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Biodiversity of rhizobia present in plant nodules of *Biserrula pelecinus* across Southwest Spain

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ABSTRACT

Biodiversity studies of native *Mesorhizobium* spp. strains able to nodulate the annual herbaceous legume *Biserrula pelecinus* L. in soils from Southwest Spain have been carried out. One or two isolates per plant, 30 in total, were randomly selected for further characterization. There was no association between the presence of mesorhizobia nodulating-*B. pelecinus* and the chemical or textural properties of the soils. The isolates were tested for their symbiotic effectiveness on this forage legume under greenhouse conditions and characterized on the basis of physiological parameters: carbon source utilisation (API 50CH), 16S rRNA sequencing and ERIC-PCR, lipopolysaccharide, protein and plasmid profiles. Our results show that in spite of the great diversity found among the native isolates, most of them belong to the genus *Mesorhizobium*, the exception being strain B24 which sequence matches 97.52% with *Neorhizobium huautlense*; this is the first description of a *Neorhizobium* strain effectively nodulating-biserrula plants. Results of a field trial indicated that some of these isolates could be recommended as inoculants for this legume.

B24 = DSM 28743 = CECT 8815; ENA (HF955513)

16S rRNA sequences of isolates B13, B18, B26, B30 and B1 are deposited at ENA under numbers LS999402 to LS999406, respectively.

Introduction

Symbiotic associations between members of the family *Leguminosae* and soil diazotrophic bacteria (rhizobia) are well known to play a determinant role in the atmospheric nitrogen fixation process on earth, named Biological Nitrogen Fixation (BNF). Nitrogen fixation potential of food legumes may range from 20 to 450 kg N/ha, with soybean the most notable crop. Nevertheless, forage and tree legumes also play an important role in total nitrogen fixation globally [1]. Taking into account the overuse of N fertilizer that results in severe pollution problems and the increasing need to regenerate degraded soils, special interest must be paid to some of these not yet well known associations.

Biserrula pelecinus L. is an annual pasture legume well adapted to both acidic soils and arid lands [17]. It is distributed around the Mediterranean basin, in the Canary Islands and in Northeast Africa [32]. Although it has been proposed as a regenerating legume in semiarid regions [29], its cropping is still scarce in the mentioned areas. However, during the last decade it has become an important forage crop in Western Australia and New South Wales [15–17] and, in the Mediterranean Region of Chile [5]. In addition, the potential of *Biserrula* crops for soil fertility restoration of contaminated soils have been demonstrated [30]. Nevertheless, little is known about the physiological characteristics and taxonomy relatedness of the rhizobia partner in the symbiosis process. *Biserrula* plants are nodulated by medium-growth rate bacteria belonging to the genus *Mesorhizobium* [7,9,20,21]. The number of validated *Mesorhizobium* species able to form nodules in this legume has increased from one (*M. ciceri* bv. *biserrulae*) [23] to three, with the descriptions of *Mesorhizobium australicum* sp. nov. and *M. opportunistum* sp. nov. [24]. In a study of the genetic diversity of root nodule bacteria isolated from endemic *B. pelecinus* plants in Northern Spain and South of Portugal, [29] it was found that most of the isolates were phylogenetically related with *M. ciceri*. However, in this study a strain related to *Rhizobium leguminosarum* bv. *phaseoli* was isolated, suggesting that lateral gene transfer between mesorhizobia and rhizobia might have occurred. A later study by the same authors, while testing the tolerance to stressful conditions of the isolates,

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as well as their BNF capacity under different fertilization levels, described moderate-growers isolates as the most promising fixers under these conditions [30]. These studies confirm that the symbiotic association between *Biserrula* and mesorhizobia is extremely specific. The aim of this study was to determine the biodiversity of *Biserrula* native rhizobia populations in Southwest Spain based on a polyphasic approach, and to select elite strains for further studies in order to develop efficient inoculants.

Material and methods

Soil sampling and rhizobia strain isolation

Thirty soil samples were collected at the depth of 0–20 cm from different locations in Southwest Spain (Fig. S1), covering a distance of 450 km from South to North and from East to West. Some chemical and textural properties from collected soils are shown in Table S1. Un-amended and air-dried soils, using one plastic pot of 200 cm^3 per soil sample, were used to perform a plant test aimed to trap specific rhizobia-nodulating biserrula. Seeds of *B. pelecinus* cultivar Cashba were surface-sterilized with 5% sodium hypochlorite for 6 min and washed several times with distilled-sterilized water. Seeds were germinated on 1% water-agar at 24 °C for two to three days. Pregerminated seeds were transferred to each plastic pot and placed in a greenhouse under natural light with a daily minimum-maximum temperature of 18/32 °C. Plants were scored for nodulation 4 weeks after emergence by the procedure of Vincent [31].

