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Phylogeny and genetic diversity of native rhizobia nodulating common bean (*Phaseolus vulgaris* L.) in Ethiopia

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ABSTRACT

The diversity and phylogeny of 32 rhizobial strains isolated from nodules of common bean plants grown on 30 sites in Ethiopia were examined using AFLP fingerprinting and MLSA. Based on cluster analysis of AFLP fingerprints, test strains were grouped into six genomic clusters and six single positions. In a tree built from concatenated sequences of *recA*, *glnII*, *rpoB* and partial 16S rRNA genes, the strains were distributed into seven monophyletic groups. The strains in the groups B, D, E, G1 and G2 could be classified as *Rhizobium phaseoli*, *R. etli*, *R. giardinii*, *Agrobacterium tumefaciens* complex and *A. radiobacter*, respectively, whereas the strains in group C appeared to represent a novel species. *R. phaseoli*, *R. etli*, and the novel group were the major bean nodulating rhizobia in Ethiopia. The strains in group A were linked to *R. leguminosarum* species lineages but not resolved. Based on *recA*, *rpoB* and 16S rRNA genes sequences analysis, a single test strain was assigned as *R. leucaenae*. In the *nodC* tree the strains belonging to the major nodulating groups were clustered into two closely linked clades. They also had almost identical *nifH* gene sequences. The phylogenies of *nodC* and *nifH* genes of the strains belonging to *R. leguminosarum*, *R. phaseoli*, *R. etli* and the putative new species (collectively called *R. leguminosarum* species complex) were not consistent with the housekeeping genes, suggesting symbiotic genes have a common origin which is different from the core genome of the species and indicative of horizontal gene transfer among these rhizobia.

Introduction

Phaseolus vulgaris L. (common bean) is the third most important legume crop growing worldwide, superseded only by *Glycine max* (soybean) and *Arachis hypogaea* (peanut). It is one of the most widely distributed food legumes in many parts of the tropics, subtropics and temperate regions [62]. In eastern and southern Africa, *Phaseolus* is a major staple food legume, nourishing more than 100 million people. Its production covers over four million hectares annually in more than 20 countries of the region [79]. In this region, common bean is considered to be the second and the third principal source of dietary protein and calories, respectively [11]. The common bean has two major centers of genetic diversity, from which the Mesoamerican center, including Mexico, Colombia, Ecuador and northern Peru is probably the primary one. The Andean center which encompasses regions from Southern Peru to northern Argentina is the second one [20,28].

Common bean establishes symbiotic associations with a wide range of root-nodule nitrogen-fixing bacteria called rhizobia. Initially, based on the cross-inoculation-group concept, all bean-nodulating rhizobia were classified as *Rhizobium leguminosarum* symbiovar (sv.) *phaseoli* [27]. The symbiovar is the new name of the term biovar proposed by [57]. Latter, due to the advancement of new molecular biological techniques and the isolation of different strains from various areas of the world, different common bean nodulating rhizobia were described. *R. tropici* [41] was described as a new bean-nodulating species based on multilocus enzyme electrophoresis (MLEE), DNA–DNA hybridization and a sequence analysis of 16S rDNA. Likewise, Segovia at al. [60] proposed *R. etli* as a separate species based on its difference in 16S rRNA gene compared with *R. leguminosarum*. Subsequently, *R. giardinii* and *R. gallicum* were reported as common bean symbionts [2]. Recently, salt tolerant *Sinorhizobium* (syn. *Ensifer*) *meliloti* sv. *mediterranense* was isolated from bean growing regions in a Tunisian oasis [44].

Sequence analyses of 16S rRNA gene and DNA–DNA hybridization have been used as regular protocols in bacterial taxonomic

Abbreviations: AFLP, amplified fragment length polymorphism fingerprints; MLSA, multilocus sequence analysis; BT, bootstrap support; NJ, neighbour-joining; ANI, average nucleotide identity; sv., symbiovar.

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studies. Nevertheless, the use of 16S rRNA gene solely as a phylogenetic marker has proven difficult because of its presence in multiple copies in a genome of some bacteria [25], susceptibility to genetic recombination and horizontal gene transfer [69] and low divergence among closely related species [1,21,72]. The DNA–DNA hybridization technique has better resolving power and strains should show 70% or greater DNA–DNA relatedness to be classified in the same species [64]. However, this technique is labour intensive and time consuming. Moreover, DNA–DNA hybridization techniques have been reported to vary between different laboratories [58] and this leads again to conflicting result for the same sets of strains. For example, *S. xinjiangensis* and *S. fredii* were reported to have 39% relatedness by Peng et al. [46] but later 74–89% similarity was reported by Martens et al. [36]. To overcome these mentioned drawbacks, protein-coding genes have been proposed as alternative phylogenetic markers to discriminate closely related species [64]. These genes have a faster evolution rate than the 16S rRNA gene but are conserved enough to retain genetic information. Multilocus sequence analysis (MLSA), using the sequences of multiple housekeeping genes have been reported to have higher discriminative power than 16S rRNA gene sequence analysis and DNA–DNA hybridization for species identification and delineation within the genus *Ensifer* (*Sinorhizobium*) [36]. Several studies revealed that MLSA can also be successfully used for phylogenetic studies of other rhizobia at species level [42,55]. Apart from its reliability, MLSA is also a rapid and economical method for species identification [6].

