Systematic and Applied Microbiology 33 (2010) 416–425

Contents lists available at ScienceDirect

Systematic and Applied Microbiology

journal homepage: www.elsevier.de/syapm

Genetic diversity of native soybean bradyrhizobia from different topographical regions along the southern slopes of the Himalayan Mountains in Nepal☆

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ARTICLE INFO

Article history:
Received 2 April 2010

Keywords:
Bradyrhizobium
Genetic diversity
16S rRNA gene
Himalaya
Nepal

ABSTRACT

Soybean-nodulating bradyrhizobia are genetically diverse and are classified into different species. In this study, the genetic diversity of native soybean bradyrhizobia isolated from different topographical regions along the southern slopes of the Himalayan Mountains in Nepal was explored. Soil samples were collected from three different topographical regions with contrasting climates. A local soybean cultivar, Cobb, was used as a trap plant to isolate bradyrhizobia. A total of 24 isolates selected on the basis of their colony morphology were genetically characterized. For each isolate, the full nucleotide sequence of the 16S rRNA gene and ITS region, and partial sequences of the *nifD* and *nodD1* genes were determined. Two lineages were evident in the conserved gene phylogeny; one representing *Bradyrhizobium elkanii* (71% of isolates), and the other representing *Bradyrhizobium japonicum* (21%) and *Bradyrhizobium yuanmingense* (8%). Phylogenetic analyses revealed three novel lineages in the *Bradyrhizobium elkanii* clade, indicating high levels of genetic diversity among *Bradyrhizobium* isolates in Nepal. *B. japonicum* and *B. yuanmingense* strains were distributed in areas from 2420 to 2660 m above sea level (asl), which were mountain regions with a temperate climate. The *B. elkanii* clade was distributed in two regions; hill regions ranging from 1512 to 1935 m asl, and mountain regions ranging from 2420 to 2660 m asl. Ten multi-locus genotypes were detected; seven among *B. elkanii*, two among *B. japonicum*, and one among *B. yuanmingense*-related isolates. The results indicated that there was higher species-level diversity of *Bradyrhizobium* in the temperate region than in the sub-tropical region along the southern slopes of the Himalayan Mountains in Nepal.

Introduction

Soybean [*Glycine max* (L.) Merril] is a leguminous crop that originates from eastern Asia. It was domesticated approximately 4000 years ago, and has been cultivated ever since. Soybean is now a major crop worldwide, and is cultivated under various climatic conditions. The high nitrogen (N) requirement of the crop is fulfilled mainly by establishing a N_2-fixing symbiosis with rhizobia. Soybeans can establish an effective N-fixing symbiosis with species of fast-growing rhizobia [16,30], as well as with species of slow-growing bradyrhizobia [15,17,45]. Members of the genus *Bradyrhizobium* are slow-growing, Gram-negative bacteria that invade and form nitrogen-fixing nodules on the roots of legumes, including soybean. To date, nine *Bradyrhizobium* species have been identified; *Bradyrhizobium japonicum* [15], *Bradyrhizobium elkanii* [17], *Bradyrhizobium liaoningense* [45], *Bradyrhizobium yuanmingense* [48], *Bradyrhizobium betae* [27], *Bradyrhizobium canariense* [38], *Bradyrhizobium iriomotense* [13], *Bradyrhizobium jicamae* [25], and *Bradyrhizobium pachyrhizi* [25]. In addition to the species division, several studies indicate that the genus *Bradyrhizobium* also contains several as yet unnamed genospecies [36,44,52].

Soybean-nodulating rhizobia are genetically diverse and are classified into different genera and species. All species described so far have been found in China [46]. The slow-growing soybean-nodulating bradyrhizobia are distributed in three species of the

☆ *Note*: Nucleotide sequence data reported are available in the DNA Data Bank Japan (DDBJ) under the following accession numbers: 16S rRNA, AB513445–AB513468; ITS region, AB513469–AB513492; *nifD* gene, AB514091–AB514114; *nodD1* gene, AB514165–AB514188; reference strains, AB563477–AB563484.

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doi:10.1016/j.syapm.2010.06.008

Bradyrhizobium genus, namely, *B. japonicum* [15], *B. elkanii* [17], and *B. liaoningense* [45]. Fast-growing rhizobia include *Sinorhizobium fredii* [7], *Sinorhizobium xinjiangense* [23], *Mesorhizobium albiziae* [42], *Mesorhizobium septentrionale*, *Mesorhizobium temperatum* [11], *Rhizobium oryzae* [24] and other unclassified rhizobia [5]. *Mesorhizobium tianshanense* nodulates soybean and shows variable generation times [6].

Nepal lies along the southern slopes of the Himalayan Mountains, between China and India. With a land area of 147,181 km^2, Nepal is 885 km from east to west and varies from 145 to 248 km from north to south. Within this short distance from north to south, the topography varies by more than 8000 m. As a result, Nepal has three distinct topographical regions; mountain, hill, and Terai (lowlands). Terraced farming is a common practice even on the high altitude slopes of Nepal. The orientation, slopes, and aspects of mountain ranges result in a number of micro-climatic regimes within short distances. Mountain regions correspond to a temperate climate, whereas hill and Terai correspond to sub-tropical and tropical climates, respectively. Despite the importance of soybean cultivation in Nepal, little is known about the genetic resources of native bradyrhizobia [39]. In particular, the genetic diversity of native soybean bradyrhizobia from different topographic regions has not been described in detail.

Factors such as horizontal gene transfer (HGT) and unequal phylogenetic information content of different genetic markers [22,26,32,36,40] can reduce the accuracy of phylogenetic analyses. Therefore, there is a growing need in systematics to analyze genetic relationships based on multiple genomic loci. In particular, analysis of symbiotic genes such as *nod* and *nif* loci often shows relationships that differ from those predicted by rRNA-based phylogenies [18,20]. Therefore, to determine the genetic diversity of native soybean bradyrhizobia obtained from different topographical regions along the southern slopes of the Himalayan Mountains in Nepal, the 16S rRNA gene, the internal transcribed spacer (ITS) region, and the *nifD* and *nodD1* genes of native soybean bradyrhizobia were analyzed. Furthermore, the relationship between phylogenetic properties and symbiotic characteristics among Nepalese *Bradyrhizobium* isolates was investigated.

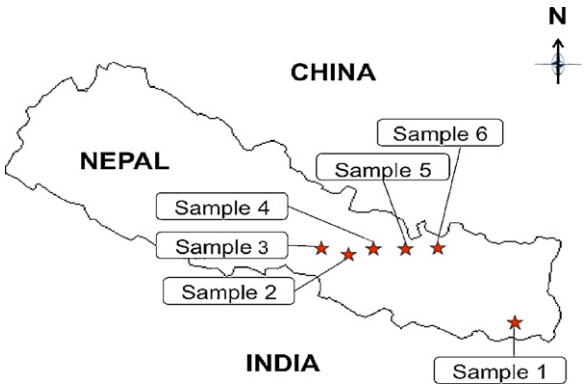

Fig. 1. Map of Nepal showing the location of soil sample collection sites. The relative position of samples 1–6 from south to north indicates the order of increased elevation.

Materials and methods

Soil samples and collection sites

Soil samples were collected in 2007 from six areas in which soybeans had been cultivated previously. These six areas were located in three topographic regions with contrasting climates (Fig. 1). Each soil sample was prepared by mixing soils obtained from 0 to 20 cm depth at each sampling location. No bacterial inoculations have been carried out in these areas and, therefore, the strains were considered to be native to Nepal. The details of the sampling sites and chemical properties of the soil samples are shown in Table 1.

Isolation of Bradyrhizobium strains

Seeds of *G. max* cv. Cobb (a local Nepalese variety) were surface-sterilized by immersion in 70% ethanol for 30 s, and then in 3% sodium hypochlorite solution for 3 min. Seeds were then exhaustively washed with sterile water. Five-fold dilutions of soil suspensions were used as inoculants. Each inoculant (5 mL/jar) was applied to sterilized vermiculite medium in 300 mL glass jars prior to sowing two surface-sterilized seeds. After sowing the seeds, the

Table 1
Sampling sites, chemical properties, and nodulation characteristics of soil samples used for bradyrhizobia isolation.

