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Microvirga tunisiensis sp. nov., a root nodule symbiotic bacterium isolated from *Lupinus micranthus* and *L. luteus* grown in Northern Tunisia

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ABSTRACT

Three bacterial strains, LmiM8^T, LmiE10 and LluTb3, isolated from nitrogen-fixing nodules of *Lupinus micranthus* (Lmi strains) and *L. luteus* (Llu strain) growing in Northern Tunisia were analysed using genetic, phenotypic and symbiotic approaches. Phylogenetic analyses based on *rrs* and concatenated *gyrB* and *dnaK* genes suggested that these *Lupinus* strains constitute a new *Microvirga* species with identities ranging from 95 to 83% to its closest relatives *Microvirga makkahensis, M. vignae, M. zambiensis*, *M. ossetica,* and *M. lotononidis*. The genome sequences of strains LmiM8^T and LmiE10 exhibited pairwise Average Nucleotide Identities (ANIb) above 99.5%, significantly distant (73–89% pairwise ANIb) from other *Microvirga* species sequenced (*M. zambiensis* and *M. ossetica*). A phylogenetic analysis based on the symbiosis-related gene *nodA* placed the sequences of the new species in a divergent clade close to *Mesorhizobium, Microvirga* and *Bradyrhizobium* strains, suggesting that the *M. tunisiensis* strains represent a new symbiovar different from the *Bradyrhizobium* symbiovars defined to date. In contrast, the phylogeny derived from another symbiosis-related gene, *nifH*, reproduced the housekeeping genes phylogenies. The study of morphological, phenotypical and physiological features, including cellular fatty acid composition of the novel isolates demonstrated their unique profile regarding close reference *Microvirga* strains. Strains LmiM8^T, LmiE10 and LluTb3 were able to nodulate several *Lupinus* spp. Based on genetic, genomic and phenotypic data presented in this study, these strains should be grouped within a new species for which the name *Microvirga tunisiensis* sp. nov. is proposed (type strain LmiM8^T = CECT 9163^T, LMG 29689^T).

Introduction

Several *Lupinus* spp. are widespread in the Mediterranean basin and are mainly nodulated, as are most lupines, by slow-growing rhizobial strains classified in the genus *Bradyrhizobium* [3,6,32]. Recently, it has been shown that endosymbionts isolated from nodules of *Lupinus micranthus* plants grown in Algeria and Spain (100% of 101 isolates) belonged to the *Bradyrhizobium* genus [10]. In Northern Tunisia, Msaddak et al. [25] reported that this legume is nodulated by bacteria belonging to the *Bradyrhizobium* (28 out of 50 isolates), *Microvirga* (20 isolates) and *Phyllobacterium* (2 out of 50 isolates) genera. The same authors obtained a collection of 43 isolates of *L. luteus* from the same region, out of which 41 belonged to the genus *Bradyrhizobium* and just two to *Microvirga* [26]. The genus *Microvirga* currently comprises eighteen species [1,4,11,13,20,21,27,30,33,34,36,37,38], of which only four have been described as effective root nodule bacteria: *M. lotononidis* and *M. zambiensis* isolated from *Listia angolensis* nodules [4], *M. lupini*, from *Lupinus texensis* [4] and *M. vignae* from *Vigna unguiculata* [27].

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Through in-depth analysis of genetic, genomic and phenotypic data, we show here that *Microvirga* strains isolated from the root nodules of Northern Tunisian *L. micranthus* and *L. luteus* plants are representatives of a new symbiotic species that we propose to designate as *Microvirga tunisiensis*.

Material and methods

The three *Microvirga* strains used in this work, LmiM8, LmiE10 and LluTb3, were isolated from *Lupinus micranthus* (Lmi strains) and *L. luteus* (Llu strain) grown in three different sites in Northern Tunisia, Mraissa, El Alia and Tabarka respectively [25,26]. These strains were selected as representatives from a previously reported collection of 22 root-nodule *Microvirga* isolates, 20 from *L. micranthus* [25] and 2 from *L. luteus* [26]. The strains were cultured in yeast mannitol agar (YMA, [35]) or tryptone yeast (TY, [7]) media at 28 °C. Cultures were conserved in glycerol (20%) at −80 °C for long-term storage.

