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Different *Mesorhizobium* species sharing the same symbiotic genes nodulate the shrub legume *Anagyris latifolia* ☆

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Abstract

The isolation and characterization of six rhizobial strains isolated from *Anagyris latifolia*, a shrub legume endemic to the Canary Islands, is reported in this study. The isolates were characterized by 16S-ARDRA, and sequencing of the ribosomal 16S rRNA gene, the 16S–23S rDNA intergenic spacer region, and the housekeeping gene for glutamine synthetase II (*glnII*). The phylogenies based on the three types of sequences matched, showing that the isolates belonged to three distinct lineages within the genus *Mesorhizobium* that could represent different species. However, the ribosomal and *glnII* phylogenies revealed some discrepancies in the relationships between the isolates and the named species in this genus. Despite their different taxonomic affiliation, all the isolates showed identical *nodC* sequences which were closely related (95% similarity) to that of the *Mesorhizobium tianshanense* type strain, indicating that they must have acquired the nodulation genes by a phenomenon of lateral gene transfer.

Keywords: Nodulation; Legume; *Mesorhizobium*; *Anagyris latifolia*; Phylogeny; Ribosomal genes; *glnII* gene; Nodulation genes; Canary Islands

Introduction

Leguminosae is one of the largest families of plants and it is distributed all around the world, from the Northern to the Southern Hemispheres. They show a great diversity in morphology, from annual herbaceous to tropical trees, as well as habitat and ecology. Legume plants are used worldwide for a wide range of purposes, mainly as grain legumes for human food and as forage, but also to reduce soil erosion, as wind breaks, for medicinal purposes and as ornamentals. Legumes are of particular agricultural, ecological and economic importance in comparison to other plants, due to their mutualistic nitrogen-fixing symbiosis with soil bacteria.

An enormous diversity has been revealed in recent years among the Gram-negative bacteria that can establish nitrogen-fixing symbiosis with legumes. Currently, 57 species of legume nodulating bacteria have been identified

Abbreviations: ARDRA, amplified ribosomal DNA restriction analysis; ITS, internally transcribed spacer

☆Note: Nucleotide sequence data of isolates Ala-1, Ala-3 and Ala-5 are available in the EMBL database under the accession numbers AM491620-AM491622 for 16S rRNA, AM491630-AM491632 for the 16S-23S ITS region, AM491623-AM491625 for *nodC*, and AM491626-AM491628 for *glnII*, respectively; and AM491629 for *glnII* of isolate Ala-6.

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[25]. Most of these species (46 species) are *Alphaproteobacteria* belonging to the genera *Rhizobium, Sinorhizobium*, *Mesorhizobium*, *Azorhizobium* and *Bradyrhizobium*, known by the general name of rhizobia, but some unexpectedly "non-rhizobial" genera of *Alphaproteobacteria* and *Betaproteobacteria* also contain legume-nodulating species [16]. The list of new rhizobial and non-rhizobial species of nodulating bacteria continues to grow, as further isolations from newly studied legumes are carried out.

The Canary Islands is a small territory (7500 km^2) characterized by an enormous richness of endemic flora (27% of endemism). Forty of these endemic species are leguminous, but there are many other wild non-endemics. They are found colonizing a great diversity of environments from the coast to the summits of the islands. Many are of interest from a biological (rare, fertility problems), ecological (colonizing sandy and salty environments) or agricultural (potential as forage) point of view. Only a small number have been studied with regard to their nodulating rhizobia. They include the characterization of several bradyrhizobial isolates from some *Genisteae* legumes (*Chamaecytisus, Teline, Adenocarpus*, *Spartocytisus* and *Lupinus*) [3,4,10,15,23] whose main genotype was described as *Bradyrhizobium canariense* [22]. Therefore, most of the legumes in the Canaries are still unexamined, and a wide diversity of rhizobia is to be expected.