	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6
Latitude	26.40°N	27.70°N	27.63°N	27.58°N	27.64°N	27.66°N
Longitude	87.28°E	85.39°E	85.07°E	85.51°E	85.70°E	86.04°E
Altitude (m asl)	135	1512	1780	1935	2420	2660
Climatic zone	Tropical	Sub-tropical	Sub-tropical	Sub-tropical	Temperate	Temperate
pH[a]	5.5	6.2	6	5.8	6.1	5.2
OC (%)[b]	1.1	1.79	2.78	0.98	2.61	0.81
Total N (%)[c]	0.09	0.16	0.24	0.09	0.22	0.07
Available P_2O_5 (kg/ha)[d]	32.7	266.9	156.5	49.7	49.7	6.2
Available K_2O (kg/ha)[e]	158.3	884.6	363.9	236.1	468.2	240.5
Total nodules	0	4	11	10	50	35
Total Isolates	0	3	4	9	19	20
Number of isolates used	0	3	4	5	6	6
Isolate name[f]		C10-1512 C11-1512 C12-1512	C6-1780 C7-1780 C8-1780 C9-1780	C1-1935 C2-1935 C3-1935 C4-1935 C5-1935	C19-2420 C20-2420 C21-2420 C22-2420 C23-2420 C24-2420	C13-2660 C14-2660 C15-2660 C16-2660 C17-2660 C18-2660

Sample 1, lowland paddy field soil; samples 2–6, upland terraced field soil.
[a] Measured with a pH meter in a 1:1 (w/v) soil and distilled water solution.
[b] Measured by the Walkley and Black method.
[c] Measured by the Kjeldahl method of digestion, distillation, and titration [14].
[d] Measured by Bray extraction followed by colorimetric determination [14].
[e] Extracted in 1 N ammonium acetate and measured by flame photometry (ELICO, CL-378) [14].
[f] The four digit number in the last part of the isolate name indicates the altitude (m asl) at the site of soil collection.

jars were transferred to a growth chamber. Sterilized N-free nutrient solution [4] was added to the jar up to the 60% moisture level and was maintained at this level throughout the growth period. Plants were grown for 4 weeks in the growth chamber under a 16 h light (28 °C)/8 h dark (18 °C) photoperiod. After 4 weeks, whole plants were removed from the medium, washed in running tap water to remove vermiculite, and the nodules were harvested. Root nodules were surface-sterilized by immersion in 70% ethanol for 30 s and then in 3% sodium hypochlorite for 3 min, and were then washed five times with sterile water. Each nodule was crushed in 200 μL glycerol solution (15%, v/v) to obtain a turbid suspension. An aliquot (10 μL) of the suspension was streaked [37] onto 1.5% yeast extract mannitol agar (YEM) [31] plates and incubated for 1 week at 28 °C. The remaining suspension was frozen at −30 °C for further isolations, if necessary. Well separated single colonies were restreaked onto fresh plates to obtain pure cultures. These isolates were reinoculated onto the host plant to verify their nodulation ability.

Plant test

Isolates were grown in 15 mL YEM broth for 1 week with shaking. To determine the cell numbers of inoculants, bacterial cells were collected by centrifugation at 28,000 × *g* for 10 min at 4 °C, and then washed twice with 1 × TNE solution. Colony forming units (CFU) were counted by the dilution plate count method. Bradyrhizobial cells at a density of 1.2×10^{10} CFU were applied to sterilized vermiculite medium containing respective host seeds. Plants were grown in axenic conditions in the growth chamber, as described for the isolation experiment. After harvesting root nodules, nodule number, nodule fresh weight, and shoot weight were determined. Plant shoots and root nodules were dried at 80 °C for 48 h to determine dry weight and were powdered for analysis of total N by the indophenol method [14].

DNA extraction

Of the 55 isolates obtained, 24 were selected for this study based on their colony morphologies (Table 1). Criteria for selection were size, shape, color, elevation, mucosity, and transparency of the colony (data not shown). DNA was extracted from isolates using the method described previously by Yokoyama et al. [51], with a slight modification related to an additional 55 μL of 10% (w/v) cetyl trimethyl ammonium bromide (CTAB).

DNA amplification and sequencing

PCR amplification and sequencing of DNA fragments of 16S rRNA, ITS, and *nifD* and *nodD1* genes were carried out as described by Yokoyama [49]. The primer sets used for PCR amplification and sequencing are shown in Table S1 (Supplementary material). PCR products were sequenced using an ABI Prism 310 Genetic Analyzer or an ABI Prism 3130 Genetic Analyzer (Applied Biosystems), according to the manufacturer's protocols. Multiple sequence alignment of nucleotide sequences and boot strapping for creating a neighbor joining phylogenetic tree were performed using Clustal X 1.81 [35] and MEGA 4.0.

Results

Root nodule frequencies in different soils

In total, 110 root nodules were harvested from six soil samples in this study. When used as an inoculant, Sample 1 (soil from the Terai region in southern Nepal; Table 1) did not result in nodule formation on soybeans. Soil from mountain regions resulted in more nodules per plant than soil from hill regions. However, legumes, including soybean, are more commonly cultivated in hill regions than in mountain regions in Nepal.

Phylogenetic analysis based on 16S rRNA genes

A total of 24 isolates and 15 reference strains belonging to nine *Bradyrhizobium* species were divided into two groups in the 16S rRNA phylogenetic analysis, as shown in Fig. 2. The GI group included 17 isolates (71% of the total) and the GII group included 7 isolates (29%) from Nepal. All isolates from hill regions (1512–1935 m asl) were in the GI group, while all of the isolates in the GII group were from mountain regions. The remaining five isolates from mountain regions were classified into the GI group. Reference strains belonging to *B. elkanii*, *B. pachyrhizi*, and *B. jicamae* were classified into GI, while reference strains belonging to the remaining six species of *Bradyrhizobium* were classified into GII. The *B. jicamae* PAC 68^T strain was well separated from other isolates in the GI group, however, *B. pachyrhizi* PAC 48^T showed very high homology (98.8–100%) to *B. elkanii* reference strains.

GI was divided into four sub-groups, as shown in Fig. 2. GIa included 15 Nepalese isolates from sub-tropical and temperate regions, and four reference strains (three *B. elkanii* strains and one *B. pachyrhizi* strain). GIb contained two temperate region isolates and no reference strains. Similarly, GII was divided into five sub-groups. GIIa contained five temperate region isolates and two *B. japonicum* reference strains. GIIb contained two temperate region isolates and four reference strains (three *B. yuanmingense* strains and one *B. liaoningense* strain). Groups GIIc, GIId, and GIIe contained the reference strains *B. iriomotense*, *B. betae,* and *B. canariense,* respectively, but no Nepalese isolates.

Phylogenetic analysis based on the ITS region

Based on differences in 770-bp DNA fragments of their ITS region, 24 isolates and 15 reference strains belonging to nine *Bradyrhizobium* species were classified into two major clusters, as shown in Fig. 3. Six reference strains, four from *B. elkanii*, and one each from *B. pachyrhizi* and *B. jicamae* were grouped into GI. Reference strains of *B. japonicum*, *B. betae*, *B. canariense*, *B. liaoningense*, *B. yuanmingense*, and *B. iriomotense* were classified into GII, as was shown in the 16S rRNA gene tree (Fig. 2).

Isolates grouped into GI were further classified into seven sub-groups. All reference strains belonging to *B. elkanii* were categorized into two sub-groups, GIa and GIb. Sub-groups GIc, GId, and GIe, contained only Nepalese isolates. Their nucleotide sequences were well separated (<94.0% homology) from their most closely related reference strains. All but one of these Nepalese strains were isolated from soil obtained from 2660 m asl. This result suggested that the isolates in these three sub-groups represented a novel lineage of bradyrhizobia unique to high altitudes of Nepal. In contrast to the 16S rRNA gene phylogeny, *B. pachyrhizi* PAC 48^T was well separated from other strains in the GI group.

Out of seven isolates belonging to GII, five were closely related to *B. japonicum* USDA32 and *B. japonicum* $USDA6^T$, and the other two were closely related to three reference strains of *B. yuanmingense* (Fig. 3). These results were consistent with those obtained from 16S rRNA data. Here, 7.0% ITS region sequence differences were observed between reference strains of *B. liaoningense* and *B. yuanmingense*, separating them into two different sub-groups. Furthermore, GIf, GIg, GIIb, GIIc, GIId, and GIIe, which were sub-groups with type strains of *B. pachyrhizi*, *B. jicamae*, *B. liaoningense*, *B. canariense*, *B. betae*, and *B. iriomotense*, respectively, did not contain any Nepalese isolates.