Genomic DNA preparation, DNA fragment amplification and Sanger sequencing were performed as previously described [25]. Sequences were edited and assembled with Geneious Pro 5.6.5 (Biomatters Ltd., Auckland, New Zealand) and were deposited in GenBank (accession numbers listed in Figs. 1–3). Phylogenetic reconstructions were performed with MEGA7 [23] using the CLUSTALW implementation for alignment [12]. Trees were inferred by the maximum likelihood (ML) method with default parameters provided by MEGA. Previous to inference, model testing was carried out within MEGA7 for each alignment, and the most favoured model was used. Tree topologies were tested using 1000 bootstrap replicates.

Genomes were sequenced externally at the Arizona State University Genomic Core Facility, Tempe, AZ, using Illumina MiSeq technology (Illumina MiSeq v.3, 2 × 300 PE libraries, over 2 million reads). Initial read quality control and adapter-trimming were done at the sequencing facility using Illumina utilities. Once received, reads were quality filtered in-house with Trimmomatic [8], assembled with SPAdes v 3.5.0 [5], and draft assemblies submitted to Genbank (Bioprojects PRJNA558674 and PRJNA558675 for LmiM8 and LmiE10, respectively). Pairwise average nucleotide identities (ANI) from genome sequences of Lmi strains and type strains of species of the genus *Microvirga* available in the GenBank database were determined using the JSpeciesWS server [28] and the BLAST algorithm (ANIb).

Results and discussion

Phylogenies of *rrs*, and concatenated *gyrB* and *dnaK* housekeeping genes from strains belonging to different *Microvirga* species are shown in Figs. 1 and 2, respectively. The three strains described in this work, isolated in locations separated by more than 100 km, had identical sequences for the three genes. *M. tunisiensis* strains appeared as a clade clearly differentiated from all the described *Microvirga* species. When the phylogenies based on the *rrs* gene or on the concatenation of *gyrB* and *dnaK* were compared, some differences were observed. On the one hand, the closest species in the analysis based on *rrs* gene were *M. makkahensis* and *M. vignae* (99 to 97% nucleotide identity; Fig. 1) whereas in the phylogeny based on the two concatenated genes the closest species were *M. ossetica*, *M. zambiensis* and *M. lotononidis* (85 to 83% amino acid identity; Fig. 2).

Genes responsible for symbiosis with the legume host can be transmitted horizontally and their phylogeny does not usually correspond to that of their bacterial host (reviewed in [3]). A phylogenetic tree based on symbiotic gene *nodA* revealed that the *M. tunisiensis* sequences cluster in a distinct branch, distantly related to sequences from a disparate group of root nodule bacteria nodulating diverse legumes, including *M. vignae* [27], *M. lotononidis* [4], *M. zambiensis* [4], *M. lupini* [4], several species of *Bradyrhizobium* [15–17,19], *Rhizobium giardinii* [2], and *Mesorhizobium plurifarium* [14] (Fig. 3A). This suggests that *nod* genes, responsible for determining the specificity of the plant-bacterial symbiosis [3], may have indeed been recently acquired by *M. tunisiensis* strains through horizontal transfer from unrelated root nodule bacteria [24]. Notably, the phylogeny inferred from another, highly conserved symbiotic gene, *nifH*, one of the structural genes for the enzyme nitrogenase [9], reproduced the species phylogeny, suggesting that *nif* genes, on the contrary, were anciently acquired by *M. tunisiensis* (Fig. 3B).

The genomes of *L. micranthus* strains LmiM8^T and LmiE10 were sequenced to a draft level (908 and 851 contigs above 1 kb, respectively; Table 1). They were highly similar and were also similar in size and composition to the genome of *M. ossetica* V5/3M^T, the only completely sequenced *Microvirga* genome, although this is a non-symbiotic strain with a larger complement of plasmids, which probably explains its larger genome (Table 1). The *L. micranthus* LmiM8^T and LmiE10 genomes exhibited pairwise ANIb values above 99.5% (Table 2) and were well separated from other described *Microvirga* species, with ANIb values ranging from below 89% for their closest relative *M. ossetica* V5/3M^T, to under 73% for *M. massiliensis* JC119^T, in all cases well below the 95–96 % threshold commonly used for species circumscription. Included among these was *M. lupini* Lut6^T, a strain nodulating *L. texensis* [4].

