzed using an atomic absorption spectrophotometer (Z-5300; Hitachi, Tokyo, Japan) afterward samples were digested. To analyze mineralizable N, we firstly used the soil incubation method [24] followed by the indophenol method [22].

2.3. Isolation of Indigenous Root Nodule Bacteria from Soil Samples of Major Black Gram (*Vigna mungo* L.) Growing Areas of Myanmar

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 one hour 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 121 °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. Myanmar Yezin-7 black gram variety, common use in Myanmar was used as trap host for all soil samples.

The culture pots using black gram Yezin-7 variety with eight soil suspensions and control pot were prepared. The seeds were surface-sterilized and planted in the sterilized vermiculite pots. 5 ml aliquot of soil suspension was inoculated per seed. The control was planted without inoculation to assess the possibility of contamination. The plants were cultivated in the incubator (25 °C and 16 hours light) for four weeks. Autoclaved deionized water was poured when the original weight of the pots decreased by around 300 g.

After carefully uprooting, the five nodules (≥2 mm in diameter) were collected per pot. For surface sterilization, the nodules were soaked in 70% ethanol for 3 min, 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. For every sample, a loopful of the suspension was streaked on Yeast Extract Mannitol Agar (YMA) plates containing 25-µg Congo red [25]. The plates were incubated at 30 °C for 1 - 3 days for fast growing bacteria and 5 - 7 days for slow growing bacteria. Finally, purified 40 indigenous root nodule bacteria isolates were isolated from soil samples of major Black gram (*Vigna mungo*

L.) growing areas of Myanmar. The collected isolates were nominated as HpaBG1 to LauBG40.

2.4. DNA Extraction, PCR Analysis and Phylogenetic Analysis

For DNA extraction, the collected isolated were streaked onto A1E agar plates and incubated at 30°C for 7 days. A single pure colony of each isolate from A1E plates was cultured in A1E liquid medium at 30°C for 5 days to obtain the required optimum density ($0.4 < OD_{600\text{ nm}} < 0.6$). Total DNA was extracted using ISOPLANT (Nippon gene, Tokyo, Japan), following instructions from the manufacturer. The DNA concentrations were calculated using NIH Image 1.62 (National Institutes of Health, Bethesda, MD, USA) after agarose gel electrophoresis (0.3% agarose gel in 1 TAE buffer), staining with ethidium bromide (Toyobo, Tokyo, Japan), and destaining in 1 TAE buffer.

The primers 16S-F (5'-AGAGTTTGATCCTGGCTCAG-3') and 16S-R2 (5'-CGGCTACCTTGTACGACTT-3') were used to amplify the 16S rRNA region of mesorhizobia. 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 [26]. The cycle was repeated for 33 cycles, followed by a final extension at 72°C for 10 min. PCR products were purified using the Wizard Gel and PCR Clean-up System (Promega, Madison, WI, USA). Purified PCR products ($\geq 50 \text{ ng}\cdot\mu\text{L}^{-1}$) were subjected to direct sequencing by Macrogen (Tokyo, Japan), using the primer set described above. Raw sequence results were edited using MEGA version 6-software [27] to create 16S sequence fragments.

For homology searches, sequences were compared with the DNA Data Bank of Japan (DDBJ) using the Basic Local Alignment Search Tool (BLAST) program [28]. To construct the phylogenetic tree, sequences of type strains and closely related strains of *Bradyrhizobium* genospecies were retrieved from the BLAST database. All selected sequences including type strains and closet strains were aligned using the CLUSTALW function of the MEGA version 6-software [27]. After alignment, a phylogenetic tree was constructed according to the neighbor-joining method [29]. The phylogenetic tree was bootstrapped with 1000 replications of each sequence to evaluate the tree topology for reliability. Genetic distances were calculated using the Kimura two-parameter model [30].

2.5. Nucleotide Sequence Accession Numbers

The nucleotide sequences of 16S rRNA genes of 40 *Bradyrhizobium* strains were deposited in the DDBJ under the set of accession numbers LC515811 to LC515850.

2.6. Myanmar Black Gram (*Vigna mungo* L.) Cultivars and *Bradyrhizobium* Strains

Myanmar black gram (*Vigna mungo* L.) cultivars, Yezin-4 and Yezin-7 were collected from Food Legumes Section, Department of Agricultural Research, Ye-

zin, Myanmar. The black gram Yezin-7 variety was the most widely grown cultivar in Myanmar and used for screening of the 40 indigenous *Bradyrhizobium* strains. For effectiveness of selected *Bradyrhizobium* strains, Black gram Yezin-4 and Yezin-7 cultivars from Myanmar were investigated. The purified forty *Bradyrhizobium* strains were cultured in A1E liquid media [31] on a rotary shaker (100 rpm) at 30°C for 7 days. The cultures were diluted with sterilized N-free half-strength modified Hoagland nutrient (MHN) solution [32] to ca 10⁷ cells mL⁻¹ for *Bradyrhizobium* strains.

