Phylogenetic diversity analysis reveals *Bradyrhizobium yuanmingense* and *Ensifer aridi* as major symbionts of mung bean (*Vigna radiata* L.) in Pakistan

Sughra Hakim^{1,2} · Asma Imran¹ · M. Sajjad Mirza¹

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Abstract

The present study was carried out to evaluate the diversity of rhizobia associated with nodules of mung bean in Pakistan, because this information is necessary for inoculum development. Based on sequence analysis of 16S rRNA gene of thirty-one bacteria, 11 were assigned to genus *Bradyrhizobium*, 17 to *Ensifer*, and 3 to *Rhizobium*. Phylogenetic analyses on the basis of 16S-23S ITS region, *atpD*, *recA*, *nifH*, and *nodA* of representative strains revealed that *B. yuanmingense* is the predominant species distributed throughout different mung bean-growing areas. Among the fast-growing rhizobia, *Ensifer aridi* was predominant in Faisalabad, Layyah, and Rawalpindi, while *E. meliloti* in Thal desert. Sequence variations and phylogeny of *nifH* and *nodA* genes suggested that these genes might have been co-evolved with the housekeeping genes and maintained by vertical gene transfer in rhizobia detected in the present study. Host infectivity assay revealed the successful nodulation of host by rhizobia related to genera *Bradyrhizobium*, *Ensifer* and *Rhizobium*. Among all, *Bradyrhizobium* and *Ensifer* spp. inoculation exhibited a significantly higher number of nodules (11–34 nodules plant⁻¹) and nitrogenase activity (nodule ARA 60–110 $\mu\text{mol g}^{-1} \text{h}^{-1}$). Contrary to the previous studies, our data reveal that *B. yuanmingense* and *E. aridi* are predominant species forming effective nodules in mung bean in Pakistan. Furthermore, to the best of our knowledge, this is the first report showing the effective symbiosis of *E. aridi*, *E. meliloti*, and *Rhizobium pusense* with mung bean. The diversity of rhizobia in different habitats revealed in the present study will contribute towards designing site-specific inocula for mung bean.

Keywords *E. meliloti* · Housekeeping genes · Intraspecies polymorphism · Predominant species · Rhizobia · Site-specific inocula

Introduction

Symbiotic interaction among legume and soil bacteria, specifically rhizobia, is of critical importance in the nitrogen cycle and agriculture production. The rhizobia interact with the roots of legume plants and lead to the formation of specialized plant organs, known as nodules, wherein they reside and fix

atmospheric nitrogen. The interactions exhibit high levels of specificity through the exchange of signal molecules between rhizobia and legumes. The symbiotic dialogue is initiated through the production and secretion of flavonoid compounds through the roots of the plant. Flavonoids interact with the transcriptional regulators which activate the synthesis of Nod-factors in rhizobia [1]. These Nod-factors activate various signaling pathways in plants for nodule formation. Legume-rhizobia symbiotic specificity depends upon the type of flavonoids and Nod-factors [1]. Besides, nodulation and host specificity are also affected by exo-lipo polysaccharides and proteins secreted by the rhizobia [2, 3].

Mung bean is an important legume crop that provides cereal-based diets to many people in China, India, Indonesia, Pakistan, Philippines, and Thailand and is well-known for its detoxification activities and regulation of gastrointestinal distress [4, 5]. Regarding nodulation, it is promiscuous in nature as it is known to be nodulated by rhizobia related to four different genera, i.e., *Bradyrhizobium*, *Ensifer*, *Mesorhizobium*, and

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✉ M. Sajjad Mirza
sajjadmirza58@gmail.com

¹ National Institute for Biotechnology and Genetic Engineering (NIBGE), P.O. Box 577, Faisalabad, Pakistan

² Pakistan Institute of Engineering and Applied Sciences (PIEAS), Nilore, Islamabad, Pakistan

Rhizobium [6–9]. However, previous studies from China, India, Nepal, and Thailand indicated that *Bradyrhizobium*-related species were predominant in the root nodules of mung bean [7–10].

For many years, 16S rRNA gene is used as a molecular marker in defining the taxonomic position of isolated bacterial strains. Due to the high level of conservation, this molecule can be widely used for defining bacteria at class and genus. However, the resolving power of this molecule at the species level is relatively low especially for the genus *Bradyrhizobium* [11]. To overcome, sequence analysis of internal transcribed spacer (ITS) regions, symbiotic genes, and housekeeping protein-coding genes is usually carried out to complement the 16S rRNA phylogenetic analysis. The ITS region between the 16S and 23S rRNA genes provides information to elucidate inter- and intraspecific relationships as it shows considerable variation in length and sequence among closely related strains [12]. It was previously used for the characterization of rhizobia associated with various legumes [13]. Symbiotic genes known to be horizontally transferred among rhizobia such as *nif* and *nod* genes have also been used for phylogenetic analyses due to variation in evolutionary history from core genes [14, 15]. Housekeeping protein-coding genes, on the other hand, have more sequence divergence and sufficiently conserved to retain genetic information for precisely characterizing the prokaryotes at the intra- and interspecific levels. Currently, most commonly used housekeeping genes for the classification of rhizobia at species level include recombinase A (*recA*), ATP synthase subunit beta (*atpD*), glutamine synthetase (*glnA*), chaperone protein (*dnaK*) and threonine synthase (*thrC*), DNA gyrase subunit beta (*gyrB*), and citrate synthase (*glcA*) [16].