Nodules were randomly excised from each plant and nodule isolates were obtained as previously described [31]. Single colonies were picked and routinely maintained on yeast-mannitol agar (YMA) slants at 4 °C for further characterization. The strain *Mesorhizobium cicer* symbiovar *biserrulae* WSM1497 was included in all tests as reference.

To quantify the indigenous biserrula-rhizobia population of soil samples, the Most Probable Number (MPN) technique was carried out as previously described [31].

Physiological characterization

Colony type was visually determined by observing individual colonies grown onto YMA plates after 5 days incubation.

Bacterial capacity to grow at different pH values was assayed in 4 mL YMB tubes supplemented with the appropriate buffer: 20 mM Homopipes for pH 4.5 and 20 mM Tris for pH 8.0 and pH 8.5. The approximate size of the inoculum was 4×10^5 bacteria. Growth response was determined by measuring the absorbance (540 nm) of the cultures six days after inoculation. Salt tolerance was determined as described before, in tubes containing YMB medium supplemented with 1.2, 1.4, 1.8 and 2.0% (w/v) NaCl.

Utilization of different carbon sources was carried out using API 50CH galleries inoculated with suspensions of each strain in a basal medium containing 0.2 $g\,L^{-1}$ $NaHPO_4$, 0.2 $g\,L^{-1}$$MgSO_4$, 0.1 $g\,L^{-1}$ NaCl, 0.8 $g\,L^{-1}$ yeast extract and 0.04 $g\,L^{-1}$ bromocresol purple adjusted to pH 7. The results were read 5 days after incubation at 28° C. The dendrogram was built using the clustering method of UPGMA (Unweighted Pair-Group Method with Arithmetic Mean) and the coefficient of Jaccard (J) with the statistics program NTSYS (Numerical Taxonomic and Multivariate Analysis System, version 1.80, Applied Biostatistics, New York).

ERIC fingerprinting

For ERIC analyses, primers ERIC 1R (5′-ATG TAA GCT CCT GGG GAT-3′) and ERIC 2 (5′-AAG TAA GTG ACT GGG GGT GAG C-3′) were used. PCR reactions were performed in a final volume of 25 μL containing the following components: 2.5 μL of 10× reaction buffer (10 mM Tris-ClH, 50 mM $MgCl_2$), 50 ng of the DNA primer, 2.5 μL of 15 mM $MgCl_2$, 2.5 μL of 2 mM dNTPs, 0.5 μL of Taq DNA-polymerase (5 U μL^{-1}), 1 μL (25 ng) of DNA template, and 15 μL ddH_20. DNA amplification was carried out in a DNA thermal cycler by using the folloing profiles: 1 cycle at 95 °C for 3 min; 35 cycles at 94 °C for 1 min, 37 °C for 1 min and 72 °C for 1 min, and 1 final cycle at 72 °C for 8 min. The PCR product was electrophoresed on 1.5% agarose gel in 0.5X TAE buffer, at 6 V cm^{-1}. SYBR green was added to the PCR product for visualizing the bands. A 100pb ladder (Favorgen FALA-100) was used as size marker.

16S rDNA gene sequencing

The 16S rDNA gene (corresponding to positions 8-1522, in *Escherichia coli*) was amplified with primers 8F (5′-AGAGTTTGATCTGGCTCAG-3′) and 1522R (5′-AAGGAGGTGATCCANCCRCA-3′). Reactions were performed in a final volume of 50 μL containing the following components: 5 μL of 10× reaction buffer, 2 μL of each DNA primer (10 μM), 5 μL of 15 mM $MgCl_2$, 5 μL of 2 mM dNTPs, 0.2 μL of Taq DNA-polymerase (5 U μL^{-1}), 2 μL (50 ng) of DNA template and 28.8 μL of ddH_2O. PCR conditions were as follows: preheating at 95° C for 9 min; 35 cycles of denaturing at 95 °C for 1 min; annealing at 59 °C for 1 min and extension at 72 °C for 2 min; and a final extension at 72 °C for 7 min. The bands corresponding to the 16S rDNA were purified from the gel using a commercial kit (Favorgen). The sequence reaction was performed on an ABI377 sequencer (Applied Biosystems Inc.) The sequence obtained was compared with those from the GenBank using the FASTA program. The 16S rDNA gene sequence was performed with the MEGA 5.03 software. The 16S rDNA gene sequence was aligned with those of phylogenetically closely related organisms (as determined by EzTaxon analyses) [4] and the alignment was confirmed and checked manually. Tree robustness was assessed by bootstrap resampling (1000 replicates each).