Table 1
Genomic features of *M. tunisiensis* strains.

	M. tunisiensis M8	*M. tunisiensis* E10	*M. ossetica* V5/3M
Assembly quality	Draft	Draft	Complete
Estimated genome size (Mb)	9.35	8.98	9.63
Average coverage	106	95	---[f]
N50 (kb)	114	104	---[f]
Contigs >1 kb	908	851	---[f]
GC (%)	61.77	61.76	62.67
rRNA operons[a]	4	4	4
tRNAs[b]	59 (+12)[c]	59 (+12)[c]	59 (+12)[c]
nod/nif symbiotic genes[d]	+	+	–
Plasmids[e]	1	1	5

[a] Since rRNA operon copies are identical or almost identical, in draft assemblies they collapse into a higher coverage consensus sequence. The number of copies was estimated from the excess read coverage of the assembled rRNA region over the average genome read coverage.
[b] Numbers of tRNAs associated to rRNA operons (three tRNAs per operon) are shown in brackets.
[c] As estimated by the tRNAscan-SE server (http://lowelab.ucsc.edu/tRNAscan-SE/).
[d] Presence or absence was estimated from results of BLAST hits against the phylogenetically close (see Fig. 3) symbiotic region from the *Mesorhizobium ciceri* genome (accession number NZ_CM002796.1).
[e] In draft sequences, this was estimated as the number of unique BLAST hits against the *M. ossetica* V5/3M plasmid replication *repAB* genes.
[f] Not applicable.

Table 2
Pairwise Average Nucleotide Identity (ANIb) among genome sequences of *M. tunisiensis* strains LmiM8^T and LmiE10 and other *Microvirga* type strains.

Strain	LmiM8^T	LmiE10
M. tunisiensis Lmi M8^T	–	99.66
M. tunisiensis Lmi E10	99.96	–
M. ossetica V5/3M^T	88.16	88.22
M. lupini Lut6^T	84.59	84.65
M. lotononidis WSM3557^T	83.86	83.84
M. flocculans ATCC BAA-817^T	82.56	85.55
M. vignae BR3299^T	79.36	79.35
M. massiliensis JC119^T	72.40	72.47

