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RESEARCH ARTICLE

Rhizobium leguminosarum symbiovar *trifolii*, *Ensifer numidicus* and *Mesorhizobium amorphae* symbiovar *ciceri* (or *Mesorhizobium loti*) are new endosymbiotic bacteria of *Lens culinaris* Medik

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One sentence summary: *Rhizobium leguminosarum* sv. *trifolii*, *Ensifer numidicus* and *Mesorhizobium amorphae* (or *M. loti*) isolates species, not considered, up to now, as a natural symbiont of lentil are reported.

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ABSTRACT

A total of 142 rhizobial bacteria were isolated from root nodules of *Lens culinaris* Medik endemic to Tunisia and they belonged to the species *Rhizobium leguminosarum,* and for the first time to *Ensifer and Mesorhizobium*, genera never previously described as microsymbionts of lentil. Phenotypically, our results indicate that *L. culinaris* Medik strains showed heterogenic responses to the different phenotypic features and they effectively nodulated their original host. Based on the concatenation of the 16S rRNA with relevant housekeeping genes (*gln*A, *rec*A, *dna*K), rhizobia that nodulate lentil belonged almost exclusively to the known *R. leguminosarum* sv. *viciae*. Interestingly, *R. leguminosarum* sv. *trifolii*, *Ensifer numidicus* (10 isolates) and *Mesorhizobium amorphae (*or *M. loti)* (9 isolates) isolates species, not considered, up to now, as a natural symbiont of lentil are reported. The *E. numidicus* and *M. amorphae* (or *M. loti*) strains induced fixing nodules on *Medicago sativa* and *Cicer arietinum* host plants, respectively. Symbiotic gene phylogenies showed that the *E. numidicus*, new symbiont of lentil, markedly diverged from strains of *R. leguminosarum*, the usual symbionts of lentil, and converged to the symbiovar *meliloti* so far described within *E. meliloti*. Indeed, the *nodC* and *nodA* genes from the *M. amorphae* showed more than 99% similarity with respect to those from *M. mediterraneum*, the common chickpea nodulating species, and would be included in the new infrasubspecific division named *M. amorphae* symbiovar *ciceri,* or to *M. loti,* related to the strains able to effectively nodulate *C. arietinum* host plant. On the basis of these data, *R. leguminosarum* sv. *trifolii* (type strain LBg_3^T), *M. loti* or *M. amorphae* sv. *ciceri* (type strain LB_4^T) and *E. numidicus* (type strain LBi_2^T) are proposed as new symbionts of *L. culinaris* Medik.

Keywords: lentil; *Ensifer numidicus*; *Mesorhizobium amorphae*; *Rhizobium leguminosarum* sv. *trifolii*; symbiotic gene; Tunisia

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INTRODUCTION

Lentil (*Lens culinaris* Medik) is an important and popular legume mainly in Central and Southwest Asia, Southern Europe, North Africa and Ethiopia countries (Tadele *et al.* 2014), due to its grain human consumption, phytochemical content, adaptation to arid and semi-arid climate and ability to fix atmospheric nitrogen through a symbiosis with soil rhizobial bacteria (Karim Mojein *et al.* 2003). Although several studies have been carried out to assess the diversity and identity of rhizobia that nodulate members of the tribe *Viciae*, there were few reports investigating rhizobia isolated from lentil. The studies performed on lentil rhizobia (Hynes and O'Connell 1990; Moawad and Beck 1991; Laguerre, Mazurier and Amarger 1992; Geniaux and Amarger 1993; Materon *et al.* 1995; Laguerre *et al.* 2003; Rashid *et al.* 2009) so far have mainly evaluated their symbiotic performance on plant growth and have described their biochemical characteristics and stress (salt and temperature) tolerance. Recently, Rashid *et al.* (2012) performed a phylogenetic analysis of housekeeping and nodulation genes of lentil bacterial isolates from originated (Turkey and Syria) and introduced (Germany and Bangladesh) lentil regions; they reported that there are four different lineages of rhizobia associated with lentil nodulation, of which three are new and endemic to Bangladesh, while Mediterranean and Central European lentil symbionts belong to the *Rhizobium leguminosarum* lineage. It is therefore important to examine lentil symbionts from other geographical regions to establish whether lentils are exceptional from other legumes of the tribe *Vicieae* in having different symbionts (Rashid *et al.* 2012). Nodulation and cross-inoculation assays are necessary to determine the host range of lentil rhizobial species, and nucleotide sequences from nodulation genes may be used to provide complementary information (Santillana *et al.* 2008).