In Ethiopia, common bean is widely cultivated as a sole crop or intercropped with mainly sorghum and maize at altitudes between 1400 and 2000 m above sea level. The Hararghe highlands in east and the Rift valley zone that covers the southeastern parts of Ethiopia are the major common bean growing areas in the country [5], where it is also widely consumed. Bean export market has increased rapidly, supplying beans to canning industries in European countries and to the neighboring Kenya [17]. The yield of common bean, however, is extremely low due to low soil fertility, mainly due to nitrogen deficiency [7] and smallholder farming systems with minimal to zero fertilizer inputs [78]. The fertility problem can partly be solved by improving the capacity of common bean to fix nitrogen using the application of effective rhizobial inoculants. The presence of native bean-nodulating rhizobia in the country has been reported [8,9]. Nevertheless, detailed information about their taxonomy and diversity in Ethiopia is very scant.

Therefore, in the present study, the MLSA technique was used to investigate the taxonomic position of native rhizobia nodulating common bean in Ethiopia. For this purpose we determined the phylogeny of housekeeping genes coding for recombination protein (*recA*), glutamine synthase II (*glnII*), RNA polymerase beta subunit (*rpoB*) and the 16S rRNA gene. The diversity of the strains was assessed using the Amplified Fragment Length Polymorphism (AFLP) fingerprinting technique. The phylogeny of symbiotic genes for nodulation (*nodC*) and nitrogen fixation (*nifH*) as well as the capacity of strains to induce effective nodules was also studied for the test strains.

Materials and methods

Isolation and sampling sites

Nodule samples were collected randomly from common bean plants (local varieties of *P. vulgaris*) grown in 28 sites in the southern (14), eastern (9), central (3) and western (2) regions of Ethiopia. The nodules were collected in August and September 2007 when the common bean was at a flowering stage. In addition, a few isolates were obtained from the nodules induced by rhizobia present in soil samples taken from two sites in Addis Ababa (central Ethiopia) on common bean variety RED WOLAYTA trap plants in the greenhouse. The cultivation and planting protocol was as described by Wolde-meskel et al. [77]. The seeds were surface sterilized with 95% ethanol for 3 min followed by 0.2% acidified mercuric chloride for 3 min. After washing several times with sterile distilled water, seeds were sown in pots containing the collected soils and plants were grown for 4 weeks.

Soils taken from the sampling sites were analyzed for pH and electric conductivity following the methods described before [3,38]. The pH of the soils varied from moderately acidic (pH 6.0) to moderately alkaline (pH 8.8), but were commonly around neutral (Table S1). The districts, geographic coordinates, electric conductivity of the soils, and mean annual rainfall and temperature of the sampling sites are summarized in Table S1.

Several nodules per plant per site were taken and kept in a vial containing the desiccant silica gel until isolation [63]. Strains were isolated from the nodules according to the standard routine laboratory techniques described by Vincent [70]. Nodules were surface-sterilized with 95% ethanol for 3 min followed by 0.2% acidified mercuric chloride for 3 min and washing several times with sterile distilled water. Sterilized nodules from each vial were crushed together in a drop of sterile distilled water. The nodule suspension was streaked onto yeast extract mannitol (YEM) and/or tryptophan yeast extract (TY) agar media. Bacterial colonies appeared after incubation at 30 °C for 3–5 days. For each sample, a single representative colony was taken among morphologically similar colonies and was further streaked several times on YEM agar medium to check the purity of the cultures.

When necessary, to separate mixed cultures, bacterial cultures grown until late log phase in YEM or TY broth were diluted in surfactant Tween 80 buffer (0.05 M phosphate, pH 6.6, 0.1% Tween 80). The first dilution was incubated at 30 °C for half an hour in a shaker-incubator and separate strains were recovered from dilutions 10^{-4} and/or 10^{-5} after growing on YEM agar medium containing 0.025 g kg^{-1} Congo red [34]. Pure cultures of the strains were maintained and preserved in 20% glycerol–YEM broth at −80 °C.

Nodulation tests

All isolated strains were tested for their ability to induce nitrogen-fixing nodules on the common bean host, variety RED WOLAYTA at National Soil Testing Center, Addis Ababa, Ethiopia. The seeds were surface sterilized as described above. Sterilized seeds were wrapped in sterilized Whatman paper in a Petri-plate and germinated at 28 °C for 2–3 days. The nodulation experiment was done in triplicate pouches. Three seedlings were transferred into each pouch and each seedling was inoculated with 1 ml of bacterial cultures grown in YEM broths to exponential phase. Non-inoculated treatments were used as negative controls. A positive control was fertilized with 0.05% KNO3 w/v solution. The seedlings were grown in a glasshouse with a 12 h day and 12 h night regime. Plants were watered alternatively with sterilized Jensen's nitrogen free medium and distilled sterilized water according to the procedure described by Vincent [70]. After six weeks, plants were uprooted and the color of the leaves, nodulation status of roots and appearance of the plants were checked. Nodulation capacity was recorded as positive when nodules were found and negative if not found. Green plants and pink nodules indicated effective nitrogen fixation. Ineffective nitrogen fixation was considered when plants looked yellowish and had white nodules.

DNA isolation

Total genomic DNA of the strains was extracted from bacterial cultures grown in YEM or TY until late log phase. Extraction of the