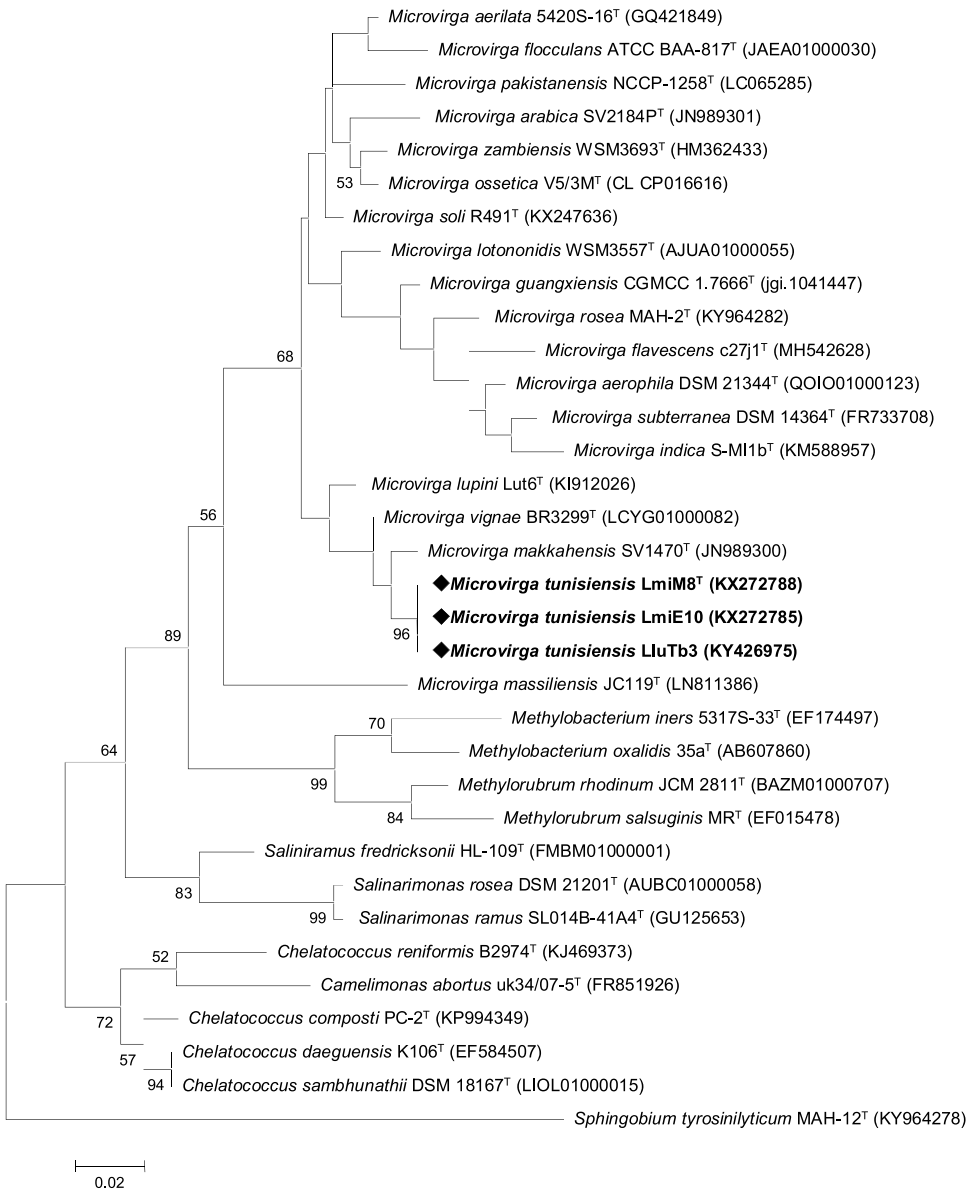

Fig. 1. Maximum Likelihood phylogenetic tree of *M. tunisiensis* isolates (highlighted in bold) and related species based on near-complete *rrs* gene sequences (1300 nt). Bootstrap values (1000 replications) above 50% are shown. GenBank accession numbers are shown in brackets. Bar, 0.02 substitutions per nucleotide position. *Sphingobium tyrosinilyticum* MAH-12^T was used as outgroup.

Phenotypic characterization of *M. tunisiensis* strains was based on characteristics previously shown to be useful for lupine microsymbionts and *Microvirga* species differentiation [16,19,38] (Table 3). The effects of temperature and pH on growth of the strains were determined by incubating cultures in YMA at 20, 25, 28, 37, 40 and 45 °C, and at pH ranging from 4.0 to 12, respectively. The optimum pH was 7–8 as it has been shown for other *Microvirga* species. *M. tunisiensis*, *M. ossetica* and *M. arabica* grew better at 28 °C whereas *M. zambiensis*, *M. lupini* and *M. lotononidis* preferred higher temperatures (35–41 °C). Salt tolerance was tested by adding NaCl to YM medium. *M. tunisiensis* strains were unable to grow with 1% NaCl (w/v), while several *Microvirga* spp. can tolerate up to 4% (Table 3). Natural antibiotic resistance was tested on YMA plates containing the antibiotics indicated in Table 3. Strains LmiM8^T, LmiE10 and LluTb3 were very sensitive to ampicillin, gentamycin, tetracycline, spectinomycin, kanamycin and streptomycin and resistant to nalidixic acid (Table 3). Six carbon sources were utilized by all the *M. tunisiensis* strains, a trait shared with *M. lupini* and *M. lotononidis*. *M. ossetica* and *M. arabica* were unable to use saccharose, as was *M. zambiensis*, a species that was also unable to use L-arabinose (Table 3).