Anagyris is a genus of legumes belonging to the *Thermopsidae*, a small tribe of six genera spread over the temperate areas of the Northern Hemisphere [17]. *Anagyris* contains two species, *Anagyris foetida*, distributed in the Mediterranean area and the Middle East, and *Anagyris latifolia* Brouss. *ex* Willd. endemic to the Canary Islands. *A. latifolia* is a shrub legume (3–5 m high) with large trifoliate leaves (5–6 cm) and spectacular yellow flowers (known in the Islands as "oro de risco," gold of the rocks). At present, only small populations survive (with very few individuals) in different, often inaccessible, habitats on the Islands of Tenerife, La Palma, La Gomera and Gran Canaria. It is considered to be one the Canary endemisms in "critical danger of extinction." Traditionally, it has been used as forage (probably the cause of its decline), traditional medicine (purgative, emetic) and recently, as an ornamental. Nodulation in this species has not previously been reported. In this study, we isolated and characterized six rhizobial strains nodulating *A. latifolia* from three natural populations, two in Tenerife, in the north (Icod) and south (Arico), and one in the northeast of La Palma (Mazo).

Materials and methods

Bacterial strains, culture conditions and rhizobia isolation

Sterilized and germinated seeds of *A. latifolia* were grown on soil samples collected where three natural populations of this legume are growing. After 8 weeks, the bacteria from the root nodules were recovered, with isolates Ala-1 and Ala-5 being from the north Tenerife population (Punta de Juan Centella, Icod), isolates Ala-3, Ala-4 and Ala-7 from the south Tenerife population (Barranco de Tamadaya, Arico) and isolate Ala-6 from the northeast of La Palma (Mazo). Redundant strains were eliminated by their ERIC-PCR profiles (data not shown). The isolates were grown at 28 °C in yeast mannitol agar (YMA) [21]. All isolates were stored at −80 °C on YM plus 20% v/v glycerol.

DNA isolation

Total genomic DNA was obtained from bacterial batch cultures grown until late exponential phase using the AquaPure Genomic DNA isolation kit (Bio-Rad). DNA concentrations were visually estimated from a 1% agarose gel electrophoresis by comparing the DNA samples with lambda-DNA *HindIII* digest.

16S ARDRA

The nearly full-length 16S rRNA gene was amplified as previously described [4]. Aliquots of the PCR products were separately digested with five endonucleases, *DdeI*, *MspI RsaI*, *HaeIII* and *HinfI* (Amersham-Pharmacia Biotech), following the manufacturer's recommendations with an excess of enzyme (4–5 U). The digests were separated by horizontal electrophoresis in 2.5% high resolution agarose gel (Sigma Chemical Co., St. Louis, Mo) in TBE buffer at 60 V for approximately 3 h. The digitalized gel images from the restriction patterns of the five endonucleases were combined and analysed with GelCompar II v. 4.2. The image was normalized with the 100 bp ladder marker (Amersham-Pharmacia Biotech) loaded at the centre and both sides of the gel. A dendrogram was constructed from the similarity matrix using the unweighted pair group method with arithmetic mean (UPGMA) and Dice's similarity coefficient. Reference strains used for ARDRA were *Mesorhizobium amorphae* LMG 18977^T, *M. huakuii* LMG 14107^T, *M. loti* LMG 6125^T, *M. tianshanense* USDA 3592^T, *M. chacoense* PR5^T, *S. fredii* USDA 205^T, *S. meliloti* LMG 6133^T, *R. leguminosarum* bv. *viciae* USDA 2370^T, *R. etli* USDA 9032^T, *R. giardinii* H152^T, *R. hainanense* I66^T, *A. tumefaciens* ATCC 23308, *B. japonicum* USDA 6^T, *B. japonicum* USDA 110 and *B. canariense* BTA-1^T.

Sequencing of the 16S rDNA, 16S–23S rDNA (ITS), *glnII* and *nodC* loci

The 16S rRNA gene was amplified as above. The internal transcribed spacer (ITS) of the 16S–23S rDNA region was amplified by using the primers FGPS 1490 and FGPL 132′