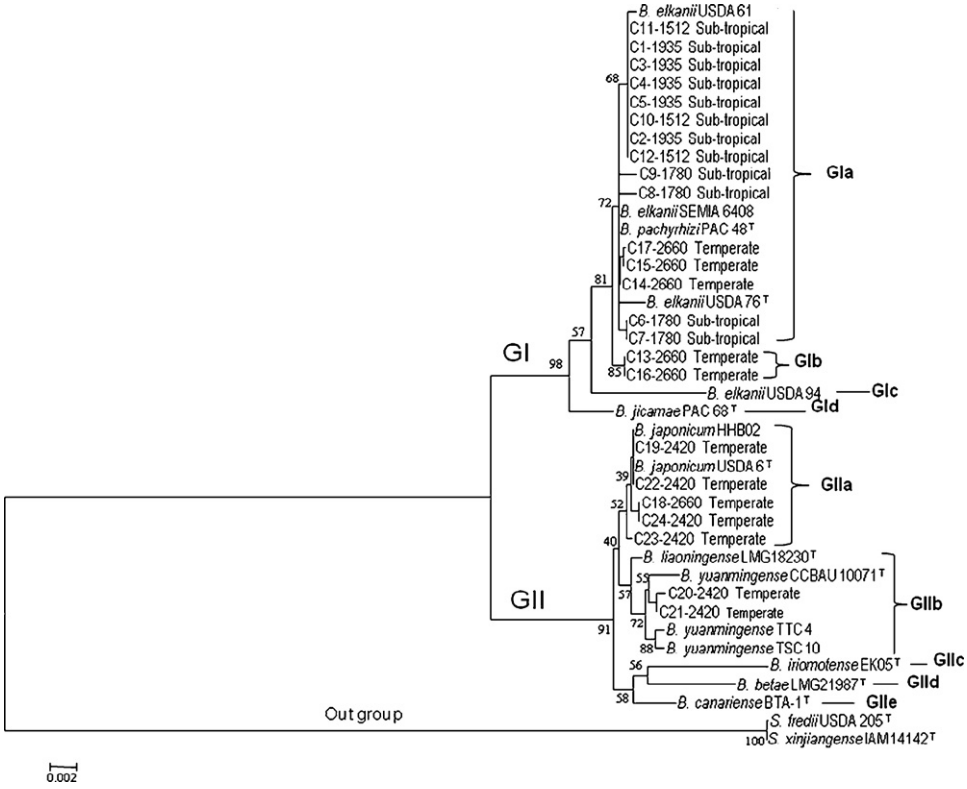

Fig. 2. Phylogenetic tree based on differences in 1440-bp DNA fragments of 16S rRNA genes obtained from 24 Nepalese *Bradyrhizobium* isolates and 17 reference strains. Numbers at the branch nodes indicate bootstrap values (%). The isolate name includes the elevation (m asl) and the respective climatic zone of the sampling site. Reference strains are as follows: *Bradyrhizobium elkanii* USDA61 (AB231916), *B. elkanii* USDA76^T (U35000), *B. elkanii* SEMIA6408 (FJ025103), *B. elkanii* USDA94 (D13429), *B. pachyrhizi* PAC48^T (AY624135), *B. jicamae* PAC68^T (AY624134), *B. japonicum* HHB02 (DQ517954), *B. japonicum* USDA6^T (AB231916), *B. liaoningense* LMG18230^T (AJ250813), *B. yuanmingense* CCBAU10071^T (AB509380), *B. yuanmingense* TSC10 (FJ540961), *B. yuanmingense* TTC4 (FJ540937), *B. iriomotense* EK05^T (AB300992), *B. betae* LMG21987^T (FM253260), *B. canariense* BTA1^T (AJ558025), *Sinorhizobium fredii* USDA205^T (AY260149), and *S. xinjiangense* IAM14142^T (D12796). The scale bar represents substitutions per nucleotide position.

Phylogenetic analysis based on partial nifD gene sequences

A total of 24 isolates and 11 reference strains were phylogenetically classified on the basis of 813-bp DNA fragments from the *nifD* region. As shown in Fig. 4, three groups were identified; GI consisted of three *B. elkanii* reference strains and 17 Nepalese isolates from the *B. elkanii* clade, including three novel lineages (shown in bold type in Fig. 3) derived from the ITS region phylogeny. However, the reference strains showed considerable sequence divergence compared with Nepalese isolates and were classified into two separate sub-clusters.

The GII cluster contained five *B. japonicum*-affiliated Nepalese isolates, four *B. japonicum* strains, and one *B. liaoningense* reference strain. However, the reference strains in this cluster separated from Nepalese isolates and produced two distinct sub-clusters. Two *B. yuanmingense*-affiliated Nepalese isolates produced a well separated GIII cluster. There were no *nifD* gene sequences from *B. yuanmingense* strains in the public databases. Therefore, we sequenced the *nifD* gene of *B. yuanmingense* TSC10, *B. yuanmingense* TTC4, and *B. yuanmingense* NBRC100594^T strains to clarify their phylogenetic relationships. *B. yuanmingense* TSC10 and *B. yuanmingense* TTC4, the closest relatives of the Nepalese *B. yuanmingense* isolates in the public databases, were isolated from nodules on cowpeas cultivated in south-west Japan [28]. Nevertheless, it was found that the *nifD* gene sequences of these reference strains were clearly separated from those of Nepalese isolates. These results indicated that *nifD* gene sequences of Nepalese isolates differed markedly from those *B. elkanii*, *B. japonicum*, *B. liaoningense*, and *B. yuanmingense* strains isolated from other regions.

Phylogenetic analysis based on partial nodD1 gene sequences

Based on differences in 674-bp DNA fragments from the *nodD1* gene region, 24 isolates and 9 reference strains were classified into two groups (Fig. 5). The branching pattern of the *nodD1* gene phylogenetic tree was similar to those of trees constructed from 16S rRNA, ITS, and *nifD* sequence data (Figs. 2–4). The G1 group contained 17 Nepalese isolates, 11 closely related to *B. elkanii* and 6 considered as novel lineages based on ITS region phylogeny. G1 was further divided into three sub-groups; GIa contained ten Nepalese isolates with *nodD1* sequences identical to that of *B. elkanii* USDA94. Two isolates in this sub-group were collected from temperate regions and the others were collected from the sub-tropical region. GIb and GIc sub-groups contained Nepalese isolates only. No closely related reference strains in the DNA Databases (DDBJ/GenBank/EMBL) were found.

GII was further divided into four sub-groups. Four Nepalese isolates showing close relationships to *B. japonicum* in Figs. 2–4 produced a different sub-group GIIb, showing considerable genetic distance from GIIa. GIIa contained one Nepalese isolate, two *B. japonicum* reference strains, and one *B. liaoningense* reference strain. The homology between C22 and *B. japonicum* USDA110 was 98.2%, whereas that between *B. japonicum* USDA110 and GIIb isolates was 97.6%. Like the GIb and GIc sub-groups, GIIb and GIIc did not contain reference strains. This indicated that isolates in these sub-groups had unique *nodD1* genes. As there were no records of *nodD1* gene sequences from *B. yuanmingense* strains in the public databases, we sequenced the *nodD1* gene of *B. yuanmingense* TSC10, *B. yuanmingense* TTC4, and *B. yuanmingense* NBRC100594^T