Cellular fatty acid composition analyses for the isolates LmiM8^T, LmiE10 and the type strains of other *Microvirga* species were performed at the Spanish Type Culture Collection (Colección Española de Cultivos Tipo, CECT, Paterna, Valencia) (Table 4). Cultures were collected after growing aerobically on TY medium at 28 °C for five days. Fatty acid methyl esters were prepared and resolved using as described by Sasser [31], and identified with the MIDI (2008) Sherlock Microbial Identification System (version 6.1), using theTSBA6 database at CECT. Fatty acids composition of strains LmiM8^T and LmiE10 were very similar (Table 4). A total of 8 fatty acids were detected in strain LmiM8^T. The higher percentages were found for $C_{16:0}$ (5.97), $C_{18:0}$ (1.27) and $C_{19:0}$ cyclo w 8*c* (7.64). Summed features 2 (2.72 %), 3 (6.13 %) and 8 (73.05 %) comprising, respectively: 1) 14:0 3OH/16:1 iso I/12:0 aldehyde; 2) 16:1 ω7c/6:1 ω6c; and 3) 18:1 ω7c/18:1 ω6c, were also relatively abundant. These data

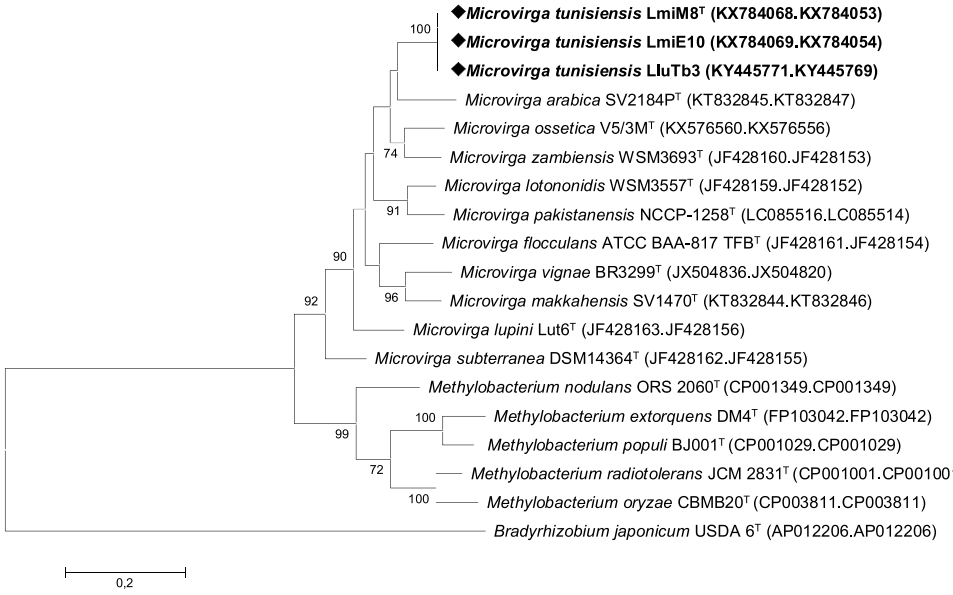

Fig. 2. Maximum Likelihood phylogenetic tree of *M. tunisiensis* isolates (highlighted in bold) and reference strains based on amino acyl sequences derived from concatenated *gyrB* (616 bp) and *dnaK* (665 bp) gene sequences. Bootstrap values were calculated for 1000 replications and those greater than 70% are shown at the internodes. GenBank accession numbers are shown in brackets. Bar, 0.2 substitutions per position. *B. japonicum* USDA6 was included as outgroup.

