Presence of a novel 16S–23S rRNA gene intergenic spacer insert in *Bradyrhizobium canariense* strains

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Abstract

Seven slow-growing bacterial strains isolated from root nodules of yellow serradella (*Ornithopus compressus*) that originated from Asinara Island on North Western Sardinia in Italy were characterized by partial 16S rRNA gene and intergenic spacer (ITS) sequencing as well as amplified fragment length polymorphism (AFLP) genomic fingerprinting. The results indicated that the *O. compressus* isolates belong to the *Bradyrhizobium canariense* species. The analysis of ITS sequences divided the branch of *B. canariense* strains into two statistically separated groups (ITS clusters I and II). All the strains in ITS cluster I showed the presence of unique oligonucleotide insert TTAGAGACTTAGGTTT CTK. This insert was neither found in other described species of the family *Rhizobiaceae* nor in any other bacterial families and can be used as a natural and high selective genetic marker for ITS cluster I of *B. canariense* strains. ITS grouping of *O. compressus* isolates was supported by the unweighted pair group method with arithmetic averages cluster analysis of their AFLP patterns, suggesting that the strains of ITS cluster II were genetically closer to each other than to isolates from the ITS cluster I. A taxonomic importance is supposed of the revealed 19 bp ITS insert for an intraspecific division within high heterogeneous *B. canariense* species.

Introduction

The genus *Bradyrhizobium* was described in 1982 (Jordan, 1982) and represents one of six described genera of root-nodule bacteria. The 16S rRNA gene sequences in *Bradyrhizobium* strains are fairly uniform, whereas DNA–DNA hybridization experiments have revealed a number of homology groups (Willems *et al*., 2001). At the moment, this genus contains six species of slow-growing rhizobia: *Bradyrhizobium japonicum* (Jordan, 1982), *Bradyrhizobium elkanii* (Kuykendall *et al*., 1992), *Bradyrhizobium liaoningense* (Xu *et al*., 1995), *Bradyrhizobium yuanmingense* (Yao *et al*., 2002), *Bradyrhizobium betae* (Rivas *et al*., 2004) and *Bradyrhizobium canariense* (Vinuesa *et al*., 2005a). The first three species were described for *Glycine* isolates. The strains of *B. yuanmingense* and *B. betae* were isolated from *Lespedeza* plants and red beet (*Beta vulgaris*), respectively. *Bradyrhizobium canariense* was recently described for strains isolated from endemic shrub genistoid legumes growing on the Canary Islands. It was shown that these strains can be clearly distinguished from other described *Bradyrhizobium* species by 16S–23S intergenic spacer (ITS), *atpD, glnII* and *recA* sequencing (Vinuesa *et al*., 1998, 2005a,b). *Bradyrhizobium canariense* strains are highly diverse phenotypically and reveal a high level of genetic distinctions. They have a wide range of host plants and nodulate different genera and species of the tribes Genisteae and Loteae. However, no correlation was found between genotypes of strains and their original host plants or geographic location (Vinuesa *et al*., 1998).

The objective of this study was to classify seven slow-growing isolates from root nodules of yellow serradella (*Ornithopus compressus*) representing a collection of rhizobia isolated from different pasture legumes native of Sardinia and Asinara Island (Italy). Yellow serradella is an economically important Mediterranean forage crop, which is well adapted to acidic soils and has a high nutritive value. It was shown that *Bradyrhizobium* strains isolated from *Lupinus* spp. can form an effective (nitrogen fixing) symbiosis with *Ornithopus* plants, but the latter plant genus has

more stringent nodulation requirements (Bottomley *et al.*, 1994; Ballard, 1996; Stepkowski *et al.*, 2005). At present, strains infecting *Ornithopus* species have been poorly classified taxonomically. In a previous work, *O. compressus* isolates were characterized by phenotypic analysis, ARDRA and ITS restriction fragment length polymorphism (RFLP; Safronova *et al.*, 2004). Here, results are reported from a study of these strains using partial 16S rRNA gene and ITS sequencing for grouping them at a genus and species level, as well as by genomic amplified fragment length polymorphism (AFLP) fingerprinting to differentiate closely related strains within one group (Vos *et al.*, 1995).