SDS-PAGE analysis

Strains were grown at 28 °C for 72 h on YMB. For protein profile, whole-cell protein extracts were prepared, and SDS-PAGE was performed [12]. Electrophoresis was carried out on 10–15% (w/v) gradient polyacrylamide gels. Gels were loaded with approximately 100 μg of protein [2] per lane and run at 10–15 mA for 4 h. Bands were visualized by silver staining [26].

Plasmid profiles analysis

Strains were grown at 28 °C for 72 h on YMB at 120 rpm. Samples (0.5 mL) of early exponential growth phase were centrifuged and washed on TBE buffer (mmol/L): Tris, (89); boric acid, (89); and EDTA (25), at pH 8.3. The cells were resuspended in 25 μL of a solution containing the following: 475 μL Tris-Ficoll (25% w/v) Ficoll dissolved in 25 mmol/L Tris-Cl, pH 8.0, 50 μL lysozyme stock (20 μg/mL, Sigma), 25 μL RNase stock (400 μg/mL RNase dissolved in 10 mmol/L Tris-Cl, pH 7.5) and 50 μL bromophenol blue loading dye (0.25% w/v) bromophenol blue in distilled water containing 40% (w/v) Ficoll.

Bacterial suspensions (25 μL) were placed in the wells of a horizontal 0.5% (w/v) agarose gel in TBE. Immediately behind the wells there was a band of 0.4% (w/v) agarose containing 1% (w/v) SDS. Plasmids were separated by electrophoresis for 1 h at 20 V (10 mA), and for 3 h at 100 V (50 mA). The gel was stained for 30 min with ethidium bromide (1 mg/mL) and the DNA was visualized with a UV transilluminator [6].

Lipopolysaccharide profiles

Polysaccharides were extracted, subjected to electrophoresis in a 16.5% polyacrylamide slab gel and run in a SDS-Tris-tricine buffer during 15 h at a constant current of 28V in a miniProtean electrophoresis system (Bio-Rad Laboratories, Richmond, Calif.) [11]. Silver staining of the LPS was performed as previously described [10].

Nodulation tests

Surface sterilized seeds of *Cicer arietinum* and *Lotus corniculatus* were axenically pre-germinated in Petri dishes. Seedlings were transferred to Leonard jars (*C. arietinum*) or to (2×20 cm) glass tubes (*L. corniculatus*) containing 15 mL of nutrient solution [25]. Seedlings were inoculated with 1 mL of 2 days-old yeast mannitol broth (YMB) cultures of rhizobia to provide approximately 10^8 cells seed^{-1}. Two non-inoculated controls were also included: non-fertilized (−N) and N-fertilized (+N). Seedlings were uniformly thinned to two per jar and covered with a layer of sterilized paraffin-coated fine gravel. Leonard jars were arranged in a randomized block design with two replicates per treatment. Plants were scored for nodulation 4 weeks after emergence.

Symbiotic efficiency

Tubes assay

Seeds of *B. pelecinus* cv. Cashba were surface-sterilized and germinated as described previously, sown in glass tubes containing 15 mL of nutrient solution (3 tubes were set up per treatment) and inoculated as described before for *L. corniculatus* plants. A negative non-inoculated control was included. *M. ciceri* bv. *biserrulae* reference strain WSM1497 (named isolate B25) was used as positive control. Tubes were placed in a greenhouse under natural light with a daily minimum-maximum temperature of 18/32 °C. Plants were harvested 6 weeks after inoculation and the symbiotic effectiveness was estimated by comparing the shoot dry weights with those of non-inoculated control plants.

Field assay

The field trial was undertaken from November to May at the Agriculture Experimental Station Las Torres in a loam soil (alluvial soil, Xerofluvent, pH 7.8, 0.89% organic matter, 12.5 mg P kg^{-1}, 194 mg K kg^{-1}, 12 mg NO_3-N kg^{-1}, 6 mg NH_4^+-N kg^{-1} and 20.5% $CaCO_3$), a site with no records of *Biserrula* cultivation. The experimental field was laid out in a randomized complete block design with four replicates. Plots ($1 \times 1.5\,\text{m}^2$) were broadcast sown at a dose of 20 kg ha^{-1} of biserrula seeds. A space of 1 m was allowed between plots and 3 m between blocks. Seeds of *B. pelecinus* cv. LO6110 were inoculated with peat-based inoculants of strains B1 and B24 before sowing. Non-inoculated controls, with N fertilizer (at a rate of 100 kg N ha^{-1}), or without fertilizer, were included. Plants were harvested 7 months later, and the N_2 fixation efficiency was evaluated by measuring the number of nodules, plant biomass and N content. The N content was determined by the Kjeldahl method [31].