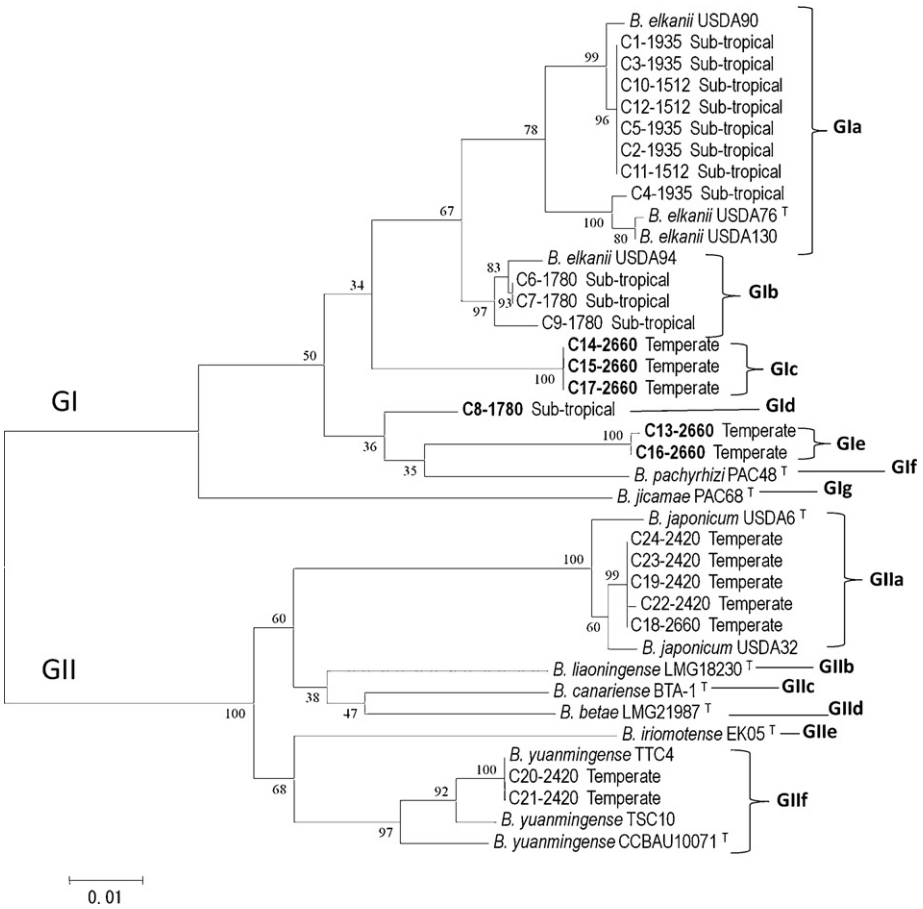

Fig. 3. Phylogenetic tree based on differences in 770-bp DNA fragments of the ITS region obtained from 24 Nepalese *Bradyrhizobium* isolates and 15 reference strains. The numbers at the branch nodes indicate bootstrap values (%). The isolate name includes the elevation (m asl) and the respective climatic zone of the sampling site. Reference strains are as follows: *B. elkanii* USDA90 (AF293380), *B. elkanii* USDA76^T (U35000), *B. elkanii* USDA130 (AF208510), *B. elkanii* USDA94 (AF208518), *B. pachyrhizi* PAC48^T (AY628092), *B. jicamae* PAC68^T (AY628094), *B. japonicum* USDA6^T (AB100741), *B. japonicum* USDA32 (AF293377), *B. liaoningense* LMG18230^T (AJ279301), *B. canariense* BTA1^T (AY386708), *B. betae* LMG21987^T (AJ631967), *B. iriomotense* EK05^T (AB300993), *B. yuanmingense* TTC4 (FJ540990), *B. yuanmingense* TSC10 (FJ541014), and *B. yuanmingense* CCBAU10071^T (AY386734). The scale bar represents substitutions per nucleotide position.

strains to clarify the phylogenetic relationships. These reference strains were well separated from Nepalese *B. yuanmingense*-related isolates. Until now, there have been no reports on *nodD1* genes in *B. yuanmingense.* Our data showed that the phylogenetic position of *nodD1* genes from Nepalese *B. yuanmingense*-related isolates clearly differed from those of *nodD1* genes from *B. elkanii*, *B. japonicum*, *B. liaoningense*, and *B. yuanmingense* strains from other regions.

Plant test

All isolates produced root nodules when inoculated onto seeds of *G. max* cv. Cobb to verify nodulation ability. The symbiotic performances of isolates with different *nifD* and *nodD1* gene sequences are shown in Table 3. For isolates with different *nifD* gene sequences, the shoot N content of host plants, amount of N fixed, and symbiotic efficiency were determined, as shown in Table 3(A). The amount of N fixed was calculated from the difference in shoot N content between test plants and controls. Isolates with different *nodD1* gene sequences were assessed for their nodulation characteristics, especially numbers of root nodules formed and their dry weight, as shown in Table 3(B). Among the three *nifD* groups, GIIIb isolates resulted in lower shoot N content, lower levels of fixed N, and lower symbiotic efficiency compared with GIb and GIIb. This group of isolates was closely related to *B. yuanmingense* in terms of 16S rRNA and ITS sequences.

The highest numbers of root nodules were formed by isolates categorized into GIIa and GIIb sub-groups in the *nodD1*-based phylogeny (belonging to *B. japonicum* in terms of 16S rRNA and ITS sequences). Of the three *B. elkanii*-related sub-groups, GIb formed the most nodules, and GIa formed the fewest. In terms of root nodule dry weight, GIIc isolates were significantly inferior to other groups, as shown in Table 3 (B). GIIc isolates showed close relationships to *B. yuanmingense* in terms of 16S rRNA and ITS sequences. Individual nodule dry weight was higher in *B. elkanii*-related isolates belonging to GIa, GIb, and GIc sub-groups than in other groups in the *nodD1* phylogeny.

Discussion

Bradyrhizobium distribution on southern slopes of Himalayan Mountains

The major objective of this study was to identify the *Bradyrhizobium* species distributed on the southern slopes of the Himalayan Mountains. Strains were isolated from six soil samples, which were collected from three topographic regions on the southern slopes of the Himalayan Mountains in Nepal. The climatic conditions at the sites from which soil was collected ranged from tropical to temperate. The optimum growing season for soybean varies significantly among the different topographic and climatic regions in Nepal. Thus, it was preferable to grow plants inoculated with soil

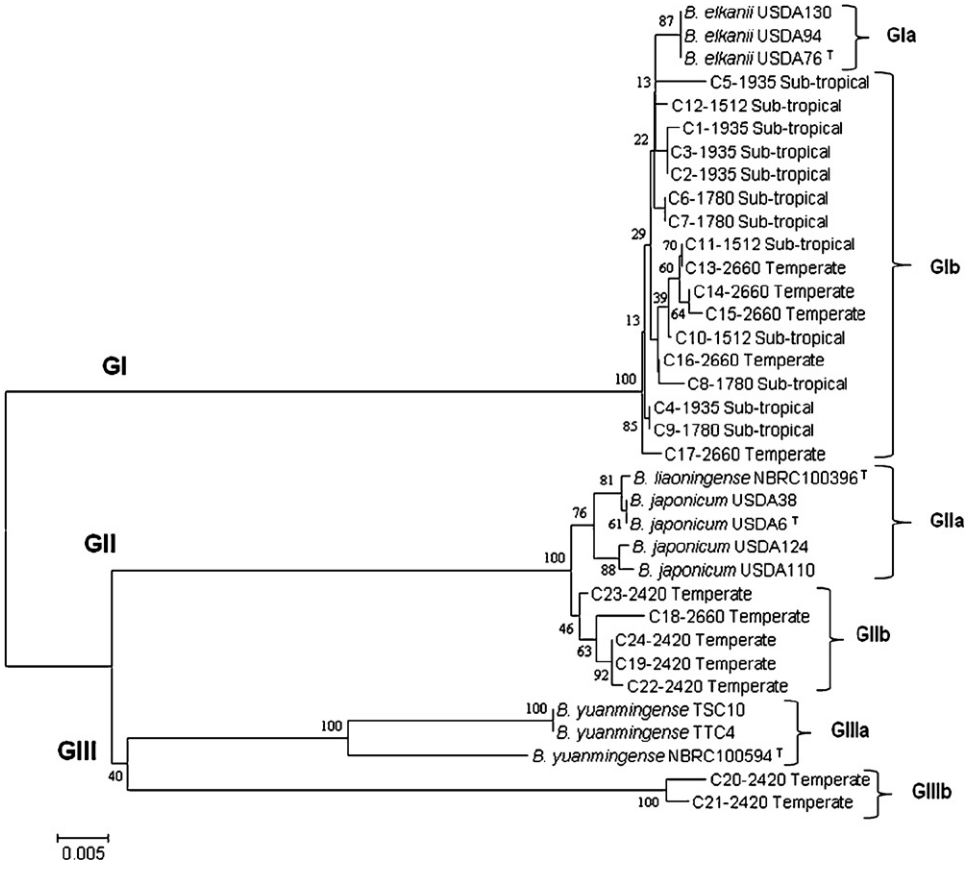

Fig. 4. Phylogenetic tree based on differences of 813-bp DNA fragments of the *nifD* gene obtained from 24 Nepalese *Bradyrhizobium* isolates and 11 reference strains. The numbers at the branch nodes indicate bootstrap values (%). The isolate name includes the elevation (m asl) and the respective climatic zone of the sampling site. Reference strains are as follows: *B. elkanii* USDA94 (AF484267), *B. elkanii* USDA76^T (AF484266), *B. elkanii* USDA130 (AF484268), *B. japonicum* USDA38 (AF484255), *B. japonicum* USDA124 (AF484259), *B. japonicum* USDA110 (BA000040), *B. japonicum* USDA6 (AF484254), *B. liaoningense* NBRC100396^T (AB563478), *B. yuanmingense* NBRC100594^T (AB563477), *B. yuanmingense* TSC10 (AB563484), and *B. yuanmingense* TTC4 (AB563483). The scale bar represents substitutions per nucleotide position.

samples in the laboratory to recover isolates, rather than recovering them directly from field-grown plants. In this study, we were unable to harvest root nodules and obtain *Bradyrhizobium* isolates from soil from the tropical region (Table 1). The sampling site in this region was lowland paddy field with a rice-wheat cropping system, with occasional inclusion of soybean as a rotation crop. Thus, it is possible that the soil sample from the tropical region used in this study contained very few bradyrhizobia. Therefore, to determine the distribution of bradyrhizobia in tropical regions of Nepal, further soil samples should be evaluated.