Mung bean-associated symbionts have been identified from various regions of South Asia through polyphasic studies, but from Pakistan, detailed studies are lacking. Few studies reported the phenotypic and biochemical characterization of mung bean-associated symbionts [17, 18] but not at molecular level. Recently, we have used 16S rRNA-based metagenomics approach to assess the diversity of microbes in the root nodules of mung bean collected from different mung bean-growing areas of Pakistan [19, 20]. Rhizobia related to three genera, i.e., *Bradyrhizobium*, *Ensifer*, and *Rhizobium*, were detected in the root nodules, of which *Bradyrhizobium* and *Ensifer* were the predominant in nodules.

The aim of the present study was, therefore, the analysis of the diversity of rhizobia isolated from the root nodules collected from different areas of Pakistan using cultivation-based techniques. Secondly, phylogenetic analyses of rhizobia were based on the 16S rRNA, 16S-23S rRNA ITS region, two symbiotic (*nifH* and *nodA*), and two additional conserved housekeeping genes (*recA* and *atpD*). The data has validated our previous metagenomics data and will be useful in finding

inter- and intraspecies variation among strains from different areas and selecting site-specific strains for inoculum production.

Materials and methods

Sample collection and isolation of root nodule endophytes (putative rhizobia)

Soil and root samples were collected from mung bean plants grown at five sites in Pakistan including Bhakkar (31° 43' 13.2" N, 71° 05' 48.4" E), Faisalabad (31° 23' 42.5" N, 73° 01' 45.5" E), Layyah (30° 59' 01.5" N, 70° 56' 27.4" E), Rawalpindi (33° 22' 45.0" N, 73° 30' 16.3" E), and Thal desert (31° 44' 31.1" N to 32° 12' 11.9" N, 71° 45' 05.6" E, 72° 01' 44.3" E) (Fig. S1). Soil samples were analyzed for physical and chemical soil quality parameter as described [19] (Table 1). From each location, five plants were sampled and 5 nodules were collected from each plant. Nodules were surface-sterilized as described previously [20] and crushed in 1 mL of sterilized water. One hundred microliters of nodule suspension was spread on Yeast Mannitol Agar (YMA) plates supplemented with Congo Red (25 µg mL⁻¹). The inoculated YMA plates were incubated at 32 ± 2 °C for 2 days and at 28 ± 2 °C for 2–8 days for the isolation of fast-growing and slow-growing rhizobia, respectively, as indicated by previous study [20]. Nodule isolates were purified by repeatedly streaking on the YMA medium, and colony and cell morphology of pure cultures was recorded.

Authentication of putative rhizobia and assessment of N₂ fixation

The nodule isolates were evaluated for their ability to form nodules on the host plant. Host inoculation experiment was conducted in the net house during mung bean-growing season under natural light. For surface sterilization, seeds of mung bean variety NM-2011 were immersed for 10 min in ethanol (70%, v/v) and then dipped into sodium hypochlorite (4%, w/v) for 4 min followed by washing 6 times with sterilized water. Twenty seeds were sown (2 seeds per sowing point) in plastic containers (40 L × 30 W × 22 H) containing 10-kg sterilized sand. Putative rhizobia purified from root nodules were cultured in 50 mL of YMA medium for 3–4 days. The cells were pelleted, washed, and resuspended in 0.85% saline (100 mL) and then 1 mL aliquot (approximately 1 × 10⁹ cells) applied to each of the germinated seedlings, while non-inoculated control received 1 mL of sterilized water. Plants were grown during mung bean-growing season; inoculated plants were irrigated with nitrogen-free Hoagland solution, while the non-inoculated plants received Hoagland with nitrogen. The plants were uprooted 28 days post-inoculation (28 dpi) to observe

Table 1 Physical and chemical properties of soil samples from mung bean-growing areas

Parameter	Bhakkar	Faisalabad	Layyah	Rawalpindi	Thal desert
Latitude ^a	31° 43' 13.2" N	31° 23' 42.5" N	30° 59' 01.5" N	33° 22' 45.0" N	31° 44' 31.1" N to 32° 12' 11.9" N
Longitude ^a	71° 05' 48.4" E	73° 01' 45.5" E	70° 56' 27.4" E	73° 30' 16.3" E	71° 45' 05.6" E to 72° 01' 44.3" E
Rainfall during crop season ^b	123 mm	79 mm	12 mm	182 mm	77 mm
Soil texture ^c /classification ^d	Silty clay loam/Inceptisol	Clay loam/Aridisol	Sandy loam/Entisol	Silty clay loam/Inceptisol	Sandy loam/Entisol
Organic matter (%) ^c	0.73 ± 0.01	0.597 ± 0.024	0.50 ± 0.01	0.629 ± 0.04	0.35 ± 0.01
pH ^c	7.89 ± 0.10	8.05 ± 0.13	7.85 ± 0.15	7.840 ± 0.15	8.10 ± 0.08
EC ^c (dSm ⁻¹)	0.28 ± 0.02	0.398 ± 0.04	0.43 ± 0.01	0.32 ± 0.03	0.41 ± 0.04
Total P ^c (μg g ⁻¹)	1054.3 ± 9.10	1148.7 ± 13.9	894.67 ± 9.30	1249.0 ± 14.20	561.7 ± 12.7
Available P ^c (μg g ⁻¹)	4.09 ± 0.11	6.873 ± 0.19	3.15 ± 0.19	3.17 ± 0.14	2.91 ± 0.03
Available K ^c (μg g ⁻¹)	115.0 ± 9.64	189.2 ± 13.25	107.3 ± 6.77	72.0 ± 3.61	65.7 ± 4.63
Available N ^c (μg g ⁻¹)	0.006 ± 0.0004	0.00856 ± 0.001	0.0052 ± 0.0002	0.0066 ± 0.001	0.0041 ± 0.0002

