Mesorhizobium olivaresii sp. nov. isolated from *Lotus corniculatus* nodules

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16S rRNA gene: FM203302

recA gene: FN556460

glnII gene: LN681554

atpD gene: FM203309

rpoB gene: KX712097

nodC gene: FM203320

Abstract

In this study four *Mesorhizobium* strains isolated from *Lotus corniculatus* nodules in Granada (Spain) were characterized. Their 16S rRNA gene sequences were closely related to those of *M. albiziae* LMG 23507^T and *M. chacoense* Pr5^T showing 99.4 and 99.2% similarity values, respectively. The analysis of concatenated *rpoB*, *recA*, *atpD* and *glnII* genes showed they formed a cluster with internal similarities higher than 97%. The closest species also were *M. albiziae* LMG 23507^T and *M. chacoense* Pr5^T showing similarity values lower than 92% in *rpoB*, *recA* and *glnII* genes and lower than 96.5% in the *atpD* gene. These results indicated that the *L. corniculatus* strains belong to a new species of genus *Mesorhizobium* which was confirmed by DNA-DNA hybridization and phenotypic characterization. Therefore a new species with the name *Mesorhizobium olivaresii* sp. nov. is proposed, and the type strain is CPS13^T (LMG 29295^T = CECT 9099^T).

The genus *Mesorhizobium* was proposed by Jarvis *et al.* [7] to accomodate several species phylogenetically divergent to those from genus *Rhizobium* and currently contains more than 30 species with *Mesorhizobium loti* as the type species of the genus (http://www.bacterio.net/mesorhizobium.html). The type strain of this species was deposited in several collections and recently it has been reported that those conserved in ATCC and USDA collections belonged to different species which have been named *Mesorhizobium erdmanii* and *Mesorhizobium jarvisii* [14]. All these species are endosymbionts of *Lotus corniculatus*, a legume worldwide distributed that establishes symbiosis with strains from genus *Mesorhizobium* in America [2, 16, 23], Europe [1, 4, 6, 10, 12], Asia [7, 21] and Oceania [17, 24].

Some strains isolated in different continents from *L. corniculatus* nodules belong to groups phylogenetically divergent to the currently described species of genus *Mesorhizobium* as was showed by Marcos *et al*. [12]. One of these groups contained some strains isolated in Granada (Spain) from *L. corniculatus* during a wide study of *Lotus* spp. endosymbionts [10]. The objective of the present work was to perform a polyphasic characterization of these strains and the proposal of a novel species named *Mesorhizobium olivaresii* sp. nov.

In this work we obtained for the strains CPS13^T, CPS1, CGS20 and CGS22 the 16S rRNA, *atpD*, *recA*, *glnII* and *nodC* gene sequences not previously obtained according the methodologies of Lorite *et al.* [10] and Turner and Young [27]. The amplification and sequencing of the *rpoB* gene was performed according to Martens *et al.* [13]. All these sequences were aligned with those of the *Mesorhizobium* species using the Clustal W program [26]. The distances were calculated according to Kimura´s two-parameter model [8]. The phylogenetic trees were inferred using the neighbour joining (NJ) and maximum likelihood (ML) models [19, 20]. MEGA5.0 [25] was used for all analyses.

The 16S rRNA gene sequences of strains CPS13^T, CPS1, CGS20 and CGS22 are identical and then only that of the strain CPS13^T was included in the NJ and ML phylogenetic analyses (Fig. 1). The results of these analyses showed that the strain CPS13^T groups with *M. albiziae* CCBAU 61158^T and *M. chacoense* Pr5^T. These strains presented similarity values of 99.4% and 99.2%, respectively, with respect to the strain CPS13^T. These high similarity values in the 16S rRNA gene sequences is a common finding among species of genus *Mesorhizobium* that are distinguishable by the analysis of housekeeping genes, from which *rpoB*, *recA*, *atpD* and *glnII* genes are available for most species of genus *Mesorhizobium*.

The NJ and ML analyses of the concatenated *rpoB*, *recA*, *atpD* and *glnII* genes showed that the strains CPS13^T, CPS1, CGS20 and CGS22 formed a cluster (Fig. 2), with internal similarities higher than 97% in the analysed genes. This cluster was related to *M. chacoense* Pr5^T (LMG 19008^T, ICMP14587^T) and *M. albiziae* LMG 23507^T with similarity values lower than 92% in *rpoB, recA* and *glnII* genes and lower than 96.5% in the *atpD* gene. The results of the phylogenetic analyses indicated that the strains CPS13^T, CPS1, CGS20 and CGS22 belong to a new species of genus *Mesorhizobium* since the distances found between the strains of this species and the remaning ones of this genus are higher than those found among most of the currently described *Mesorhizobium* species (Fig. 2).

