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Genetic Diversity of Rhizobia Associated with *Acacia longifolia* in Two Stages of Invasion of Coastal Sand Dunes[▽]

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We examined the genetic diversity of root nodule bacteria associated with the Australian legume *Acacia longifolia* in two stages of invasion of a coastal sand dune system. All isolates belonged to the genus *Bradyrhizobium*. A higher diversity was found in the long-established trees. The results suggest the introduction of exotic bradyrhizobia with the plant.

A high level of promiscuity in the symbiosis with nitrogen-fixing bacteria can be essential for the invasion of new areas by exotic legumes (11, 15). Native plants can be the source of symbiotic rhizobia for the invasive species (14), but coexisting exotic and native woody legumes can also harbor different and unrelated rhizobial groups (5, 25). In any case, well-established populations of invasive legumes seem to have distinctive rhizobia which can significantly increase the growth of the invader in new areas (5, 14). In order to elucidate whether this is the case for the Australian woody legume *Acacia longifolia*, we surveyed the rhizobia associated with this plant in a site with long-established trees (site L) and a site where invasion is in progress (site P) in the Natural Reserve of São Jacinto, Portugal.

Root nodules were obtained from nine young *A. longifolia* plants randomly selected from the two areas, which were about 800 m apart. The isolation of the rhizobia was conducted following standard procedures (21). Single colonies appeared between 6 and 12 days and were restreaked onto fresh yeast mannitol agar plates. Cell lysates were obtained from single colonies picked from the new yeast mannitol agar plates (16) and stored at −20°C.

PCR with the primer BOX A1R (24) (PCR-BOX) was used to amplify the DNA of all isolates in a GeneAmp 9700 system (Applied Biosystems, Perkin Elmer, CA). The PCR conditions used were published previously (16). Aliquots (9 μl) of each PCR mixture were examined by electrophoresis in 1.8% agarose gels stained with ethidium bromide. Digital images of the gels were processed with GelCompar II software (Applied Maths, Belgium). A cluster analysis was performed using the unweighted pair-group method with arithmetic mean algorithm and the Pearson product-moment correlation coefficient. The Shannon index of diversity (19) was used to calculate the diversity of the bacterial isolates from each area.

The 16S gene was amplified using primers 63f and 1837r based on the *Escherichia coli* numbering system (10, 16). The internal transcribed spacer (ITS) region was amplified using primers R16-1 and R23-3R (4). PCR products were purified using the QiaQuick PCR purification kit (QIAGEN, Hilden, Germany) and sequenced using an ABI PRISM dye terminator cycle sequence reaction kit (Perkin Elmer, CA). Partial sequences were obtained for the 16S rRNA gene using primer 63f. Full-length sequences of the ITS region were obtained using the CLC Gene Workbench 2 software to assemble the partial sequences obtained with primers R16-1, R23-3R, and ILEF (4).

Sequence alignment and tree construction were performed using MEGA3 (www.megasoftware.net). The evolutionary distances were computed as described by Jukes and Cantor (3), and the evolutionary trees were inferred by the neighbor-joining method (17). A bootstrap analysis based on 1,000 resamplings of the neighbor-joining data was performed.

The 44 isolates obtained in this study, 24 from the long-established trees (site L) and 20 from the site with invasion in progress (site P), were slow-growing *Bradyrhizobium* strains. Although *A. longifolia* can establish symbiosis with both *Rhizobium* and *Bradyrhizobium* species (1, 8), our data confirm that bradyrhizobia are more common symbionts of *A. longifolia* (1, 25).

There was a higher genetic diversity of rhizobia associated with *A. longifolia* in site L than in site P. More than 90% of the isolates from site P clustered together with a similarity of 85% (Fig. 1). Using a value of 90% similarity in the PCR-BOX data, the 44 isolates could be ascribed to 13 different fingerprints, 5 from site P and 8 from site L. Based on this assumption, the genetic rhizobial diversity, measured using the Shannon index, was 1.37 in site P and 1.83 in site L. Although rhizobia can persist as free-living bacteria in the soil, rhizobial populations increase drastically in the presence of the host legume (20). The impact of legumes on the genetic structure of bacterial populations within natural environments is not very well understood, but a higher density of rhizobia might increase lateral gene transfer and therefore promote genomic diversity (13).