Considering the importance of *L. culinaris* Medik in forage production, for human nutrition and the insufficient study on the diversity of rhizobia associated with this legume in Tunisia, the aim of this study is to analyze, using both phenotypic and genotypic methods, the taxonomic diversity of 142 nodules isolates from this legume grown in different areas of Tunisia.

MATERIALS AND METHODS

Determination of soil properties and Rhizobial isolation

Bacteria were isolated, as described by Rejili *et al.* (2012), from naturally occurring root nodules sampled on *Lens culinaris* Medik plants growing in various geographical regions of Tunisia distributed into saharian (Ben Guerdene, Tataouine, Kebili, Park Bouhedma), arid (Limawa, Menzel Habib, Matmata, Medenine, Djerba, Zarzis, Skhira, Sidi Bouzid), semi-arid (Nabeul) and humid (Beja, Bizerte) climates (Fig. 1). Soil pH and electrical conductivity (EC) were determined by pH meter (Matest, Italia) and conductivity meter (Bibby Scientific, UK), respectively. The carbonate content of the soil samples was determined using a Bernard calcimeter, according to the method described by Vatan (1967). For rhizobial isolation, four plants were considered for each location and 8–11 lentil pink nodules were selected for each site. Nodules from Sidi Bouzid, Nabeul, Beja and Bizerte locations are collected by trapping experiment and for the remaining locations nodules are collected from field-grown lentil. The purity of each isolate was ensured by repeated streaking of single colonies onto yeast extract mannitol agar plates (YMA) (Vincent 1970). After purification, a single colony was preserved in 20% glycerol at −80°C until further analysis. The new isolates used in this study and the soil properties are listed in Table 1.

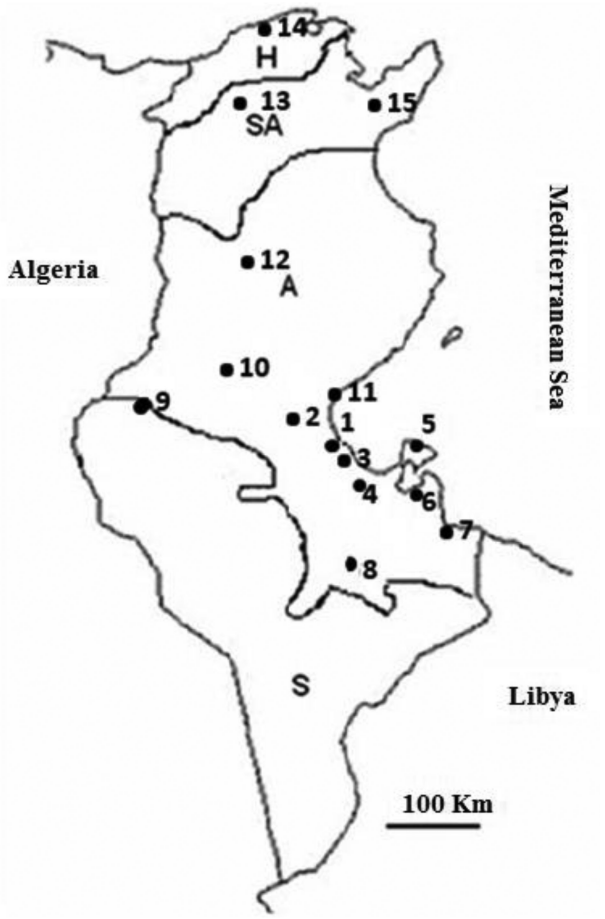

Figure 1. Bioclimatic map of Tunisia showing the location of studied sites. 1, Gabes; 2, Menzel Habib; 3, Matmata; 4, Medenine; 5, Djerba; 6, Zarzis; 7, Ben Guerdene; 8, Tataouine; 9, Kebili; 10, Park Bouhedma; 11, Skhira; 12, Sidi Bouzid; 13, Beja; 14, Bizerte; 15, Nabeul. Climate: H, humid; SA, semi-arid; A, arid; S: saharian.