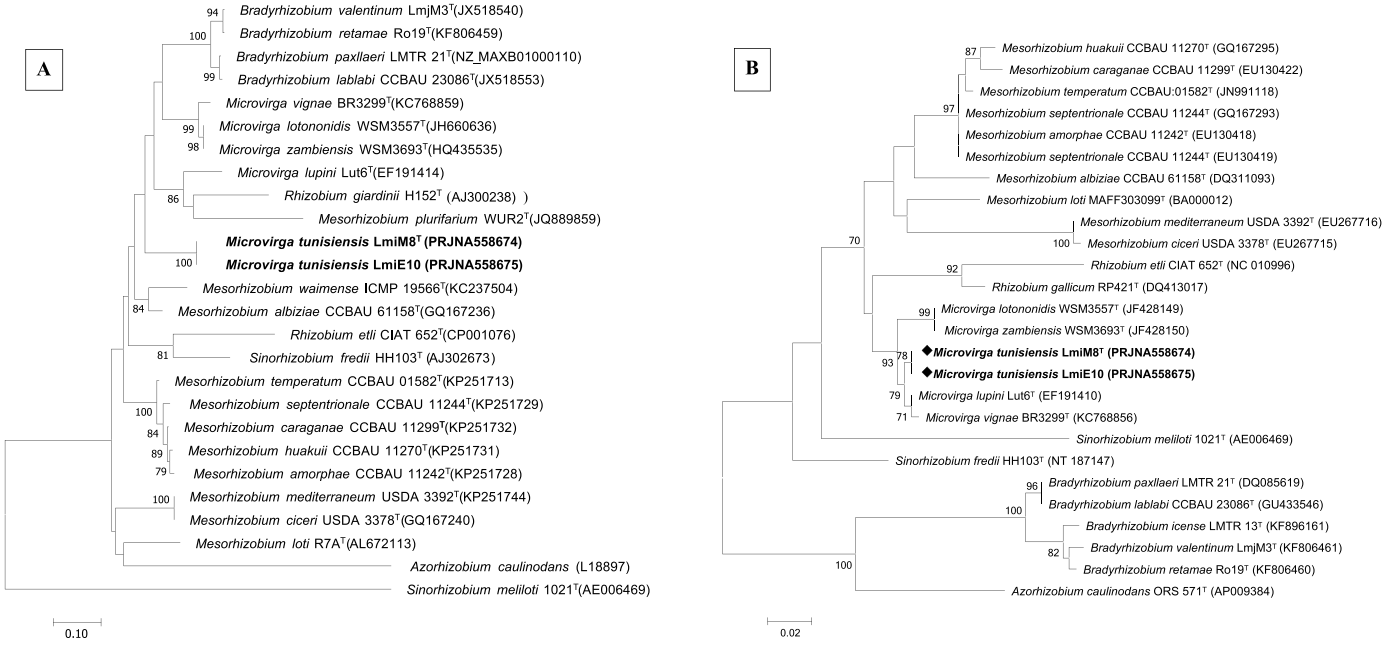

Fig. 3. Maximum Likelihood phylogenetic tree of *M. tunisiensis* isolates (highlighted in bold) and reference strains based on *nodA* (panel A) or *nifH* (panel B) gene sequences. Only bootstrap values (1000 replications) above 70% are indicated at the internodes. GenBank accession numbers are shown in brackets. Bars, 0.1 and 0.02 substitutions per nucleotide position, respectively. *Azorhizobium caulinodans* ORS571 (panel A) and *Sinorhizobium meliloti* 1021 (panel B) were included as outgroups.

were compared to those from the closest *Microvirga* species type strains (*M. ossetica, M. arabica, M. makkahensis* and *M. zambiensis*) (Table 4), and differences among the five strains at the level of percentages and in the presence/absence of certain fatty acids were found. Among other, we can point out that LmiM8 and *M. ossetica* differed in 5 fatty acids, 2 present only in LmiM8, and 3 only present in *M. ossetica*. *M. arabica* and *M. makkhaensis* lacked $C_{19:0}$ cyclo ω8c, which is abundant in the rest of the strains. *M. zambiensis* differed from the rest by having $C_{20:0}$ ω6,9c, and also differed from LmiM8 by having $C_{14:0}$ and $C_{18:1}$ ω7c 11-methyl. The obtained patterns are consistent with previous reports for *Microvirga* strains [30,34] and observed differences may be due to the different cultivation conditions used.