The standard analysis procedures were used for the estimation of soil organic matter (Loss-on-Ignition method) [21], available nitrogen (Kjeldahl method) [22], total and available phosphorus (Bray's method) [23], and potassium (Knudsen method) [24]

Data represent the mean of three independent replicates; ± standard deviation

^a Google Earth

^b Pakistan Metrological Department

^c Data from this study

^d FAO (Food and Agriculture Organization of the United Nations) 2017, Soil Fertility Atlas of Pakistan: The Punjab Province

nodule formation and different growth parameters. Nitrogenase activity was estimated through acetylene reduction assay (ARA) which is the most commonly used method of indirect quantification of N₂ fixation rate [25]. ARA is based on reduction of acetylene to ethylene by nitrogenase enzyme, instead of reducing N₂ to NH₃ [26]. The nodulated roots were incubated at room temperature in 10 mL air-tight glass vials to which acetylene (10% v/v) was injected. After 24 h, 200 μL of the gas sample was analyzed for ethylene production by gas chromatography (Thermoquest, Trace G.C, Model K, Rodono Milan, Italy) using a Porapak Q column with hydrogen flame ionization detector. Samples of root nodule and plant shoots were dried for 2 days at 60 °C and weighed to record the data of nodule and plant dry weight.

Identification of rhizobia by 16S rRNA gene sequence analysis

Rhizobia were cultured in TY medium at 28 ± 2 °C for 2–4 days, and total genomic DNA was extracted using the CTAB method [27]. PCR amplification of 16S rRNA gene from genomic DNAs was carried out using fD1/rD1 primers (Table 2). The PCR products were purified using QIAquick spin Columns (Qiagen, Germany, Hilden) and sequenced from Macrogen Inc., Seoul, South Korea. Forward and reversed sequences of 16S rRNA gene were aligned using Clustal X [28], and the Basic Local Alignment Search Tool

(BLAST) [29] on the EMBL/GenBank databases was used to find out the similar sequences. 16S rRNA gene from isolates and reference sequences retrieved from GenBank were aligned using Clustal W software [30]. Maximum likelihood-based phylogenetic trees were constructed using MEGA 7 software [31]. The bootstrap values (≥ 50%) were shown in the phylogenetic tree.

Sequence and phylogenetic analyses of 16S-23S ITS, *recA*, *atpD*, *nifH*, and *nodA* genes

Amplification of internal transcribed spacer region, symbiotic, and housekeeping genes was performed from the representative strains related to different genera of rhizobia from each site. 16S-23S ITS region, *atpD*, *recA*, *nifH*, and *nodA* region were amplified using primers and PCR conditions described in Table 2. Phylogenetic analyses of gene sequences were performed as described above for 16S rRNA gene, and sequences were submitted to EMBL-EBI and accession numbers were obtained (Fig. 1 and Table S2).

Detection of nucleotide polymorphism and statistical analyses

Nucleotide polymorphism among strains related to genera *Bradyrhizobium*, *Ensifer*, and *Rhizobium* was calculated using DNASP v5 software. Sequences related to

Table 2 Primers and PCR conditions used in the present study

Primer	Sequence (5'–3')	Target gene/bp amplified	PCR conditions	References
fD1	AGAGTTTGATCCTGGCTCAG	16S rRNA	2 min 95 °C, 30 × (1 min 93 °C, 45 s 55 °C, 2 min 72 °C)	[32]
rD1	AAGGAGGTGATCCAGCC	(~ 1500)	and 5 min 72 °C	
ITS-For2	TACACACCGCCCGTCACACC	16S-23S ITS	2 min 95 °C, 30 × (1 min 93 °C, 45 s 55 °C, 2 min 72 °C)	[33]
ITS-Rev2	TGGTCCGCGTTCGCTCGCC	region	and 5 min 72 °C	
		(~ 1100–1450)		
<i>atpD</i> 255F	GCTSGGCCGCATCMTSAACGTC	<i>atpD</i>	3 min 95 °C, 30 × (1 min 93.5 °C, 40s 55 °C, 1 min 72 °C)	[34]
<i>atpD</i> 782R	GCCGACACTTCMGAACCGCCTG	(~ 550)	and 5 min 72 °C	
<i>recA</i> 41F	TTCGGCAAGGGMTCGRTSATG	<i>recA</i>	3 min 95 °C, 30 × (1 min 93.5 °C, 1 min 58 °C, 1 min	[34]
<i>recA</i> 640R	ACATSACRCCGATCTTCATGC	(~ 500)	72 °C)	
			and 5 min 72 °C	
POL F	TGCGAYCCSAARGCBGACTC	<i>nifH</i>	2 min 95 °C, 30 × (1 min 94 °C, 1 min 55 °C, 2 min 72 °C)	[35]
POL R	ATSGCCATCATYTCRCCGGA	(~ 370)	and 5 min 72 °C	
<i>nodA</i> -1	TGCRGTGGAARNTRNNCTGGGAAA	<i>nodA</i>	2 min 93 °C, 35 X (45 s 93 °C, 1 min 49 °C, 2 min 72 °C)	[36]
<i>nodA</i> -2	GGNCCGTCRTCRAAWGTCARGTA	(~ 666)	and 5 min 72 °C	

each gene fragment were aligned using the Clustal X and manually trimmed to keep the equal length. These homologous aligned sequences were used as input for the estimation of nucleotide variation. The sequence types (STs) representing sequence type for each gene and nucleotide diversity (Pi) were detected through the nucleotide polymorphism. Nucleotide diversity at synonymous and non-synonymous sites was estimated by assigning the coding frame of the gene.

