Mesorhizobium ciceri biovar *biserrulae*, a novel biovar nodulating the pasture legume *Biserrula pelecinus* L.

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Biserrula pelecinus L. is a pasture legume species that forms a highly specific nitrogen-fixing symbiotic interaction with a group of bacteria that belong to *Mesorhizobium*. These mesorhizobia have >98.8% sequence similarity to *Mesorhizobium ciceri* and *Mesorhizobium loti* for the 16S rRNA gene (1440 bp) and >99.3% sequence similarity to *M. ciceri* for the *dnaK* gene (300 bp), and strain WSM1271 has 100% sequence similarity to *M. ciceri* for *GSII* (600 bp). Strain WSM1271 had 85% relatedness to *M. ciceri* LMG 14989^T and 50% relatedness to *M. loti* LMG 6125^T when DNA–DNA hybridization was performed. WSM1271 also had a similar cellular fatty acid profile to *M. ciceri*. These results are strong evidence that the *Biserrula* mesorhizobia and *M. ciceri* belong to the same group of bacteria. Significant differences were revealed between the *Biserrula* mesorhizobia and *M. ciceri* in growth conditions, antibiotic resistance and carbon source utilization. The G+C content of the DNA of WSM1271 was 62.7 mol%, compared to 63–64 mol% for *M. ciceri*. The *Biserrula* mesorhizobia contained a plasmid (~500 bp), but the symbiotic genes were detected on a mobile symbiosis island and considerable variation was present in the symbiotic genes of *Biserrula* mesorhizobia and *M. ciceri*. There was <78.6% sequence similarity for *nodA* and <66.9% for *nifH* between *Biserrula* mesorhizobia and *M. ciceri*. Moreover, the *Biserrula* mesorhizobia did not nodulate the legume host of *M. ciceri*, *Cicer arietinum*, and *M. ciceri* did not nodulate *B. pelecinus*. These significant differences observed between *Biserrula* mesorhizobia and *M. ciceri* warrant the proposal of a novel biovar for *Biserrula* mesorhizobia within *M. ciceri*. The name *Mesorhizobium ciceri* biovar *biserrulae* is proposed, with strain WSM1271 (=LMG 23838 =HAMBI 2942) as the reference strain.

Biserrula pelecinus L. is an annual herbaceous legume suited to arid land, which is rapidly gaining popularity as a forage species in Australia (Howieson *et al.*, 1995, 2000; Loi *et al.*, 2005). The Mediterranean basin is the centre of origin of this monospecific genus (Allen & Allen, 1981). *B. pelecinus* forms a highly specific nitrogen-fixing symbiotic association with bacteria that have been classified within the genus *Mesorhizobium* (Howieson *et al.*, 1995; Nandasena *et al.*, 2001, 2004).

Four strains (WSM1271, WSM1283, WSM1284 and WSM1497) isolated from *B. pelecinus* growing in the Mediterranean basin shared >98.8% sequence similarity to *Mesorhizobium ciceri* and *Mesorhizobium loti* for the 16S

rRNA gene (1440 bp) (Nandasena *et al.*, 2001; K. G. Nandasena, G. W. O'Hara, R. P. Tiwari and J. G. Howieson, unpublished results). To investigate whether phylogenetically diverse strains nodulate *B. pelecinus* growing in its centre of origin, we sequenced variable regions of two housekeeping genes, *dnaK* and *GSII*, for these four strains and for another ten strains isolated from *B. pelecinus* growing in undisturbed locations within the Mediterranean basin, including Morocco, several Greek islands in the Cyclades group and several different sites on Sardinia (Table 1).

