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Phylogenetic diversity and symbiotic functioning in mungbean (*Vigna radiata* L. Wilczek) bradyrhizobia from contrast agro-ecological regions of Nepal☆

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ABSTRACT

Nepal consists wide range of climatic and topographical variations. Here, we explored the phylogeny of native mungbean bradyrhizobia isolated from different agro-ecological regions of Nepal and accessed their nodulation and nitrogen fixation characteristics. Soil samples were collected from three agro-ecological regions with contrasting climate and topography. A local mungbean cultivar, Kalyan, was used as a trap plant. We characterized isolates based on the full nucleotide sequence of the 16S rRNA, ITS region, and *nodA* genes; and partial sequences of *nodD1* and *nifD* genes. We found 50% of isolates phylogenetically related to *B. yuanmingense*, 13% to *B. japonicum*, 8% to *B. elkanii*, and 29% to novel phylogenetic origin. Results of the inoculation test suggested that expression of different symbiotic genes in isolates resulted in different degrees of symbiotic functioning. Our results indicate *B. yuanmingense* and novel strains are more efficient symbiotic partners than *B. elkanii* for the local mungbean cv. Kalyan. We also found most mungbean rhizobial genotypes were conserved across agro-ecological regions. All the strains from tropical Terai region belonged to *B. yuanmingense* or a novel lineage of *B. yuanmingense*, and dominance of *B. japonicum* related strains was observed in the Hill region. Higher genetic diversity of *Bradyrhizobium* strains was observed in temperate and sub-tropical region than in the tropical region.

Introduction

Mungbean [*Vigna radiata* (L.) Wilczek] is an important leguminous crop domesticated in the Indian sub-continent for over 3500 years [25], and spread to other parts of Asia, Australia, and Africa [39]. Approximately, 5.8 million ha areas in the world are under mungbean cultivation with 3.0 million tonnes of production of which Asia alone shares almost 90 percent of the acreage and production [41]. It complements cereal based diets as a nutritious pulse and as a sprouted salad vegetable. It is also a valuable green manure crop for improving soil fertility. Inoculation of effective rhizobia to boost up nodulation and nitrogen fixation is an established agronomic practice [10,20].

Mungbean-nodulating rhizobia are genetically diverse. In the past, they were generally ascribed to the "cowpea miscellany", strains consisted of uncharacterized slow growing bradyrhizobia [1]. Recent studies show mungbeans can also establish an effective N-fixing symbiosis with species of fast-growing rhizobia. They include *Rhizobium vignae* [21], *Rhizobium alkalisoli* [14], and *Mesorhizobium shangrilense* [15]. Previous study analyzing genetic diversity of Nepalese soybean bradyrhizobia revealed that one-fourth of the analyzed strains belonged to novel strain lines [22]. Furthermore, the *nodD1* and *nifD* genes of Nepalese isolates were considerably different from others with different levels of symbiotic efficiencies. However, the genetic diversity of Nepalese mungbean bradyrhizobia has not been described yet. Since, Soybean and Mungbean are phylogenetically closely related [5], rhizobia from Mungbean might be useful genetic resources for the improvement of the soybean symbiotic system and vice versa.

The 16S rRNA gene is a universal genetic marker for taxonomic identification of bacteria that allows phylogenetic studies and facilitates comparisons between different laboratories. However, in the genus *Bradyrhizobium*, limited taxonomic information can be deduced from 16S rDNA sequences because there is high

☆ *Note*: Nucleotide sequence data reported are available in the DNA Data Bank Japan (DDBJ) under the following accession numbers: 16S rRNA, AB601624–AB601671; ITS region, AB601672–AB601719; *nifD* gene, AB601777–AB601824; *nodD1* gene, AB601720–AB601767; *nodA* gene, AB610422–AB610462.

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degree of conservation among species and sequence similarity is relatively high [43]. The phylogeny based on 16S–23S rRNA internal transcribed spacer (ITS) regions was shown to be coherent with results from DNA–DNA hybridizations [44] and often, shows higher resolution than the 16S rRNA gene phylogeny in *Bradyrhizobium*. Therefore, to determine the phylogeny of native mungbean bradyrhizobia obtained from different agro-ecological regions in Nepal, we analyzed the 16S rRNA gene, the internal transcribed spacer (ITS) region, *nodD1*, *nodA*, and *nifD* genes of native mungbean bradyrhizobia. Furthermore, we investigated the relationship between phylogeny and symbiotic functioning including host-specificity of Nepalese mungbean bradyrhizobia.