The effect of rhizobial inoculation on nodulation and growth parameter of mung bean plants was determined through analysis of variance (ANOVA) at a level of significance ($p = 0.05$) using Statistix 8.1 software.

Results

Soil analyses indicated that soils sampled from Bhakkar, Faisalabad, and Rawalpindi were silty clay loam with more organic matter, total P, and available N contents as compared to the sandy loam soil from Layyah and Thal desert (Table 1). The soil of Thal desert showed maximum pH, i.e., 8.10, followed by Faisalabad (8.05).

A total of 31 putative rhizobia were isolated from surface-sterilized root nodules in the present study. These isolates included both slow-growing and fast-growing bacteria. Fifteen rhizobial isolates were obtained from the root nodules collected from Faisalabad, 5 from Bhakkar, 4 from Layyah, 5 from Rawalpindi, and 2 isolates from the Thal desert.

Authentication of rhizobia

Host infectivity assay was performed to assess the nodulation ability of isolates. The seedlings were grown in sterilized sand

contained in rectangular plastic containers. All the isolates formed nodules on the host plant (Supplementary Table S1). The maximum number of nodules (33.3 nodules plant⁻¹) were formed in mung bean plants inoculated with *Ensifer* sp. Vr33 followed by *Ensifer* sp. Vr25 (28.5 nodules plant⁻¹) at 21 days post-inoculation (dpi). Among bradyrhizobia, maximum nodulation was observed in plants inoculated with *Bradyrhizobium* sp. Vr50 (24.4 nodules plant⁻¹) followed by *Bradyrhizobium* sp. Vr61 (23.6 nodules plant⁻¹). *Rhizobium* strains formed very less number of nodules (2–5 nodules plant⁻¹). The maximum plant dry weight (0.46 g) was observed in plants inoculated with *Ensifer* sp. Vr33 followed by *Bradyrhizobium* sp. Vr50 (0.40 g). Nitrogen fixation in the root nodules was estimated through the acetylene reduction assay (ARA). The results indicated that all the rhizobia-induced effective nodulation and maximum activity (109.3 $\mu\text{mol}^{-1} \text{g}^{-1} \text{h}^{-1}$) were observed in nodules produced by the slow-growing isolate Vr50. Among fast-growing isolates, Vr33 showed maximum ARA activity (102.7 $\mu\text{mol}^{-1} \text{g}^{-1} \text{h}^{-1}$) followed by Vr34 (99.0 $\mu\text{mol}^{-1} \text{g}^{-1} \text{h}^{-1}$). The maximum number of nodules and the nitrogenase activity was observed in plants inoculated with the isolates Vr33 and Vr50.

Sequence and phylogenetic analysis of 16S rRNA gene

Sequence analysis of 16S rRNA gene sequence indicated that 17 rhizobia were related to genus *Ensifer*, 11 were assigned to genus *Bradyrhizobium*, while 3 strains belonged to genus *Rhizobium*. None of the isolate purified from root nodule belonged to genus *Mesorhizobium*.

All the *Ensifer* strains obtained from Faisalabad, Layyah, and Rawalpindi showed identical 16S rRNA gene sequence and clustered in the first clade with *Ensifer aridi* LEM 451 and *Ensifer kostiense* LMG 19225^T in phylogenetic analysis (Fig. 1), while two

Fig. 1 Phylogenetic tree of mung bean rhizobia based on 16S rRNA sequences (~ 1300 bp) showing the relationship of rhizobia isolated in this study with already published strains. The tree was constructed using ML method, and bootstrap values (1000 replicates) higher than 50% are shown at the nodes. Bacterial isolates obtained in the present study are shown in bold

Ensifer strains Vr77 and Vr78 from the Thal region clustered with *Ensifer meliloti* IAM 12611^T and formed the second clade.

16S rRNA gene sequences from all the bradyrhizobia were identical and formed single cluster with *Bradyrhizobium yuanmingense* B071^T (clade 4). Two *Rhizobium* strains

Vr69 and Vr76 purified from root nodules collected from Rawalpindi clustered with *Rhizobium pusense* NRCPB10^T while one isolate Vr65 from Layyah showed different gene sequence and clustered with *Rhizobium* sp. UCCB 145 (clade 3).

To further clarify the taxonomic position of these strains at the species level and find the variations at intra- and interspecific levels, 14 representative strains were selected from different genera for further analysis.

Phylogeny of rhizobia based on 16S-23S rRNA ITS region

The 16S-23S rRNA ITS region of 1100 to 1500 bp was amplified from 14 representative strains (Supplementary Table S2) and sequenced. The ITS region amplified from bradyrhizobia has a sequence length of 1100 to 1160 bp while the ITS region of *Ensifer*- and *Rhizobium*-related strains have a sequence length of 1300 to 1420 bp. The alignment of the ITS region indicated the deletion of base at various sites in *Bradyrhizobium* strains. tRNA gene present in the ITS region was scanned through tRNAscan-SE [37]. Two types of tRNA genes for Ile and Ala were present in the 16S-23S rRNA ITS regions amplified from all the representative rhizobia strains. Five *Bradyrhizobium* strains had sequence variation in the ITS region and clustered with *B. yuanmingense* B071^T in the phylogenetic tree (Fig. 2). Out of 6 rhizobia related to genus *Ensifer*, ITS sequence from five strains, i.e., Vr25, Vr28, Vr33, Vr66, and Vr70, from Faisalabad, Layyah, and Rawalpindi have 100% homology and clustered with *E. aridi* LEM 451. *Ensifer* sp. Vr77 from Thal region has 21.8% sequence variation from other *Ensifer* isolates. All the three *Rhizobium*-related strains have sequence variation in their ITS region. Two *Rhizobium* strains Vr66 and Vr76 clustered with *Rhizobium pusense* strain CFEB5875, while *Rhizobium* sp. Vr65 showed the highest homology with *Rhizobium* sp. RBG74.