Materials and methods

Bacterial strains and cultural conditions

The strains Oc1, Oc4, Oc6, Oc7, Oc9, Oc3A and Oc39 were isolated by a standard method (Novikova & Safronova, 1992) from nodules of different *O. compressus* plants that originated from Asinara Island on North Western Sardinia, Italy. The reference strains of *B. japonicum* USDA6^T and USDA110, *B. elkanii* USDA76^T, *Mesorhizobium loti* USDA3471^T, *Mesorhizobium mediterraneum* USDA3392^T and *M. mediterraneum* strains 1801, 1802, 1803, 1804, 1809 from the CIAM Collection (All-Russia Research Institute for Agricultural Microbiology, St-Petersburg) were used for AFLP genomic fingerprint analysis. For all experiments, the strains were uniformly grown on yeast extract mannitol agar (YMA) at 28 °C (Vincent, 1970).

DNA extraction

For 16S rRNA gene and ITS sequence analysis, total genomic DNA was isolated using the sodium dodecyl sulfate-proteinase K lysis procedure and CTAB-precipitation (Sambrook & Russell, 2001). For AFLP fingerprinting, UltraClean Microbial DNA Isolation Kits (MO BIO Laboratories, Inc.) were used to extract the ultra pure DNA.

Sequencing of partial 16S rRNA gene and ITS fragments

Amplification of a 16S rRNA gene fragment (about 500 bp) was performed with primers b341 (5′-CCTACGGGAGGCAGCAG-3′) and b758 (5′-CTACCAGGGTATCTAATCC-3′) (Juck *et al.*, 2000) by a standard method. The DNA fragments were purified and sequenced according to the manufacturer's recommendation (Amersham Biosciences, CyDye Terminator sequencing kit and Visible Genetics Long Read Tower DNA sequencer, Visible Genetics, Toronto Canada).

An ITS region (about 800 bp) was amplified with primers FGPS1490-72 (5′-TGCGGCTGGATCCCCTCCTT-3′) and FGPL-132 (5′-CCGGGTTTCCCCATTCGG-3′) (Normand *et al.*, 1992) and cloned in pTZ57R/T using the PCR Product Cloning Kit (Fermentas). Sequencing was performed with the ABI PRISM BIGDYE TERMINATOR v3.1 Cycle Sequencing Kit (Applied Biosystems).

Nucleotide sequence data reported are available in the DDBJ/EMBL/GenBank databases under the accession numbers AY526225, AY526226, AY526227, AY526228, AY526229, AY526230 and AY527139 for the partial 16S rRNA gene sequences, and DQ646569, DQ646570, DQ646571, DQ646572, DQ646573, DQ646574 and DQ646575 for the ITS sequences.

Sequence manipulation

The BLAST program (Altschul *et al.*, 1990) was used for homology searches. Sequence alignment was performed with CLUSTALX (Thompson *et al.*, 1997). Phylogenetic trees were constructed with MEGA3 software (Kumar *et al.*, 2004), using the number of nucleotide differences (including indels treatment) for distance estimation, Neighbour-Joining for clustering and bootstrapping (500 replicates).

AFLP fingerprinting

For AFLP, two endonucleases (ApaI, TaqI) and corresponding adapters (5′-TCGTAGACTGCGTACAGGCC-3′ and 5′-TGTACGCAGTCTAC-3′ (for ApaI) and 5′-GACGATGAG TCCTGAC-3′ and 5′-CGGTCAGGACTCAT-3′ (for TaqI) were used (Huys *et al.*, 1996). Selective primers (one selective base added) for final amplification were A01 (5′-GACTGCGTACAGGCCC**A**-3′) and T01 (5′-CGATGAGT CCTGACCG**A**-3′) labelled with Cy5. The amplified fragments were separated on 8% polyacrylamide gel. The 100–500 bp ladder labelled with CY5 was used as a molecular weight marker. The gels were scanned using a high-resolution densitometry scanner (Typhoon, Amersham Int.). The AFLP patterns were normalized by the software package IMAGEQUANT TL (version 2003.02, Amersham Biosciences). The Dice coefficient was used for the calculation of pair-wise similarity matrix. The matrix was imported into the NTSYS-pc program (EXETER software, 1992), and cluster analysis was performed by the unweighted pair group method with arithmetic averages (UPGMA).

Results and discussion

The *O. compressus* isolates showed almost 100% identity to the partial (about 500 bp) 16S rRNA gene sequences of *B. canariense* strains available in the GeneBank database (differences in a single nucleotide were observed). Absolute 16S rRNA gene fragment identity was found between *O. compressus* isolates and 11 unclassified bradyrhizobial microsymbionts of Spanish shrubs, tropical trees and *Vigna unguiculata*. The similarity between *O. compressus* isolates