Twenty isolates representing all the subgroups obtained in the PCR-BOX analysis were selected for PCR sequencing of the 16S gene and the ITS region. Most evolutionary and phylogenetic studies of bacteria are based on the sequence of the 16S rRNA gene (9). Within the bradyrhizobia, however, this gene presents a limited divergence (23). The ITS region, which has a greater sequence variation, provides a better alternative for taxonomic and phylogenetic studies of this bacterial group

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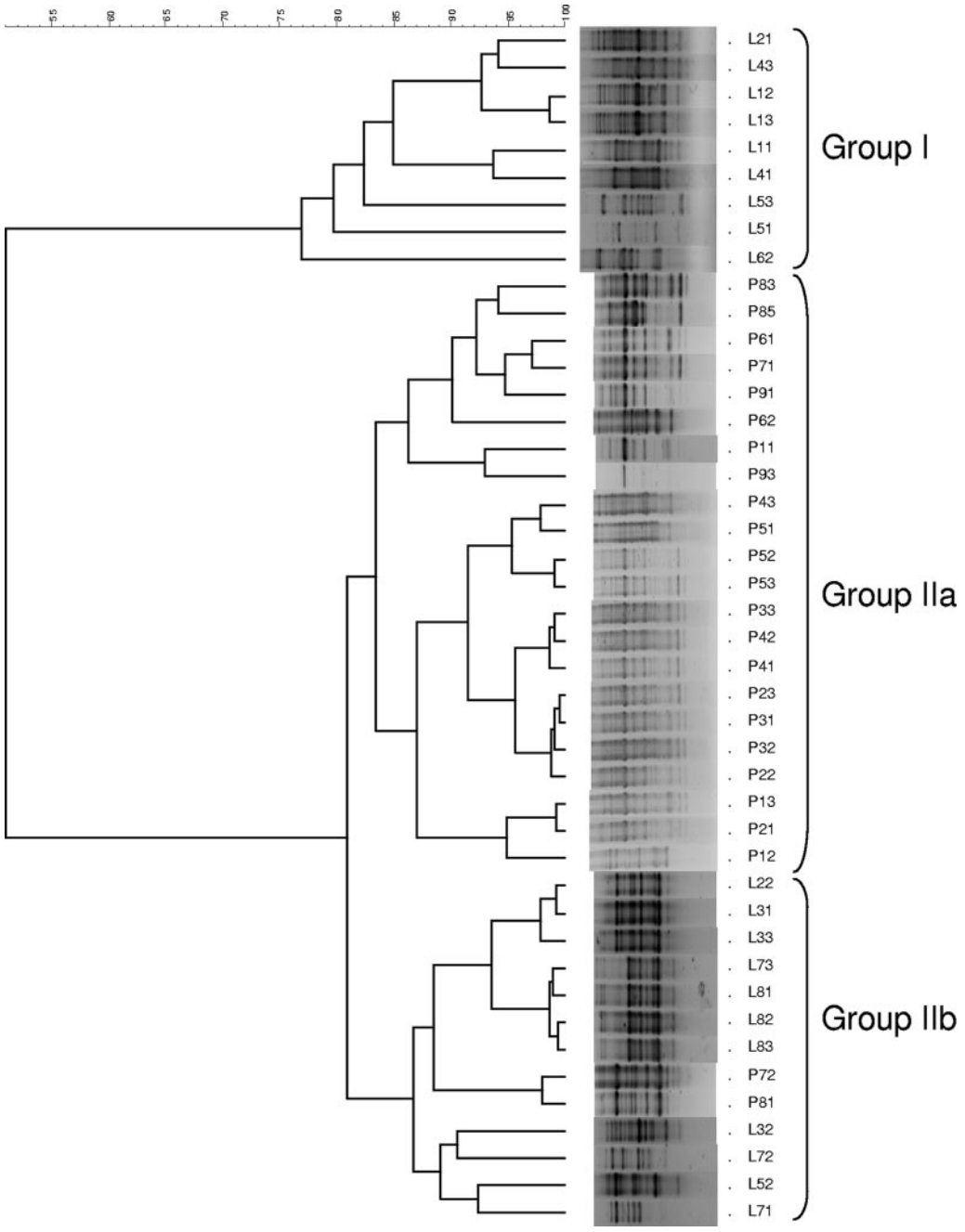