Analyses of protein-coding housekeeping genes *atpD* and *recA*

The *atpD* gene fragment of approximately 550 bp and *recA* gene of 500 bp were amplified from representative strains. *Ensifer* strains Vr25, Vr28, Vr33, Vr66, and Vr70 have identical *atpD* gene sequence and show maximum similarity with *E. aridi* LEM451 while Vr77 with *E. meliloti* IAM 12611^T (Supplementary Table S2). All the *Rhizobium* strains showed variation in their gene sequence; however, two strains Vr69 and Vr76 showed 99–100% sequence similarity with *R. pusense* strain NRCPB10^T. *Rhizobium* sp. Vr65 has 11–12% sequence variation from other two isolates and showed 98.3% homology with *Rhizobium* sp. GMF8. *Bradyrhizobium*-related strains Vr50, Vr57, Vr63, and Vr73 showed identical gene sequence,

while Vr61 has 1.25% sequence variation from other *Bradyrhizobium* strains.

Based on *recA* gene sequence analysis, *Ensifer* strains Vr25, Vr28, Vr33, Vr66, and Vr70 showed maximum sequence similarity with *E. aridi* LEM451 while Vr77 with *E. meliloti* IAM 12611^T (Supplementary Table S2). Similarly, Vr69 and Vr76 showed maximum homology with *R. pusense* NRCPB10^T, while Vr65 showed maximum sequence homology with *Rhizobium* sp. NCSU 2642. Interestingly, *recA* gene amplified from *Bradyrhizobium* strains showed variations in the gene sequences and divided these strains into three subgroups.

Maximum likelihood phylogenetic tree was constructed using housekeeping genes (*recA* and *atpD*) concatenation. Based on the two gene-concatenated analysis, strains Vr25, Vr28, Vr33, Vr66, and Vr70 clustered together with *E. aridi* LEM451 while Vr77 clustered with *E. meliloti* IAM 12611^T (Fig. 3). *Rhizobium* strains Vr69 and Vr76 clustered together with *R. pusense* NRCPB10^T and Vr65 formed cluster with *Rhizobium* sp. NCSU 2642. *Bradyrhizobium* strains Vr50 and Vr57 clustered with *B. yuanmingense* CCBAU 10071^T, Vr61 *Bradyrhizobium* sp. JNVU VA7 and Vr63 and Vr73 were placed with *B. yuanmingense* CCBAU 33112 in phylogenetic tree.

Phylogeny of rhizobia based on *nifH* and *nodA* gene sequences

Phylogenetic analyses of *nifH* gene (370 bp) amplified from 14 rhizobial strains revealed that bradyrhizobia were clustered into three groups, *Ensifer* strains into two groups, and *Rhizobium* strains into three groups. *Bradyrhizobium* sp. Vr50 originating from Faisalabad had *nifH* gene sequence variation from other strains and clustered with *B. yuanmingense* CCBAU 10071^T in the phylogenetic tree (Fig. 4). The isolates Vr57, Vr63, and Vr73 sampled from Bhakkar, Layyah, and Rawalpindi have identical *nifH* sequences and showed homology with *B. yuanmingense* M7. *nifH* sequences amplified from Vr61 isolated from Bhakkar was different from all other bradyrhizobia and showed homology with *Bradyrhizobium* sp. JNVU VA7. The *nifH* gene sequences amplified from five *Ensifer* isolates Vr25, Vr28, Vr33, Vr66, and Vr70 were identical and clustered with *E. aridi* JNVU TP6 in phylogenetic analysis, while sequence from *Ensifer* sp. Vr77 showed homology with *E. meliloti* CCBAU 83493. All the isolates related to genus *Rhizobium* have variation in their *nifH* gene sequence and formed three clusters in the phylogenetic tree. *Ensifer* sp. Vr65 showed the highest homology with *Rhizobium* sp. ORS571, Vr69 was clustered with *R. tropici* CFN ESH25, and Vr76 placed with *R. pusense* AmV15 in phylogenetic tree.

The *nodA* gene sequences were amplified from 12 strains while the sequences were not amplified from Vr61 and Vr65

Fig. 2 Phylogenetic tree of selected rhizobia in this study based on ITS sequences (~ 1100 bp) showing the relationship of rhizobia isolated in this study with already published strains. The tree was constructed using ML method, and bootstrap values (1000 replicates) higher than 50% are shown at the nodes. Bacterial isolates obtained from this study are shown in bold

using the primers given in Table 2. Phylogenetic analysis showed the clustering of *Bradyrhizobium* strains in two groups. *Bradyrhizobium* strains Vr57, Vr63, and Vr73 shared one *nodA* gene sequence, which differed by *Bradyrhizobium* sp. Vr50 clustered with *B. yuanmingense* CCBAU 10071^T (Fig. 5). Similarly, *Ensifer* strains were clustered in two group, of which Vr25, Vr28, Vr33, Vr66, and Vr70 have identical *nodA* sequence and showed homology with *E. aridi* JNVU TP6, while *Ensifer* sp. Vr77 clustered with *E. meliloti* CCNWSX0020. The *nodA* gene sequence obtained from two *Rhizobium* strains Vr69 and Vr76 formed a cluster with *Rhizobium pusense* YIC4072 in phylogenetic tree.