2.7. Screening of Effective Bradyrhizobial Strains by Myanmar Black Gram Yezin-4 for Nitrogen Fixation

The purified forty indigenous bradyrhizobial strains were screened for nitrogen fixing effectiveness on Myanmar black gram Yezin-7 in pots with vermiculite and MHN solution were covered with aluminum foil and autoclaved at 121°C for 20 min. Black gram Yezin-7 seeds were surface sterilized [33] and germinated on sterile petri dishes with filter paper. The germinated seeds were sown into the sterilized pot with vermiculite. In the non-inoculated treatment, a control treatment was also provided. The weights of original pots were measured. The plants were cultivated in incubator (25°C and 70% relative humidity). During the growing period, sterilized water was irrigated. The plants in each pot were uprooted and carefully washed with water so as not to detach the nodules. The acetylene reduction assay (ARA) was performed according to Haider *et al.*, 1991 [34] to measure nitrogenase activity. The black gram plants were cut at the cotyledonary nodes. Then, the black gram roots with intact nodules were placed in a 100 mL conical flask and sealed with a serum stopper. A 12 mL aliquot of acetylene (C₂H₂) gas was injected into the flask to replace the air with acetylene. The flasks containing roots with intact nodules were incubated at room temperature and 1.0 mL subsamples were analyzed at 5 and 65 min, respectively. The ARA value, in terms of ethylene (C₂H₄) production per plant, was measured using a flame ionization gas chromatograph (GC-14A, Shimadzu, Kyoto, Japan) equipped with a stainless steel column (3 mm diameter, 0.5 m length). The column was filled with Porapak R 60 - 80 mesh (Nicalai Tesque, Inc., Kyoto Japan). Column, injection and detection temperatures were 35°C, 45°C and 170°C, respectively. N₂ gas was used as the carrier gas at a flow rate of 45 mL·min⁻¹. The number of nodules was counted after the assay. Shoots, roots and nodules were collected separately and oven dried for at 70°C for 48 hours to record the dry weight determination.

2.8. Evaluation the Effectiveness of Selected *Bradyrhizobium* Strains on Two Myanmar Black Gram Cultivars: Yezin-4 and Yezin-7

The five *Bradyrhizobium* strains of *B. elkanii* HpaBG5, *B. liaoningense* HpaBG6, *B. liaoningense* HpaBG7, *B. liaoningense* HpaBG12, *B. elkanii* LauBG38 (Table 3) were selected based on the results of the above screening experiment in their

Table 3. The morphological characteristics of black gram bradyrhizobial isolate from Myanmar.