Statistical analyses were performed using Statistix sofware (NH Analytical Sofware, USA). Results were expressed at the $P < 0.05$ level of significance.

Results and discussion

The first description of rhizobial-nodulating *B. pelecinus* plants consist of three strains (WSM 1283, WSM 1284, WSM 1497), that were isolated from nodules on roots of *B. pelecinus* growing in different countries in the Mediterranean basin, and according to their physiology, 16S rRNA sequences and symbiotic characteristics, they were included into the genus *Mesorhizobium* and denominated *Biserrula* mesorhizobia [20]. Looking at the cellular fatty acids analysis, these mesorhizobia strains and *M. ciceri* shared similar profiles, however, the former strains were later differentiated into a new biovar (*M. ciceri* bv. *biserrulae*) on the basis of considerable differences in genetic, phenotypic and symbiotic characteristics with *M. ciceri* [23].

There is scarce information about the abundance, physiology and genetic characteristics of the native mesorhizobia-nodulating *Biserrula* in Spain, apart from the studies of the Portuguese group [29,30] that included one Spanish soil. Thus, the aim of the present study was to investigate the abundance and the diversity of *Biserrula* mesorhizobia in 30 soil samples collected across 450 km in the Southwest Spain (Fig. S1 and Table S1). Interesting, although the sampled area had never been cultivated with *B. pelecinus*, 28 out of 30 *Biserrula* plants grown with the different soils had pink effective nodules, and the natural soil population of rhizobia nodulating-*Biserrula* ranged between 0 and 10^5 rhizobia g^{-1} soil (data not shown).

Physiological characterization

A set of 30 isolates and *M. ciceri* bv. *bisserrulae* WSM 1497 (B25) as reference strain were used for further characterization. All the isolates, apart from the B24 strain, are moderately fast-growing rhizobia, with generation times ranging from 4 to 6 h in YMB at 28 °C. In comparison, strain B24 is a fast-grower, with a generation time around 2 h at the same conditions, and it has been assigned to *Neorhizobium huautlense* (97.52% 16S rDNA level of similarity). The *Biserrula* mesorhizobia described previously share similarities with regards to distinct phenotypic characteristics such as salt or pH tolerance and the range of carbon source utilization. The set of strains isolated in this work reveal clearly differentiable performances under such conditions (Table 1 and Table S2), where seven groups can be separated, each group sharing the same sensitivity/tolerance (0/1) to the growth conditions. The most frequent phenotype (acid-S, alkaline-T, salt-S up to 2% and temperature-S at 37 °C) (bar code 0100) is shared by 15 isolates (48%), including the reference strain WSM 1497, and the isolate B30 assigned to *M. japonicum*. Some isolates present a unique code, as B10 (0101), B16 (0111) and B24 (1101) assigned to *N. huautlense*. Seven isolates: B9, B11, B21, B27, B28, B29 and B31 did not grow at any of the set up conditions (code 0000). *M. ciceri* bv. *biserrulae* strains are able to utilize a wide range of carbon sources (Table S2 and Fig. S2), however most of the biserrula isolates in this study were able to catabolize xylitol but did not grow in the presence of erythritol, methyl β-xyloside (none of them), arbutina or N-acetylglucosamine, thus differing from known species that nodulate *Biserrula* [23]. Moreover, *M. ciceri* bv. *biserrulae* strains cannot grow at 2% NaCl or pH 4.5 [23,24]. In contrast, four of our isolates were tolerant to 2% NaCl and five grew at pH 4.5 (one of these was B24). Our results are in accordance with the characteristics of Portuguese strains already described [30], where all of them were considered tolerant to severe acidity (pH 4.5–5.5), and around 40% were able to tolerate NaCl concentrations up to 2.5%. These contrasting results could indicate a huge diversity among these mesorhizobia isolates, at least in the sampled areas. Surprisingly, the more acidic- or salt-tolerant isolates did not come from the most acidic or the most saline soils of the study.

