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Multilocus sequence analysis of bradyrhizobia isolated from *Aeschynomene* species in Senegal

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Abstract

This study reports the multilocus sequence analysis (MLSA) of nine house-keeping gene fragments (*atpD*, *dnaK*, *glnA*, *glnB*, *gltA*, *gyrB*, *recA*, *rpoB* and *thrC*) on a collection of 38 *Bradyrhizobium* isolated from *Aeschynomene* species in Senegal, which had previously been characterised by several phenotypic and genotypic techniques, allowing a comparative analysis of MLSA resolution power for species delineation in this genus. The *nifH* locus was also studied to compare house-keeping and symbiotic gene phylogenies and obtain insights into the unusual symbiotic properties of these *Aeschynomene* symbionts. Phylogenetic analyses (maximum likelihood, Bayesian) of concatenated nine loci produced a well-resolved phylogeny of the strain collection separating photosynthetic bradyrhizobial strains (PB) from non-photosynthetic bradyrhizobial (NPB) ones. The PB clade was interpreted as the remains an expanding ancient species that presently shows high diversification, giving rise to potential new species. *B. denitrificans* LMG8443 and BTAi1 strains formed a sub-clade that was identified as recently emerging new species. Congruence analyses (by Shimodaira–Hasegawa (S–H) tests) identified three gene-fragments (*dnaK*, *glnB* and *recA*) that should be preferred for MLSA analyses in *Bradyrhizobium* genus. The nine loci or *nifH* phylogenies were not correlated with the unusual symbiotic properties of PB (nod-dependent/nod-independent). Advantages and drawbacks of MLSA for species delineation in *Bradyrhizobium* are discussed.

Keywords: *Bradyrhizobium*; *Aeschynomene*; MLSA; Rhizobia; Nodulation; Phylogeny; Nod-independent nodulation

Introduction

Rhizobia are a functional group of soil bacteria characterised by their ability to form nitrogen-fixing symbioses with leguminous plants. Rhizobia are polyphyletic and do not correspond to a taxonomic unit [34]. They are currently divided into alpha and beta sub-classes of Proteobacteria and the names alpha and beta rhizobia have been proposed to distinguish them [25]. The alpha rhizobia include more than 50 bacterial species representing 10 genera *Sinorhizobium*, *Mesorhizobium*, *Rhizobium*, *Methylobacterium*, *Devosia*, *Azorhizobium*, *Bradyrhizobium*, *Ochrobactrum*, *Bosea* and *Phyllobacterium* (website of the ISCP sub-committee on the taxonomy of *Rhizobium* and *Agrobacterium*; http://edzna.ccg.unam.mx/rhizobial-taxonomy/).

The *Bradyrhizobium* genus occurs worldwide and is associated with economically important legumes such as soybean, cowpea, peanut or acacias. Species delineation in this genus has proven difficult because of very low 16S

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rRNA sequence divergence among heterogeneous bradyrhizobial strains and limited consensus between traditional taxonomic methods [51,53]. To date, the *Bradyrhizobium* genus has been shown to harbour six recognised species: *B. japonicum* [15,17], *B. elkanii* [20], *B. liaoningense* [54], *B. yuanmingense* [55], *B. betae* [29] and *B. canariense* [47]. Correlations between AFLP, DNA/DNA hybridisation and 16S–23S internal transcribed spacer (ITS) sequence analysis were found that led to the description of 11 genomic species in *Bradyrhizobium* [53]. However, phenotypic data were not congruent between or within genomic groups and no additional species names were proposed.

