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Lotus endemic to the Canary Islands are nodulated by diverse and novel rhizobial species and symbiotypes☆

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ABSTRACT

Genetic and symbiotic characterization of 34 isolates from several *Lotus* species endemic to the Canary Islands showed extraordinary diversity, with bacteria belonging to different species of the genera *Mesorhizobium* (17 isolates), *Sinorhizobium* (12 isolates) and *Rhizobium*/*Agrobacterium* (5 isolates). In a previous report, we showed that the *Sinorhizobium* isolates mostly belonged to *S. meliloti*. Here, we focused on the remaining isolates. The *Lotus* mesorhizobial strains were distributed in the *rrs* tree within six poorly resolved branches. Partial sequences from *atpD* and *recA* genes produced much better resolved phylogenies that were, with some exceptions, congruent with the ribosomal phylogeny. Thus, up to six different mesorhizobial species were detected, which matched with or were sister species of *M. ciceri*, *M. alhagi*, *M. plurifarium* or *M. caraganae*, and two represented new lineages that did not correspond to any of the currently recognized species. Neither *M. loti* nor *Bradyrhizobium* sp. (*Lotus*), recognized as the typical *Lotus*-symbionts, were identified among the Canarian *Lotus* isolates, although their nodulation genes were closely related to *M. loti*. However, several subbranches of mesorhizobia nodulating *Lotus* spp. could be differentiated in a *nodC* tree, with the isolates from the islands distributed in two of them (A1 and A3). Subbranch A1 included reference strains of *M. loti* and a group of isolates with a host range compatible with biovar loti, whereas A3 represented a more divergent exclusive subbranch of isolates with a host range almost restricted to endemic *Lotus* and it could represent a new biovar among the *Lotus* rhizobia.

Introduction

The diversity of rhizobia nodulating *Lotus* spp. is starting to be elucidated. Until recently, most isolates had been collected from a small number of *Lotus* species used as pasture-forage in a few locations of South America and New Zealand [11,16,18,26,38]. *Mesorhizobium loti* and *Bradyrhizobium* sp. (*Lotus*) have been traditionally recognized as the typical *Lotus* rhizobia [32]. *M. loti* establish effective symbiosis with some *Lotus* species, which include *L. corniculatus*, *L. japonicus* or *L. filicaulis*, whereas the bradyrhizobial strains either do not nodulate or form ineffective nodules in these species, although they effectively nodulate species such as *L. uliginosus* or *L. angustissimus*, which form ineffective symbioses with *M. loti* [3,4,32,35]. Broad host range mesorhizobia, such as NZP 2037, that are able to nodulate both groups of *Lotus* effectively [34] are rare. Many strains classified as *M. loti* have been poorly characterized [6,31] and some are probably misclassified, which may explain the wide variability in total DNA–DNA homology [6], the different phylogenies [7,50] and the great differences in N_2-fixing effectiveness [3,32] detected among "*M. loti*" strains. Recently, strain "*M. loti* MAFF303099" was re-classified as *M. huakuii* biovar loti [42]. Similarly, the broad host range "*M. loti* NZP2037" strain needs to be better characterized and probably reclassified.

Lotus is a genus of legume plants with 120–180 species of herbs and small shrubs distributed mainly across the Northern Hemisphere [36], and most *Lotus* species remain unexplored from the point of view of their rhizobial partners. Thus, larger diversity than that currently recognized can be expected for the *Lotus* rhizobia, as new isolates from less well known species are studied and better characterized genetically. Recent studies are starting to change our knowledge about the genetic diversity of the bacteria able to establish symbiosis with *Lotus* spp. Characterization of root nodule bacteria from *Lotus creticus*, *L. argenteus* and *L. roudairei* in an

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arid region of South Tunisia detected rhizobia closely related to *Mesorhizobium*, *Rhizobium*, *Sinorhizobium* and *Bradyrhizobium* [51]. Mesorhizobial strains described as two new species, *M. tarimense* and *M. gobiense*, were isolated from *L. frondosus* and *L. tenuis* in the Xinjiang desert soils of China [15]. Bacteria belonging to the three genera *Rhizobium*, *Mesorhizobium* and *Aminobacter* were identified during the characterization of 103 isolates from *L. tenuis* in Argentina [10].