Isolates	Genus and species	Dendrogram cluster	Shape	Size (mm)
HpaBG1	<i>Bradyrhizobium elkanii</i>	Be1	UF	1.5
HpaBG2	<i>Bradyrhizobium elkanii</i>	Be1	UF	1.5
HpaBG3	<i>Bradyrhizobium elkanii</i>	Be1	UF	1.5
HpaBG4	<i>Bradyrhizobium</i> sp.	Bs1	UF	1.5
HpaBG5	<i>Bradyrhizobium elkanii</i>	Be1	UF	1.5
ChaBG6	<i>Bradyrhizobium liaoningense</i>	B11	EP	1.5
ChaBG7	<i>Bradyrhizobium liaoningense</i>	B12	EP	1.5
ChaBG8	<i>Bradyrhizobium liaoningense</i>	B11	EP	1.5
ChaBG9	<i>Bradyrhizobium japonicum</i>	Bo1	EP	1.5
ChaBG10	<i>Bradyrhizobium liaoningense</i>	B11	EP	1.5
NyaBG11	<i>Bradyrhizobium liaoningense</i>	B11	EP	1.5
NyaBG12	<i>Bradyrhizobium liaoningense</i>	B11	EP	1.5
NyaBG13	<i>Bradyrhizobium liaoningense</i>	B11	EP	1.5
NyaBG14	<i>Bradyrhizobium liaoningense</i>	B11	EP	1.5
NyaBG15	<i>Bradyrhizobium liaoningense</i>	B11	EP	1.5
DanBG16	<i>Bradyrhizobium liaoningense</i>	B12	EP	2.0
DanBG17	<i>Bradyrhizobium japonicum</i>	Bo1	EP	2.0
DanBG18	<i>Bradyrhizobium japonicum</i>	Bo1	EP	2.0
DanBG19	<i>Bradyrhizobium japonicum</i>	Bo1	EP	2.0
DanBG20	<i>Bradyrhizobium japonicum</i>	Bo1	EP	2.0
SalBG21	<i>Bradyrhizobium</i> sp.	Bs2	UF	1.5
SalBG22	<i>Bradyrhizobium yunamingense</i>	By1	UF	1.5
SalBG23	<i>Bradyrhizobium</i> sp.	Bs2	UF	1.5
SalBG24	<i>Bradyrhizobium</i> sp.	Bs2	UF	1.5
SalBG25	<i>Bradyrhizobium</i> sp.	Bs2	UF	1.5
SitBG26	<i>Bradyrhizobium liaoningense</i>	B12	EP	1.5
SitBG27	<i>Bradyrhizobium liaoningense</i>	B12	EP	1.5
SitBG28	<i>Bradyrhizobium liaoningense</i>	B11	EP	1.5
SitBG29	<i>Bradyrhizobium japonicum</i>	Bo1	EP	1.5
SitBG30	<i>Bradyrhizobium liaoningense</i>	B12	EP	1.5
PyiBG31	<i>Bradyrhizobium japonicum</i>	Bo1	EP	2.0
PyiBG32	<i>Bradyrhizobium japonicum</i>	Bo1	EP	2.0
PyiBG33	<i>Bradyrhizobium japonicum</i>	Bo1	EP	2.0
PyiBG34	<i>Bradyrhizobium</i> sp.	Bs2	EP	2.0
PyiBG35	<i>Bradyrhizobium japonicum</i>	Bo1	EP	2.0
LauBG36	<i>Bradyrhizobium elkanii</i>	Be1	UF	1.5
LauBG37	<i>Bradyrhizobium elkanii</i>	Be1	UF	1.5
LauBG38	<i>Bradyrhizobium elkanii</i>	Be1	UF	1.5
LauBG39	<i>Bradyrhizobium liaoningense</i>	B12	UF	1.5
LauBG40	<i>Bradyrhizobium elkanii</i>	Be1	UF	1.5

B11 = *Bradyrhizobium liaoningense* cluster 1; B12 = *Bradyrhizobium liaoningense* cluster 2; Bo1 = *Bradyrhizobium ottawaense* cluster 1; Bs1 = *Bradyrhizobium* sp. cluster 1; Bs2 = *Bradyrhizobium* sp. cluster 2; By1 = *Bradyrhizobium yunamingense* cluster 1; Be1 = *Bradyrhizobium elkanii* cluster 1; UP = Undulated-pulvinate, EP = Entirely-pulvinate.

potential efficiency on ARA per plant. The experiment was performed in completely randomized design with three replicates. The inoculation and growing condition of pot experiment were also conducted as the above experiment. ARA per plant, nodule, root and shoot dry weight were determined after four weeks. Data were analyzed using the STATISTIX 8 software (Analytical Software, Tallahassee, FL, USA), and treatment means were compared by Tukey's HSD test ($P < 0.05$) for the collected parameters.

3. Results

3.1. Diversity of Indigenous *Bradyrhizobium* Strains for Myanmar Black Gram (*Vigna mungo* L.) Cultivars

The forty root nodule bacteria were isolated from eight different soil samples from major black gram growing areas in Myanmar (Table 1, Table 2 and Figure 1). According to Somasegaran and Hoben, 1994 [25], these strains were proved as pure *Bradyrhizobium* strains. In YMA plates, the bradyrhizobial colonies reached 1 - 3 mm diameter with undulated pulvinate and entirely pulvinate shapes after 5 - 7 days incubation (Table 3).

Neighbor-joining trees for each gene had similar overall tree topologies. Groups were selected on the basis of the minimum standard changes between named species in the 16S rRNA phylogram (Figure 2), and all groups were well supported in neighbor-joining analyses which had less than 50% bootstrap support in the neighbor-joining tree. The results of the phylogenetic analysis based on the 16S rRNA sequence, indicated that all the 40 isolates belonged to the genus *Bradyrhizobium* (Table 3 and Figure 2).