A total of 24 *Bradyrhizobia* isolates collected from sub-tropical and temperate regions in Nepal were phylogenetically categorized (Table 2). In the sub-tropical region, *B. elkanii*-related isolates were the predominant group, and no *B. japonicum*-related isolates were detected. In the temperate region, *B. japonicum* accounted for 42% of the isolates detected. This result is consistent with those reported previously [2,50,51]. In these studies, RFLP analysis was used to examine Thai *Bradyrhizobia* on the basis of common *nod* gene regions, and the results showed that *B. japonicum* is the predominant species in temperate regions, whereas *B. elkanii* is widely distributed from temperate to tropical regions.

Phylogenetic analysis of the 16S rRNA gene region is a common method for determining generic names of unknown micro-organisms [1], while phylogenetic analysis of the ITS region is well used to determine species names of micro-organisms within some taxa, including *Bradyrhizobium* [41,44]. The 16S rRNA sequence of *B. elkanii* SEMIA 6408 was identical to that of *B. pachyrhizi* PAC48^T, whereas that of *B. elkanii* USDA94 differed markedly from those of other *B. elkanii* strains (Fig. 2). On the other hand, the ITS-based phylogeny did not show such anomalies, and gave more definitive information for species determination than the 16S rRNA-based phylogeny. In a study by Willems et al. [44], when two strains showed 95.5% or more ITS sequence similarity they were always found to belong to the same genospecies. In line with this result, it is possible that the six isolates shown in bold type in Table 2 and Fig. 3 are a novel lineage of *Bradyrhizobium*. The closest relative for these isolates showed more than 5% sequence divergence.

Among isolates obtained from the temperate region, two were categorized as *B. yuanmingense* on the basis of phylogenetic analyses of DNA fragments corresponding to 16S rRNA and ITS regions. *B. yuanmingense* was first reported by Yao et al. [48] and was isolated from root nodules of *Lespedeza* spp. Since then, several studies have reported nodulation of various legumes by *B. yuanmingense*-related rhizobia [21,45]. Studies of host specificity showed that these *B. yuanmingense* isolates could not effectively nodulate soybean plants [47,48]. However, the isolates that showed close relationships to *B. yuanmingense* in the present study nodulated soybeans, and the nodules fixed N_2 effectively. Recently, Chinnaswami et al. [8,9] reported that 36% of soybean isolates and 95% of *Vigna* isolates in different agro-ecological–climatic regions of India were closely related to *B. yuanmingense*.

Symbiotic gene diversity between Nepalese Bradyrhizobium and Bradyrhizobium isolated from other regions

Nepalese isolates were categorized into three clusters based on phylogenetic analysis of the *nifD* gene region, and they were compared with type strains belonging to *B. elkanii*, *B. japonicum*,

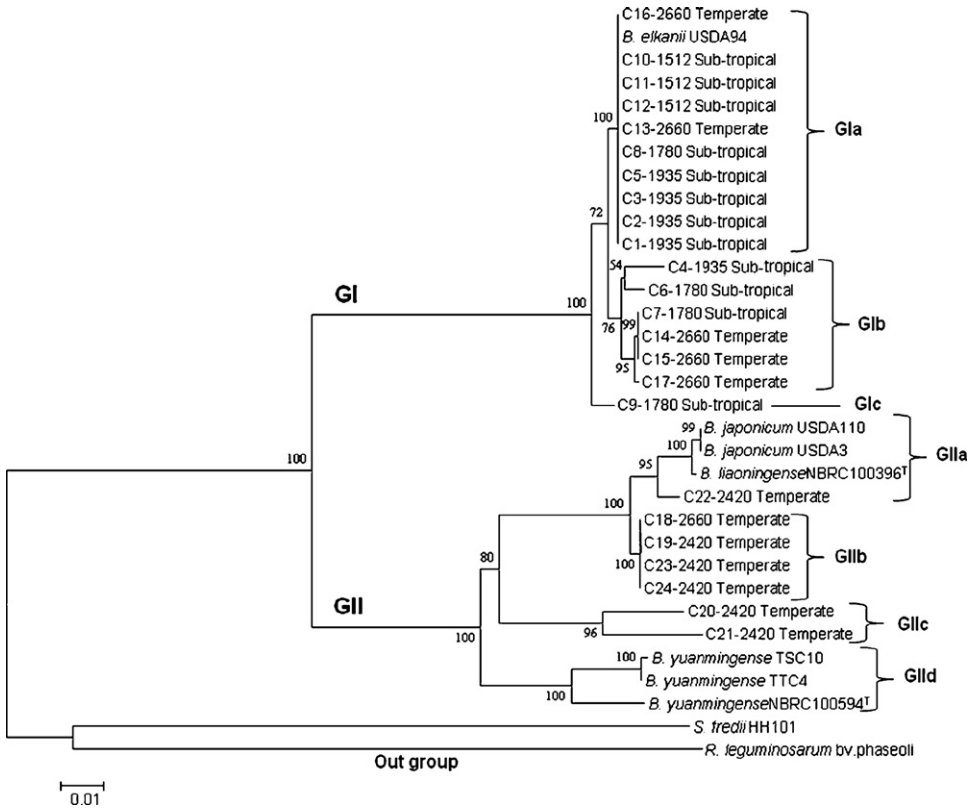

Fig. 5. Phylogenetic tree based on differences in 674-bp DNA fragments of the *nodD1* gene obtained from 24 Nepalese *Bradyrhizobium* isolates and 9 reference strains. The numbers at the branch node indicate bootstrap values (%). The isolate name includes the elevation (m asl) and the respective climatic zone of the sampling site. Reference strains are as follows: *B. elkanii* USDA94 (U04609), *B. japonicum* USDA3 (M81825), *B. japonicum* USDA110 (BA000040), *B. liaoningense* NBRC100396T (AB563479), *B. yuanmingense* NBRC100594T (AB563480), *B. yuanmingense* TSC10 (AB563481), *B. yuanmingense* TTC4 (AB563482), *R. leguminosarum* bv. phaseoli (X54214), and *S. fredii* HH101 (Y08938). The scale bar represents substitutions per nucleotide position.

and *B. yuanmingense*. Although *nifD* genes are considered as highly homologous genes, we observed considerable *nifD* gene sequence divergence among Nepalese isolates and reference strains. The amino acid sequences of 75% (18 out of 24) of the Nepalese isolates were identical to those of their type strains; however, there was considerable amino acid sequence divergence among three *B. elkanii*, one *B. japonicum*, and two *B. yuanmingense*-related isolates from Nepal (Fig. S1, Supplementary material). This could be one of the causes for functional differences among these isolates.

Phylogenetic analysis of the *nodD1* region of 24 Nepalese isolates produced two clusters, as in the phylogenetic analyses of the 16S rRNA and ITS regions. Unlike *nifD* genes, *nodD1* gene sequences of 59% of GI cluster isolates were identical to that of *B. elkanii* USDA94. The branch patterns of phylogenetic trees produced from sequence data of conserved DNA regions (16S rRNA and ITS), were similar to those produced from *nifD* and *nodD1* sequence data. This result suggests that the evolution of symbiotic genes of Nepalese isolates has progressed similarly to that of their conserved genes. Horizontal transfer of symbiotic genes has been a topic of interest for a long time [3,19,33,34]. In general, the incongruence among phylogenetic trees produced from different loci has been considered as evidence for horizontal gene transfer [10,12,43,53]. However, our results suggest that horizontal gene transfer is not a major factor in the evolution of *nifD* and *nodD1* genes among Nepalese *Bradyrhizobium* isolates.

Ten multi-locus genotypes were detected among Nepalese isolates (Table 2). Multi-locus genotype diversity was higher within the Nepalese *B. elkanii* (seven types, including three novel types) lineage than within the *B. japonicum* (two types) and *B. yuanmingense* (single type) lineages. Five multi-locus genotypes were detected among tropical region isolates, and five among temperate region isolates. However, species-level diversity was higher among isolates from the temperate region. Of all the isolates from the sub-tropical region, 92% were *B. elkanii* isolates and 8% were novel isolates. Among isolates from the temperate region, 42% were novel isolates, 42% were related to *B. japonicum,* and 12% were related to *B. yuanmingense* (Table 2). In other studies, taxonomic relationships among *Bradyrhizobium* isolates have been explored using DNA-DNA hybridization combined with analysis of several housekeeping genes (*atpD*, *recA*, *dnaK*, *glnII*, *gyrB*, and *rpoB*) apart from the sequences of the 16S rRNA gene and ITS region [13,21,26,32,39,40]. However, this information was not available for species identity of our isolates (as mentioned in Table 2).

Symbiotic performance of isolates

In terms of the phylogenetic groups based on differences in *nifD* gene sequences, it was found that GIIIb isolates fixed small amounts of atmospheric N and, therefore, showed lower symbiotic efficiency than other isolates (Table 3(A)). Isolates in this group were closely related to *B. yuanmingense*. These results show that symbiotic N_2 fixation by *B. yuanmingense* in soybean "Cobb" is clearly inferior to that of other isolates.