Nucleotide diversity inferred from different genes

Nucleotide polymorphisms among different genera of all isolates for 16S rRNA gene and 14 representative strains for ITS, *atpD*, *recA*, and *nifH* and 12 strains for *nodA* were calculated (Table 3). Two sequence types (STs) were detected in genus *Ensifer* on the basis of nucleotide diversity in the targeted genes, i.e., 16S rRNA (0.0144), ITS (0.0398), *atpD* (0.0294), *recA* (0.03713), *nifH* (0.0464), and *nodA* (0.1064). These sequence types represent two species of this genus, i.e.,

E. aridi and *E. meliloti*. No nucleotide polymorphism was detected in any of the genes amplified from five *E. aridi* strains originating from three different sites, i.e., Faisalabad, Layyah, and Rawalpindi. *Rhizobium* strains showed high level of nucleotide diversity and represent three STs based on ITS (0.1678), *atpD* (0.0794), and *nifH* (0.1824) and two STs based on *nodA* (0.0058). *Bradyrhizobium* strains represent single sequence type based on the 16S rRNA gene while showing nucleotide polymorphism in all other genes and represent three STs based on ITS, *recA*, and *nifH* genes, while two STs based on *atpD* gene and *nodA*.

Discussion

Present study demonstrates the diversity of mung bean nodule rhizobia from different regions of Pakistan at species level using cultivation-based sequence analyses of multiple genes. The study further reveals few novel rhizobial symbionts found in mung bean. Symbiotic bacteria especially rhizobia fix atmospheric nitrogen within root nodules of legumes, and this symbiosis is among the most beneficial process in the agriculture system. Exploration of different biogeographic regions

Fig. 3 Concatenated tree using *atpD* and *recA* gene sequences showing the relationship of rhizobia isolated in this study with already published strains. The tree was constructed using ML method, and bootstrap values (1000 replicates) higher than 50% are shown at the nodes. Bacterial isolates obtained from this study are shown in bold

for the isolation and identification of rhizobia can lead to the selection of elite microsymbionts which could be further used for site-specific inoculum production.

Previous 16S rRNA-based metagenomics studies revealed that two genera *Bradyrhizobium* and *Ensifer* are co-dominant (> 60% of total rhizobia) in mung bean nodules from Faisalabad [20] while *Bradyrhizobium* is the single dominant genus in Bhakkar, Layyah, and Rawalpindi regions (82–94% of total rhizobia) and *Ensifer* is the dominant genus (99.9%) at Thal desert. A very small fraction of sequences were related to the genus *Rhizobium* [19]. Furthermore, the occurrence of *Ensifer* in nodules was found to be positively correlated with soil pH. The results of the present study validate the promiscuity of the mung bean plant as revealed previously by the cultivation-independent analysis [19, 20].

During this study, a total of 31 putative rhizobia were isolated from different sites of Pakistan. Fifteen rhizobial strains were

isolated from the root nodules collected from the local site in Faisalabad (soil pH = 8.05) in several attempts at different time intervals. Out of these, 13 strains belonged to genus *Ensifer* and two strains were related to genus *Bradyrhizobium*. All the isolated strains from Bhakkar (soil pH = 7.89) belonged to genus *Bradyrhizobium*, while the strains related to three genera, i.e., *Bradyrhizobium* (50–60%), *Ensifer* (20–25%), and *Rhizobium* (25–40%), were isolated from Layyah (soil pH = 7.85) and Rawalpindi (soil pH = 7.84). Isolates originated from Thal desert (pH = 8.10) were related to genus *Ensifer*. The present study indicated the occurrence of *Ensifer* in root nodules where the soil pH was relatively higher which validated the finding of previous study based on culture-independent approach [19]. Soil pH has been found to influence rhizobial diversity in legumes [38–40], due to the influence of soil pH on the bioavailability of mineral nutrients for growth. Soybean nodulation by *B. japonicum* and *B. elkanii* is favored when pH is below 7.96,

Fig. 4 Phylogenetic tree of selected rhizobia in this study based on *nifH* gene sequences showing the relationship of rhizobia isolated in this study with already published strains. The tree was constructed using ML method, and bootstrap values (1000 replicates) higher than 50% are shown at the nodes. Bacterial isolates obtained from this study are shown in bold

while *B. yuanmingense* when pH is high pH > 8.19 [41]. Similarly, both *Bradyrhizobium* and *Ensifer* were symbionts of legumes in Indian Thar desert, but *Ensifer* was found to be preferred symbiont-nodulating legumes in alkaline soil [42], while *Bradyrhizobium* nodulate legumes in acidic soil [43]. All the rhizobial strains effectively nodulated the host plant. Among all, *Bradyrhizobium* and *Ensifer* formed significantly higher number of nodules (11–34 nodules plant⁻¹) and exhibited maximum ARA activity (60–110 $\mu\text{mol g}^{-1} \text{h}^{-1}$). Maximum plant dry weight was observed in plants inoculated with *Ensifer* sp. Vr33 and *Bradyrhizobium* sp. Vr50, while most of other plants produced less dry weight as compared to the control irrigated with Hoagland with available N. It might be due to higher efficiency of abovementioned strains and vice versa and/or early harvesting of plants. *Rhizobium*-inoculated plants showed less number of nodules (2–5 nodules plant⁻¹) and ARA activity (13–25 $\mu\text{mol g}^{-1} \text{h}^{-1}$) which imply them as less effective symbionts of mung bean. Various studies have reported the ability of *Bradyrhizobium*, *Ensifer*, and *Rhizobium* to nodulate mung bean but bradyrhizobia were predominantly isolated from nodules and assumed to be the major symbionts of mung bean [7–9], which might be due to the biasness of the isolation protocols and/or growth conditions. Our current and previous studies [20] show that the optimum temperature for fast-growing *Ensifer* strains is