The seven clusters were identified in the phylogenetic tree including four clusters of *B. liaoningense* (Bl1 and Bl2), two clusters of *B. sp.* (Bs1 and Bs2), one cluster of each *B. japonicum* (Bj1), *B. yunamingense* (By1) and *B. elkanii* (Be1) (Figure 2). Among these clusters, two clusters of Bl1 and Bl2 were 98% sequence similarity with *B. liaoningense* CCNWSX 0360^T and *B. liaoningense* LMG 18230^T while two clusters of Bs1 and Bs2 were 98% sequence similar to *B. sp.* CI 110^T and *B. sp.* JNVU DC11^T. Among the clusters belonged to *B. japonicum*, Bj1 was showing 97% sequence similarity with *B. japonicum* LMG 6138^T, *B. japonicum* N2 225^T, *B. japonicum* CCBAU 83623^T and *B. japonicum* R33^T. The last two clusters of By1 and Be1 groups were related to *B. yunamingense* 65010^T and *B. elkanii* Cte 503^T with at least 96% and 98% sequence similarity.

Almost all the collected isolates from Nyaunglebin Bago Region, Chaungzon Mon State and Sittwe Rakhine State black gram growing regions were identified as *B. liaoningense*. At Danubyu Ayeyarwady Region and Pyinmanar Nay Pyi Taw Region, most of the strains were identified as *B. japonicum*. On the other hand, more or less all the isolates from Launglon Tanintharyi Region and Hpa-an Kayin State were related to *B. elkanii*. However, all *B. sp.* strains were found in Salingyi Sagaing Region black gram growing region (Table 3 and Figure 2).