In terms of the phylogenetic groups based on differences in *nodD1* gene sequences, it was found that GIa isolates formed the fewest root nodules. The root nodule dry weight per plant was clearly divided into two groups; one group contained GIa, GIb, GIc, GIIa, and GIIb, which consisted of isolates related to *B. japonicum* and *B. elkanii,* and isolates in the novel lineage. The other group contained GIIc, which consisted of isolates that were closely related to *B. yuanmingense*. This result also suggested that nodulation performance of Nepalese isolates related to *B. yuanmingense*

Table 2
Summary of phylogenetic analysis of native soybean root nodule *Bradyrhizobium* isolates from Nepal.

MLS type	Strain[a]	Conserved gene types		Symbiotic gene types		Climate	Identity	Source of nucleotide sequence
		16S rRNA[b]	ITS[c]	*nifD*[d]	*nodD1*[e]			
	USDA 61	GIa	GIa	–	–	–	*B. elkanii*	AB231916, EU634736
	USDA 90	–	GIa	–	–	–	*B. elkanii*	AF293380
1	**USDA 76^T**	GIa	GIa	GIa	–	–	*B. elkanii*	U35000, AF484266
	USDA 130	–	GIa	GIa	–	–	*B. elkanii*	AF208510, AF484268
	SEMIA 6408	GIa	–	–	–	–	*B. elkanii*	FJ025103
	Cl-1935	GIa	GIa	GIb	GIa	Sub-tropical	*B. elkanii*[f]	This study
	C2-1935	GIa	GIa	GIb	GIa	Sub-tropical	*B. elkanii*[f]	This study
	C3-1935	GIa	GIa	GIb	GIa	Sub-tropical	*B. elkanii*[f]	This study
2	C5-1935	GIa	GIa	GIb	GIa	Sub-tropical	*B. elkanii*[f]	This study
	C10-1512	GIa	GIa	GIb	GIa	Sub-tropical	*B. elkanii*[f]	This study
	C11-1512	GIa	GIa	GIb	GIa	Sub-tropical	*B. elkanii*[f]	This study
	C12-1512	GIa	GIa	GIb	GIa	Sub-tropical	*B. elkana*[f]	This study
3	C4-1935	GIa	GIa	GIb	GIb	Sub-tropical	*B. elkanii*[f]	This study
4	C6-1780	GIa	GIb	GIb	GIb	Sub-tropical	*B. elkanii*[f]	This study
	C7-1780	GIa	GIb	GIb	GIb	Sub-tropical	*B. elkanii*[f]	This study
5	C9-1780	GIa	GIb	GIb	GIc	Sub-tropical	*B. elkanii*[f]	This study
6	**C8-1780**	GIa	GId	GIb	GIa	Sub-tropical	*Bradyrhizobium* sp.[f]	This study
	C17-2660	GIa	GIc	GIb	GIb	Temperate	*Bradyrhizobium* sp.[f]	This study
7	**C14-2660**	GIa	GIc	GIb	GIb	Temperate	*Bradyrhizobium* sp.[f]	This study
	C15-2660	GIa	GIc	GIb	GIb	Temperate	*Bradyrhizobium* sp.[f]	This study
8	**C13-2660**	GIb	GIe	GIb	GIa	Temperate	*Bradyrhizobium* sp.[f]	This study
	C16-2660	GIb	GIe	GIb	GIa	Temperate	*Bradyrhizobium* sp.[f]	This study
9	USDA 94	GIc	GIb	GIa	GIa	–	*B. elkanii*	D13429, AF208518, AF484267, U04609
10	PAC 48^T	GIa	GIf	–	–	–	*B. pachyrhizi*	AY624135, AY628092
11	PAC 68^T	GId	GIg	–	–	–	*B. jicamae*	AY624134, AY628094
	HHBO2	GIIa	–	–	–	–	*B. japonicum*	DQ517954
	USDA 3	–	–	–	GIIa	–	*B. japonicum*	M81825
	USDA 6^T	GIIa	GIIa	GIIa	–	–	*B. japonicum*	AB231916, AB100741, AF484254
12	USDA 32	–	GIIa	–	–	–	*B. japonicum*	AF293377
	USDA 38	–	–	GIIa	–	–	*B. japonicum*	AF484255
	USDA 110	–	–	GIIa	GIIa	–	*B. japonicum*	BA000040
	USDA 124	–	–	GIIa	–	–	*B. japonicum*	AF484259
13	C22-2420	GIIa	GIIa	GIIb	GIIa	Temperate	*B. japonicum*[f]	This study
	C18-2660	GIIa	GIIa	GIIb	GIIb	Temperate	*B. japonicum*[f]	This study
14	C19-2420	GIIa	GIIa	GIIb	GIIb	Temperate	*B. japonicum*[f]	This study
	C23-2420	GIIa	GIIa	GIIb	GIIb	Temperate	*B. japonicum*[f]	This study
	C24-2420	GIIa	GIIa	GIIb	GIIb	Temperate	*B. japonicum*[f]	This study
	CCBAU10071^T	GIIb	GIIf	–	–	–	*B. yuanmingense*	AB509380, AY386734
15	NBRC 100594^T	–	–	GIIIa	GIId	–	*B. yuanmingense*	This study
	TSC10	GIIb	GIIf	GIIIa	GIId	–	*B. yuanmingense*	FJ540961, FJ541014, this study
	TTC4	GIIb	GIIf	GIIIa	GIId	–	*B. yuanmingense*	FJ510937, FJ540990, this study
16	C20-2420	GIIb	GIIf	GIIIb	GIIc	Temperate	*B. yuanmingense*[f]	This study
	C21-2420	GIIb	GIIf	GIIIb	GIIc	Temperate	*B. yuanmingense*[f]	This study
17	LMG18230^T	GIIb	GIIb	–	–	–	*B. liaoningense*	AJ250813, AJ279301
	NBRC 100396^T	–	–	GIIa	GIIa	–	*B. liaoningense*	This study
18	EK05^T	GIIc	GIIe	–	–	–	*B. iriomotense*	AB300992, AB300993
19	LMG21987^T	GIId	GIId	–	–	–	*B. betae*	FM253260, AJ631967
20	BTA-1^T	GIIe	GIIc	––	–	–	*B. canariense*	AJ558025, AY386706

(–) Not applicable.
( ) Phylogenetic positions of isolates do not match those any reference strains used in this study.
[a] Strain name corresponds to that in a DNA Database (DDBJ/GenBank/EMBL).
[b] Group name corresponds to the group number shown in Fig. 2.
[c] Group name corresponds to the group number shown in Fig. 3.
[d] Group name corresponds to the group number shown in Fig. 4.
[e] Group name corresponds to the group number shown in Fig. 5.
[f] Further confirmation needed for species determination.

is inferior to those of other isolates. To clarify aspects of nodulation performance, it is important to investigate the ability of each isolate's NodD1 protein to recognize flavonoid compounds, which is an essential part of initiating the legume-*Rhizobium* interaction [49].

The results of the plant test suggested that expression of different symbiotic genes in these isolates resulted in different degrees of symbiotic performance. Our results suggested that *B. japonicum* and *B. elkanii* might be more efficient symbiotic partners than *B. yuanmingense* for the local soybean cv. Cobb cultivated at high altitudes

Table 3
Symbiotic characteristics of groups of Nepalese isolates based on *nifD* and *nodD1* gene sequences.

(A) Nitrogen-fixing characteristics of isolates classified as differences in *nifD* gene sequences

nifD group[a]	Shoot N content (mg/plant)	N fixed (mg/plant)	Symbiotic efficiency[b]
GIb (39)	14.5 ± 5.6a	12.7 ± 5.6a	33.2 ± 8.4a
GIIb (15)	14.2 ± 5.3a	12.4 ± 5.3a	31.8 ± 9.7a
GIIIb (6)	5.2 ± 0.35b	3.4 ± 0.35b	17.8 ± 0.91b
Fishers *F* value	8.131	8.131	7.751
Level of significance (*p*)	<0.01	<0.01	<0.01

(B) Nodulation characteristics of isolates classified as differences in *nodD1* gene sequences

nodD1 group[c]	Nodule no./plant	Nodule dry wt (mg/plant)	Nodule dry wt (mg/nodule)
GIa (27)	19.33 ± 6.16a	32.74 ± 11.32a	1.80 ± 0.24a
GIb (12)	24.25 ± 4.72bc	49.58 ± 9.37b	2.06 ± 0.30b
GIc (3)	19.90 ± 1.56ab	36.75 ± 1.77a	1.86 ± 0.23ab
GIIa (3)	29.0 ± 2.05cd	38.30 ± 2.71a	1.32 ± 0.19c
GIIb (12)	28.25 ± 4.57d	37.60 ± 4.59a	1.53 ± 0.18c
GIIc (6)	20.0 ± 3.07ab	18.8 ± 0.78c	1.0 ± 0.17d
Fishers *F* value	6.662	5.424	6.048
Level of significance (*p*)	<0.01	<0.01	<0.01

Different letters following the values in each column represent significant differences ($p < 0.05$, pairwise Fisher's *F*-test). Three replicates of each isolate were analyzed. Figures in parentheses indicate the number of samples analyzed in each group.