Phenotypic characterization

Twenty six phenotypic features were used for characterization of studied isolates. The determination of NaCl tolerance (2%, 3% and 4%) and different growth temperature (15°C, 35°C and 40°C) were tested on YMA medium. The tolerance of isolates to extreme pH was tested on YEM agar medium set at different pH values, using the buffers HOMOPIPES (25 mM) for pHs ranging between 4 and 5, MES (20 mM) for pHs ranging between 5.5 and 7 (Priefer *et al.* 2001), MOPS (20 mM) for pHs ranging between 7 and 8 and adjusted with NaOH for pHs up to 9 (Rejili *et al.* 2009). Antibiotic resistance (ampicillin 100 μg ml^{-1}, streptomycin 100 μg ml^{-1} and nalidixic acid 100 μg ml^{-1}) was assessed also in YMA as described by Mohamed *et al.* (2000). Acid and alkali production was determined in YMA medium with bromothymol blue indicator (0.0025%). In all experiments, growth was recorded after 5 days in triplicate.

DNA extraction and sequencing of the rrs, recA, dnaK, glnA, nodA and nodC loci

Bacterial genomic DNAs were obtained from batch cultures grown until late exponential phase using the protocol type k from i-genomic CTB DNA Extraction Mini Kit (iNtRON Biotechnology, Inc., Korea). For the PCR-RFLP of 16S rRNA gene, 16S rRNA gene was amplified as previously described by Rejili *et al.*

Table 1. Isolates used in this study, their geographical origin and soil characteristics (EC, pH, % total carbonate), and their RFLP clusters.

Isolates	Geographical origin	EC (ms cm^{-1})	pH	% Total carbonate	RFLP clusters
$LLi_1 \rightarrow LLi_{10}$	Limawa (Gabes)	01.63	08.45	03.63	1
$LMh_1 \rightarrow LMh_{10}$	Menzel Habib (Gabes)	03.39	08.09	08.63	1
$LMa_1 \rightarrow LMa_{10}$	Matmata (Gabes)	04.00	08.56	17.27	1
$LMe_1 \rightarrow LMe_{10}$	Medenine	05.11	08.08	10.45	1
$LDj_1 \rightarrow LDj_9$	Djerba	03.95	08.24	19.54	1
$LZ_1 \rightarrow LZ_{10}$	Zarzis	06.57	08.13	14.54	1
$LBg_1 \rightarrow LBg_{10}$	Ben Guerdene	01.25	08.35	04.54	1
$LT_1 \rightarrow LT_{10}$	Tataouine	01.81	08.18	21.36	1
$LKe_1 \rightarrow LKe_8$	Kebili	02.82	08.12	05.45	1
$LB_1 \rightarrow LB_9$	Park Bouhedma	02.41	08.26	03.63	2
$LSk_1 \rightarrow LSk_{10}$	Skhira (Sfax)	01.37	08.36	09.09	1
$LSb_1 \rightarrow LSb_8$, LSb_{10}	Sidi Bouzid	02.01	08.25	02.72	1
$LBe_1 \rightarrow LBe_{10}$	Beja	07.35	08.41	06.77	1
$LBi_1 \rightarrow LBi_{10}$	Bizerte	07.62	08.64	06.84	3
$LNa_1 \rightarrow LNa_3$, $LNa_5 \rightarrow LNa_9$	Nabeul	07.22	08.37	05.36	1

(2012). Aliquots of 10 μl of the amplified 16S rDNA were digested with the restriction endonucleases MspI, HaeIII and HinfI provided by Sigma and the PCR-RFLP patterns were resolved on 3% agarose gels during 2 h at 80 mV (4V cm^{-1}) and analyzed. Comparative analysis of electrophoretic 16S rRNA PCR-RFLP patterns was performed with InfoQuest FP from Bio-Rad using Pearson's product–moment correlation analysis. Similarity matrices were clustered using the unweighted pair–group method with averages (UPGMA) algorithm (Sneath and Sokal 1973). Gel normalization, background substraction and zone definition were performed as previously described (Rademaker *et al.* 1997). For *rec*A, *gln*A and *dna*K genes, PCR amplifications were used as previously reported by Martens *et al.* (2007). PCR amplification of *nod*C and *nod*A genes was performed according to Haukka *et al.* (1998). The PCR amplification product was purified from agarose gel using Illustra GFXTM PCR DNA and Gel Band Purification Kit (GE Healthcare, UK). Gene cycle sequencing was performed using the ABI PRISM Big Dye terminator cycle sequencing kit according to the manufacturer's protocol and analyzed on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). The results of gene sequences were analyzed for homologies to sequences deposited in the GenBank. A neighbor-joining tree was reconstructed and bootstrapped with replications (1000 replications) of each sequence using Mega6 software (Tamura *et al.* 2013). The sequences were aligned using ClustalW software (Chenna *et al.* 2003), and the distances were calculated according to the model of Kimura (1980). The GenBank accession numbers for the sequenced genes reported in this paper are included in Table S1 (Supporting Information).