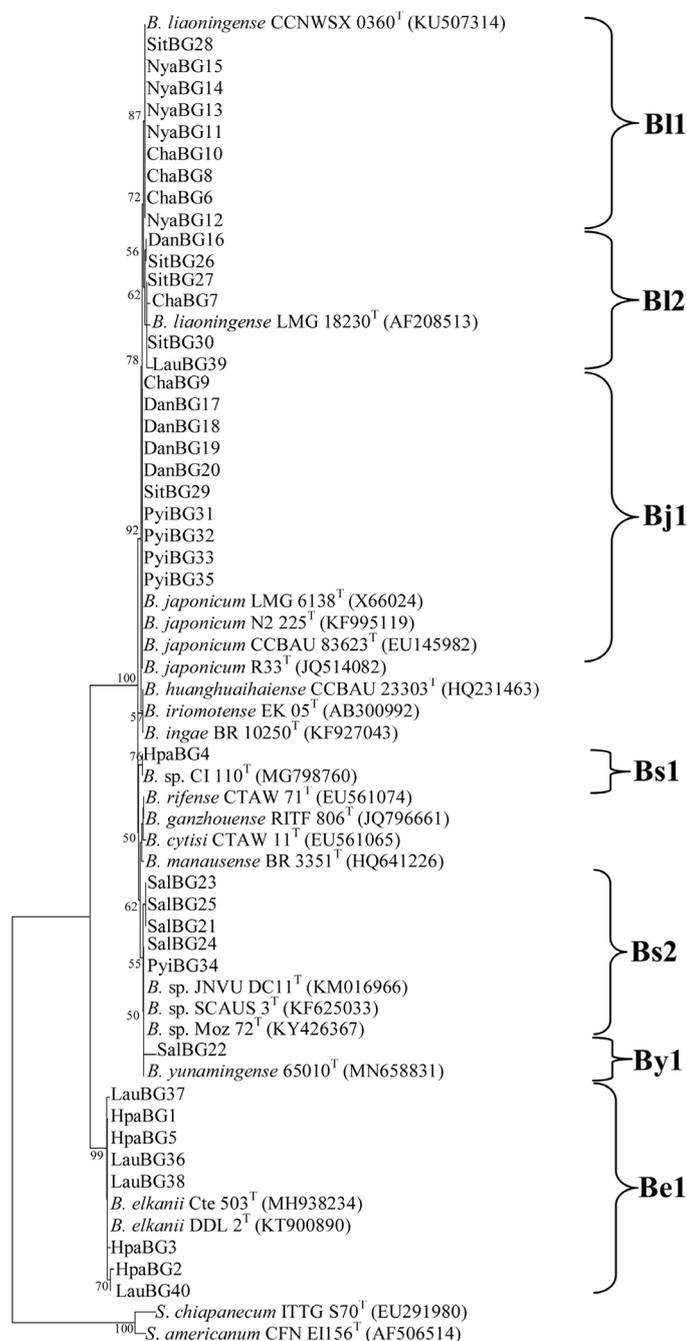


Figure 2. Position of the 40 strains in the phylogenetic tree based on the 16S rRNA sequences of related *Bradyrhizobium* strains (in italics) retrieved from GenBank. The tree was constructed by the neighbor-joining method with the Kimura 2-parameter (K2P) distance correlation model and 1000 bootstrap replications. Bootstrap values above 50% are indicated at the nodes. Bar, 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 into the tree: B11 = *Bradyrhizobium liaoningense* cluster 1; B12 = *Bradyrhizobium liaoningense* cluster 2; Bj1 = *Bradyrhizobium japonicum* cluster 1; Bs1 = *Bradyrhizobium sp.* cluster 1; Bs2 = *Bradyrhizobium sp.* cluster 2; By1 = *Bradyrhizobium yunamingense* cluster 1; Be1 = *Bradyrhizobium elkanii* cluster 1.

In this study, cluster B11 and B12 were observed in major black gram growing areas Nyaunglebin Bago Regio, Chaungzon Mon State, Sittwe Rakhine State, Danubyu Ayeyarwady Region and Launglon Tanintharyi Region. In Hpa-an Kayin State, Salingyi Sagaing Region and Pyinmanar Nay Pyi Taw Region, cluster Bs1 and Bs1 were found but in Chaungzon Mon State, Danubyu Ayeyarwady Region, Sittwe Rakhine State and Pyinmanar Nay Pyi Taw Region, cluster Bj1 was distributed. However, By1 cluster was only distributed in Salingyi Sagaing Region and Be1 cluster was scattered in both Hpa-an Kayin State and Launglon Tanintharyi Region of black growing areas of Myanmar (Figure 2).

3.2. Screening of Effective Bacterial Strains by Yezin-7 for Nitrogen Fixation

In the screening experiment, the effective strains were determined their potential ability in the N fixation analyzed by means of ARA per plant. Each bacterial strain that responded on black gram Yezin-7 was expressed in Figure 3. The higher ARA per plant was found in plants inoculated by *B. elkanii* HpaBG5, *B. liaoningense* ChaBG6, *B. liaoningense* ChaBG7 and *B. elkanii* LauBG38 (Figure 3). From this experiment, five indigenous Bradyrhizibium strains were screened out for further studies based on their effectiveness in ARA per plant. There were four Bradyrhizobial strains from the highest ARA per plant value of *B. elkanii* HpaBG5, *B. liaoningense* ChaBG6, *B. liaoningense* ChaBG7, *B. elkanii* LauBG38 and one strain from the middle ARA per plant value of *B. liaoningense* NyaBG12.