[a] Group name corresponds to the group number shown in Fig. 4.

[b] Symbiotic efficiency was calculated as follows: (mg N fixed/mg dry nodule) × 100.

[c] Group name corresponds to the group number shown in Fig. 5.

of Nepal. Symbiotic performances in legume-*Rhizobium* symbioses have been reported to be cultivar-dependent [28,29]. Therefore, further investigations including different cultivars and reference strains are required. Such data will be useful for selecting the best candidate for bio-fertilizer suitable for soybean cultivation at high altitudes in Nepal.

Soybean is one of the main legume crops in Nepal. However, the average yield is much lower than the world average, and improving crop performance is a major challenge. The effectiveness of symbiotic N_2 fixation might be an important factor for increasing productivity through successful management of the soybean and native bradyrhizobia symbiosis. Vinuesa et al. [39] characterized bradyrhizobia from soybean cultivated at two sites in the humid temperate climate zone in Nepal, and showed that all isolates were members of highly epidemic and well differentiated *B. japonicum* of the DNA homology group Ia. In the present study, we determined the genetic diversity and symbiotic effectiveness of native soybean-nodulating *Bradyrhizobium* from different topographic–climatic regions in Nepal. Our results will be useful for the development of effective bio-fertilizers, which is a key project targeted by the Nepalese government. However, further studies are necessary to investigate in detail the relationship between genetic diversity and functional variability of the soybean-*Bradyrhizobium* symbiosis.

Acknowledgments

We thank Dhruba Dhakal, Soil Management Directorate Hariharbhawan, Nepal, for providing soybean seeds and for collection and analysis of soil samples. We thank Takeo Yamakawa and Papa S. Sarr of Kyushu University, Japan, for providing pure cultures. We are grateful to the Ministry of Education, Japan, for a research fellowship, and the Government of Nepal for providing study leave to complete this research.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.syapm.2010.06.008.

References

[1] Amann, R.I., Ludwig, W., Schleifer, K.H. (1995) Phylogenetic identification and *in situ* determination of individual microbial cells without cultivation. Microbiol. Rev. 59, 143–169.
[2] Ando, S., Yokoyama, T. (1999) Phylogenetic analysis of *Bradyrhizobium* nodulating soybean (*Glycine max*) in Thailand with reference to the USDA strains of *Bradyrhizobium*. Can. J. Microbiol. 45, 639–645.
[3] Barcellos, F.G., Menna, P., Batista, J.S.S., Hungria, M. (2007) Evidence of horizontal transfer of symbiotic genes from a *Bradyrhizobium japonicum* inoculant strain to indigenous diazotrophs *Sinorhizobium* (*Ensifer*) *fredii* and *Bradyrhizobium elkanii* in a Brazilian savannah soil. Appl. Environ. Microbiol. 73, 2635–2643.
[4] Broughton, W.J., Dilworth, M.J. (1970) Methods in legume-*rhizobium* technology: plant nutrient solutions. In: Somasegaran, P., Hoben, H.J. (Eds.), Handbook for *Rhizobium*, NifTAL Project and University of Hawaii, Paia, Hawaii, pp. 245–249.
[5] Chen, L.S., Figueredo, A., Pedrosa, F.O., Hungria, M. (2000) Genetic characterization of soybean rhizobia in Paraguay. Appl. Environ. Microbiol. 66, 5099–5103.
[6] Chen, W., Wang, E., Wang, S., Li, Y., Chen, X., Li, Y. (1995) Characteristics of *Rhizobium tianshanense* sp. nov., a moderately and slowly growing root nodule bacterium isolated from an arid saline environment in Xinjiang, People's Republic of China. Int. J. Syst. Bacteriol. 45, 153–159.
[7] Chen, W.X., Yan, G.H., Li, J.L. (1988) Numerical taxonomic study of fast-growing soybean rhizobia and a proposal that *Rhizobium fredii* be assigned to *Sinorhizobium* gen. nov. Int. J. Syst. Bacteriol. 38, 392–397.
[8] Chinnaswamy, A., N'Zoue, A., Laguerre, G. (2008) Genetic diversity of native bradyrhizobia isolated from soybeans (*Glycine max* L) in different agricultural–ecological–climatic regions of India. Appl. Environ. Microbiol. 74, 5991–5996.
[9] Chinnaswamy, A., N'Zoue, A., Moulin, L., Depret, G., Laguerre, G. (2009) *Vigna mungo*, *V. radiata* and *V. unguiculata* plants sampled in different agronomical–ecological–climatic regions of India are nodulated by *Bradyrhizobium yuanmingense*. Syst. Appl. Microbiol. 32, 460–470.
[10] Dykhuizen, D.E., Green, L. (1991) Recombination in *Escherichia coli* and the definition of biological species. J. Bacteriol. 173, 7257–7268.
[11] Gao, J.L., Turner, S.L., Kan, F.L., Wang, E.T., Tan, Z.Y., Qiu, Y.H., Gu, J., Terefework, Z., Young, J.P.W., Lindström, K., Chen, W.X. (2004) *Mesorhizobium septentrionale* sp. nov. and *Mesorhizobium temperatum* sp. nov., isolated from *Astragalus adsurgens* growing in the northern regions of China. Int. J. Syst. Evol. Microbiol. 54, 2003–2012.
[12] Haukka, K., Lindstrom, K., Young, J.P.W. (1998) Three phylogenetic groups of *nodA* and *nifH* genes in *Sinorhizobium* and *Mesorhizobium* isolates from leguminous trees growing in Africa and Latin America. Appl. Environ. Microbiol. 64, 419–426.
[13] Islam, M.S., Kawasaki, H., Muramatsu, Y., Nakagawa, Y., Seki, T. (2008) *Bradyrhizobium iriomotense* sp. nov., Isolated from a tumor-like root of the legume *Entada koshunensis* from Iriomote Island in Japan. Biosci. Biotechnol. Biochem. 72, 1416–1429.
[14] Jones, J.B. 2001 Laboratory Guide for Conducting Soil Tests and Plant Analysis , pp. 191–239 (Chapter 3).
[15] Jordan, D.C. (1982) Transfer of *Rhizobium japonicum* Buchanan 1980 to *Bradyrhizobium* gen. nov., a genus of slow growing root nodule bacteria from leguminous plants. Int. J. Syst. Bacteriol. 32, 136–139.

