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Biodiversity of Root-Nodule Bacteria Associated With the Leguminous Plant *Biserrula pelecinus*

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Abstract: *Biserrula pelecinus* is a noteworthy forage legume known for its drought- and acid-resistant properties and nitrogen-fixing ability that forms an extremely specific symbiotic relationship with bacteria from genera *Mesorhizobium*. To investigate the biodiversity of root-nodule bacteria associated with *B. pelecinus*, a total of 88 bacterial strains were examined using rep–polymerase chain reaction amplification. The 16S rRNA gene sequencing was also used to determine the phylogenetic relationship with type strains of different genera of rhizobia. Our results show that although a considerable genetic variability among *Biserrula* isolates was found, most isolates were phylogenetically related to *Mesorhizobium ciceri*. Curiously, a new symbiotic partner was found to nodulate *Biserrula*, suggesting possible lateral gene transfer of nodulation and nitrogen fixation genes between *Mesorhizobium* and *Rhizobium*.

Key words: *Mesorhizobium*, biodiversity, pasture, *Biserrula pelecinus*

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The successful establishment of legume plants is strongly affected by inoculation with symbiotic bacteria, which, in turn, should be able to effectively colonize the rhizosphere, being highly competitive with other soil rhizobia and to have genetic compatibility with the legume host. Considering the genetic variation of legume populations, the genetic diversity of rhizobia and its influence on host-rhizobia relationships should also be considered in the development of new legume species (Loi et al., 1999; 1997).

Native to the Mediterranean basin, *Biserrula pelecinus* is an annual legume adapted to desert-like conditions, surviving in acidic sandy soils, with poor nutrient-content and low precipitation conditions (Loi et al., 1999; 2001). Thus, *Biserrula* is an ideal plant to be introduced into semiarid Mediterranean pastures as a regenerating legume. The symbiotic bacteria associated with *B. pelecinus* have been, recently, identified as *Mesorhizobium ciceri* biovar *biserrulae*. This new species is distinguished from *M. ciceri* by significant differences in growth conditions, antibiotic resistance, carbon source utilization, and as well in similarity between symbiotic genes (Nandasena et al., 2007). In addition, the symbiotic relationship established between *Biserrula* and mesorhizobia is defined as being extremely specific. Nandasena et al. (2004) found that specific strains originated from *B. pelecinus* had a specific host range and did not nodulate several legumes, such as *Amorpha fruticosa* L., *Astragalus sinicus* L., *Cicer arietinum* L., *Hedysarum spinosissimum* L., *Lotus parviflorus* Desf., *Macroptilium atropurpureum* (DC) Urb, and *Trifolium lupinaster* L.

In parallel to breeding and improvement of *Biserrula* legume varieties, this study concerns the first approach to the development of a new commercial rhizobia strain specific to *Biserrula* and efficiently adapted to semiarid Mediterranean regions of Iberian Peninsula. In this work, we analyzed the genetic diversity of root-nodule bacteria isolated from endemic *B. pelecinus* collected in geographically distinct locations across northern Spain and southern Portugal (Table 1). We intend to select, based on DNA fingerprinting techniques, different root-nodule bacteria for further phenotypic characterization and evaluation of infective and effectiveness potential of rhizobia isolates.

MATERIALS AND METHODS

Sampling Procedures and Isolation of Bacterial Strains

Within each field site, undisturbed spots were randomly chosen. Topsoil samples were collected from a depth of 0 to 15 cm, and healthy plants of *B. pelecinus* were screened for fresh and effective nodulation. Individual plant roots with nodules were sealed in plastic bags and transferred to the laboratory. Standard procedures of collection and isolation of root-nodule bacteria were performed as described by Vincent (1970). After surface sterilization, nodules were individually macerated, and the milky suspension was streaked onto yeast mannitol agar plates (Vincent, 1970). Pure cultures were obtained by selection of a single colony with typical morphology of rhizobia (Jordan, 1984).