The Canary Islands constitute a hot spot of plant biodiversity in the Mediterranean area [25]. The genus *Lotus* is represented by 24 species (17 endemics) on the islands [1], where they usually show an insular distribution pattern (exclusive to a single island and with a limited distribution area) [2]. Characterization of the *Lotus* rhizobia on these islands has been limited until now to the description of a group of strains isolated mostly from *L. lancerottensis* [21]. In this study, we characterized another 23 new isolates from seven *Lotus* species (*L. kunkelii*, *L. arinagensis*, *L. callis-viridis*, *L. sessilifolius*, *L. pyranthus*, *L. berthelotii* and *L. campylocladus*). Most isolates characterized in the study were recovered from the arid coastal environments of the Canaries (average annual rainfall 250 mm in the north and 150 mm in the south) [24], under the direct influence of the sea. The isolation of compatible and efficient strains is of special interest for the endemic *Lotus* species, because their growth in a restricted area could be associated with specific rhizobial strains only found on a particular island or in a characteristic environment. It is worth noting that many of these *Lotus* species are threatened or in danger of extinction, so the use of appropriate bacterial inoculants might become essential for recovering specific *Lotus* populations. Natural populations of *L. kunkelii* and *L. arinagensis* are only found in sand-soils (about 20 a.s.l.) on the southeast coast of Gran Canaria. *L. kunkelii* is currently in extreme danger of extinction. *L. callis-viridis* only grows on the high cliffs in southwest (500 a.s.l.) Gran Canaria. *L. sessilifolius* is a frequent species on the coasts and lowlands of Tenerife, Gomera, Hierro and Gran Canaria. *L. pyranthus*, *L. berthelotii* and *L. campylocladus* grow in the pine forests (with higher annual rainfall of 500–800 mm). Only a few natural individuals of *L. pyranthus* (La Palma, 1300 a.s.l) and *L. berthelotii* (Tenerife, 1200–1500 a.s.l.) are known, but fortunately they are widely propagated as cuttings due to their ornamental value (red-orange flowers and bright-red flowers, respectively) which has likely contributed to their conservation. *L. campylocladus* grows in the subalpine zone (1800–2200 a.s.l.) of Tenerife. Therefore, the aim of this study was to characterize the bacterial diversity associated with *Lotus* species endemic to the Canary Islands, to establish whether special genotypes were symbionts of particular *Lotus* species or associated with particular environments and to select good N_2-fixing bacteria which could be of use in the future recovery of endangered populations.

Materials and methods

Isolation of rhizobia and growth conditions

Sterilized and germinated *Lotus* seeds or cuttings were grown in soil samples collected where natural populations were growing. The root nodule bacteria were recovered 8 weeks later. Alternatively, some strains were obtained directly from root nodules of plants growing at these locations. The strains isolated are listed in Table 1. The isolates were grown at 28 °C on yeast mannitol agar

Table 1
Lotus isolates used in this study, origin of host *Lotus*, 16S ARDRA and symbiotic types.