ERIC profile and 16S rRNA sequencing

The majority of the isolates have a distinctive ERIC profile and the number of fragments obtained ranged between one and 13,

Table 1
Classification of *Biserrula* isolates based on codes generated from ERIC, API and protein dendograms plus an arbitrary code assigned respect tolerance/sensitiveness behaviour to several physiological traits.

Strain	Soil	ERIC code	API code	Protein code	Dendograms code[a]	Physiological code[b]	N° plasmids
B1	Herreruela	E	C	D	**ECD**(1)	**0100**(1)	1
B2	El garrobo	D	C	C	**DCC**(2)	0100	1
B3	Castilblanco	E	C	C	**ECC**(3)	0100	1
B4	Cazalla de la Sierra	C	C	D	**CCD**(4)	0100	1
B5	Puebla de los Infantes	E	C	D	ECD	**1100**(2)	3
B6	Garlitos	E	C	B	**ECB**(5)	1100	1
B7	Villanueva del Fresno	E	C	B	ECB	0100	3
B8	Burguillos	E	C	B	ECB	1100	1
B9	Hornillos	E	C	D	ECD	**0000**(3)	1
B10	Logrosan	C	C	A	**CCA**(6)	**0101**(4)	3
B11	Puerto Moral	D	C	D	**DCD**(7)	0000	1
B12	Aroche	E	C	D	ECD	1100	0
B13	Sta. Barbara	A	C	D	**ACD**(8)	0100	0
B14	Valverde del Camino	E	C	D	ECD	0100	1
B15	Cardeña	E	C	D	ECD	0100	1
B16	Pozoblanco	E	C	D	ECD	**0111**(5)	1
B17	Espiel	E	A	D	**EAD**(9)	0100	1
B18	Espiel	C	C	D	CCD	0100	2
B19	Cañamero	E	C	D	ECD	**0110**(6)	3
B20	Salvatierra de los Barros	E	C	D	ECD	0100	1
B21	Alburquerque	E	C	D	ECD	0000	1
B22	Esparragosa de Lares	E	C	D	ECD	0110	1
B23	Trujillo	D	B	D	**DBD**(10)	0100	0
B24	Valencia de Mombuey	E	C	D	ECD	**1101**(7)	0
B25	Reference strain	D	C	D	DCD	0100	1
B26	Elvas	E	C	D	ECD	0110	1
B27	Aracena	D	C	D	DCD	0000	0
B28	La Orden	C	C	D	CCD	0000	0
B29	Torremegía	D	C	D	DCD	0000	0
B30	Fuente de Cantos	B	C	D	**BCD**(11)	0100	1
B31	Fuente de Cantos	B	C	D	BCD	0000	1

*Bold values with a superscript number in brackets indicate the diferent dendograms and physiological codes found.

[a] Bar code for: ERIC, API and Protein dendograms.

[b] Bar code definition for: acid-, alkaline-, salt-up to 2% and temperature-, sensitive = 0 and tolerant (1).

with a size from 250 to 3000 bp (Fig. S3), suggesting a huge genetic diversity.

The isolates grouped in five main clusters (Fig. 1), from which several isolates belonging to each group were selected (B1, B13, B18, B24, B26 and B30) for further 16S rRNA sequencing.

Strains B13 (unique from group A) and B30 (as representative from group B) were linked with *M. opportunistum* and *M. japonicum*, at a 99.85% and 99.93% level of similarity, respectively. The isolate B18 was chosen from the cluster C and belongs to *M. australicum* (100% identity). The cluster D encloses six isolates, including the strain B25 which is the reference strain of *M. ciceri* bv. *biserrulae*. Finally, the lower cluster (E) contains 18 isolates, of which outer strains B1 and B24 showed 99.86% of identity with *M. ciceri* and 97.52% with *N. huautlense*, respectively and B26 from the middle of the cluster, matched at 99.2% with *M. opportunistum*.

The 16S rRNA phylogenetic tree mostly resumes the grouping derived from ERIC fingerprinting (Fig. 2). Clearly, isolate B24 was confirmed as the only strain in the *Neorhizobium* branch. B30 and B31 isolates grouped with *M. japonicum*, however we only have included the sequence of B30 since it matches 100% with B31. Isolates B13 and B26, who show different ERIC profiles match at 98% among them and both are closed to *M. opportunistum*. Strain B18 conforms a single subdivision with *M. australicum*, and finally strain B1 from the most diverse group, has been included in the same group with *M. ciceri* and the type strain B25 *M. ciceri* bv *biserrulae*.