This was confirmed by DNA-DNA hybridization experiments carried out following the method of Ezaki *et al.* [5] with the recommendations of Willems *et al*. [29]. The strain CPS13^T was hybridized with *M. albiziae* LMG 23507^T and *M. chacoense* Pr5^T showing 50% (± 9%) and 54% (± 6%) DNA-DNA relatedness, respectively. Both values are lower than the threshold value of 70% DNA-DNA similarity for definition of bacterial species [28] supporting that the *L. corniculatus* strains isolated in Granada belong to a new species of genus *Mesorhizobium.*

DNA for analysis of DNA base composition was prepared according to Chun and Goodfellow [3]. The mol % G+C content of DNA was determined using the thermal denaturation method [11]. The G+C content of strain CPS13^T was 62.7 mol %.

The cellular fatty acids were analysed by using the Microbial Identification System (MIDI; Microbial ID) Sherlock 6.1 and the library RTSBA6 according to the technical instructions provided by this system [22]. The strains were cultured aerobically on TY plates at 28ºC and cells were collected after 48h incubation. The major fatty acids of strain CPS13^T are summed feature 8 ($C_{18:1}\omega7c$/ $C_{18:1}\omega6c$) and C18:1ω7c 11-methyl as in their closest related *Mesorhizobium* species (Table 1).

The phenotypic characterization was performed using API 20NE and API ID32GN galleries inoculated according to the manufacturer's instructions and adding sterile $MgSO_4.7H_2O$ to the supplied medium up to a concentration of $0.2gl^{-1}$ with the aid of a disposable Pasteur pipette. The results were read after 7 days incubation. Growth temperature range was determined by incubating cultures in Yeast Mannitol Agar (YMA) medium at 4, 15, 28, 37 and 45ºC. Growth pH range was determined in the same medium with final pH 4.0, 6, 7, 8, 9 and 10. Salt tolerance was tested in the same medium containing 0.5, 1, 1.5, 2 and 2.5% (w/v) NaCl. To test the natural antibiotic

resistance, the disc diffusion method on YMA medium was used. The discs contained the following antibiotics: ampicillin (2 μg), erythromycin (2 μg), ciprofloxacin (5 μg), penicillin (10 IU), polymyxin (300 IU), cloxacillin (1 μg), oxytetracycline (30 μg), gentamycin (10 μg), cefuroxime (30 μg), netilmicin (30 μg) and neomycin (5 μg), (Becton Dickinson, BBL). The type strains of *M. albiziae* LMG 23507^T and *M. chacoense* Pr5^T were included in the phenotypic study as reference. Phenotypic characteristics of the new species are reported below in the species description and the differences with respect to the closest species of *Mesorhizobium* are recorded in Table 2.

Despite symbiotic genes do not offer taxonomic information because they are located in easily interchangeable elements (plasmids or symbiotic islands), the analysis of the *nodC* gene sequences allowed the identification of strains at symbiovar level [15, 18]. Rhizobial symbiovars are constituted by different symbiotic groups within a single species [18] that in the case of genus *Mesorhizobium* have been described on the basis of the *nodC* gene phylogenetic analyses [9]. In the previous work of Lorite *et al.* [10] the *nodC* gene of the type strain CPS13^T was analysed showing that it belongs to the same symbiovar that the type strain of *M. loti* LMG 6125^T (NZP 2213^T). In this work we analysed the other strains from the new species *M. olivaresii* CPS1, CGS20 and CGS22 showing that they were phylogenetically related to the strain CPS13^T after the *nodC* gene NJ and ML phylogenetic analyses (Fig. 3). These results confirmed that the strains isolated in Granada from *L. corniculatus* belong to the symbiovar loti, although they belong to a cluster phylogenetically divergent to those formed by the type strains of other *Mesorhizobium* species nodulating this host, particularly *M. jarvisii* (Fig. 3).

The results from the phylogenetic analyses of core genes, DNA-DNA hybridization experiments and phenotypic and chemotaxonomic characterization showed that the strains isolated from *L. corniculatus* nodules in Granada (Spain) represent a novel species for which we propose the name *Mesorhizobium olivaresii* sp. nov.

Description of *Mesorhizobium olivaresii* sp. nov.

Mesorhizobium olivaresii (o.li.va.res'i.i N.L. masc. gen. n. olivaresii to honour José Olivares, Spanish microbiologist, for his valuable contributions in rhizobial research).