Nodulation assays and symbiotic effectiveness

Nodulation assays were done in a growth chamber set to 25°C with 14 h light/10 h dark cycles. Seeds germination and plant inoculation were performed as described by Rejili *et al.* (2010, 2012). Cross-nodulation test of the two isolates LBi_2 and LB_4 were performed with *Medicago sativa* and *Cicer arietinum* host plants, respectively. Inoculation was performed with 10^8–10^9 cells of each isolate. The uninoculated plants (T_N: N-fertilized and uninoculated plants and T_0: non-fertilized and uninoculated plants) were included as controls. N-fertilized plants were maintained with Jensen's medium containing 0.1 M KNO_3. Plants were harvested 7 weeks after planting and observed for nodulation. Shoots were cut-off, dried at 70°C for 48 h, and then weighed. Nitrogen-fixing effectiveness of nodules was expressed in percent of dry weight of the aerial biomass of the test plant to that of nitrogen control plants, which were maintained with Jensen's medium containing 0.1 M KNO_3 as described by Rejili *et al.* (2012).

RESULTS

Bacterial collection and soil characteristics

A total of 142 bacterial isolates were obtained and purified from root nodules (8–10 isolates from each site). Soils from all sampling sites were alkaline, with pH 8.08 to 8.64 (Table 1). Carbonate content varied from 2.72% (Sidi Bouzid soil) to 21.36% (Tataouine soil) and EC varied from 01.37 (Skhira soil) to 07.62 ms cm^{-1} (Bizerte soil) (Table 1). Annual rainfall varies from humid superior (>350 mm per annum) to saharian inferior (<50 mm per annum), and the mean temperature range in summer is 35°C –40°C and in winter 8°C –15°C.

Infectivity and symbiotic efficiency

The ability of new bacterial isolates to renodulate *Lens culinaris* Medik host was tested. Results showed that all isolates were able to induce nodules in their host plant. Symbiotic properties of 11 isolates, belonging to each distinct PCR-RFLP genotype, reported significant differences in the capacity to infect the host plant and to fix atmospheric nitrogen (Table 2). The two strains, LZ_1 and LMa_7 (affiliated to *Rhizobium leguminosarum*), showed the highest nodule numbers per plant, 38.25 (±00.77) and 29.63 (±01.73), respectively (Table 2). The two strains, LZ_1 and LBi_2, described taxonomically as *R. leguminosarum* and *Sinorhizobium* (= *Ensifer) numidicus*, respectively, can be considered the most effectives with 92.73 (±00.46%) and 89.09% (±00.75%) dry biomass, respectively, of the T_N control (Table 2).

PCR-RFLP and phylogeny of the 16S rRNA gene analysis

The 16S rRNA gene of all isolates was amplified, resulting in a single band of about 1500 pb. This size corresponded well to the expected size of the 16S rRNA gene of most members of the Rhizobiaceae (Laguerre *et al.* 1994). PCR products were digested with three restriction enzymes MspI, HaeIII and HinfI. The obtained profiles were combined and analyzed using the UPGMA

Table 2. Infectivity and symbiotic efficiency of the representative 16S rRNA gene sequenced isolates.