BOX and ERIC-PCR Fingerprinting

Genomic DNA of each root-nodule bacteria was extracted as described by alkaline hydrolysis method (Sambrook and Russel, 2001). Molecular fingerprint of all isolates, including a sample of the commercial strain of *B. pelecinus*, *Mesorhizobium* species, were carried out with BOX and ERIC primers (De Bruijn, 1992; De Bruijn et al., 1996). The amplification reactions contained 40 ng of DNA, 1× buffer Taq polymerase (Fermentas), 1 U Taq DNA polymerase (Fermentas), 0.2 mmol/L from each dNTP, 0.5 μmol/L from each primer, in a final volume of 25 μL. Amplification was conducted in a Biometra UNO II thermocycler. The polymerase chain reaction (PCR) amplification program consisted of a first cycle at 95 °C for 5 minutes; 35 cycles of 94 °C for 30 seconds, 53 °C (BOX A1R) or 52 °C (ERIC-R and ERIC-F) for 1 minute, 72 °C for 1.5 minutes, and a final extension at 72 °C for 7 minutes. The reproducibility of the PCR amplifications was validated twice. The PCR products were separated by electrophoresis on 1.5% agarose gel in TBE 0.5× buffer, stained with ethidium bromide, and photographed using the Electrophoresis Documentation and Analysis System 120 Kodak. The size of the DNA fragments was estimated by comparison with a 100-bp molecular marker (Gene Ruler DNA ladder, MBI, Fermentas) and recorded in a binary form

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TABLE 1. Geographic Data From Collection Sites in Spain and Portugal

Site	Location	Geographic Coordinates		Altitude, m	Soil Type	Climate Classification
		Latitude	Longitude			
1	Ávila (Sp)	40° 27′ 27″	5° 19′ 33″	1062	Cambisol	Continental
2	Elvas (Pt)	39° 32′ 45″	7° 35′ 43″	268	Luvisol	
3	Monsaraz (Pt)	38° 26′ 53″	7° 24′ 8″	191	Luvisol	
4	Ajuda (Pt)	38° 46′ 43″	7° 10′ 16″	166	Cambisol	Semiarid Mediterranean
5	Arronches (Pt)	39° 04′ 07″	7° 15′ 36″	277	Cambisol	
6	Terena (Pt)	38° 38′ 00″	7° 15′ 36″	277	Luvisol	
7	Safara (Pt)	38° 06′ 44″	7° 14′ 7″	178	Luvisol	

Pt: Portugal; Sp: Spain.

(0, absence of band; 1, presence of band). The binary matrices from BOX and ERIC primers were combined using NTedit from the software NTSYSpc version 2.0b (Rohlf, 1990). DNA fingerprint analysis was inferred by a dendrogram constructed using the unweighted pair group method with arithmetic mean algorithm (UPGMA) (Backeljau et al., 1996) and the Jaccard similarity coefficient (J) (Mantel, 1967). Goodness of fit of the cluster analysis was determined by a cophenetic value matrix (CCC) (Romesburg, 1984).

Partial 16S rRNA Sequence Analysis

Amplification of 16S rRNA gene was conducted using the universal primers 63f and 1387r (Marchesi et al., 1998). The PCR reactions contained 40 ng of DNA, 1× buffer Taq polymerase, 1 U Taq DNA polymerase (Fermentas), 0.2 mmol/L from each dNTP, 0.5 μmol/L from each primer, for a volume of 25 μL. The amplification program was 94 °C for 1 minute (1 cycle); 30 cycles of 94 °C for 1 minute, 55 °C for 1 minute, 72 °C for 1.5 minutes; and 72 °C for 7 minutes (1 cycle). The PCR products were purified by the PureLink Quick Gel Extraction Kit (Invitrogen, Paisley, UK) and sequenced at STABVIDA Lda (Oeiras, Lisbon, Portugal). Partial sequences of 16S rRNA gene obtained using primer 1387r were submitted to BLASTN search (NCBI) for significant nucleotide sequence alignments and to GenBank for assignment of accession numbers. An unrooted phylogenetic tree was constructed by MEGA4 software (Tamura et al., 2007), using nucleotide sequences of *Biserrula* bacterial isolates and five species representative of the five main genera of rhizobia (*Mesorhizobium*, *Sinorhizobium*, *Azorhizobium*, *Bradyrhizobium*, and *Rhizobium*). The evolutionary distance was computed as described by Jukes and Cantor (1969), and the evolutionary tree was inferred by the neighbor-joining method (Saitou and Nei, 1987). A bootstrap analysis based on 1000 replicates of neighbor-joining data was performed (Felsenstein, 1985).

RESULTS

Isolation of Root-Nodule Bacteria

Eighty-eight bacterial isolates exhibited similar phenotypic characteristics of rhizobia culture, expressed by milky translucence with moderate-to-copious extracellular polysaccharides colonies (Jordan, 1984) after a period of incubation ranging between 3 and 8 days.