Gram-negative, aerobic rods as for the other species of the genus. Colonies on YMA are white, circular and convex with diameter of 1-2 mm within 4-5 days at 28ºC. It grows

from 15ºC to 37ºC and optimally at 28ºC. The pH range for growth is 6.5 to 8 with optimum growth at pH 7. They grow up to 1.5% NaCl. Nitrate reduction, arginine dehydrolase and gelatinase were negative and urease and β-galactosidase were positive. Esculin hydrolysis was positive. Assimilation of glucose, L-arabinose, L-rhamnose, D-ribose, L-fucose, D-mannose, mannitol, inositol, D-sorbitol, maltose, sucrose, melibiose, valerate, 3-hydroxi-butyrate, L-histidine and L-proline was positive. Assimilation of salicin, gluconate, caprate, adipate, citrate, phenylacetate itaconate, suberate, malonate, 2 keto-gluconate, glycogen, 3 and 4 hydroxi-benzoate and L-serine was negative. Assimilation of N-acetyl-glucosamine, malate and D,L-lactate was variable. Acetate, L-alanine, 5 keto-gluconate and propionate were weakly assimilated. Sensitive to neomycin, gentamycin, netilmycin and tetracyclin and resistant to ampicillin, cefuroxime, cloxacillin, penicillin, and erythromycin. Variable results were found in the case of cyprofloxacin and polymyxin B. G+C content was 62.7 mol %. The type strain CPS13^T (=LMG 29295^T = CECT 9099^T) was isolated from root nodules of *Lotus corniculatus*.

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Figure legends

Figure 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences (1270 nucleotides) showing the position of *Mesorhizobium olivaresii* CPS13^T within genus *Mesorhizobium*. Bootstrap values calculated for 1000 replications are indicated. Bar, 1 nt substitution per 100 nt. The nodes marked with filled circles were also obtained with the maximum likelihood algorithm.

Figure 2. Neighbour-joining phylogenetic tree based on concatenated *recA* and *glnII* gene sequences (2000 nucleotides) showing the position of *Mesorhizobium olivaresii* strains within genus *Mesorhizobium*. Bootstrap values calculated for 1000 replications are indicated. Bar, 1 nt substitution per 100 nt. The nodes marked with filled circles were also obtained with the maximum likelihood algorithm.

Figure 3. Neighbour-joining phylogenetic tree based on *nodC* gene sequences (390 positions) showing the position of *Mesorhizobium olivaresii* strains within genus *Mesorhizobium*. Bootstrap values calculated for 1000 replications are indicated. Bar, 2 nt substitution per 100 nt. The nodes marked with filled circles were also obtained with maximum the likelihood algorithm.

Table 1

Table 1. Cellular fatty acid composition of *M. olivaresii* CPS13^T and its most closely related species *M. albiziae* LMG 23507^T and *M. chacoense* Pr5^T, and the type strain of the type species of the genus *Mesorhizobium*, *M. loti* NZP 2213^T.
Strains: 1, *M. olivaresii* sp. nov. CPS13^T; 2, *M. albiziae* LMG 23507^T; 3, *M. chacoense* Pr5^T; 4, *M. loti* NZP 2213^T. Fatty acids present in amounts lower than 1% are not shown. nd, not detected. Data are from this study.

	1	2	3	4
Characteristics				
$C_{16:0}$	3.4	5.1	5.3	12.1
$C_{17:0}$	3.3	0.8	2.1	1.4
$C_{18:0}$	2.7	3.1	4.7	5.6
$C_{15:0}$ iso	2.9	6.4	2.9	nd
$C_{15:0}$ iso 3 OH	3.3	2.2	4.6	nd
$C_{17:0}$ iso	5.7	5.1	8.2	4.6
$C_{17:1}\omega 8c$	3.4	0.4	1.2	nd
summed feature 3 ($C_{16:1}\omega 7c$/ $C_{16:1}\omega 6c$)	2.3	1.5	1.1	nd
summed feature 8 ($C_{18:1}\omega 7c$/ $C_{18:1}\omega 6c$)	37.6	62.8	40.3	43.6
$C_{18:1}\omega 7c$ 11-methyl	24.0	11.1	19.9	16.0
$C_{19:0}$ cyclo $\omega 8c$	8.8	0.4	8.5	16.3

Table 2

Table 2. Phenotypic differences between the new species *M. olivaresii* and its most closely related species *M. albiziae* LMG 23507^T and *M. chacoense* Pr5^T, and the type strain of the type species of the genus *Mesorhizobium*, *M. loti* NZP 2213^T.
Strains: 1, *M. olivaresii* sp. nov. CPS13^T; 2, *M. olivaresii* sp. nov. CPS1; 3, *M. olivaresii* sp. nov. CGS20; 4, *M. olivaresii* sp. nov. CGS22; 5, *M. albiziae* LMG 23507^T; 6, *M. chacoense* Pr5^T; 7, *M. loti* NZP 2213^T. +: positive, -: negative, w: weak. Data are from this study.