Plant host	Isolates	Nodule number (nodules plant^{-1})	Aerial part dry weight (mg plant^{-1})	Relative effectiveness (%)
	LBg_3	07.67 ±1.03^{a}	45 ±0.53^{a}	81.82 ±0.93^{a}
	LBg_4	08.26 ±0.46^{a}	44 ±1.17^{a}	80.00 ±0.31^{a}
	LDj_4	18.84 ±0.85^{b}	46 ±0.48^{a}	83.64 ±0.34^{a,b}
	LDj_5	19.23 ±0.63^{b}	46 ±0.67^{a}	83.64 ±0.54^{a,b}
	LZ_1	38.25 ±0.77^{c}	51 ±0.63^{b}	92.73 ±0.46^{d}
Lens culinaris	LMh_1	16.07 ±0.88^{b}	45 ±0.82^{a}	81.82 ±0.65^{a}
	LMa_7	29.63 ±1.73^{d}	48 ±0.65^{a,b}	87.27 ±0.25^{c}
	LKe_1	08.42 ±0.83^{a}	44 ±0.93^{a}	80.00 ±0.85^{a}
	LB_4	13.72 ±0.34^{e}	46 ±0.22^{a}	83.64 ±0.78^{a,b}
	LBi_2	14.83 ±0.24^{e}	49 ±0.68^{a,b}	89.09 ±0.75^{c,d}
	LNa_6	11.85 ±0.78^{e}	43 ±0.47^{a}	78.18 ±0.28^{e}
Control − N (T0)		0	25 ±0.73^{c}	45.45 ±0.48^{f}
Control + N (TN)		0	55 ±0.93^{d}	100 ±0.00^{g}

Letters on behind of the numbers show statistical differences between isolates at the level of $P < 0.05$ for nodule number. Six replicates were considered for each treatment.

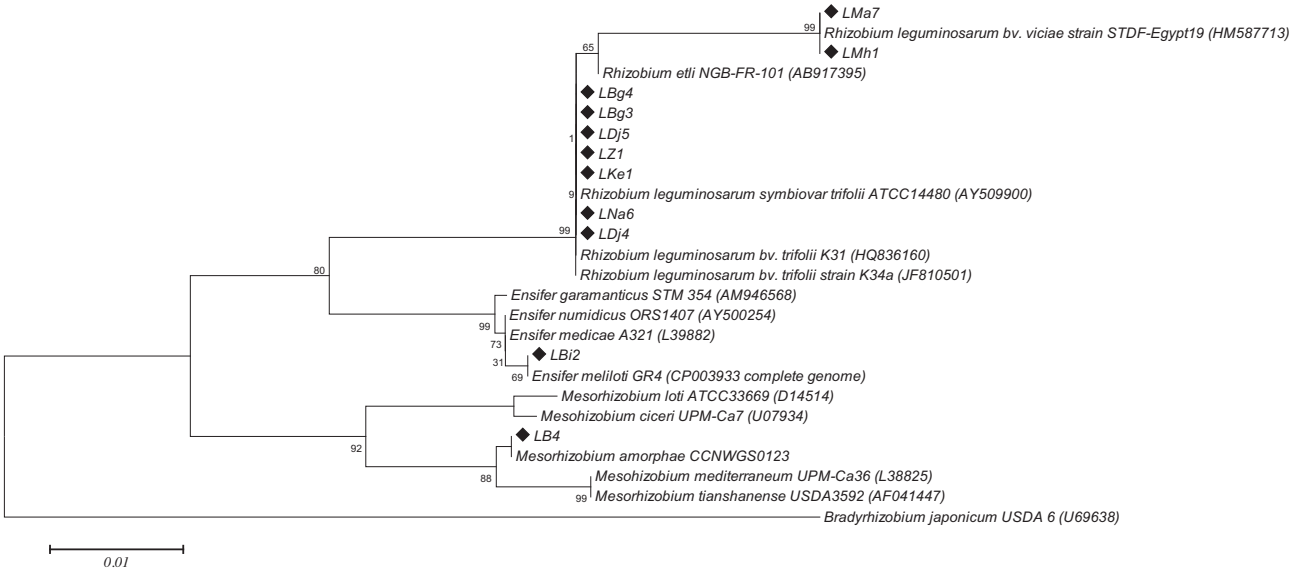

Figure 2. Neighbor-joining tree based on 16S rRNA gene partial sequences showing the relationships of the Tunisian *L. culinaris* Medik isolates and reference strains of *Ensifer, Rhizobium* and *Mesorhizobium*. Bootstrap values >70% (using 1000 replicates) are indicated at branching points. Bar, 0.01% estimated substitutions.