Isolates	Original *Lotus* host	Location	16S RFLP group	*nodC* symbiotype	Symbiotic phenotype on *L. corniculatus*	Reference
LSE1	*L. sessilifolius*	Tenerife	*Mesorhizobium*	A1	Nod+/Fix+	This study
LSE2	*L. sessilifolius*	Tenerife	*Sinorhizobium*	bv. lancerottense	Nod+/Fix±	[21]
LSE4	*L. sessilifolius*	Tenerife	*Mesorhizobium*	n.d.	n.d.	This study
LSE5	*L. sessilifolius*	Tenerife	*Agrobacterium*	n.d.	Nod−	This study
LSE6	*L. sessilifolius*	Tenerife	*Sinorhizobium*	n.d.	Nod−	[21]
LKU1	*L. kunkelii*	Gran Canaria	*Sinorhizobium*	n.d.	Nod−	[21]
LKU2	*L. kunkelii*	Gran Canaria	*Agrobacterium*	n.d.	Nod−	This study
LKU3	*L. kunkelii*	Gran Canaria	*Mesorhizobium*	A3	Nod−	This study
LKU4	*L. kunkelii*	Gran Canaria	*Mesorhizobium*	n.d.	n.d.	This study
LKU6	*L. kunkelii*	Gran Canaria	*Mesorhizobium*	A3	Nod−	This study
LAR2	*L. arinagensis*	Gran Canaria	*Sinorhizobium*	n.d.	Nod+/Fix±	This study
LAR5	*L. arinagensis*	Gran Canaria	*Mesorhizobium*	A3	Nod+/Fix−	This study
LAR8	*L. arinagensis*	Gran Canaria	*Mesorhizobium*	A3	Nod−	This study
LAR9	*L. arinagensis*	Gran Canaria	*Mesorhizobium*	n.d.	Nod−	This study
LAR12	*L. arinagensis*	Gran Canaria	*Mesorhizobium*	n.d.	n.d.	This study
LCV1	*L. callis-viridis*	Gran Canaria	*Rhizobium*	n.d.	Nod−	This study
LCV2	*L. callis-viridis*	Gran Canaria	*Agrobacterium*	n.d.	Nod−	This study
LCV3	*L. callis-viridis*	Gran Canaria	*Mesorhizobium*	n.d.	Nod+/Fix+	This study
LCV5	*L. callis-viridis*	Gran Canaria	*Agrobacterium*	n.d.	Nod−	This study
LCA1	*L. campylocladus*	Tenerife	*Mesorhizobium*	A1	Nod+/Fix+	This study
LMAX	*L. maculatus*	Tenerife	*Sinorhizobium*	bv. lancerottense	Nod+/Fix±	[21]
LPYR1	*L. pyranthus*	La Palma	*Mesorhizobium*	A1	Nod+/Fix+	This study
LPYR2	*L. pyranthus*	La Palma	*Mesorhizobium*	A1	Nod+Fix+	This study
LBER1	*L. bertheloti*	Tenerife	*Mesorhizobium*	A1	Nod+Fix+	This study
LBER2	*L. bertheloti*	Tenerife	*Mesorhizobium*	n.d.	Nod+/Fix+	This study
LCO1b	*L. corniculatus*	La Palma	*Mesorhizobium*	n.d.	Nod+/Fix−	This study
LCO2b	*L. corniculatus*	La Palma	*Mesorhizobium*	n.d.	Nod+/Fix+	This study
LLAN2	*L. lancerottensis*	Lanzarote	*Sinorhizobium*	bv. lancerottense	Nod+/Fix−	[21]
LLAN3	*L. lancerottensis*	Lanzarote	*Sinorhizobium*	bv. lancerottense	Nod+/Fix−	[21]
LLAN4	*L. lancerottensis*	Lanzarote	*Sinorhizobium*	bv. lancerottense	Nod+/Fix−	[21]
LLAN6	*L. lancerottensis*	Lanzarote	*Sinorhizobium*	bv. lancerottense	Nod+/Fix−	[21]
LLAN7	*L. lancerottensis*	Lanzarote	*Sinorhizobium*	bv. lancerottense	Nod+/Fix−	[21]
LLAN8	*L. lancerottensis*	Lanzarote	*Sinorhizobium*	bv. lancerottense	n.d.	[21]
LLAN9	*L. lancerottensis*	Lanzarote	*Sinorhizobium*	bv. lancerottense	n.d.	[21]

L. corniculatus seeds grown on soil from *L. pyranthus* natural population, n.d.: not determined.