Materials and methods

Soil samples and collection sites

Soil samples were collected in 2007 from five areas in which mungbeans had been included in the cropping history. These areas were located in three agro-ecological regions with contrasting climates (Fig. 1). Soil samples from each area were the composite of eight sub-samples prepared by mixing soils obtained from 0 to 20 cm depth. 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 S3 (Supplementary material).

Isolation of Bradyrhizobium strains

Seeds of *Vigna radiata* cv. Kalyan (a local Nepalese variety) were surface-sterilized and inoculated with fivefold dilutions of soil suspensions as described previously [22]. After sowing seeds, the 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, the nodules were harvested, surface-sterilized, and crushed in 200 μl glycerol solution (15%, v/v) to obtain a turbid suspension. An aliquot (10 μl) of the suspension was streaked [36] onto 1.5% Yeast Extract Mannitol agar [26] 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.

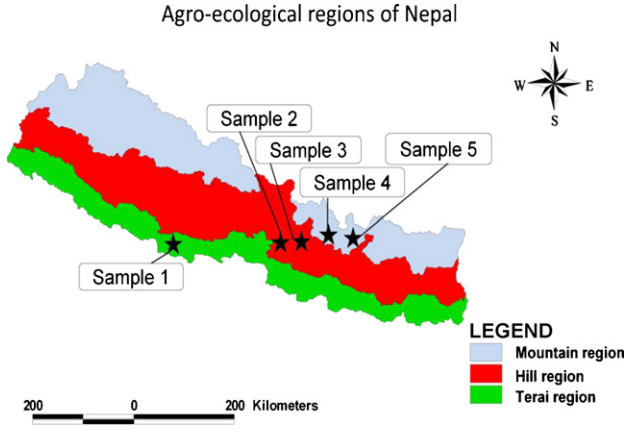

Fig. 1. Map of Nepal showing the different Agro-ecological regions and the location of soil sample collection sites.

Symbiotic characterization

Isolates were grown in 15 ml YEM broth for 1 week with shaking. Inoculation of bradyrhizobial cells were performed as described previously [22]. Plants were grown in axenic conditions in the growth chamber as described for the isolation experiment. After four weeks, the intact root nodules were assessed for Acetylene Reduction Assay (Gas Chromatograph GC-2014, Shimadzu Corporation, Kyoto, Japan), followed by the determination of nodule numbers, nodule fresh weights, and shoot weights. 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 [13]. All the treatments were performed in three replications, and data were subjected to statistical analysis as mentioned in the footnotes (Figs. 4 and 5).

DNA extraction

Of the 129 isolates obtained 48 were selected, based on their colony morphologies, for DNA extraction to have representatives of diverse groups (Table S3, Supplementary material). Criteria for selection were size, shape, color, and transparency of the colony (data not shown). DNA was extracted from isolates using the method described by Djedidi et al. [8].

DNA amplification and sequencing

PCR amplification and sequencing of DNA fragments of 16S rRNA, ITS region, *nodA*, *nodD1* and *nifD* genes were carried out as described by Yokoyama [46]. The primer sets used for PCR amplification and sequencing are shown in Table S1 (Supplementary material). PCR products were sequenced using an ABI Prism 3500 Genetic Analyzer (Applied Biosystems), according to manufacturer's protocols. Multiple sequence alignment of nucleotide sequences and boot-strapping for creating the N-J phylogenetic tree were performed using Clustal X 1.81 [33] and MEGA 4.0 [32].

Results

In total, we harvested 343 root nodules from seven soil samples. From these nodules we isolated 129 strains, all of which were slow growers. Out of them 48 strains were selected for this study and found soil samples from Mountain regions resulted in less nodules per plant than soil from Hill and Terai regions (Table S3, Supplementary material). Mungbean is an important pulse crop in Nepal and is more commonly cultivated in Hill and Terai regions than in Mountain regions.

Phylogenetic analysis based on 16S rRNA genes

The tree divided the mungbean isolates into two groups (Fig. 2). GI group included 8% isolates and GII group included 92% isolates. All isolates from Terai region produced very high 16S rRNA gene homology (>99.6) among them and were placed in the GII 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. *B. jicamae* PAC 68^T strain was well separated from other isolates in the GI group, however *B. pachyrhizi* PAC 48^T showed closeness to *B. elkanii* reference strains.

However, the overall 16S rRNA gene homology among the Nepalese mungbean isolates was high. Particularly, the reference strain *B. pachyrhizi* PAC 48^T showed >99.9% homology with another reference *B. elkanii* strain C-11. Most of the isolates in GII