algorithm, and a similarity dendrogram (data not shown) was performed. All strains were delineated into three clusters at 100% similarity level as shown in Table 1. Cluster 1 was the largest group with 123 isolates. Cluster 2 consists of nine strains. Cluster 3 includes 10 strains. The PCR-amplified 16S rRNA genes of 11 isolates belonging to each distinct PCR-RFLP genotype were sequenced. The 16S rRNA neighbor-joining tree (Fig. 2) showed that all *L. culinaris* endosymbiotic bacteria belonged phylogenetically to distinct group rhizobia. The 16S rRNA sequences of strains LMh_1 and LMa_7 shared 99% similarity with 16S rRNA reference sequence of *R. leguminosarum* sv. *viciae* STDF-Egypt19. Surprisingly, the strains LNa_6, LDj_4, LDj_5, LBg_3, LBg_4, LZ_1 and LKe_1 formed a homogeneous group (bootstrap value of 99%) with different *R. leguminosarum* sv. *trifolii* reference strains, while the strain LB_4 had 16S rRNA gene sequence that was identical to *M. amorphae* (99% bootstrap support), and LBi_2 shared low 16S rRNA gene sequences similarity with *E. meliloti, E. numidicus* and *E. medicae* (Fig. 2). Notably, the 16S rRNA gene sequences of *M. amorphae* CCNWGS0123 and LB_4 strains shared 92% similarity with *M. loti* ATCC33669.

Sequence analysis of concatenated housekeeping genes: rrs, glnA, recA and dnaK

Since the resolution of 16S rRNA analysis reported the description of *R. leguminosarum* sv. *trifolii* and *M. amorphae* as new symbionts of *L. culinaris*, and an ambiguous affiliation of the strain LBi_2, a phylogenetic analysis was performed based on the relevant housekeeping genes *gln*A, *rec*A, *dna*K and 16S rDNA in order to define a more robust phylogenetic position of the *L. culinaris* rhizobia. PCR amplifications resulted in DNA fragments with average length of 1500 (16S rDNA), 498pb (*rec*A), 975pb (*gln*A) and 285pb (*dna*K). Phylogenetic analyses were performed with these new and reference sequences. The aligned sequences for 16S rDNA, *rec*A, *dna*K and *gln*A were concatenated, and an alignment