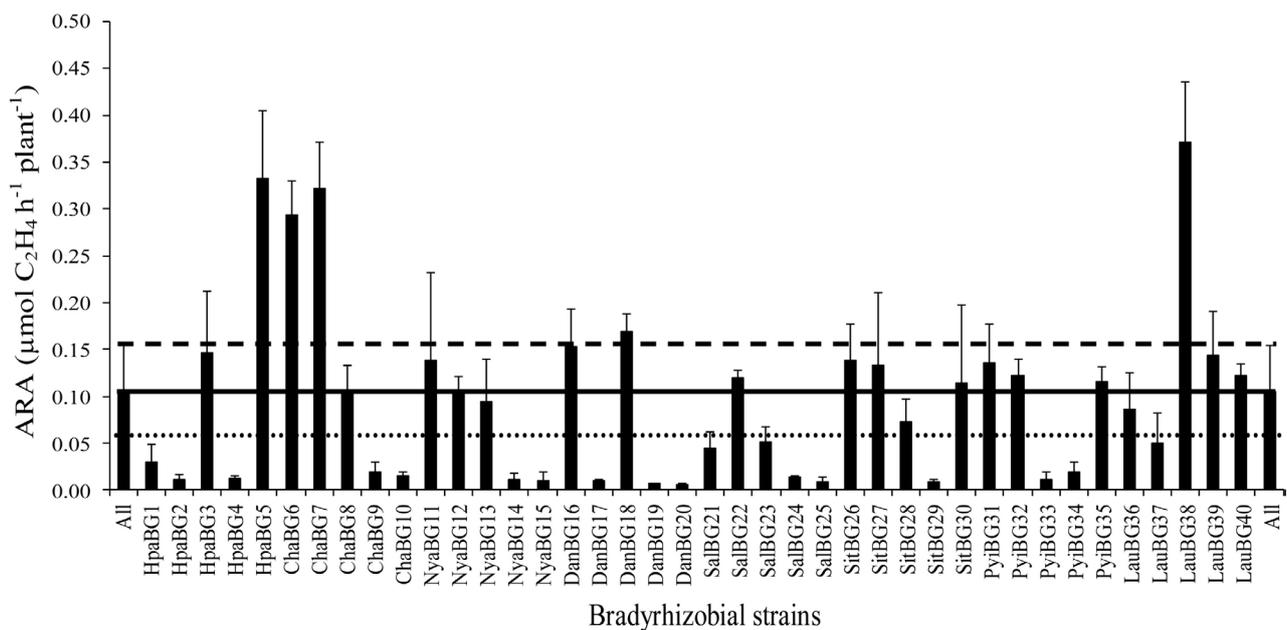


Figure 3. Acetylene reduction activity per plant of Yezin-7 Black gram (*Vigna mungo*) variety inoculated with 40 purified *Bradyrhizobium* strains after four weeks, Bars mean standard deviation levels. The middle line indicates average acetylene reduction activity per plant of all strains; the upper line means the higher acetylene reduction activity per plant and the lower line refers the lower acetylene reduction activity per plant of all strain.

3.3. Effectivity of Selected *Bradyrhizobium* Strains on Yezin-4 and Yezin-7 of Two Myanmar Black Gram Varieties

Table 4 shows the effectiveness of selected Bradyrhizobial strains on ARA per plant, nodule and shoot dry weight of Yezin-4 and Yezin-7 black gram varieties. The ARA per plant of selected five Bradyrhizobial strains was significant affected on Yezin-4 and Yezin-7 black gram varieties (**Table 4**). It was found that significant higher in ARA per plant ($P < 0.05$) was observed by *B. elkanii* LauBG38 than *B. liaoningense* NyaBG12 in both Yezin-4 and Yezin-7 black gram varieties. In Yezin-4 black gram variety, the *B. elkanii* LauBG38 was insignificant differences that compared with *B. elkanii* HpaBG5, *B. liaoningense* ChaBG6 and *B. liaoningense* ChaBG7 in ARA per plant in **Table 4**.

On the other hand, the *B. elkanii* LauBG38 showed the highest nodule and shoot dry weights of 9.17 mg plant⁻¹, 0.17 g plant⁻¹ in Yezin-4 and 10.97 mg plant⁻¹, 0.18 g plant⁻¹ in Yezin-7 black gram variety, respectively. In Yezin-4 variety, inoculation with *B. elkanii* LauBG38 gave significant higher in nodule dry weight than *B. liaoningense* ChaBG6, *B. liaoningense* ChaBG7, and *B. liaoningense* NyaBG12. In Yezin-7 variety, *B. elkanii* LauBG38 gave the highest nodule dry weight among the tested strains and this strain was significant difference from those given by *B. liaoningense* ChaBG6 and *B. liaoningense* NyaBG12 (**Table 4**). However, shoot dry weight of tested *B. elkanii* LauBG38 was only significant difference than *B. liaoningense* NyaBG12 in both Myanmar black gram varieties (**Table 4**).

4. Discussion

The symbiotic association between legumes and rhizobia is one of the most important contributors to the world's supply of biologically fixed nitrogen to agriculture. Effective symbiosis can only be achieved when the nodules are formed

Table 4. Response of selected Myanmar bradyrhizobial strains on acetylene reduction activity, nodule and shoot dry weight of Yezin-4 and Yezin-7 black gram varieties after four weeks.

Treatment	Yezin-4			Yezin-7		
	NDW (mg.plant ⁻¹)	SDW (g.plant ⁻¹)	ARA (μmole C ₂ H ₄ h ⁻¹ .plant ⁻¹)	NDW (mg.plant ⁻¹)	SDW (g.plant ⁻¹)	ARA (μmole C ₂ H ₄ h ⁻¹ .plant ⁻¹)
HpaBG5	6.83 ab	0.15 ab	0.19 ab	7.83 ab	0.17 ab	0.23 ab
ChaBG6	5.50 b	0.14 ab	0.17 ab	5.93 b	0.15 ab	0.15 b
ChaBG7	5.77 b	0.14 ab	0.18 ab	7.30 ab	0.16 ab	0.22 ab
NyaBg12	4.63 b	0.11 b	0.10 b	4.80 b	0.12 b	0.12 b
LauBG38	9.17 a	0.17a	0.36 a	10.97 a	0.18 a	0.41 a

Means in each column followed by different letters differed significantly at $P < 0.05$ (Tukey's test), NDW, SDW means nodule and shoot dry weight per plant and ARA means C₂H₄ produced per hour per plant, NDW and ARA of non-inoculated treatment is zero for both black gram varieties, SDW of non-inoculated treatment is 0.05 g plant⁻¹ for both black gram varieties.

by effective rhizobia. The symbiotic relationship between rhizobia and black gram has not been extensively analyzed. Therefore, this is the first report investigation of addressing the genetic diversity and evaluation the effectiveness of indigenous *Bradyrhizobium* strains for Myanmar black gram cultivars.