Several *Microvirga* species can form effective nodules with legumes. *M. tunisiensis* strains were isolated from *Lupinus micranthus* and *L. luteus*, two legumes usually nodulated by members of the genus *Bradyrhizobium* [10,26]. Many bradyrhizobia [22] and lupines [18] prefer acidic soils. Therefore, it is interesting that *M. tunisiensis* strains adapted to growth at higher pH (Table 3), effectively nodulate lupines in alkaline soils from Tunisia. Symbiotic characteristics of these *M. tunisiensis* strains were examined by means of cross-inoculation tests (Table 5). Effective, N_2-fixing nodules were observed in *L. micranthus*, *L. luteus*, *L. angustifolius* and

Table 3
Phenotypic differences of *M. tunisiensis* strains as compared to closely-related *Microvirga* species type strains.

Characteristic	*M.Tunisiensis*[a]	*M. Ossetica*[b]	*M. zambiensis*[c]	*M. lupini*	*M. Arabica*[d]	*M.lotononidis*
Isolation source	Root nodule	Root nodule	Root nodule	Root nodule	Soil sample	Root nodule
Colony colour	Pink	Transparent	Cream	Pale orange	Pink	Light pink
Temperature for growth (°C)						
Optimum	28	28	35	39	28–30	41
Range	20–37	20–37	15–38	10–43	20–37	15–44
pH for growth						
Optimum	6.8–8.0	ND	7–8.5	7.0–8.5	7	7.0–8.5
Range	4–12	ND	6–9.5	4–12	6–9	4–12
Salt tolerance:						
1% (w/v) NaCl	–	+	–	+	+	+
2% (w/v) NaCl	–	+	–	–	–	+
4% (w/v) NaCl	–	+	–	–	–	ND
Symb. N_2 fixation	+	–	+	+	ND	+
Resistance to antibiotics ($\mu g/mL^{-1}$)						
Ampicillin (100)	–	ND	ND	–	ND	–
Gentamycin (30)	–	ND	ND	–	ND	±
Tetracycline (5)	–	ND	ND	–	ND	–
Spectinomycin (50)	–	ND	ND	±	ND	±
Kanamycin (50)	–	ND	–	–	ND	±
Chloramphenicol (20)	–	ND	ND	±	ND	±
Nalidixic acid (20)	+	ND	ND	±	ND	+
Rifampicin (5)	–	ND	ND	–	ND	+
Streptomycin (10)	–	ND	ND	+	ND	–
Utilization of C sources						
Mannitol	+	–	+	+	–	+
D-glucose	+	+	+	+	+	+
D-galactose	+	ND	ND	+	ND	+
L-arabinose	+	ND	–	+	+	+
D-fructose	+	+	+	+	ND	+
Saccharose	+	–	–	+	–	+

+, Positive; -, negative; ±, weakly positive.
ND, Not Determined.
[a] All three *M. tunisiensis* strains exhibited the same phenotype.
[b] Data taken from Safranova et al. [30].
[c] Data taken from Ardley et al. [4].
[d] Data taken from Veyisoglu et al. [34].

Table 4
Fatty acid composition of *Microvirga tunisiensis* LmiM8^T and related strains.

Fatty acid/strains	1	2	3	4	5	6
12:0	–	–	0.87	–	–	–
14:0	–	–	0.71	0.48	0.44	0.60
16:0	5.97	6.38	8.23	9.80	8.79	10.56
15:0 3OH	–	–	–	–	0.30	–
17:1 ω8c	0.46	0.68	–	–	0.71	–
17:1 ω6c	–	–	–	–	0.95	–
17:0 cyclo	0.81	1.20	–	–	1.37	2.13
17:0	0.58	1.04	1.64	1.27	2.27	1.02
18:0	1.27	1.06	3.30	3.71	2.07	2.45
18:1 ω7c 11-methyl	–	–	–	0.72	–	0.84
18:1 ω9c	–	–	1.04	–	–	–
19:0 cyclo ω8c	7.64	7.95	6.18	–	–	19.02
19:0 10-methyl	0.95	0.97	1.08	1.18	0.96	0.69
18:0 3OH	0.43	0.51	1.03	1.17	1.67	1.55
20:0 ω6,9c	–	–	–	–	–	0.64
Summed feature 2	2.72	2.75	3.41	3.22	2.90	4.01
Summed feature 3	6.13	6.82	3.12	5.34	4.25	2.73
Summed feature 8	73.05	70.63	69.39	73.12	65.35	53.74