The locus *dnaK* encodes a highly conserved chaperone protein with multiple cellular functions, and this gene has proven to be a useful diagnostic tool for root-nodule bacteria (Stepkowski *et al.*, 2003; Eardly *et al.*, 2005). A 300 bp intragenic fragment from the variable region of *dnaK* was amplified and sequenced using the primers TSdnaK2 and TSdnaK3 (Stepkowski *et al.*, 2003). Ten strains, WSM1283, WSM1448, WSM1449, WSM1493, WSM1497,

The GenBank/EMBL/DDBJ accession numbers for the *dnaK* gene sequences of the *Biserrula* mesorhizobia are EF035140–EF035149 and DQ485740–DQ485743, and those for the *GSII* gene sequences of the *Biserrula* mesorhizobia are EF035150–EF035159 and DQ485748–DQ485751.

Table 1. Collection details of *Biserrula* mesorhizobia from the Mediterranean basin

Strain	Location	Altitude (m)	Rainfall (mm)	Soil type	Soil pH	Habitat	Grazing	Collector(s)
WSM1271	Bottida, Sardinia	295	–	Sandy loam	5.5	Fallow	Heavy	J. G. Howieson
WSM1283	Oulmes, Morocco	1100	600	Sand	6	Pasture	Heavy	J. G. Howieson
WSM1284	Siniscola, Sardinia	30	–	Sand	6.2	Grassland	Light	J. G. Howieson
WSM1448	Falatados, Greece	300	600	Loamy sand	7	Pasture	Light	S. Carr and B. Nutt
WSM1449	Frantato, Greece	150	600	Sand	7	Fallow	Nil	S. Carr and B. Nutt
WSM1450	Frantato, Greece	150	600	Sand	7	Fallow	Nil	S. Carr and B. Nutt
WSM1493	Ios, Greece	0	350	Sandy loam	7	Pasture	Light	S. Carr and B. Nutt
WSM1497	Mykonos, Greece	30	450	Sandy loam	8	Pasture	Light	S. Carr and B. Nutt
WSM1533	Aggius-Luogosanto, Sardinia	300	850	Sand	5.5/6.0	Pasture	Heavy	A. Loi, S. Carr and C. Porqueddu
WSM1539	Cagliari-Muravera, Sardinia	345	–	Sandy loam	7	Pasture	Heavy	A. Loi, S. Carr and C. Porqueddu
WSM1540	Cagliari-Muravera, Sardinia	345	–	Sandy loam	7	Pasture	Heavy	A. Loi, S. Carr and C. Porqueddu
WSM1548	Pattada-Bentutti, Sardinia	265	750	Sandy loam	6	Pasture	Heavy	A. Loi, S. Carr and C. Porqueddu
WSM1562	Quarta, Sardinia	50	–	Sandy loam	7	Pasture	Light	A. Loi, S. Carr and C. Porqueddu
WSM1568	Ozieri, Sardinia	430	650	Sandy loam	5.5/6.0	Pasture	Light	A. Loi, S. Carr and C. Porqueddu

–, No information available.

WSM1533, WSM1539, WSM1548, WSM1562 and WSM1568, had identical partial *dnaK* sequences, while the other four strains (WSM1271, WSM1284, WSM1450 and WSM1540) differed by only 1 or 2 bp. These *Biserrula* mesorhizobia had >99.3 % sequence similarity to *M. ciceri* and <91.9 % sequence similarity to *M. loti* and, as a consequence, all 14 *Biserrula* mesorhizobia given in Table 1 clustered with *M. ciceri* when a phylogenetic tree was developed (not shown) using the available partial *dnaK* sequences of type strains of root-nodule bacteria.

Sequences for two glutamine synthetases (*GSI* and *GSII*) have been used to determine genetic relationships among root-nodule bacteria (Turner & Young, 2000). To provide further evidence for the grouping of the *Biserrula* mesorhizobia and *M. loti*, a 600 bp intragenic fragment of a glutamine synthetase gene (*GSII*) was amplified and sequenced using the primers *GSII*-1 and *GSII*-2 (Turner & Young, 2000). Eight of the strains, WSM1283, WSM1284, WSM1448, WSM1450, WSM1533, WSM1539, WSM1540 and WSM1548, shared identical sequences for *GSII*, and these sequences gave 100 % sequence similarity to *M. ciceri*, while they were only 90.4 % similar to *M. loti*. The other six *Biserrula* mesorhizobia, WSM1271, WSM1449, WSM1493, WSM1497, WSM1562 and WSM1568, had 1 or 2 bp differences from the *GSII* sequence of the first eight strains. All 14 *Biserrula* mesorhizobia given in Table 1 clustered together with *M. ciceri* when a phylogenetic tree was developed (not shown) using the *GSII* sequences of available type strains of 21 root-nodule bacteria, thereby confirming the phylogenetic relationships developed with the 16S rRNA gene and *dnaK*.