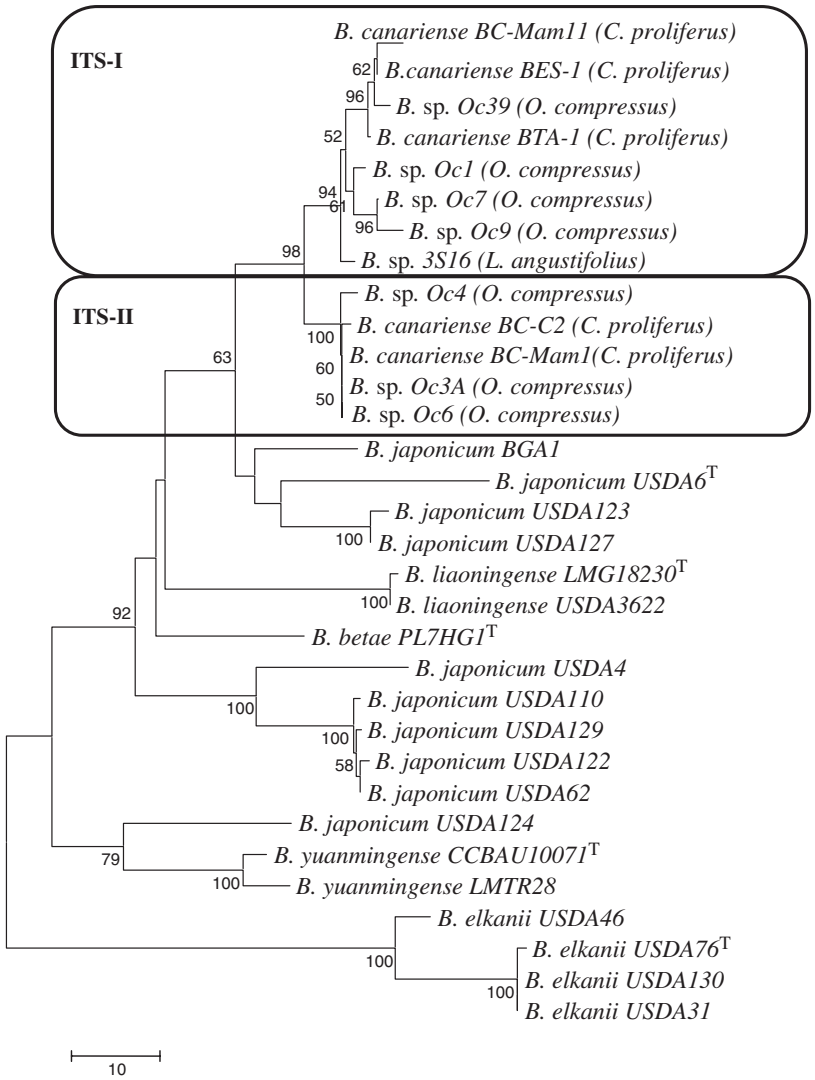

Fig. 1. Neighbour-Joining/number of differences phylogenetic tree based on ITS sequencing of the *Ornithopus compressus* isolates and reference *Bradyrhizobium* strains. The abbreviations for the host generic names correspond to: *C., Chamaecytisus; L., Lupinus; O., Ornithopus*.

and the closest representatives of *B. elkanii* and *B. japonicum* strains was 99.5% or less.

An ITS-based phylogenetic tree is presented in Fig. 1. It confirms that *O. compressus* isolates belong to *B. canariense* species. They are intermingled with five reference *B. canariense* strains and together form a statistically supported branch that is well distinguished from all other *Bradyrhizobium* species. A detailed description of the symbiotic and metabolic characteristics of *O. compressus* microsymbionts has been presented in a previous paper (Safronova *et al.*, 2004). It can be concluded that *O. compressus* isolates exhibit the main distinctive phenotypic features of the *B. canariense* species. They are able to nodulate effectively legumes of tribes Genisteae and Loteae (*Lupinus, Scorpiurus* and *Lotus* spp.) but do not nodulate *Glycine max*.

The analysis of ITS sequences divided the branch of *B. canariense* strains into two statistically separated groups (> 90% bootstrap support), which were named ITS clusters I and II (Fig. 1). To confirm this divergence, all available *B. canariense* strains from GeneBank database were included in the ITS analysis as well as the strains isolated from *Ornithopus* spp. by Stepkowski *et al.* (2005) and described as *B. canariense*. The ITS-tree presented in Fig. 2 shows that the same main clusters (I and II) were formed when supplementary 20 strains were added.