	1	**2**	**3**	**4**	**5**	**6**	**7**
Characteristics							
Growth at 37ºC	+	+	+	+	+	-	-
Growth in presence 2% NaCl	-	-	-	-	+	+	-
Hydrolysis of:							
PNP-β-L-arabinopyranoside	-	-	-	-	+	+	+
PNP-α-D-maltopyranoside	-	-	-	-	+	+	-
PNP-α-D-mannopyranoside	-	-	-	-	-	+	-
PNP-β-D-mannopyranoside	-	-	-	-	-	+	-
Assimilation of (API ID32GN):							
Malate	-	-	w	+	+	+	-
N-acetyl-glucosamine	-	+	+	+	+	+	+
Assimilation of (API ID32GN):							
Valerate	+	+	+	+	+	-	w
D,L-lactate	+	-	+	+	+	+	w
Alanine	-	-	-	-	+	-	-
Melibiose	+	+	+	+	+	-	+
Resistance to:							
Netilmicin	-	-	-	-	+	-	+
Penicillin	+	+	+	+	+	-	+

Figure 1

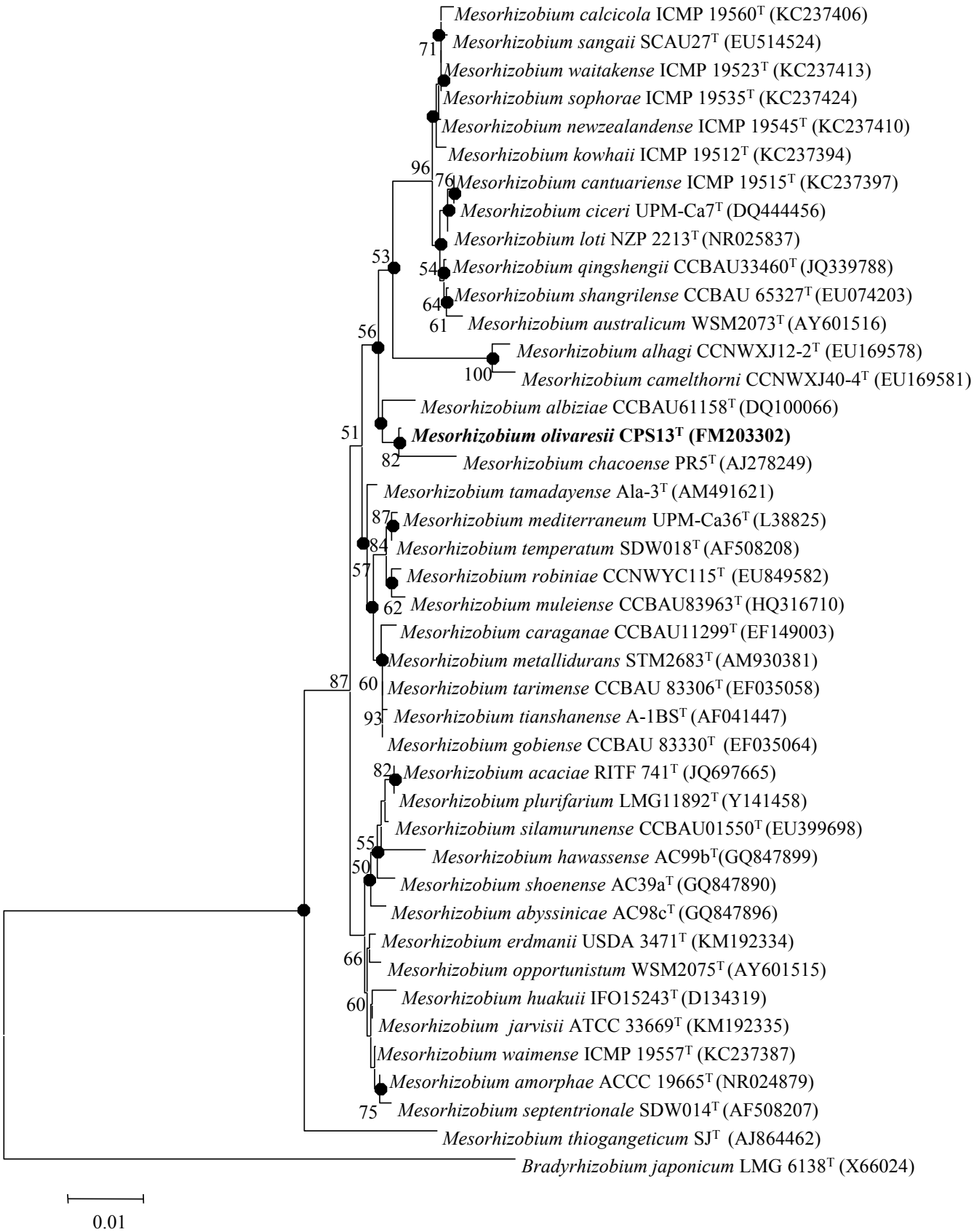

Mesorhizobium calcicola ICMP 19560^T (KC237406)
Mesorhizobium sangaii SCAU27^T (EU514524)
Mesorhizobium waitakense ICMP 19523^T (KC237413)
Mesorhizobium sophorae ICMP 19535^T (KC237424)
Mesorhizobium newzealandense ICMP 19545^T (KC237410)
Mesorhizobium kowhaii ICMP 19512^T (KC237394)
Mesorhizobium cantuariense ICMP 19515^T (KC237397)
Mesorhizobium ciceri UPM-Ca7^T (DQ444456)
Mesorhizobium loti NZP 2213^T (NR025837)
Mesorhizobium qingshengii CCBAU33460^T (JQ339788)
Mesorhizobium shangrilense CCBAU 65327^T (EU074203)
Mesorhizobium australicum WSM2073^T (AY601516)
Mesorhizobium alhagi CCNWXJ12-2^T (EU169578)
Mesorhizobium camelthorni CCNWXJ40-4^T (EU169581)
Mesorhizobium albiziae CCBAU61158^T (DQ100066)