FIG. 1. Dendrogram based on cluster analysis of PCR-BOX products, using the unweighted pair-group method with arithmetic mean algorithm and the Pearson product-moment correlation coefficient, of the strains isolated from nodules of *A. longifolia* plants. L, isolate from the long-term established area; P, isolate from the area with progressing invasion.

than the 16S rRNA gene does (4, 23). Nevertheless, the 16S rRNA gene is still useful because most available studies on the genetic diversity of nodulating bacteria associated with Australian woody legumes, including *Acacia* spp., have been based on this gene (6, 7).

The phylogenetic tree based on partial 16S rRNA sequences grouped all the isolates within the *Bradyrhizobium* genus and showed a clear difference between isolates from the site with a long-established population of *A. longifolia* and those from the site with invasion in progress (Fig. 2). The nearest relatives of most isolates from the long-established *A. longifolia* population were bradyrhizobia obtained from Australia, either from pastureland, native legumes, or the introduced *Cytisus scoparius*, and from *A. longifolia* from New Zealand (5, 7, 18, 25). The second cluster included the five recognized species of *Bradyrhizobium* and most isolates from site P. These grouped with *Bradyrhizobium elkanii* and *Bradyrhizobium yuanmingense* and were more closely related to isolates obtained from different legumes in Central America, North America, Japan, and the Canary Islands.

FIG. 2. Phylogenetic tree based on partial sequences of the 16S rRNA gene for the isolates obtained in this study and related bradyrhizobia determined by using the neighbor-joining method. Numbers at the nodes are bootstrap percentages for 1,000 resamplings; values below 50% are not shown. For an explanation of L and P prefixes, see the legend to Fig. 1. GenBank accession numbers for the isolates obtained in this study are given in parentheses.

The phylogenetic analysis of the ITS region also placed the isolates within the *Bradyrhizobium* genus and divided them into two groups supported by high bootstrap values (Fig. 3). Fourteen of the 20 ITS sequences obtained in this study (10 from site L and 4 from site P) were more closely related to *Bradyrhizobium japonicum* and *Bradyrhizobium canariense* than to *B. elkanii*. Within this big group, isolates L22, L32, L81, and P81 clustered with isolates obtained from native *Bossiaea* species in Australia (22). In contrast, most isolates from site P and an isolate from site L were more closely related to *B. elkanii* and to isolates from North America and Korea (4, 12). These isolates might, therefore, represent cosmopolitan bradyrhizobia related to *B. elkanii*.

Isolates P11, P42, P85, and P91 probably represent the same bradyrhizobial genospecies because they had almost identical 16S rRNA and ITS sequences and an overall genetic similarity of 85%. In some cases, as for P21, P11, and P12, L51 and L52, or L43 and L72, there were discrepancies between the sequence results for 16S rRNA and ITS. This was also observed for P71, which had a 16S rRNA sequence that was 99.6% similar to that of *B. canariense*, and for L43 and L72, which had ITS sequences identical to that of *B. canariense* DQ646570.