Alignment of ITS sequences showed the presence of a unique oligonucleotide insert TTAGAGACTTAGGTTTCTK in all strains forming ITS cluster I of the *B. canariense* branch. This cluster included isolates Oc1, Oc7, Oc9 and Oc39 of *O. compressus* (Fig. 2). The insert was neither present in other described species of the family *Rhizobiaceae* nor in any other bacterial families and can be used like a genetic marker for group I of *B. canariense* strains. The ITS sequence divergence of *B. canariense* strains can easily be

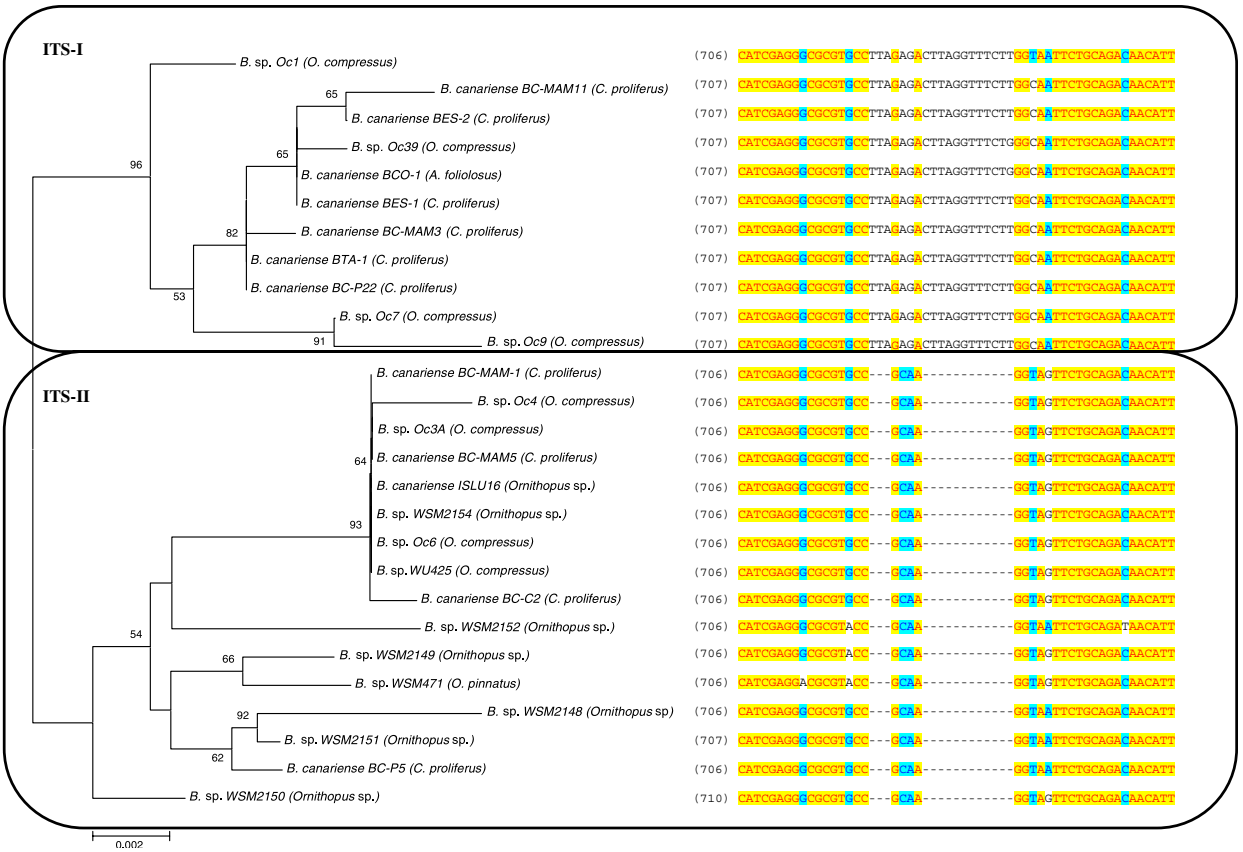

Fig. 2. Neighbour-Joining/number of differences ITS-tree showing two statistically separated groups (ITS clusters I and II) formed by strains from *Bradyrhizobium canariense – B*. sp. *(Ornithopus)* branch. The abbreviations for the host generic names correspond to: *A., Adenocarpus; C., Chamaecytisus; O., Ornithopus*.

observed by ITS–RFLP analysis as well. Strains from two *B. canariense* clusters generated different restriction patterns of the PCR amplified ITS region digested with DdeI having a recognition site of CTNAG (Vinuesa *et al*., 1998; Safronova *et al*., 2004).