The similarities observed for 16S rRNA, *dnaK* and *GSII* genes are strong evidence that *B. pelecinus* growing in its centre of origin is nodulated by a very similar group of bacteria currently classified within the rhizobial species *M. ciceri*. At present, 11 species of *Mesorhizobium* have been proposed, ten of which, *Mesorhizobium amorphae*, *M. chacoense*, *M. ciceri*, *M. huakuii*, *M. loti*, *M. mediterraneum*, *M. plurifarum*, *M. septentrionale*, *M. temperatum* and *M. tianshanense*, have been shown to form nitrogen-fixing symbiotic associations with leguminous plants (Gao *et al.*, 2004; Chen *et al.*, 2005; Ghosh & Roy, 2006). All ten species share >97.7 % sequence similarity for the 16S rRNA gene and it is recommended that DNA–DNA hybridization values be included when describing novel species within this genus (Chen *et al.*, 2005). Therefore, DNA–DNA hybridization was performed [microplate method described by Ezaki *et al.* (1989) as modified by Goris *et al.* (1998)] between *Biserrula* mesorhizobium strain WSM1271 and the type strain of *M. ciceri* LMG 14989^T. A relatedness value of 85 % between the two strains was obtained, providing evidence that they can be regarded as members of the same bacterial species. DNA–DNA hybridization values between *Biserrula* mesorhizobium strain WSM1271 and the type strain of *M. loti* LMG 6125^T were 50 %.

Cellular fatty acid analysis provides a useful tool in identification of root-nodule bacteria (Tighe *et al.*, 2000). Therefore, cellular fatty acid analysis was undertaken for strains WSM1271 and WSM1497 as described by Tighe *et al.* (2000). The fatty acids of WSM1271 and WSM1497 were straight-chain saturated fatty acids, unsaturated fatty acids, 11-methyl-branched unsaturated fatty acids and 3-hydroxy

fatty acids. Although the fatty acid patterns of these two strains were diagnostic of root-nodule bacteria in general, the strains could not be assigned to any of the published root-nodule bacterial species when the fatty acid patterns were compared with all the bacterial strains available in the MIS fatty acid database (MIDI Inc.). However, similar fatty acid profiles were observed for *M. ciceri* and the two strains of *Biserrula* mesorhizobia for the fatty acids that were present in amounts >0.25 % (Table 2). This observation further confirms that the *Biserrula* mesorhizobia are members of *M. ciceri*.

However, there are significant variations between the *Biserrula* mesorhizobia and *M. ciceri* for a number of phenotypic and symbiotic characteristics. As we have concluded that *B. pelecinus* is being nodulated by a very similar group of phylogenetically related bacteria, further descriptions and comparisons of *Biserrula* mesorhizobia were based on the initial four strains (WSM1271, WSM1283, WSM1284 and WSM1497). *Biserrula* mesorhizobia cannot grow at 2 % (w/v) NaCl (Nandasena *et al.*, 2001), while *M. ciceri* can (Jarvis *et al.*, 1997). *M. ciceri* is resistant to chloramphenicol (Nour *et al.*, 1994), while the *Biserrula* mesorhizobia are sensitive to this antibiotic (K. G.