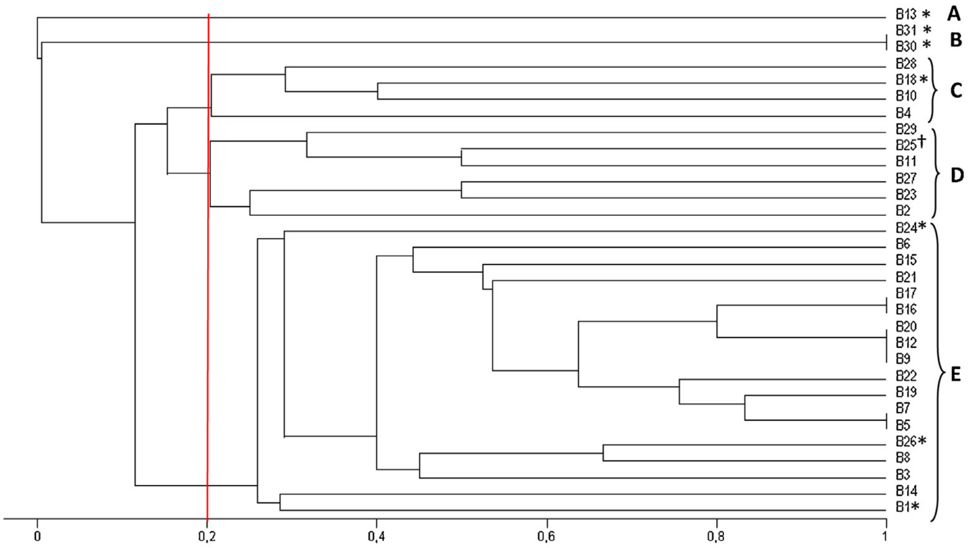
The profiles obtained with the ERIC fingerprint products have not allowed the grouping of the isolates according to their phylogeny, indicating that each profile is unique and correlated with the strain level. Therefore, the results confirm previous reports that amplification with specific and arbitrary primers may be quite useful to detect genetic diversity at the strain level, but do not work for phylogenetic characterization [8,13].

Plasmid profiles

Earlier studies on other rhizobial strains have included the analysis of distinct molecular patterns to establish differences among bacterial field isolates [27]. Electrophoretic mobility of plasmid content is a hallmark that determine plant preference to trap specific bacterial strains. The number of plasmids visualized among the isolates of this study ranged between 0 and 3 (Table 1). Most of the isolates (61%) have a single plasmid (~500 pb) as described for the reference strain of *M. ciceri* bv. *biserrulae* [22]. No plasmids were detected in seven of the isolates, while 2 plasmids were found in B18 strain, and three in isolates B5, B7, B10 and B19. Rhizobial isolates carrying more than one plasmid have shown a low symbiotic efficiency. This phenomenon has been previously described for *R. trifolii* [28] and for rhizobia nodulating-chickpea, presumably assigned to genus *Mesorhizobium* [14], however the presence of several plasmids might indicate horizontal gene transfer events which might result in additional helpful performances to the recipient strain such as increased survival to toxic compounds in rhizosphere. We have not found low molecular weight-plasmids among these *Biserrula* isolates, which on some occasions appear as stable indigenous plasmids in other rhizobial strains and that could be used as genetic tools [3].

Protein profiles

Protein profiles also showed a high diversity (Fig. S4A). Isolates clustering based on this feature (Fig. S4B) fit neither with ERIC, nor with API dendrograms. Isolates showing relatively similar protein profiles were quite different based on ERIC profile (i.e. B21 and B19, or B15 and B16) (Fig. S4C) and, in contrary, isolates with similar ERIC profiles exhibited very different protein ones (B9, B12, B20). Some-



†Type strain WSM1497
*Sequenced strains in this work

Fig. 1. Dendrogram showing the relatedness of *B. pelecinus* isolates as determined by ERIC-PCR profiles. The dendrogram was built using the clustering method of UPGMA (Unweighted Pair-Group Method with Arithmetic Mean) and the coefficient of Jaccard (J). A cut-off value of 80% has been applied.