To analyse the genetic diversity of the Sardinian *O. compressus* isolates, an AFLP fingerprinting analysis was performed. Nine reference strains of *Bradyrhizobium* and *Mesorhizobium* species were used. Twenty-three to 34 bands per pattern, whose sizes ranged from 40 to 400 bp, were observed in the electrophoresis of AFLP products (data not shown). The pattern of each strain was unique, proving that all isolates were not just clones of the same and indicating a high discriminative power of the AFLP technique at the intraspecific level. The UPGMA dendrogram of the AFLP patterns (Fig. 3) shows that the *O. compressus* isolates form a distinct cluster with a similarity level of more then 50%, which is arbitrarily chosen to distinguish rhizobial AFLP clusters (Janssen *et al*., 1996; Willems *et al*., 2000). The level of genomic heterogeneity among *O. compressus* isolates is obviously much higher than among *M. mediterraneum* reference strains and is comparable with that between two *B. japonicum* strains representing the most distant groups of highly heterogeneous *B. japonicum* species (Willems *et al*., 2000). AFLP clustering of the strains from *O. compressus* shows that the isolates Oc3A, Oc6 and Oc4 (ITS cluster II) are closely positioned in the dendrogram, suggesting that these three strains are genetically closer to each other than to the other *B. canariense* strains Oc9, Oc7, Oc39 and Oc1 (Fig. 3). In this respect, the data of AFLP fingerprints analysis are in agreement with the results obtained by ITS sequencing.

The genomic diversity of *B. canariense* strains isolated from genistoid legumes of the Canary Islands has earlier been studied using of BOX-, ERIC- and REP-PCR fingerprinting (Vinuesa *et al*., 1998). It was shown that strains BTA-1^{T} and BES-1 of ITS group I formed in the combined fingerprintints analysis a cluster statistically different from the other *B. canariense* strains tested. Recently, Vinuesa *et al*. (2005b) performed a genetic characterization of the *B. canariense* strains using REP-PCR fingerprinting and analyses of *atpD, glnII* and *recA* sequences. As a rule, the strains from ITS clusters I and II were members of different clades (more or less resolved) in all trees presented. In particular, all *B. canariense* strains having an ITS insert

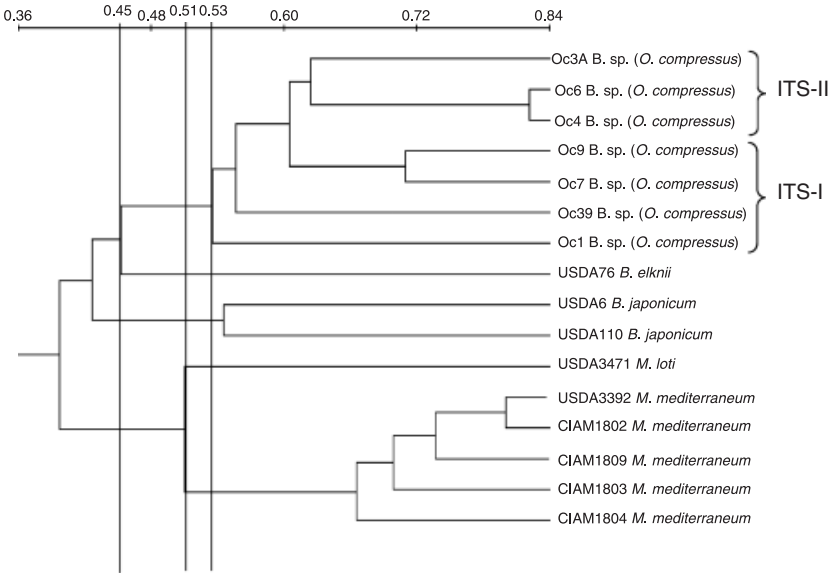

Fig. 3. UPGMA cluster analysis of AFLP patterns of the *Ornithopus compressus* isolates in comparison with the reference rhizobial strains.

formed a fairly well-resolved *atpD*-cluster (80% bootstrap support), while the strains without this insert formed a separate *glnII*-clade (95% bootstrap support).

In conclusion, the data presented in this paper confirm that seven slow-growing strains isolated from root nodules of yellow serradella (*O. compressus*) native of Asinara Island on North Western Sardinia belong to the newly described *B. canariense* species. Twenty-seven *B. canariense* strains analysed have clear differences in ITS sequences and form two well-separated ITS clusters according to the presence or the absence of the unique oligonucleotide insert TTAGAG ACTTAGGTTTCTK. Representatives of different ITS-groups display significant genetic divergence discovered by BOX, ERIC, REP and AFLP genomic fingerprinting as well as *atpD, glnII* and *recA* sequences analyses. On the bases of the data presented, a considerable importance can be supposed of the novel 19 bp ITS insert for an intraspecific division within *B. canariense* species and further molecular ecology investigations.

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