***Mesorhizobium olivaresii* CPS13^T (FM203302)**
Mesorhizobium chacoense PR5^T (AJ278249)
Mesorhizobium tamadayense Ala-3^T (AM491621)
Mesorhizobium mediterraneum UPM-Ca36^T (L38825)
Mesorhizobium temperatum SDW018^T (AF508208)
Mesorhizobium robiniae CCNWYC115^T (EU849582)
Mesorhizobium muleiense CCBAU83963^T (HQ316710)
Mesorhizobium caraganae CCBAU11299^T (EF149003)
Mesorhizobium metallidurans STM2683^T (AM930381)
Mesorhizobium tarimense CCBAU 83306^T (EF035058)
Mesorhizobium tianshanense A-1BS^T (AF041447)
Mesorhizobium gobiense CCBAU 83330^T (EF035064)
Mesorhizobium acaciae RITF 741^T (JQ697665)
Mesorhizobium plurifarium LMG11892^T (Y141458)
Mesorhizobium silamurunense CCBAU01550^T (EU399698)
Mesorhizobium hawassense AC99b^T(GQ847899)
Mesorhizobium shoenense AC39a^T (GQ847890)
Mesorhizobium abyssinicae AC98c^T (GQ847896)
Mesorhizobium erdmanii USDA 3471^T (KM192334)
Mesorhizobium opportunistum WSM2075^T (AY601515)
Mesorhizobium huakuii IFO15243^T (D134319)
Mesorhizobium jarvisii ATCC 33669^T (KM192335)
Mesorhizobium waimense ICMP 19557^T (KC237387)
Mesorhizobium amorphae ACCC 19665^T (NR024879)
Mesorhizobium septentrionale SDW014^T (AF508207)
Mesorhizobium thiogangeticum SJ^T (AJ864462)
Bradyrhizobium japonicum LMG 6138^T (X66024)
71
96
76
53
54
64
61
56
100
51
82
87
84
57
62
87
60
93
82
55
50
66
60
75
0.01