32 °C, while for slow-growing *Bradyrhizobium* isolates, it is 28 °C. Few *Ensifer* colonies appear at 28 °C but they are dominated by bradyrhizobia and vice versa.

The dataset shows that 16S rRNA gene sequences from the same genus show relatively less variation among the strains related to different genera, e.g., all the slow-growing rhizobia from different sites showed identical 16S rRNA gene sequences and showed high sequence similarity ($\geq 99\%$) with reference strains for 25 *Bradyrhizobium* species. This shows that a single *Bradyrhizobium* species is prevalent throughout mung bean-growing regions of Pakistan. Similarly, all the *Ensifer* strains from Faisalabad, Layyah, and Rawalpindi showed identical 16S rRNA gene sequence and sequence similarity ($\geq 99\%$) with reference strains for 12 *Ensifer* species. It is well-reported that 16S rRNA gene is widely used for determining the phylogenetic relationship and taxonomic position at the genus level but has limited resolving power to differentiate closely related species of bacteria [44]. In such cases, sequence analyses of the 16S–23S ITS region and housekeeping protein genes such as *recA*, *atpD*, *dnaK*, *glnII* are frequently used to resolve the species status of bacteria, especially rhizobia [16, 45–49]. The sequence analyses of the ITS region and *atpD* and *recA* genes of rhizobia further validated the species status as well as detected the polymorphism among the strains.

Fig. 5 Phylogenetic tree of selected rhizobia in this study based on *nodA* gene sequences showing the relationship of rhizobia isolated in this study with already published strains. The tree was constructed using ML method, and bootstrap value (1000 replicates) higher than 50% are shown at the nodes. Bacterial isolates obtained from this study are shown in bold

Sequence analysis of the ITS region and housekeeping genes revealed that these slow-growing rhizobia belong to *B. yuanmingense*. Nucleotide polymorphism observed in the ITS sequences divided these strains into three sequence types (STs) (Table 3). The ITS region of *Bradyrhizobium* strains has been widely used as a useful marker to differentiate closely related species as it exhibited high sequence variation [48, 49]. The differentiation of two *atpD* STs and three *recA* STs among the isolates related to *B. yuanmingense* evidenced the existence of different strains in the *Bradyrhizobium* population obtained in this study. The higher variability of housekeeping genes in rhizobia as compared to 16S rRNA for better delineation of species and differentiation of subspecies and strains is well-reported [16, 50–52]. Furthermore, bradyrhizobia are divided into three and two STs based upon *nifH* and *nodA* gene sequences, respectively, and clustered with different strains of *B. yuanmingense*. The *nodA* gene was not amplified from *Bradyrhizobium* sp. Vr61 and *Rhizobium* sp. Vr65 using primer pairs *nodA*-1/*nodA*-2. It is previously documented that *nodA* gene is under direct positive selection due to involvement in symbiosis process; thus, it is less conserved than housekeeping genes [53]. Sequence analyses collectively revealed that mung bean nodules in different areas of Pakistan host multiple strains of *B. yuanmingense*,

although multiple species of *Bradyrhizobium*, i.e., *B. japonicum*, *B. elkanii*, *B. liaoningense*, and *B. yuanmingense*, were reported to nodulate mung bean in China and Nepal [7–9]. *B. yuanmingense* was identified as a major slow-growing symbiont of mung bean in India [10], while some novel strains of *Bradyrhizobium* originating from acidic soils of the sub-Himalayan regions of India were found to be compatible with mung bean [54]. So, the cultivation-based study corroborated the findings of previous reports [7–9] and the bradyrhizobial community associated with mung bean may vary in different regions. However, nucleotide polymorphism was observed among these bradyrhizobia on the basis of ITS, *atpD*, *recA*, *nifH*, and *nodA* genes. So, sequence analysis of multiple genes clarified the species as well as intraspecies polymorphism.

Likewise, two *Rhizobium* strains Vr69 and Vr76 from Rawalpindi and one isolate Vr65 originating from Layyah showed 98.6–99.7% 16S rRNA gene sequence similarity with *R. pusense* previously obtained from chickpea rhizosphere in India [55]. The definition of these isolates as *R. pusense* enlarged the host range of this species, while the division of three STs in analysis of ITS, *atpD*, and *nifH* and two STs in *nodA* gene sequences evidenced the existence of other *Rhizobium* species as nodule endophytes of mung bean. Symbiotic genes

Table 3 Nucleotide polymorphism detected among different genes amplified from rhizobia