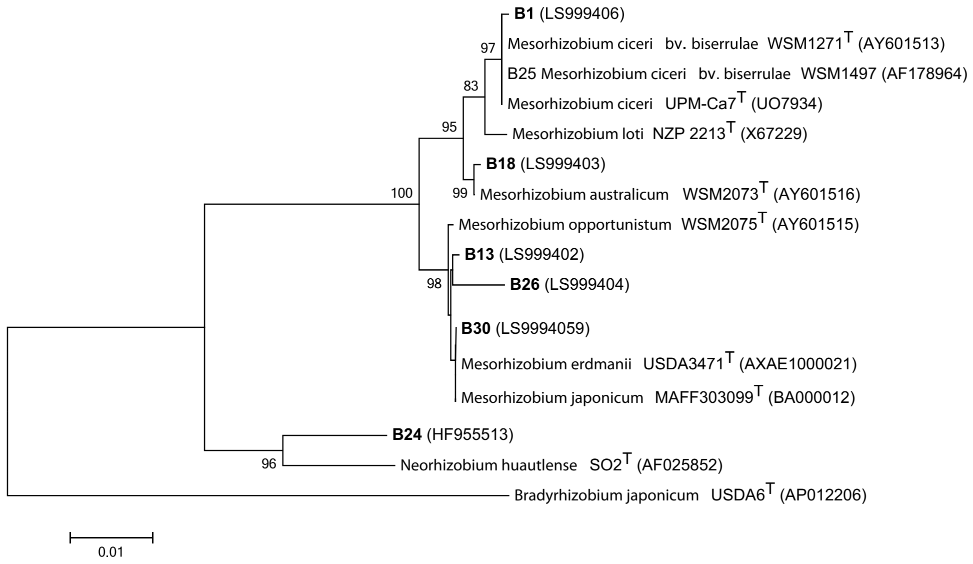


Fig. 2. Phylogenetic tree based on 16S rRNA gene sequences showing the relationship between several isolates and related type strains. The tree was constructed using the neighbor-joining method based on comparison of aprox. 1400 nt. Bootstrap values are expressed as percentages of 1000 replicates; values <70% are not shown. Bar, 0,01 nt substitution per site. *Bradyrhizobium japonicum* USDA6 (AP012206) was used as out group.

times isolates collected nearby showed similar protein profiles (i.e isolates B19, B20, B21and B22, or B15, B16 and B17 or B30 and B31).

A combination of the information derived from ERIC fingerprinting, carbon usage plus protein profiles allowed us to cluster the isolates into 11 groups, which basically correlate with those derived from ERIC fingerprinting alone.

LPS profiles

Electrophoretic mobility of surface polysaccharides is an additional hallmark to be considered in order to establish differences among isolates from legume nodules. In our study, most strains share a typical LPS pattern, with the exception of strains B10, B14 and clearly B24, a strain that was lately identified as distinct bacterial species (Fig. S5). However, a high molecular weight polysaccharide with distinct electrophoretic mobility was present in some isolates, such as B14 or B16, but absent in others, such as B24, B28 and B29. Nevertheless, these variations do not correlate with the arrangement derived from protein profiling or API dendrograms; only the strain B10, that belongs to a lone group of protein profiles, correlates with a slightly different LPS migrating pattern.

Symbiotic characteristics

All the isolates were able to form effective nodules with biserrula plants, although symbiotic efficiency varied between them. All of them yielded significantly higher dry shoot weights than the un-inoculated control (Fig. 3), being strain B18 (assigned to

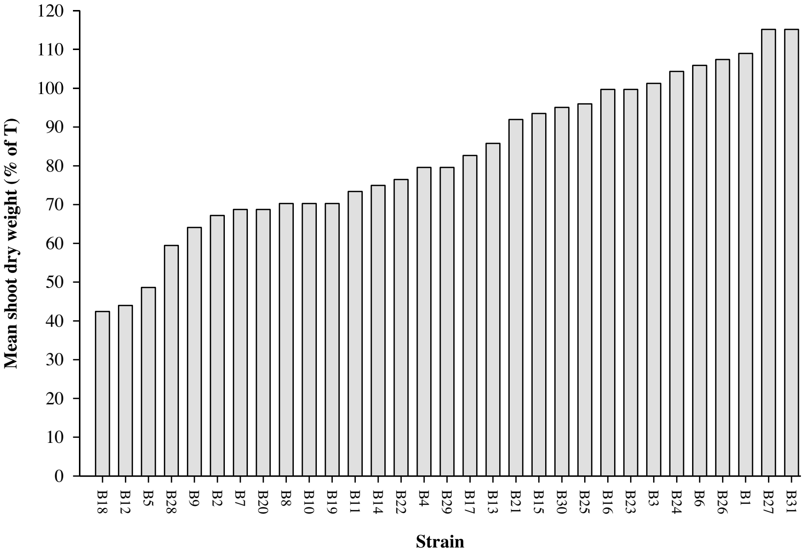


Fig. 3. Mean shoot dry weight of *Biserrula pelecinus* cv. Cashba inoculated with 30 isolates from Spain expressed as a percentage of uninoculated control plants. Data are means of three plants harvested 42 days after sowing. SDW of non-inoculated control = 3.27 mg plant^{-1}.