Figure 2

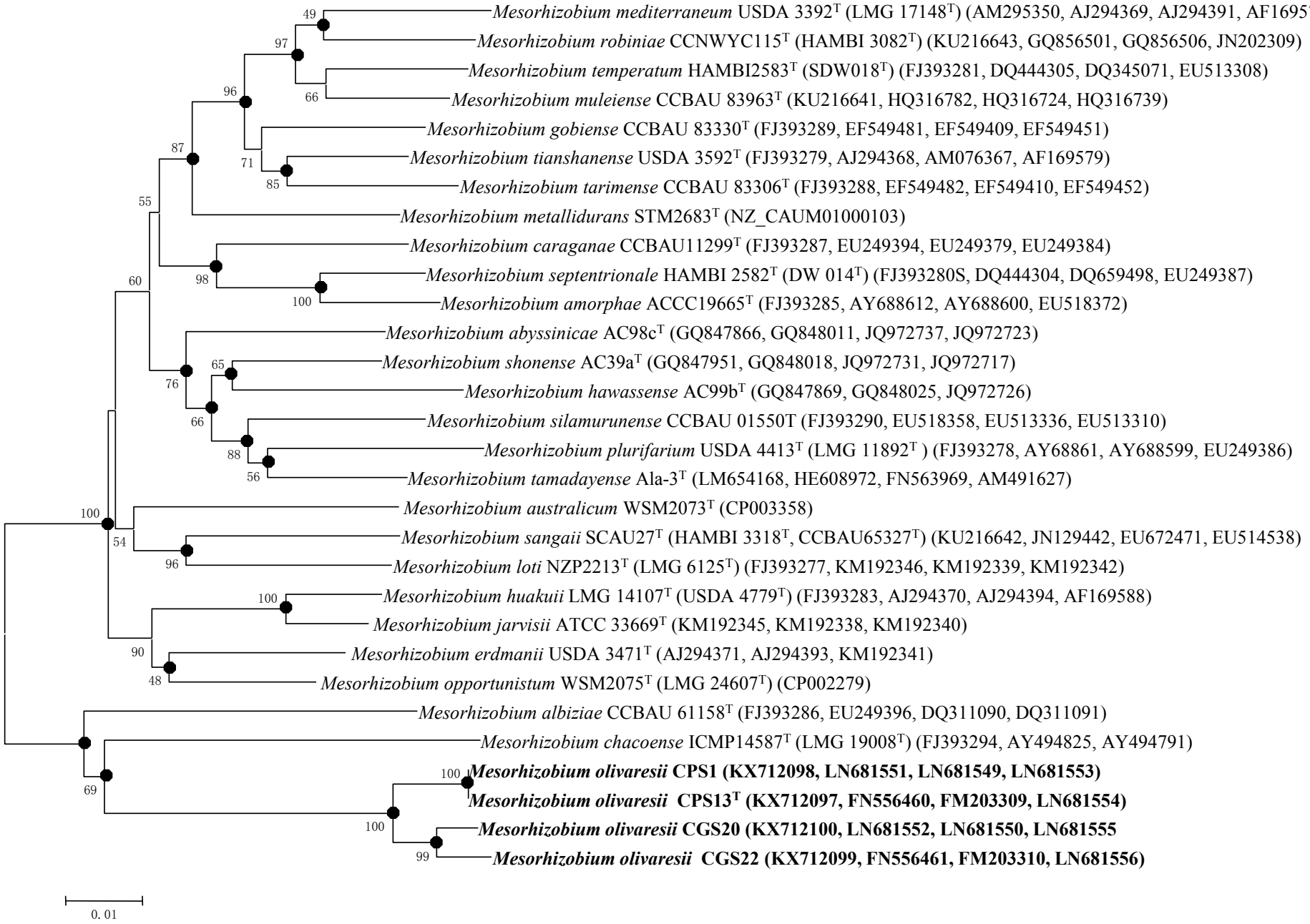
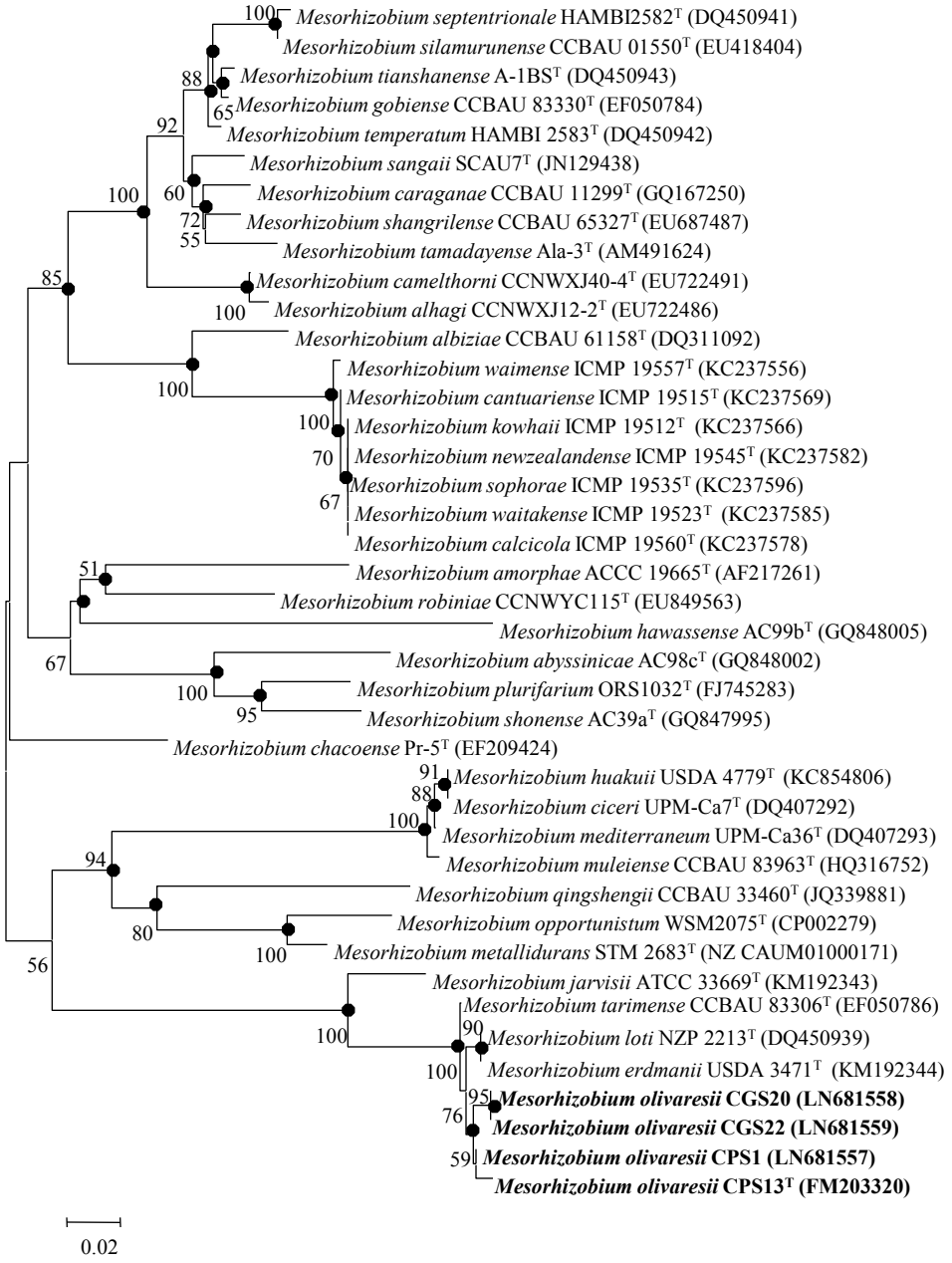

