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Phylogenetically diverse groups of *Bradyrhizobium* isolated from nodules of tree and annual legume species growing in Ethiopia

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ABSTRACT

Bacteria belonging to the genus *Bradyrhizobium* nodulate various leguminous woody plants and herbs, including economically important crops such as soybean, peanut and cowpea. Here we analysed 39 *Bradyrhizobium* strains originating from root nodules of the leguminous trees and crops *Acacia saligna*, *Faidherbia albida*, *Erythrina brucei*, *Albizia gummifera*, *Millettia ferruginea*, *Cajanus cajan*, *Vigna unguiculata* and *Phaseolus vulgaris*, growing in southern Ethiopia. Multilocus sequence analyses (MLSA) of the 16S rRNA, *glnII*, *recA*, *gyrB* and *dnaK* genes and the ITS region grouped the test strains into seven well-supported genospecies (I–VII), six of which occupied distinct positions excluding all hitherto defined *Bradyrhizobium* species. Analyses of the *nodA*, *nodC* and *nifH* genes suggested different evolutionary history of the chromosomal and symbiosis-related genes. Our study corroborates earlier findings that Ethiopia is a hotspot for rhizobial biodiversity, justifying further search for novel strains from this region and calling for intensified research on the ecology and biochemistry of these organisms.

Introduction

The genus *Bradyrhizobium* circumscribes bacteria capable of establishing N_2-fixing symbioses with different legume species, including herbaceous and woody species distributed over tropical and temperate regions. They are well known as microsymbionts of economically important legumes such as soybean (*Glycine max*), peanut (*Arachis hypogaea*) and cowpea (*Vigna unguiculata*) but bacteria within this genus are also known to nodulate aquatic legumes such as *Aeschynomene* species, as well as the non-legume *Parasponia andersonii* [1,22,33,44]. Furthermore, some *Bradyrhizobium* strains exist as non-nodulating endophytes of non-legumes such as rice (*Oryza sativa* L.) [39] and sugar beet (*Beta vulgaris* L.) [29]. Bradyrhizobia are also found as free-living bacteria and are commonly reported from metagenomics analyses of soils where they may be detected among the dominant sequences [11], although not necessarily being able to fix nitrogen [47]. Despite the great genetic and symbiotic heterogeneity among bradyrhizobia, along with their different modes of existence, only 29 species have been described at the moment [42]. The descriptions of the hitherto defined species of *Bradyrhizobium* were based on several commonly used taxonomic techniques such as numerical taxonomy, Biolog, SDS-PAGE, AFLP fingerprinting and multilocus sequence analyses (MLSA) using the 16S rRNA gene and several protein coding housekeeping genes [16,45,54,55,61].

In the recent past, MLSA revealed phylogenetically diverse groups of *Bradyrhizobium* genospecies among strains isolated from root nodules of *Crotalaria* spp., *Indigofera* spp., *Erythrina brucei* and *G. max* growing in Ethiopia [3]. In line with this, an earlier study of similar nature demonstrated a large metabolic and genomic diversity among 39 Ethiopian *Bradyrhizobium* test strains [58]. The strains were isolated from root nodules of the leguminous trees *Acacia saligna*, *Faidherbia albida*, *Erythrina brucei*, *Millettia ferruginea* and *Albizia gummifera*, and the legume crops *Cajanus cajan*, *V. unguiculata* and *Phaseolus vulgaris*, growing at eight geographic locations representing diverse agro-climatic zones in Ethiopia, ranging from dry hot semi-arid to moist cold conditions [57,58]. The diversity of the isolates was further explored by phylogenetic analysis of partial 16S rRNA gene sequences of 21 representative strains from that study, which delineated eight distinct groups comprising 12 different genotypes [59]. In the present investigation we exploited the power of MLSA of different housekeeping genes to further elucidate the taxonomic positions of all 39 *Bradyrhizobium* strains in our collection of Ethiopian rhizobia [59]. Their genetic diversity was investigated using sequence analysis of the 16S rRNA

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gene, the 16S–23S rRNA intergenic transcribed spacer (ITS) and four additional conserved housekeeping genes including *glnII* (encoding glutamine synthetase), *recA* (encoding the recombinase A protein), *gyrB* (encoding DNA gyrase subunit B) and *dnaK* (encoding a 70 kDa molecular Hsp70 class chaperone; heat shock protein). In addition to the analysis of individual housekeeping genes, three genes were concatenated in order to determine the exact phylogenetic position of the new test strains. Furthermore, the symbiosis related genes including *nodA* and *nodC*, which encode the enzymes NodA and NodC involved in the synthesis of the core of the Nod factor molecule [26], as well as the gene *nifH*, encoding the Fe protein of the dinitrogenase enzyme [15], were analyzed for selected strains as these genes are important for determining the host ranges of rhizobia [26].

Materials and methods

Bacterial strains and growth conditions

The taxonomy and phylogeny of 39 test strains, which in an earlier study of rhizobia from southern Ethiopia [59] were classified as belonging to *Bradyrhizobium* using PCR-RFLP of the 16S rRNA gene and internal transcribed sequence (ITS) regions, AFLP and sequencing of partial 16S rRNA gene, were further analysed using MLSA. Host plant, geographic origin and grouping based on the earlier and present investigation are presented in Table 1. The strains were kept in 15% (v/v) glycerol at −80 °C and cultured in 10 ml YMB at 28 °C for 7–10 days.