Nandasena, G. W. O'Hara, R. P. Tiwari and J. G. Howieson, unpublished results). When compared with *M. ciceri*, *Biserrula* mesorhizobia are able to utilize a wider range of carbon sources (as the sole source of carbon). These include erythritol, L-xylose, adonitol, methyl β -xyloside, galactose, sorbitol, aesculin, cellobiose, melibiose, β -gentiobiose, D-lyxose, D-tagatose and D-fucose [API 50CH and API 50CHE/B medium (bioMérieux) were used to determine carbohydrate utilization profiles]. The G + C content of the DNA of WSM1271 is 62.7 mol%, while this value is between 63 and 64 mol% for *M. ciceri*.

The symbiotic genes of *Biserrula* mesorhizobia are located on a mobile symbiosis island and integrate into a phenylalanine tRNA gene (Nandasena *et al.*, 2006), while such an element is not reported for *M. ciceri*. Furthermore, the symbiosis island of the *Biserrula* mesorhizobia contains a gene that encodes a phage P4-like integrase (*intS*) that is responsible for the excision and insertion of symbiosis islands (Nandasena *et al.*, 2006). To investigate whether *M. ciceri* had a similar *intS* adjacent to a phenylalanine tRNA gene, we performed PCR with primers phetRNAf and intS522r (Nandasena *et al.*, 2006). *M. ciceri* failed to give an amplification product, while *M. loti* and *M. tianshanense* produced an expected 800 bp PCR product (data not shown). This indicates that *M. ciceri* may not have an *intS* adjacent to a phenylalanine tRNA as found in the *Biserrula* mesorhizobia.

The nodulation gene *nodA* determines the type of N-acyl substitution transferred onto the oligosaccharide backbone of Nod factor and thus plays a significant role in determining the symbiotic specificity of root-nodule bacteria (Downie, 1998). A 567 bp intragenic fragment of *nodA* was sequenced for *Biserrula* mesorhizobial strains WSM1271, WSM1283, WSM1284 and WSM1497 (Nandasena *et al.*, 2006), and there was <78.6 % sequence similarity between the *nodA* sequences of *Biserrula* mesorhizobia and that of the type strain of *M. ciceri*. Nitrogenase is the key enzyme in nitrogen fixation, and *nifH* is responsible for the structural development of component II of nitrogenase (Rubio & Ludden, 2000). A 710 bp intragenic fragment of *nifH* was sequenced for *Biserrula* mesorhizobia strains WSM1271, WSM1283, WSM1284 and WSM1497 (Nandasena *et al.*, 2006), and there was <66.9 % sequence similarity between the *nifH* sequences of *Biserrula* mesorhizobia and that of the type strain of *M. ciceri*. All *Biserrula* mesorhizobia clustered separately from all other species of *Mesorhizobium* when phylogenetic trees were developed separately for *nodA* and *nifH* sequences (Nandasena *et al.*, 2006). Furthermore, *M. ciceri* is considered a monospecific cross-nodulation system, as it is unable to nodulate 88 plant species belonging to the Fabaceae and Mimosaceae and only nodulates chickpea (*Cicer arietinum*) (Gaur & Sen, 1979). Our previous work has shown that *M. ciceri* is unable to nodulate *B. pelecinus* and *Biserrula* mesorhizobia are unable to induce nodules on *Cicer arietinum* (Nandasena *et al.*, 2004), indicating that there is no cross-nodulation compatibility

Table 2. Fatty acid compositions (%) of *M. ciceri* and *Biserrula* mesorhizobium strains WSM1497 and WSM1271

Data for *M. ciceri* are mean contents for nine strains taken from Tighe *et al.* (2000).