Mesorhizobium mediterraneum USDA 3392^{T} (LMG 17148^{T}) (AM295350, AJ294369, AJ294391, AF169578)
Mesorhizobium robiniae CCNWYC115^{T} (HAMBI 3082^{T}) (KU216643, GQ856501, GQ856506, JN202309)
Mesorhizobium temperatum HAMBI2583^{T} (SDW018^{T}) (FJ393281, DQ444305, DQ345071, EU513308)
Mesorhizobium muleiense CCBAU 83963^{T} (KU216641, HQ316782, HQ316724, HQ316739)
Mesorhizobium gobiense CCBAU 83330^{T} (FJ393289, EF549481, EF549409, EF549451)
Mesorhizobium tianshanense USDA 3592^{T} (FJ393279, AJ294368, AM076367, AF169579)
Mesorhizobium tarimense CCBAU 83306^{T} (FJ393288, EF549482, EF549410, EF549452)
Mesorhizobium metallidurans STM2683^{T} (NZ_CAUM01000103)
Mesorhizobium caraganae CCBAU11299^{T} (FJ393287, EU249394, EU249379, EU249384)
Mesorhizobium septentrionale HAMBI 2582^{T} (DW 014^{T}) (FJ393280S, DQ444304, DQ659498, EU249387)
Mesorhizobium amorphae ACCC19665^{T} (FJ393285, AY688612, AY688600, EU518372)
Mesorhizobium abyssinicae AC98c^{T} (GQ847866, GQ848011, JQ972737, JQ972723)
Mesorhizobium shonense AC39a^{T} (GQ847951, GQ848018, JQ972731, JQ972717)
Mesorhizobium hawassense AC99b^{T} (GQ847869, GQ848025, JQ972726)
Mesorhizobium silamurunense CCBAU 01550T (FJ393290, EU518358, EU513336, EU513310)
Mesorhizobium plurifarium USDA 4413^{T} (LMG 11892^{T}) (FJ393278, AY68861, AY688599, EU249386)
Mesorhizobium tamadayense Ala-3^{T} (LM654168, HE608972, FN563969, AM491627)
Mesorhizobium australicum WSM2073^{T} (CP003358)
Mesorhizobium sangaii SCAU27^{T} (HAMBI 3318^{T}, CCBAU65327^{T}) (KU216642, JN129442, EU672471, EU514538)
Mesorhizobium loti NZP2213^{T} (LMG 6125^{T}) (FJ393277, KM192346, KM192339, KM192342)
Mesorhizobium huakuii LMG 14107^{T} (USDA 4779^{T}) (FJ393283, AJ294370, AJ294394, AF169588)
Mesorhizobium jarvisii ATCC 33669^{T} (KM192345, KM192338, KM192340)
Mesorhizobium erdmanii USDA 3471^{T} (AJ294371, AJ294393, KM192341)
Mesorhizobium opportunistum WSM2075^{T} (LMG 24607^{T}) (CP002279)
Mesorhizobium albiziae CCBAU 61158^{T} (FJ393286, EU249396, DQ311090, DQ311091)
Mesorhizobium chacoense ICMP14587^{T} (LMG 19008^{T}) (FJ393294, AY494825, AY494791)
***Mesorhizobium olivaresii* CPS1 (KX712098, LN681551, LN681549, LN681553)**
***Mesorhizobium olivaresii* CPS13^{T} (KX712097, FN556460, FM203309, LN681554)**
***Mesorhizobium olivaresii* CGS20 (KX712100, LN681552, LN681550, LN681555**
***Mesorhizobium olivaresii* CGS22 (KX712099, FN556461, FM203310, LN681556)**
0. 01