Sequence analysis of 16S ribosomal RNA (rRNA) has been developed used as one of the most important methods in taxonomy and phylogenic analysis of bacteria [35] [36] [37] [38]. The phylogenetic investigates based on 16S rRNA gene sequences showed that the rhizobial strains nodulating black gram in India were dispersed under the genus *Bradyrhizobium* [39]. These results were dependable with former reports that showed black gram rhizobia were more closely associated with *Bradyrhizobium* species [39] [40] [41]. The present study was focused on investigation of 16S rRNA region of 40 *Bradyrhizobium* strains that were successfully isolated from the different soil samples of major black gram growing areas in Myanmar and proved as pure *Bradyrhizobium* strains [25].

The genus *Bradyrhizobium* was proposed by Jordan (1982) [42] for the slow-growing root nodule bacteria on yeast extract-mannitol agar (YMA) medium. The *Bradyrhizobium* genus contained 12 defined species: *Bradyrhizobium japonicum* [42], *B. elkanii* [43], *B. liaoningense* [44], *B. yuanmingense* [45], *B. betae* [46], *B. canariense* [47], *B. denitrificans* [48], *B. iriomotense* [49], *B. jicamae*, *B. pachyrhizi* [50], *B. lablabi* [51] and *B. cytisi* [52]. With the exception of *B. betae*, all species are symbiotic nitrogen fixing bacteria associated with different legumes.

Previously, Soe *et al.*, 2013 [52] and Htwe *et al.*, 2015 [53] confirmed that the collected root nodule bacteria isolates from different soybean growing of Myanmar were categorized and identified as the *B. japonicum*, *B. elkanii*, *B. yuanmingense* and *Bradyrhizobium* sp. and *B. liaoningense* from Myanmar soybean growing areas. Several scientists found the *B. japonicum*, *B. elkanii*, *B. liaoningense* and *B. yuanmingense* [54] [55] [56] [57] have been isolated from root nodules of soybean grown in different regions of China as well. Clear biogeographic patterns have been revealed in the soybean rhizobia and soil types appeared to be the main factor of the biogeography of these bacteria [58]. Cowpea bradyrhizobia isolated in Africa [59], in China [60], and in Brazil [61] were identified as *B. elkanii*, *B. japonicum*, *B. liaoningense*, *B. yuanmingense* or as novel *Bradyrhizobium* lineages. Less information is available for indigenous rhizobia nodulating green gram and black gram. Also, unidentified slow growers with different 16S rRNA gene haplotypes were isolated from black gram plants growing in the south of India [62], while some strains isolated from green gram and black gram plants growing in Thailand were closely related to *B. japonicum* [63]. In our present investigation, *B. liaoningense*, *B. japonicum*, *B. sp.*, *B. yuanmingense* and *B. elkanii* were collected from major black gram growing areas of Myanmar with different locations, weathers and soil conditions.

The agro-ecological origin of rhizobial inoculants and thus most possibly edaphic and climatic variation are often not considered sufficiently to make in-

oculation successful. A variety of biotic and abiotic factors, such as host plant, cultivation history, drought, soil pH, salinity, mineral nutrient availability, soil organic carbon content and texture, are known to affect rhizobial diversity and distribution [64]. This is evidence that the collected 40 Bradyrhizobia isolates from major black gram growing areas of Myanmar with various soil types, soil pH and soil nutrients were affected to bradyrhizobial strains diversity and distribution.

A phylogenetic analysis of present study showed that indigenous *B. liaoningense* as a dominant strain was distributed throughout the five major black gram growing areas of Myanmar with a pH range of 4.69 - 6.70. *B. liaoningense* strain was isolated from soybean growing areas in China [44] and in Myanmar [53].

Symbiotic N₂ fixation can recompense for absent soil nitrogen (N) and thus potentially save costly mineral N fertilizer [65]. Rhizobial inocula for inoculating legumes increasingly account for differences in symbiotic specificity and effectivity, two parameters that are often correlated [66]. Several studies also reported significant increase growth parameters and yield due to the inoculation of rhizobial isolates in chickpea in Myanmar [14], green gram and black gram in Thailand [67]. In addition, indigenous rhizobial strains also play important role since they have adapted to local environmental conditions.