BOX and ERIC-PCR Fingerprinting

BOX and ERIC-PCR amplification generated complex DNA patterns for all root-nodule isolates. To assure a correct molecular characterization, a total of 12 polymorphic bands with a size ranging between 630 and 1600 bp (BOX) and 180 and 850 bp (ERIC) were analyzed (Fig. 1). The dendrogram (Fig. 2) revealed considerable genetic diversity among *Biserrula* root-nodule isolates, with a cophenetic coefficient of 0.81. Two main groups were described with approximately 72% similarity to *Mesorhizobium* species–type strain. Groups A and B contained, respectively, 59% and 41% of *Biserrula* isolates. Six subgroups were also defined in Group A. Isolates ElvPt18 and SafPt1, within subgroup A1, shared the highest similarity percentage with *Mesorhizobium* species. Subgroups A3 and A4 had a low polymorphism between root-nodules because the great majority shared the same DNA banding pattern. Group B gradually branches with bacterial isolates with clearly distinct patterns, resulting in a decrease of similarity with the type strain. Seven subgroups and two isolates, ArrPt11 and ArrPt12, which share

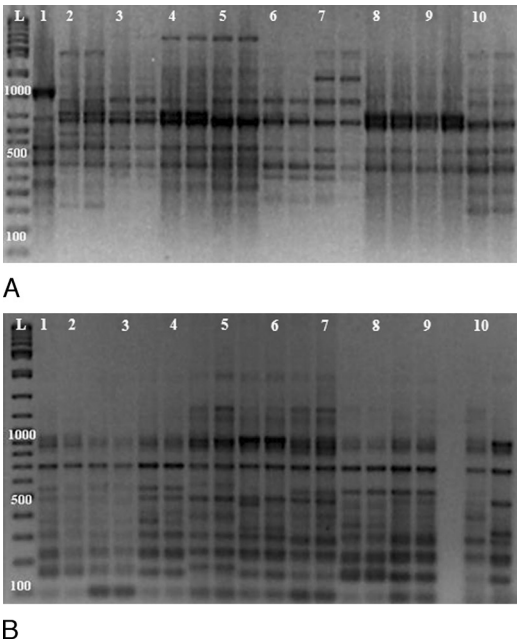

FIG. 1. Molecular fingerprints of *Biserrula* root-nodule bacteria using BOX (A) and ERIC (B) primers. DNA samples of each bacterial isolate were amplified in duplicate. L (DNA ladder 100 bp). The lanes are labeled by number and not by strain type: (A): 1, *Mesorhizobium* species; 2, MonPt12; 3, MonPt13; 4, MonPt14; 5, MonPt15; 6, MonPt16; 7, MonPt17; 8, MonPt18; 9, AjuPt1; 10, AjuPt2. (B) 1, AjuP6; 2, AjuPt7; 3, AjuPt8; 4, AjuPt10; 5, AjuPt11; 6, AjuPt12; 7, AjuPt13; 8, AjuPt15; 9, AjuPt16; 10, AjuPt17.