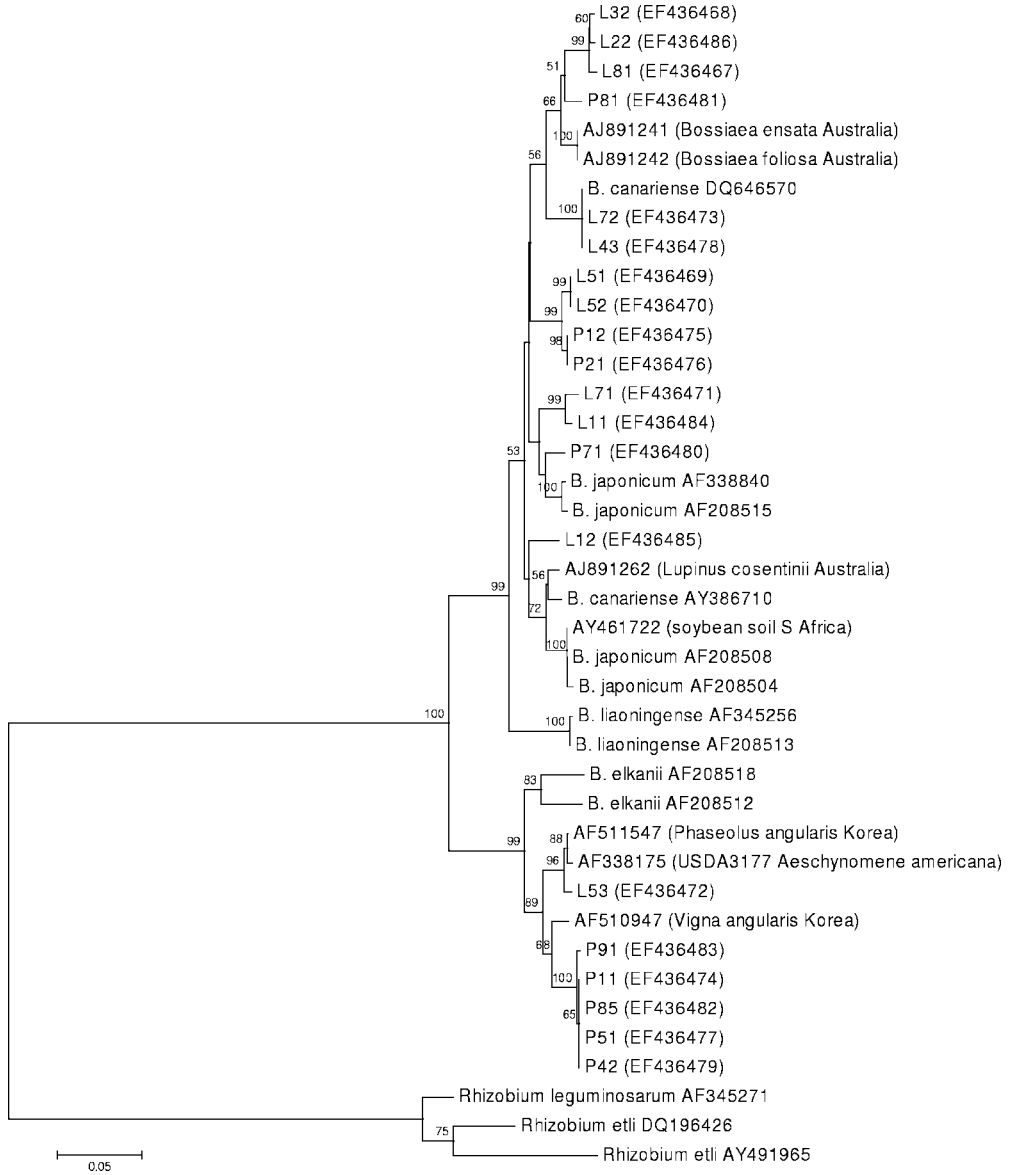

FIG. 3. Phylogenetic tree based on the full-length ITS sequences for the isolates obtained in this study and related bradyrhizobia determined by using the neighbor-joining method. Numbers at the nodes are bootstrap percentages for 1,000 resamplings; values below 50% are not shown. For an explanation of L and P prefixes, see the legend to Fig. 1. GenBank accession numbers for the isolates obtained in this study are given in parentheses.

Although not tested in the present study, these results might indicate an active lateral gene transfer between *Bradyrhizobium* isolates in the studied site (13). Some isolates that were phylogenetically closely related had a low overall genetic similarity, suggesting a higher genetic and physiological diversity than what is indicated by the ribosomal genes (2).

The differences found between the two sites could be partially explained by soil spatial heterogeneity or by the differences in tree age. However, the similarity of most isolates from site L to *Bradyrhizobium* from Australia suggests that exotic bradyrhizobia might have been introduced during the planting of *A. longifolia* in Portugal. Whether these bradyrhizobia play a key role in the invasion of sand dunes by *A. longifolia* is currently under study.

Nucleotide sequence accession numbers. All sequences have been deposited in GenBank under accession numbers EF436467 to EF436486, EF436494 to EF436498, EF471332 to EF471353, and EF493831.

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