Table 2
Field experiment: response of *Biserrula pelecinus* cv. LO6110 to inoculation with selected strains B1 y B31, related to *M. ciceri* and *M. japonicum* respectively. NDW: nodule dry weight. LSD: least significative difference.

Treatment	Nodules plant^{-1}		Yield (kg ha^{-1})	
	Number	NDW (mg)	Dry matter	N content
Non inoculated	1.00 c	21.9 c	3276 c	91.6 c
Non inoculated +N	0.70 c	10.7 c	5675 a	158.9 a
B1	6.89 a	138.6 b	5508 a	151.7 ab
B31	3.60 b	143.2 a	4225 bc	122.8 bc
LSD ($p > 0.05$)	1.85	45.1	1041	33.3

M. australicum) the less effective one. The most effective isolates B1, B27 and B31 achieved more than twice the dry shoot weight value than the non-inoculated control plants. It is noteworthy the symbiotic effectiveness showed by strains B13 and B26 (assigned to *M. opportunistum*), species described as ineffective with biserrula plants [24]. On the other hand, none of the isolates nodulate chickpea nor *Lotus corniculatus* plants, except isolate B24 (assigned to *N. huautlense*) that induced pink nodules on *Lotus*. According to the description of the *Neorhizobium*, its species can nodulate *Lotus* [19], thus, the effective nodulation of *B. pelecinus* plants by a *Neorhizobium* strain has been found by first time in this study.

Isolates B1 and B31, which showed the best symbiotic performance (shoot dry weight accumulation) in the test tubes assay, were tested under field conditions with *Biserrula* cv. LO6110. The effect of inoculation with peat-based inoculants on nodulation and crop yield is shown in Table 2. Inoculant-strain B1 (this strain was assigned to *M. ciceri*, 99.86% level of similarity) induced a significantly higher number of nodules, but they were smaller than those developed by strain B31 (strain assigned to *M. japonicum*, 99.93% level of similarity). Concerning forage yields, strain B1 showed no significant statistical differences with the N-fertilizer control, reaching the same dry matter and N content. Inoculant-strain B31 was significantly less productive than both treatments (B1 and N+ control).

The symbiotic performance of our isolates agree with that of Nandasena et al. [23] who described *M. australicum* strains as poor N_2-fixers with biserrula plants, and strongly contrast with those characterized as *M. opportunistum* which were completely ineffective with this host legume [24]. However, our results match those previously described for *Biserrula* mesorhizobia strains, as able to effectively nodulate biserrula plants but inefficient with chickpea plants [20]. Interestingly, these authors found a strain (WSM 1284) showing a broad host range, and able to form nodules on *L. corniculatus* among others legumes, as the isolate B24 did. However, WSM 1284 strain belongs to *M. ciceri* bv. *biserrulae* while B24 matches with *N. huautlense*. The work of Vicente et al., [29] describing the biodiversity of root-nodule bacteria associated to biserrula plants in Portugal, found a strain (ArrPt12) that was aligned with *R. leguminosarum* bv. *phaseoli*. Later on, these authors [30] described 23 fast-growing biserrula-isolates out of 42 analyzed. Therefore, while it is not the first time that fast-growing strains have been isolated from nodules of *B. pelecinus* plants, this study has linked these rhizobial strains with different legume-symbiotic genders. In addition, the alignment of the isolates B30 and B31 with *M. japonicum* [18] is a novelty with respect to the most frequent species found in biserrula nodules.

In conclusion, this paper shows the extraordinarily high diversity of rhizobia able to nodulate *B. pelecinus* plants present in the Southwest soils of Spain. To our knowledge, this is the first time that a member of genus *Neorhizobium* and *M. japonicum* species have been isolated from nodules of biserrula plants and described as good N_2-fixers with this legume. Physiological analyses, ERIC and whole protein profiling were found to be technical tools more suitable for illustrating this high biodiversity. These results also show the potential of the Spanish soils as a source of mesorhizobia-nodulating *Biserrula* with striking characteristics. In a preliminary field trial some isolates from this collection have provided promising results, under our field conditions, with a commercial *Biserrula* cultivar supporting the interest of these strains to be used as commercial inoculants in agricultural practice.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.syapm.2019.03.005.

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