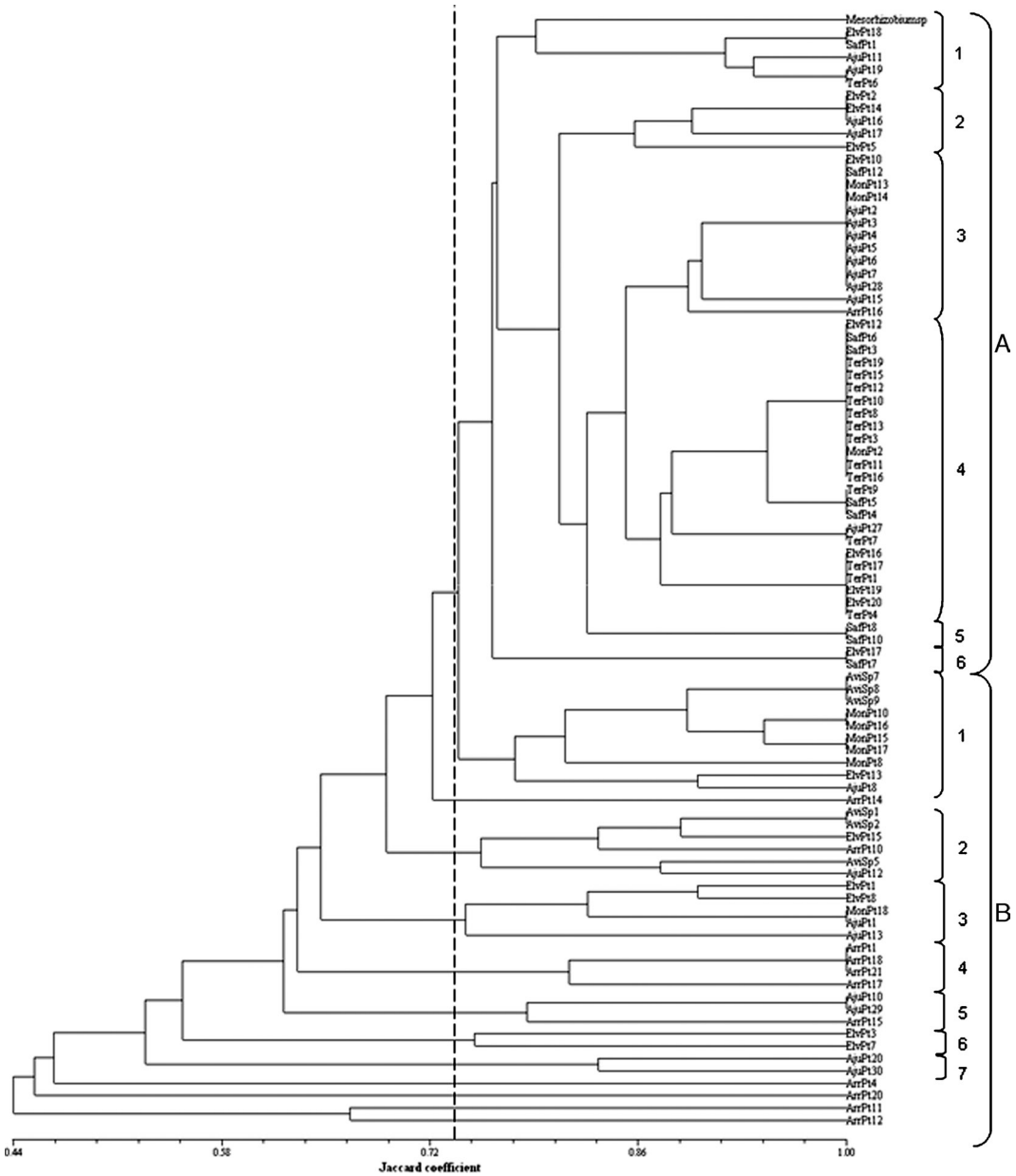

FIG. 2. Dendrogram showing the relatedness of *B. pelecinus* isolates as determined by BOX + ERIC combined fingerprint data analysis. Relationship was determined using Jaccard similarity coefficient and UPGMA clustering method.

only 44% of similarity with the other bacteria, were described. From the 88 root-nodule bacteria initially characterized, 56 bacteria with different band patterns were selected for phenotypic and effectiveness tests.

Partial 16S rRNA Sequence Analysis

A partial fragment of 16S rRNA gene, approximately 1000 bp, was amplified and sequenced for five representative bacterial isolates belonging to the main groups of the dendrogram (Group A, AjuPt16, SafPt12, and SafPt6; Group B, AviSp7 and ArrPt12). Nucleotide sequences were first submitted to NCBI BLASTN to search for close relations through sequence similarity. The isolate AjuPt16, with a sequence length of 1008 bp, shared 94% of maximum identity with bacterial strains belonging to the genera *M. ciceri* and *M. ciceri* bv. *biserrulae* strain WSM1271 (AY601513). Identical results were also obtained for strains SafPt12, SafPt6, and AviSp7. For isolate ArrPt12, a BLASTN search retrieved 97% maximum identity with *Rhizobium leguminosarum* bv. *phaseoli*. An unrooted phylogenetic tree was developed using the model by Jukes and Cantor (1969) and the neighbor-joining method (Saitou and Nei, 1987) (Fig. 3). The reliability of the evolutionary tree was assessed by the bootstrap test (confidence values estimated from 1000 replication of each sequence) (Felsenstein, 1985). The phylogenetic tree was divided in two groups: Group 1, where *Biserrula* bacteria AjuPt6, SafPt6, SafPt12, and AviSp7 were grouped with *M. ciceri* bv. *biserrulae* (AY601513), *M. ciceri* (U07934), and *M. loti* (X67229); and Group 2, which presented ArrPt12, clearly