Fatty acid	WSM1497	WSM1271	<i>M. ciceri</i>
12:0 3-OH	0.37	0.53	0.27
13:0 iso 3-OH	0.42	0.45	0.24
Unknown (ECL 14.780)			0.16
15:0 iso			0.18
15:0			0.02
16:0	10.92	12.56	13.41
17:0 iso	5.13	4.18	4.16
17:1 ω 8c	0.45	0.62	0.15
17:0 cyclo	0.86	0.58	0.84
17:0	1.59	2.14	1.29
Unknown (ECL 17.875)			0.17
18:0	2.84	2.65	3.25
11-methyl 18:1 ω 7c	12.55	15.52	11.92
18:1 ω 9cis			0.12
Unknown (ECL 18.794)			0.21
19:0 cyclo ω 8c	41.52	35.29	37.27
10-methyl 19:0	0.5	0.81	0.18
20:0			0.05
20:1 ω 9t			0.14
20:2 ω 6,9c	0.72	0.87	0.95
Summed feature 4*			0.34
Summed feature 7*	22.13	23.78	24.69

*Summed feature 4 contains 15:0 iso 2-OH and/or 16:1 ω 7c. Summed feature 7 contains 18:1 ω 7c/ ω 9t/ ω 12t.

between *M. ciceri* and the *Biserrula* mesorhizobia. These results strongly indicate that the symbiotic genes of *M. ciceri* and the *Biserrula* mesorhizobia are significantly different. The considerable differences observed for phenotypic and symbiotic characteristics between *M. ciceri* and the *Biserrula* mesorhizobia warrant the *Biserrula* mesorhizobia to be regarded as a novel biovar, for which we propose the name *M. ciceri* biovar *biserrulae*.

Description of *Mesorhizobium ciceri* biovar *biserrulae*

Mesorhizobium ciceri biovar *biserrulae* is named after the legume host, *Biserrula pelecinus* L., with which these bacteria form a nitrogen-fixing symbiotic relationship.

Gram-negative rods ($0.5 \times 1 \mu\text{m}$) with a polar or subpolar flagellum and containing granules of poly- β -hydroxybutyrate. They are moderately fast-growing, forming 2–4 mm diameter colonies within 4–5 days on YMA, and have a mean generation time of 4–6 h when grown in YMB at 28 °C. Colonies on YMA are white–opaque, slightly domed, moderately mucoid with smooth margins. Strains grow on YMA containing 1.5 % (w/v) NaCl, but not with 2.0 % (w/v) NaCl, and are able to tolerate a pH range between 5.5 and 8.0. Utilizes the following compounds as sole sources of carbon: glycerol, erythritol, D-arabinose, L-arabinose, ribose, D-xylose, L-xylose, adonitol, methyl β -xyloside, galactose, D-glucose, D-fructose, D-mannose, rhamnose, dulcitol, inositol, mannitol, sorbitol, aesculin, cellobiose, melibiose, β -gentiobiose, D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol and L-arabitol. Cannot utilize the following compounds as sole sources of carbon: methyl α -D-mannoside, methyl α -D-glucoside, amygdalin, inulin, melezitose, glycogen, xylitol, gluconate, 2-ketogluconate and 5-ketogluconate. Strains are sensitive to ($\mu\text{g ml}^{-1}$) ampicillin (50), chloramphenicol (40), spectinomycin (50) and tetracycline (20) while they are resistant to gentamicin (40), kanamycin (50), nalidixic acid (50) and streptomycin (100). Synthesizes the following fatty acids: 12:0 3-OH (0.53 %), 13:0 iso 3-OH (0.45 %), 16:0 (12.56 %), 17:0 iso (4.18 %), 17:1 ω 8c (0.62 %), 17:0 cyclo (0.58 %), 17:0 (2.14 %), 18:0 (2.65 %), 11-methyl 18:1 ω 7c (15.52 %), 19:0 cyclo ω 8c (35.29 %), 10-methyl 19:0 (0.81 %), 20:2 ω 6,9c (0.87 %) and summed feature 7 (18:1 ω 7c/ ω 9t/ ω 12t) (23.78 %). The G+C content of the DNA is 62.7 mol%. Contains a single plasmid (~ 500 kb). Symbiotic genes of this organism are located on a mobile symbiosis island and it is able to nodulate *B. pelecinus* and *Astragalus membranaceus*, while strains do not nodulate *Cicer arietinum* (Nandasena *et al.*, 2004).

The reference strain is WSM1271 (= LMG 23838 = HAMBI 2942).

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