Genus (no. of strains)	Length	S	Eta	STs	Pi	Pi(s)	Pi(n)
16S rRNA							
<i>Ensifer</i> (17)	1323	19	19	2	0.0144	0.0225	0.0120
<i>Rhizobium</i> (3)	1076	16	16	2	0.0149	0.01211	0.0159
<i>Bradyrhizobium</i> (11)	1307	0	0	1	0.00	0.00	0.00
ITS							
<i>Ensifer</i> (6)	1282	153	153	2	0.0398	-	-
<i>Rhizobium</i> (3)	1295	322	330	3	0.1678	-	-
<i>Bradyrhizobium</i> (5)	1077	2	2	3	0.0009	-	-
<i>atpD</i>							
<i>Ensifer</i> (6)	443	39	39	2	0.0294	0.1004	0.0040
<i>Rhizobium</i> (3)	466	55	56	3	0.0794	0.2355	0.0254
<i>Bradyrhizobium</i> (5)	479	6	6	2	0.0050	0.0206	0.0000
<i>recA</i>							
<i>Ensifer</i> (6)	393	44	44	2	0.0373	0.1269	0.0052
<i>Rhizobium</i> (3)	393	45	45	2	0.0763	0.2667	0.0069
<i>Bradyrhizobium</i> (5)	393	14	14	3	0.0182	0.0681	0.0000
<i>nifH</i>							
<i>Ensifer</i> (6)	309	43	43	2	0.0464	0.1601	0.0079
<i>Rhizobium</i> (3)	318	87	91	3	0.1824	0.5084	0.0825
<i>Bradyrhizobium</i> (5)	284	7	7	3	0.0099	0.0293	0.0037
<i>nodA</i>							
<i>Ensifer</i> (6)	522	167	167	2	0.1064	0.2195	0.0662
<i>Rhizobium</i> (2)	519	3	3	2	0.0058	0.0217	0.0000
<i>Bradyrhizobium</i> (4)	554	55	55	2	0.0496	0.1182	0.0257

S, total number of polymorphic sites; Eta, number of mutations; STs, sequence types; Pi, nucleotide diversity; Pi(s), Pi at synonymous sites; Pi(n), Pi at non-synonymous sites

are commonly transferred horizontally as these are carried on mobile genetic elements [56]. However, in present study, the topologies of *nifH* and *nodA* genes were congruent to the phylogeny of housekeeping genes suggesting that these strains might have evolved through vertical gene transfer. The coherence of symbiotic and housekeeping gene phylogenies has been reported in several studies [53, 57].

All the fast-growing rhizobia related to genus *Ensifer* originated from three sites (Faisalabad, Layyah, and Rawalpindi) exhibit 100% sequence identity of 16S rRNA gene and clustered with *E. aridi* LEM 451, whereas, *Ensifer* strains from Thal desert show 1.51% sequence variation and belonged to *E. meliloti* IAM 12611^T. Similar to 16S rRNA gene sequence, representative *Ensifer* strains (Vr25, Vr28, Vr33, Vr67, and Vr70) from Faisalabad, Layyah, and Rawalpindi contain identical ITS, *atpD*, *recA*, *nifH*, and *nodA* gene sequences and belonged to *E. aridi*. These findings suggest that a single strain of *Ensifer* might be distributed among the mung bean at all sites/regions. *E. aridi* is newly described species, which has been isolated in Asian, African, and American deserts [58], from root nodules of *Tephrosia purpurea* in the Thar desert in India [59]; *Acacia gummifera* in Merzouga desert,

Morocco [60]; and *Phaseolus filiformis* in the Mexican desert [58]. More recently, the strains of *E. aridi* were isolated from nodules of *Retama monosperma* in Eastern Morocco [61]. Among *Ensifer* strains, only *E. fredii* has been reported as a symbiont of mung bean from China [8, 9]. Rhizobial strain from Thal desert was related to *E. meliloti* which is dominantly isolated from root nodules of *Acacia tortilis*, *Argyrobolium uniflorum*, *Cicer arietinum*, *Genista saharae*, *Hedysarum carnosum*, *Hippocrepis bicontorta*, *Medicago* spp., *Lotus* spp., *Ononis natrix*, *Phaseolus vulgaris*, and *Retama raetam* [62]. *Ensifer* strains have also been identified as major symbionts of legume in Thar desert of India which are closely related to *E. saheli*, *E. arboris*, *E. kostiensis*, *Ensifer teranga* [63], and *E. aridi* [43]. The findings of the present study suggest that *E. aridi* and *E. meliloti* are among the major symbionts of mung bean in Pakistan. The occurrence of both of these symbionts has not been reported in mung bean earlier.

In conclusion, the diversity analyses of symbionts of mung bean through the cultivation-based method showed predominance of *Bradyrhizobium* and *Ensifer*. Sequence analyses of multiple genes indicated that *B. yuanmingense* and *E. aridi* are the two major symbionts of mung bean at different sites

(Bhakkar, Faisalabad, Layyah, and Rawalpindi), while *E. meliloti* seems specific to Thal desert of Pakistan. This study has contributed to the global database on the distribution of rhizobial species and reported the presence of *Bradyrhizobium yuanmingense*, *Ensifer aridi*, *E. meliloti*, and *R. pusense*, and a novel *Rhizobium* sp. as nodulating microsymbionts of mung bean. Furthermore, intraspecies polymorphism detected through the sequence analyses of housekeeping genes validated that these genes could be used for the differentiation of isolates at the strain level. Moreover, two representative strains from *B. yuanmingense* and *E. aridi* could be used as universal inoculum as they were the dominant groups in almost all the sampling sites. However, *Ensifer* sp. Vr77 related to *Ensifer meliloti* could be used as inoculum in Thal desert. These strains could be further evaluated under field conditions to improve the productivity of plants and fertility of soil.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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