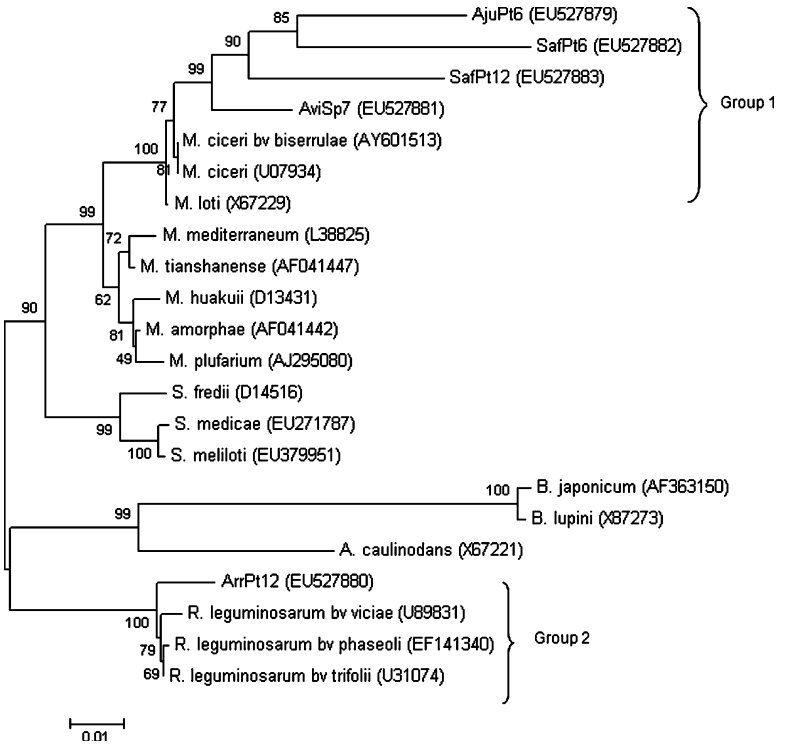

FIG. 3. Phylogenetic tree showing relationships between root-nodule bacteria (AjuPt6, SafPt6, SafPt12, AviSp7, and ArrPt12) and reference strains of rhizobia. The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.

separately from the other *Biserrula* isolates, and distinguished between *R. leguminosarum* subspecies *phaseoli* (EF141340), *R. leguminosarum* bv. *trifolii* (U31074), and *R. leguminosarum* bv. *viciae* (U89831). *Azorhizobium caulinodans* (X67221), *Bradyrhizobium japonicum* (AF363150), and *Bradyrhizobium lupini* (X87273), more phylogenetically related, played an outgroup role.

DISCUSSION

Molecular fingerprints produced were very complex for all bacterial isolates, showing as documented, good discrimination power of rep-PCR amplification (De Bruijn, 1992; De Bruijn et al., 1996; Wang et al., 2008). The BOX + ERIC combined dendrogram distributed bacterial isolates along a Jaccard coefficient ranging from 44% to 100% similarity, denoting therefore considerable genetic diversity among root-nodule bacteria and an association with *B. pelecinus*. The dendrogram analysis was based on the level of similarity of *Biserrula* isolates to the reference microsymbiont *Mesorhizobium* species (Nandasena et al., 2001). The most related root-nodule bacteria to *Mesorhizobium* species were primarily located in Group A, whereas Group B gradually enclosed the most dissimilar bacterial strains. To confirm the cluster distribution, the cophenetic correlation coefficient was calculated and found to be high (between 80% and 90%), therefore, suggesting a good fit of the original data (Romesburg, 1984). The topology of the dendrogram showed no relationship between the disposition of *Biserrula* isolates and geographic sites. Similar studies with mesorhizobia isolated from pulse crop *Cicer arietinum* L. (chickpea) indicated that the high genetic diversity found among chickpea isolates did not correlate with their geographic origin within Portugal (Laranjo et al., 2004 and 2008). Jarabo-Lorenzo et al. (2003) obtained the same result working with bradyrhizobial populations of *Lupinus* species and *Ornithopus* species that originated in different countries and as well as continents.