The *Bradyrhizobium* sp. (*Aeschynomene*) group contains photosynthetic bradyrhizobial (PB) and non-photosynthetic bradyrhizobial (NPB) strains with distinct host-ranges on *Aeschynomene* spp. [24]. Alazard [1] defined three cross-inoculation groups (CI) in *Aeschynomene*: Cross-inoculation group 1 is root-nodulated by NPB strains only; CI group 3 is root- and stem-nodulated by PB strains only, while CI group 2 is nodulated by both PB and NPB, but with the distinction that PB strains nodulate both stem and roots while NPB nodulate only roots of CI group 2. *Aeschynomene* symbionts have recently attracted particular attention since some of the PB strains (ORS278 or BTAi1) which nodulate roots and stems of plants of CI group 3 (*A. indica*, *A. sensitiva*) lack the canonical nodulation genes [10]. Other PB strains carry both Nod factor-dependent and Nod factor-independent systems to nodulate *Aeschynomene* CI groups 2 and 3 species (*A. afraspera*/Nod-dependent; *A. sensitiva*/Nod-independent) [10].

PB strains belong to genospecies VI and VIII that form a homogenous clade in 16S rRNA gene and ITS phylogenies [24,51]. However this group exhibits very low sequence divergence of 16S rRNA genes but high diversity in ITS sequences (with multiple copies) compared to other bradyrhizobia, making this usual marker of *Bradyrhizobium* diversity less useful for genetic screening in the PB group [51,52].

To overcome limitations of conventional molecular methods in rhizobial taxonomy, several authors have suggested integrating phylogenetic analysis of protein-encoding genes, with a higher level of sequence divergence than rRNA genes, but sufficient conservation to retain phylogenetic signal and to allow primer design (references are listed below). The *ad hoc* committee for redefinition of bacterial species concept recommended the use of five genes [38]. In rhizobia, several housekeeping genes such as *glnA* and *glnB* [43], *atpD* and *recA* [9,49], *dnaK* [40], *recA* and *glnB* [39,47] combined with *atpD* and *rpoB* [48] or larger studies using *atpD*, *dnaK*, *gap*, *glnA*, *gltA*, *gyrB*, *pnp*, *recA*, *rpoB* and *thrC* for multilocus sequence analysis (MLSA) in *Sinorhizobium* [22] were used to study the evolutionary relationships among strains and evaluate these data as an alternative to DNA/DNA hybridisation methodology for bacterial classification. Vinuesa et al. [47,48] were pioneers in application of multilocus sequence analysis using phylogenetic methodologies to delineate species in *Bradyrhizobium*. More recently, Rivas et al. [30] used a combination of five loci (*atpD*, *recA*, *gyrB*, *rpoB*, and *dnaK*) to resolve species delineation among genomic species of *Bradyrhizobium* by comparing with DNA/DNA hybridisation values. The authors found that hybridisation values were well reflected in the five gene concatenate phylogeny. However, cut-off levels of sequence similarities for species delineation could not be set for several markers (*gyrB*, *rpoB* and *dnaK*) as no clear-cut gap was found between intra- and interspecific sequences.

The aim of this study was to produce a well-revolved phylogeny of photosynthetic bradyrhizobia and to evaluate the use of MLSA for species delineation in *Bradyrhizobium*. Nine gene fragments (*atpD*, *dnaK*, *glnA*, *glnB*, *gltA*, *gyrB*, *recA*, *rpoB* and *thrC*) were chosen to perform MLSA on a collection of 38 *Bradyrhizobium* spp. (*Aeschynomene* spp.) strains previously characterized by ITS sequencing, DNA/DNA hybridisation and AFLP fingerprinting [52,53] allowing a comparison between MLSA and conventional taxonomic methodologies. In addition, one symbiotic gene (*nifH*) that reflects the bacterial symbiotic properties of rhizobia [2,14,21,49] was studied for comparison with core gene phylogenies and strain host range in *Aeschynomene–Bradyrhizobium* symbiosis.

Materials and methods

Bacterial strains

All *Bradyrhizobium* strains are listed in Table 1. Bacteria were grown on yeast mannitol (YM) medium [46] at 37 °C, and conserved at −80 °C in the same medium supplemented with glycerol (20% final concentration).

Molecular methods

Genomic DNA was extracted from 4-day cultures at 37 °C in 20 ml of YM broth using standard procedures [33].

PCR amplification was carried out as described in [26], and the primers used are listed in Table S2. Some listed primers were redesigned for better PCR amplification and sequencing.

For each gene-fragment amplification, the following cycles were used: initial denaturation step