Strains: 1, *M. tunisiensis* LmiM8^T; 2,*M. tunisiensis* LmiE10; 3,*M. ossetica* V5/3M^T ; 4, *M. arabica* SV2184P^T; 5, *M. makkhaensis* SV1470^T ; 6, *M. zambiensis* WSM3693^T. Summed Feature 2 comprises 14:0 3OH/16 :1 iso I/12 :0 aldehyde ; Summed Feature 3 comprises 16:1 ω7c/16:1 ω6c; Summed feature 8 comprises 18:1 ω7c/18:1 ω6c.

Macroptilium atropurpureum plants inoculated with the three *M. tunisiensis* strains; in contrast, only white nodules were observed when these strains were used to inoculate *L. mariae-josephae* (very low nitrogen fixation activity) or *Vigna unguiculata* (no nitrogen fixation activity) (Table 5).

Description of M. tunisiensis *sp. nov*

Microvirga tunisiensis (tu.ni.si.en'sis. N.L. fem. adj. *tunisiensis* pertaining to Tunisia, where the type strain was isolated).

Table 5
Legume host-range analysis of *M. tunisiensis* strains.

Legume	*Lupinus micranthus*	*L. luteus*	*L. angustifolius*	*L. mariae-josephae*	*V. unguiculata*	*M. atropurpureum*	*Lupinus micranthus*	*L. luteus*	*L. angustifolius*	*L. mariae-josephae*	*V. unguiculata*	*M. atropurpureum*
Strains	Nod	NF	Nod	NF	Nod	NF	Nod	NF	Nod	NF	Nod	NF
LmiM8^T	+	20.0	+	15.4	+	3.7	+W	0.3	+W	0	+	12.5
LmiE10	+	18.4	+	14.4	+	9.1	+W	0.2	+W	0	+	6.4
LluTb3	+	15.2	+	17.5	+	ND	+W	0	+W	0	+	ND

Nod: (+) red nodules, (+W) white nodules. Nitrogen fixation (NF) was determined by the acetylene reduction test and expressed as μmol of acetylene reduced × (h × g of nodules)$^{-1}$. Values are the average of two replicates and standard deviations were less than 15%.

The isolates were obtained from surface-sterilized, effective nodules from *Lupinus* plants grown in Tunisian soils, and grown on Yeast Mannitol Agar (YMA). They are Gram negative, non spore-forming rods, with mean generation times of 5 h in TY. Colonies grown at 28 °C for 4 days on YMA medium are circular, beige, opaque, and 2 mm in diameter. After 8 days of growth a brown/pinky spot is observed at the centre of the colony. Optimum growth occurs at pH 6.8–8.0 (range 4 to 12). They do not tolerate 1% NaCl (w/v) and are sensitive to ampicillin, chloramphenicol, gentamicin and kanamycin, while they are resistant to nalidixic acid. They utilize D-mannitol, D-glucose, D-galactose, L-arabinose, D-fructose and saccharose as carbon sources. Eight fatty acids were detected in LmiM8^T, and $C_{19:0}$ cyclo ω8c, summed feature 8 ($C_{18:1}$ ω7c/18:1 ω6c) and $C_{16:0}$ were the predominant species.

M. tunisiensis strains are genomically divergent from the closest species within the *Microvirga* genus, sharing less than 89% ANI with their closest relative, *Microvirga ossetica* V5/3M^T. They establish diazotrophic root nodule symbioses with *Lupinus micranthus*, *L. luteus*, *L. angustifolius* and *Macroptilium atropurpureum* but not with *Vigna unguiculata* or *L. mariae-josephae*. The type strain Lmi M8^T (CECT 9163^T = LMG 29689^T) was isolated from *L. micranthus* nodules collected from Mraissa, Tunisia.

The Digital Protologue TaxoNumber (Rosselló-Móra et al. [29]) for strain LmiM8^T is TA00627.

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Conflict of interest

The authors declare that there are not conflicts of interest.

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