An effective *Rhizobium*-legume symbiosis largely depends on the presence of a specific and compatible strain in the soil for a particular legume. Several studies have reported a significant increase in green gram and black gram growth parameters and yield due to the inoculation of bradyrhizobial isolates [68]. In the present study, the effectiveness of indigenous 40 *Bradyrhizobium* strains in symbiosis association with Yezin-7 Myanmar black gram variety by using sterilized vermiculite with MHN as a growth media. When the nitrogen fixation in terms of ARA per plant with the test *Bradyrhizobium* strains was compared, four *Bradyrhizobium* strains designated as *B. elkanii* HpaBG5, *B. liaoningense* ChaBG6, *B. liaoningense* ChaBG7 and *B. elkanii* LauBG38 were found to be more effective than other tested strains. The effectiveness of a strain of rhizobia is due to the genetic interaction with the host plant, which is known as host-strain specificity. Thus, the selection of strains of rhizobia for cultivated legume varieties is a critical step in the production of the legume inoculants [69].

In this study, the evaluation the effectiveness of selected *Bradyrhizobium* strains on two Myanmar black gram varieties, Yezin-4 and Yezin-7 were investigated. It was observed that the inoculation of *Bradyrhizobium elkanii* LauBG38 gave significantly higher in ARA per plant for nitrogen fixation and nodule dry weight in both black gram varieties. ARA per plant for nitrogen fixation and nodule dry weight of Yezin-7 variety was higher than those of Yezin-4 black gram. Therefore based on the results of nodulation efficiency of nodule dry weight of the plants Yezin-7 could be used in future experiment as superior host genotypes for high nitrogen fixation. Symbiotic nitrogen fixation depends on interactions among the genotype of the host plant, rhizobial strain genotype and

environment. In grain legume species, genotypic variability affected nodule number or mass or nitrogenase activity [70] pointed out that, through the use of plant genotypes in symbiotic ability, it is possible to identify genes responsible for a particular part of the process, depending on a particular rhizobial strain used. The selection of effective rhizobial strains for cultivated legumes is a critical step in the production of high quality legume inoculant.

The effectiveness of indigenous *Bradyrhizobium* strains was observed in Myanmar black gram cultivars using the correct varieties, and proper nodulated bacteria. This is the first report of phylogenetic diversity and evaluation the effectiveness of indigenous *Bradyrhizobium* strains for Myanmar black gram cultivars. The selected *Bradyrhizobium elkanii* LauBG38 strain might be considered for rhizobial inoculants to use as Biofertilizer in Myanmar near future.

5. Conclusion

This is the first report on describing *Bradyrhizobium* strains that were isolated from soil samples of major black gram growing areas of Myanmar and the effectiveness of those strains for plant growth and nitrogen fixation of Myanmar Black gram varieties. In total, 40 indigenous bradyrhizobia were successfully isolated and their geographic distribution was determined based on the analysis of the 16S rRNA region. Our results indicated that *B. liaoningense* strain was widely distributed in the major black gram growing regions of Myanmar whereas *B. japonicum* was found more abundant in Danubyu Ayeyarwady Region and Pinyinmanar Nay Pyi Taw Region. However, *B. elkanii* was found more abundant in Launglon Tanintharyi Region and Hpa-an Kayin State. The effectiveness of those strains for plant growth and nitrogen fixation of Myanmar black gram varieties was investigated in the present study. The 40 *Bradyrhizobium* strains were screened for their effectiveness on Yezin-7 black gram variety and five *Bradyrhizobium* strains were selected. These selected strains were tested for their effectiveness on Yezin-4 and Yezin-7 black gram varieties. The *Bradyrhizobium elkanii* LauBG38 was significantly superior in both black gram varieties. All of these experiments were conducted under the control conditions by growing the plants in the sterilized vermiculite with MHN solution. So, the bradyrhizobial strains selected in the control room trials then must be evaluated in the field. Although it is a preliminary study, it can help for the future study in the inoculants production. The further investigation of symbiotic effectiveness of selected indigenous bradyrhizobia strains on Myanmar black gram cultivars will be examined in the field condition. We do hope that Myanmar *Bradyrhizobium* strains will be able to use as Biofertilizer for black gram cultivars that enhance crop production through nitrogen fixation and yield.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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