DNA preparation PCR amplification and gene sequencing

Total genomic DNA was isolated using the procedure described previously [5], with a slight modification using diatomaceous earth or Celite analytical filter aid as a DNA binding solid support [40]. The sequences chosen for MLSA included the larger fragments of the 16S rRNA gene [52], the ITS gene loci [36], and internal fragments of the protein coding chromosomal genes *glnII* [48], *recA* [13], *gyrB* [20] and *dnaK* [35]. In addition, the symbiosis related genes *nodA* [36], *nodC* [17] and *nifH* [28] were analysed. The PCR conditions specified in the respective references were followed. In several cases, this did not result in successful amplification. Despite multiple attempts to optimize the conditions by varying the annealing temperature, and testing different primer sets, amplicons for the targeted genes could not be obtained for some test strains. It was especially difficult to amplify the *nodC* and *nifH* genes, for which amplicons were obtained only from 17 and 20 strains, respectively.

Phylogenetic analyses

The 16S rRNA, ITS, *recA*, *gyrB*, *glnII* and *dnaK* gene sequences of the type strains within each of the hitherto defined 29 *Bradyrhizobium* species were retrieved from the NCBI database, if they were available, and aligned with the test strains for construction of a phylogenetic tree. The Jukes-Cantor distance was calculated to determine the closest phylogenetic neighbours to our entire set of test strains. Furthermore, formerly published sequences of two housekeeping genes including *recA* and *glnII* (nine strains for each gene) from Ethiopian *Bradyrhizobium* strains isolated from root nodules of *Crotalaria* spp., *Indigofera* spp., *E. brucei* and *G. max* [3] were included in our dataset for comparative purposes. For the phylogenetic inference, multiple nucleotide alignments for the test isolates, the previously published Ethiopian *Bradyrhizobium* strains and the reference species in the genus, were carried out using CLUSTAL W program [43] from the MEGA version 6 [38]. The phylogenetic trees were constructed based on the maximum likelihood (ML) and neighbour joining (NJ) methods using the MEGA version 6 [38]. However, since there were no topological differences in the relative placements of the test isolates on the phylogenetic trees (both ML and NJ), only ML trees are presented in this paper. The robustness of the topology of the ML trees were calculated from bootstrap analysis with 500 replications. For concatenation, sequences of the same gene, of all strains were aligned using MEGA version 6 [38], and saved as "fasta" file, then joined using online Fasta alignment joiner tool [49] to form a matrix of concatenated gene sequences. The average nucleotide identity (ANI), from the concatenated gene matrix were calculated from pair-wise comparisons of all sequences shared between the test strains and the reference *Bradyrhizobium* reference species.

Results

Comparative grouping of the test strains based on different core genes

In the present study, all test strains and strains of the defined *Bradyrhizobium* reference species were clearly differentiated based on MLSA of the housekeeping genes *glnII*, *recA*, *gyrB* and *dnaK*. The separation into genospecies was based on analyses of the individual housekeeping genes and on concatenated gene sequences. Except for three single strains (AC62a, AC97a and AC104c2), all test strains for which the target genes which were successfully amplified and sequenced, occupied seven different positions (Figs. 2 and S1a–d). Hereafter, these are referred to as genospecies I–VII, while the three single strains occupied their own independent branch and thus were designated as un-clustered (U) for convenience.

After extensive efforts to optimize PCR conditions for the various genes that were targeted, aiming at covering as many as possible of the test strains, 267 new sequences were generated in total. The 16S rRNA gene was amplified from 39 strains, *recA* from 31, *glnII* from 32, *gyrB* from 38, ITS region from 26, and *dnaK* from 30 of the strains. The success in amplifying the symbiosis related genes was even more variable. Of these, *nodA* was amplified from 34, *nodC* from 17 and *nifH* from 20 of the test strains. The nucleotide sequences of each of the core genes from the test strains and from the *Bradyrhizobium* reference strains, if available in the NCBI database, were aligned to reconstruct the phylogenetic trees (Figs. 1 a and b; S1a–d). In addition, we concatenated three housekeeping genes including the 16S rRNA, *glnII* and *recA* genes to reconstruct a composite tree (Fig. 2). The reason for choosing these core genes was that we wanted to include the Ethiopian *Bradyrhizobium* strains from an earlier study [3], and these genes were the only overlapping ones. In addition to this, we also concatenated the four housekeeping genes including *glnII*, *gyrB*, *recA* and *dnaK* by excluding 16S rRNA genes and Ethiopian *Bradyrhizobium* strains from earlier study [3] to construct a composite tree (Fig. S2), and to examine if there might be any topological differences in the relative placement of the test strains with the respect to the defined *Bradyrhizobium* reference species. In all housekeeping genes trees reconstruction, *Rhizobium etli* was included as an out group.

Phylogenetic analysis based on the 16S rRNA and ITS gene sequences

Based on the 16S rRNA gene sequence analysis, all the new isolates were clearly delineated within the cluster that defines the genus *Bradyrhizobium* (Fig. 1a). The new strains from the present study shared identical, or nearly identical, 16S rRNA gene sequence similarities (99–100%) to eighteen *Bradyrhizobium* reference strains, including *B. refense*, *B. ganzhouense*, *B. huanmingense*, *B. ingae*, *B. arachidis*, *B. ottawaense*, *B. daqingense*, *B. icense*, *B. paxllaeri*, *B. lablabi*, *B. yuanmingense*, *B. liaoningense*, *B. betae*, *B. cytisi*,