[16] Keyser, H.H., Bohlool, B.B., Hu, T.S., Weber, D.F. (1982) Fast growing rhizobia isolated from root nodules of soybeans. Science 215, 1631–1632.
[17] Kuykendall, L.D., Saxena, B., Devine, E.E., Udell, S.E. (1992) Genetic diversity in *Bradyrhizobium japonicum* Jordan (1982) and a proposal for *Bradyrhizobium elkanii* sp. nov. Can. J. Microbiol. 38, 501–505.
[18] Laguerre, G., Nour, S.M., Macheret, V., Sanjuan, J., Drouin, P., Amargar, N. (2001) Classification of rhizobia based on *nodC* and *nifH* gene analysis reveals a close phylogenetic relationship among *Phaseolus vulgaris* symbionts. Microbiology 147, 981–993.
[19] Minamisawa, K., Itakura, M., Suzuki, M., Ichige, K., Isawa, T., Yuhashi, K., Mitsui, H. (2002) Horizontal transfer of nodulation genes in soils and microcosms from *Bradyrhizobium japonicum* to *B. elkanii*. Microbes Environ. 17, 82–90.
[20] Moulin, L., Béna, G., Boivin-Masson, C., Stepkowski, T. (2004) Phylogenetic analysis of symbiotic nodulation genes support vertical and lateral gene co-transfer within the *Bradyrhizobium* genus. Mol. Phylogenet. Evol. 30, 720–732.
[21] Ormeno-Orrillo, E., Vinuesa, P., Zuniga-Dávila, D., Martínez-Romero, E. (2006) Molecular diversity of native bradyrhizobia isolated from lima bean (*Phaseolus lunatus* L.) in Peru. Syst. Appl. Microbiol. 29, 253–262.
[22] Parker, M.A., Doyle, J.L., Doyle, J.J. (2004) Comparative phylogeography of *Amphicarpaea* legumes and their root nodule symbionts in Japan and North America. J. Biogeogr. 31, 425–434.
[23] Peng, G.X., Tan, Z.Y., Wang, E.T., Reinhold-Hurek, B., Chen, W.F., Chen, W.X. (2002) Identification of isolates from soybean nodules in Xinjiang region as *Sinorhizobium xinjiangense* and genetic differentiation of *S. xinjiangense* from *Sinorhizobium fredii*. Int. J. Syst. Evol. Microbiol. 52, 457–462.
[24] Peng, G.X., Yuan, Q.H., Li, H.X., Zhang, W., Tan, Z.Y. (2008) *Rhizobium oryzae* sp. nov., isolated from the wild rice *Oryza alta*. Int. J. Syst. Evol. Microbiol. 58, 2158–2163.
[25] Ramírez-Bahena, M.H., Peix, A., Rivas, R., Camacho, M., Rodríguez-Navarro, D.N., Mateos, P.F., Martínez-Molina, E., Willems, A., Velázquez, E. (2009) *Bradyrhizobium pachyrhizi* sp. nov. and *Bradyrhizobium jicamae* sp. nov., isolated from effective nodules of *Pachyrhizus erosus*. Int. J. Syst. Evol. Microbiol. 59, 1929–1934.
[26] Rivas, R., Martens, M., de Lajudie, P., Willems, A. (2009) Multilocus sequence analysis of the genus *Bradyrhizobium*. Syst. Appl. Microbiol. 32, 101–110.
[27] Rivas, R., Willems, A., Palomo, J.L., García-Benavides, P., Mateos, P.F., Martinez-Molina, E., Gillis, M., Velázquez, E. (2004) *Bradyrhizobium betae* sp. nov. isolated from roots of *Beta vulgaris* affected by tumor-like deformations. Int. J. Syst. Evol. Microbiol. 54, 1271–1275.
[28] Sarr, P.S., Yamakawa, T., Fujimoto, S., Saeki, Y., Thao, H.T.B., Myint, A.K. (2009) Phylogenetic diversity and symbiotic effectiveness of root-nodulating bacteria associated with cowpea in the south-west area of Japan. Microbes Environ. 24, 105–112.
[29] Shutsrirung, A., Sutigoolabud, P., Santasup, C., Senoo, K., Tajima, S., Hisamatsu, M., Bhromsiri, A. (2002) Symbiotic efficiency and compatibility of native rhizobia in northern Thailand with different soybean cultivars. Soil Sci. Plant Nutr. 48, 491–499.
[30] Scholla, M.H., Elkan, G.H. (1984) *Rhizobium fredii* sp. nov., a fast growing species that effectively nodulates soybeans. Int. J. Syst. Bacteriol. 34, 484–486.
[31] Somasegaran, P., Hoben, J.H. 1994 Handbook for Rhizobia: Methods in Legume—Rhizobium Technology, Springer-Verlag, New York.
[32] Stepkowski, T., Moulin, L., Krzyzanska, A., McInnes, A., Law, I.J., Howieson, J. (2005) European origin of *Bradyrhizobium* populations infecting lupins and serradella in soils of western Australia and South Africa. Appl. Environ. Microbiol. 71, 7041–7052.
[33] Sullivan, J.T., Patrick, H.N., Lowther, W.L., Scott, D.B., Ronson, C.W. (1995) Nodulating strains of *Rhizobium loti* arise through chromosomal symbiotic gene transfer in the environment. Proc. Natl. Acad. Sci. U.S.A. 92, 8885–8889.
[34] Sullivan, J.T., Eardly, B.D., van Berkum, P., Ronson, C.W. (1996) Four unnamed species of non-symbiotic rhizobia isolated from the rhizosphere of *Lotus corniculatus*. Appl. Environ. Microbiol. 62, 2818–2825.
[35] Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., Higgins, D.G. (1997) The CLUSTAL_X Windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 25, 4876–4882.
[36] van Berkum, P., Fuhrmann, J.J. (2000) Evolutionary relationships among the soybean bradyrhizobia reconstructed from 16S rRNA gene and internally transcribed spacer region sequence divergence. Int. J. Syst. Evol. Microbiol. 50, 2165–2172.
[37] Vincent, J.M. 1970 A Manual for the Practical Study of the Root Nodule Bacteria, Blackwell Scientific Publications, Ltd, Oxford, UK.
[38] Vinuesa, P., León-Barrios, M., Silva, C., Willems, A., Jarabo-Lorenzo, A., Pérez-Galdona, R., Werner, D., Martínez-Romero, E. (2005) *Bradyrhizobium canariense* sp. nov., an acid-tolerant endosymbiont that nodulates endemic genistoid legumes (Papilionoideae: Genisteae) growing in the Canary Islands, along with *B. japonicum* bv. *genistearum*, *Bradyrhizobium* genospecies α and *Bradyrhizobium* genospecies β. Int. J. Syst. Evol. Microbiol. 55, 569–575.
[39] Vinuesa, P., Rojas-Jiménez, K., Contreras-Moreira, B., Mahna, S.K., Prasad, B.N., Moe, H., Selvaraju, S.B., Thierfelder, H., Werner, D. (2008) Multilocus sequence analysis for assessment of the biogeography and evolutionary genetics of four *Bradyrhizobium* species that nodulate soybeans on the Asiatic continent. Appl. Environ. Microbiol. 74, 6987–6996.
[40] Vinuesa, P., Silva, C., Werner, D., Martinez-Romero, E. (2005) Population genetics and phylogenetic inference in bacterial molecular systematics: the roles of migration and recombination in *Bradyrhizobium* species cohesion and delineation. Mol. Phylogenet. Evol. 34, 29–54.
[41] Volker, G., Vilma, A.S. (1996) New approaches to typing and identification of bacteria using the 16S-23S rDNA spacer region. Microbiol. Rev. 142, 3–16.
[42] Wang, F.Q., Wang, E.T., Liu, J., Chen, Q., Sui, X.H., Chen, W.F., Chen, W.X. (2009) *Mesorhizobium albiziae* sp. nov., a novel bacterium that nodulates *Albizia kalkora* in a subtropical region of China. Int. J. Syst. Evol. Microbiol. 57, 1192–1199.
[43] Wernegreen, J.J., Harding, E.E., Riley, M.A. (1997) *Rhizobium* gone native: unexpected plasmid stability of indigenous *Rhizobium leguminosarum*. Proc. Natl. Acad. Sci. U.S.A. 94, 5483–5488.
[44] Willems, A., Munive, A., de Lajudie, P., Gillis, M. (2003) In most *Bradyrhizobium* groups sequence comparison of 16S-23S rDNA internal transcribed spacer regions corroborates DNA–DNA hybridizations. Syst. Appl. Microbiol. 26, 203–210.
[45] Xu, L.M., Ge, C., Cui, Z., Li, J., Fan, H. (1995) *Bradyrhizobium liaoningense* sp. nov., isolated from the root nodules of soybean. Int. J. Syst. Bacteriol. 45, 706–711.
[46] Yang, J.K., Zhang, W.T., Yuan, T.Y., Zhou, J.C. (2006) Genotypic characteristics of the *rrn* operon and genome of indigenous soybean bradyrhizobia in cropping zones of China. Can. J. Microbiol. 52, 968–976.
[47] Yang, J.K., Zhou, J.C. (2008) Diversity, phylogeny and host specificity of soybean and peanut bradyrhizobia. Biol. Fertil. Soils 44, 843–851.
[48] Yao, Z.Y., Kan, F.L., Wang, E.T., Wei, G.H., Chen, W.X. (2002) Characterization of rhizobia that nodulate legume species of the genus *Lespedeza* and description of *Bradyrhizobium yuanmingense* sp. nov. Int. J. Syst. Evol. Microbiol. 52, 2219–2230.
[49] Yokoyama, T. (2008) Flavonoid-responsive *nody-lacz* expression in three phylogenetically different *Bradyrhizobium* groups. Can. J. Microbiol. 54, 401–410.
[50] Yokoyama, T., Ando, S., Tsuchiya, K. (1999) Serological properties and intrinsic antibiotic resistance of soybean *Bradyrhizobia* isolated in Thailand. Can. J. Microbiol. 42, 1209–1218.
[51] Yokoyama, T., Ando, S., Murakami, T., Imai, H. (1996) Genetic variability of the common *nod* gene in soybean bradyrhizobia isolated in Thailand and Japan. Soil Sci. Plant Nutr. 45, 505–515.
[52] Zhang, W.T., Yang, J.K., Yuan, T.Y., Zhou, J.C. (2007) Genetic diversity and phylogeny of indigenous rhizobia from cowpea [*Vigna unguiculata* (L.) Walp.]. Biol. Fertil. Soils 44, 201–210.
[53] Zhou, J., Bowler, L.D., Spratt, B.G. (1997) Interspecies recombination, and phylogenetic distortions, within the glutamine synthetase and shikimate dehydrogenase genes of *Neisseria meningitidis* and commensal *Neisseria* species. Mol. Microbiol. 23, 799–812.