Figure 3


100 Mesorhizobium septentrionale HAMBI2582T (DQ450941)
Mesorhizobium silamurunense CCBAU 01550T (EU418404)
88 Mesorhizobium tianshanense A-1BST (DQ450943)
65 Mesorhizobium gobiense CCBAU 83330T (EF050784)
92 Mesorhizobium temperatum HAMBI 2583T (DQ450942)
Mesorhizobium sangaii SCAU7T (JN129438)
100 60 Mesorhizobium caraganae CCBAU 11299T (GQ167250)
72 Mesorhizobium shangrilense CCBAU 65327T (EU687487)
55 Mesorhizobium tamadayense Ala-3T (AM491624)
85 Mesorhizobium camelthorni CCNWXJ40-4T (EU722491)
100 Mesorhizobium alhagi CCNWXJ12-2T (EU722486)
Mesorhizobium albiziae CCBAU 61158T (DQ311092)
100 Mesorhizobium waimense ICMP 19557T (KC237556)
Mesorhizobium cantuariense ICMP 19515T (KC237569)
100 Mesorhizobium kowhaii ICMP 19512T (KC237566)
70 Mesorhizobium newzealandense ICMP 19545T (KC237582)
Mesorhizobium sophorae ICMP 19535T (KC237596)
67 Mesorhizobium waitakense ICMP 19523T (KC237585)
Mesorhizobium calcicola ICMP 19560T (KC237578)
51 Mesorhizobium amorphae ACCC 19665T (AF217261)
Mesorhizobium robiniae CCNWYC115T (EU849563)
Mesorhizobium hawassense AC99bT (GQ848005)
67 Mesorhizobium abyssinicae AC98cT (GQ848002)
100 Mesorhizobium plurifarium ORS1032T (FJ745283)
95 Mesorhizobium shonense AC39aT (GQ847995)
Mesorhizobium chacoense Pr-5T (EF209424)
91 Mesorhizobium huakuii USDA 4779T (KC854806)
88 Mesorhizobium ciceri UPM-Ca7T (DQ407292)
100 Mesorhizobium mediterraneum UPM-Ca36T (DQ407293)
94 Mesorhizobium muleiense CCBAU 83963T (HQ316752)
Mesorhizobium qingshengii CCBAU 33460T (JQ339881)
80 Mesorhizobium opportunistum WSM2075T (CP002279)
100 Mesorhizobium metallidurans STM 2683T (NZ CAUM01000171)
56 Mesorhizobium jarvisii ATCC 33669T (KM192343)
Mesorhizobium tarimense CCBAU 83306T (EF050786)
100 90 Mesorhizobium loti NZP 2213T (DQ450939)
100 Mesorhizobium erdmanii USDA 3471T (KM192344)
95 Mesorhizobium olivaresii CGS20 (LN681558)
76 Mesorhizobium olivaresii CGS22 (LN681559)
59 Mesorhizobium olivaresii CPS1 (LN681557)
Mesorhizobium olivaresii CPS13T (FM203320)
0.02