The 16S rRNA gene sequencing revealed that the randomly chosen root-nodule bacteria from Group A, AjuPt6, SafPt12, SafPt6, and AviSp7 from group B were recognized, at strain level, as being similar to *M. ciceri* bv. *biserrulae*. This result is supported by Nandasena et al. (2007), who identified the specific microsymbiont of *B. pelecinus* belonging to *Mesorhizobium* genus and who more recently proposed *M. ciceri* bv. *biserrulae* because of significant divergences with strains of *M. ciceri* (Nandasena et al., 2007). The most interesting result obtained by BLASTN sequence comparison lays on isolate ArrPt12 (Group B), which is the most unrelated strain to *Mesorhizobium* and which was designated as *R. leguminosarum* bv. *phaseoli*. Howieson et al. (1995) classified the symbiotic relationship established between *Biserrula* and acid-tolerant rhizobia as extremely specific. They demonstrated through cross-inoculation tests that *R. leguminosarum* bv. trifolii, *R. leguminosarum* bv. viciae, *Sinorhizobium meliloti*, and *Bradyrhizobium* species were unable to infect *Biserrula*. Whereas in our study, we have identified *R. leguminosarum* as one of the strains extracted from a *Biserrula* nodule.

The phylogenetic tree distribution corroborates the previous results. The isolate AviSp7 was clustered closely with *M. ciceri* subspecies *biserrulae*, emphasizing the low percent nucleotide mismatches (approximately 0.792%). The AjuPt6 and SafPt6 group clustered with SafPt2 and gradually branched with AviSp7, explaining the nucleotide mismatches obtained in relation to the reference strain. Sahgal and Johri (2006) suggests that the 5% nucleotide mismatch used in practice to place individual strains in separate genera has become questionable in

current rhizobia taxonomy because of lateral gene transfer (LTG) and recombination between rhizobia strains. The fact that the 16S rRNA gene sequencing was based only in a partial fragment of the total gene sequence may also help to justify the percents obtained by AjuPt6 (6.83%), SafPt (5.05%), and SafPt6 (4.15%). The main genera of rhizobia, *Mesorhizobium*, *Sinorhizobium*, *Bradyrhizobium*, and *Rhizobium*, were disposed in accordance with Kwon et al. (2005), who, using 16S rRNA gene and internally transcribed spacer region sequencing, inferred the phylogenetic relationships between these genera. In agreement with the previous discussion, isolate ArrPt12 clustered with *R. leguminosarum* bv. *phaseoli*, *R. leguminosarum* bv. *Trifolii*, and *R. leguminusarum* bv. *viciae* in the phylogenetic tree. Although the genetic diversity of *Biserrula* root-nodule bacteria was substantial, 16S rRNA gene sequencing showed that the most related isolates with *Mesorhizobium* species in the dendrogram were taxonomically identified as *M. ciceri* bv. *biserrulae* and that the most distant isolate belonged to other genera of rhizobia. Several studies have suggested LTG of nodulation and nitrogen fixation genes between different species as the most likely hypothesis explaining the promiscuity of rhizobia bacteria (Boucher et al., 2003; Wang et al., 2008). Lu et al. (2009) proved the existence of LTG of symbiotic genes from *Mesorhizobium* to *Bradyrhizobium* and among *Mesorhizobium* species in *Caragana* species. In addition, LTG has also been reported in species of *Mesorhizobium* in *B. pelecinus* (Nandasena et al., 2007). It is in our best interest to infer the ability of ArrPt12 to nodulate *Biserrula* by analysis of symbiosis and core genes.

The increasing interest in this legume, extensively studied for agricultural uses in southern Australia (Carr et al., 1999; Howieson et al., 1995; Loi et al., 1999; 1997; 2001), is progressively expanding to Europe and South American countries (Barradas et al., 2002; Simões et al., 2004; Ovalle et al., 2004, Tamura et al., 2007). Its good adaptation in the agricultural systems, under Mediterranean climate influence, of the Iberian Peninsula depends greatly on the discovery of native elite mesorhizobia for nitrogen fixation. In the present study, we emphasize, once more, the specificity of the symbiosis rhizobia-*Biserrula*. From the analysis conducted, 56 different bacterial isolates were already chosen for the selection of native bacteria for inoculant development.

Nucleotide Sequence Accession Numbers

All sequences have been deposited in GenBank under the accession numbers EU527879, EU527880, EU527881, EU527882, and EU527883.

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