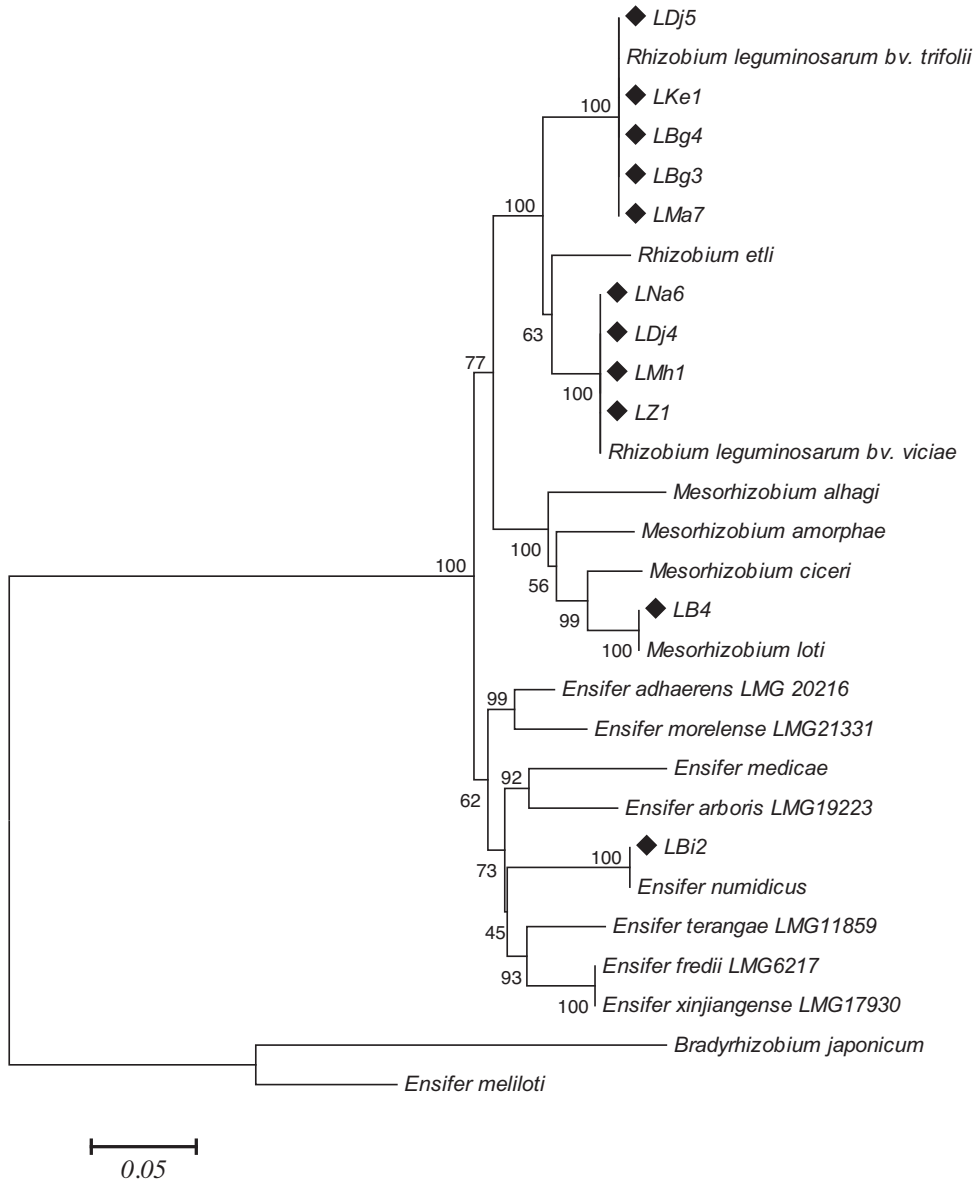

Figure 3. Phylogenetic trees based on concatenated sequences (3258 nucleotides) of 16S rDNA, recA, dnaK and glnA, calculated using neighbor joining (NJ). Bootstrap values (using 1000 replicates for NJ) are indicated at branch points. Bars, 0.01 estimated nucleotide substitutions per site (NJ).

of approximately 3258 nucleotides was obtained. The concatenated tree showed that *L. culinaris* isolates are grouped in three different clades within the *R. leguminisarum, Mesorhizobium* sp. and *Ensifer* sp. genus. The two strains LB_4 and LBi_2 had concatenated sequences that were identical to *M. loti* and *E. numidicus*, respectively, (Fig. 3) with a high bootstrap support (100%). The other strains were distributed between *R. leguminosarum* sv. *trifolii* and *R. leguminosarum* sv. *viciae* with a 100% bootstrap value (Fig. 3).

Symbiotic gene sequence analysis: nodC and nodA genes

*Nod*C and *nod*A are proteins required for synthesis of the rhizobial nodulation factors involved in legume infection signaling. To characterize the nodulation genes of the *L. culinaris* rhizobia, partial *nod*A (Fig. 4A) and *nod*C (Fig. 4B) sequences from the 16S RFLP-PCR representative strains were obtained. According to our results, the *nod*C and *nod*A gene sequences of strains LBg_3, LBg_4 and LKe_1 shared 100% *nod*C and *nod*A sequences similarity with *R. leguminosarum* sv. *trifolii* (97%–100% bootstrap values) (Fig. 4A and B). The *nod*C and *nod*A sequences of the strains LDj_4, LDj_5 and LMa_7 revealed that these strains are similar to *R. leguminosarum* sv. *viciae*; while the strains LMh_1, LNa_6 and LZ_1 shared *nod*C and *nod*A sequences similarity with *R. leguminosarum* (Fig. 4A and B). Surprisingly, the strain LB_4 had *nod*C and *nod*A gene sequences that were identical to *M. mediterraneum*. In addition, the strain LBi_2 shared *nod*A sequence similarity between *E. numidicus* and *E. meliloti* sv. *meliloti* with a high bootstrap support of 92% (Fig. 4A). However, the *nod*C of this strain sequence showed low similarity with *E. meliloti* (Fig. 4B). No *nod*C sequence of *E. numidicus* is available in databanks.

Since our LBi_2 and LB_4 strains had *nod*A and *nod*C gene sequences that were identical to *M. mediterraneum* and

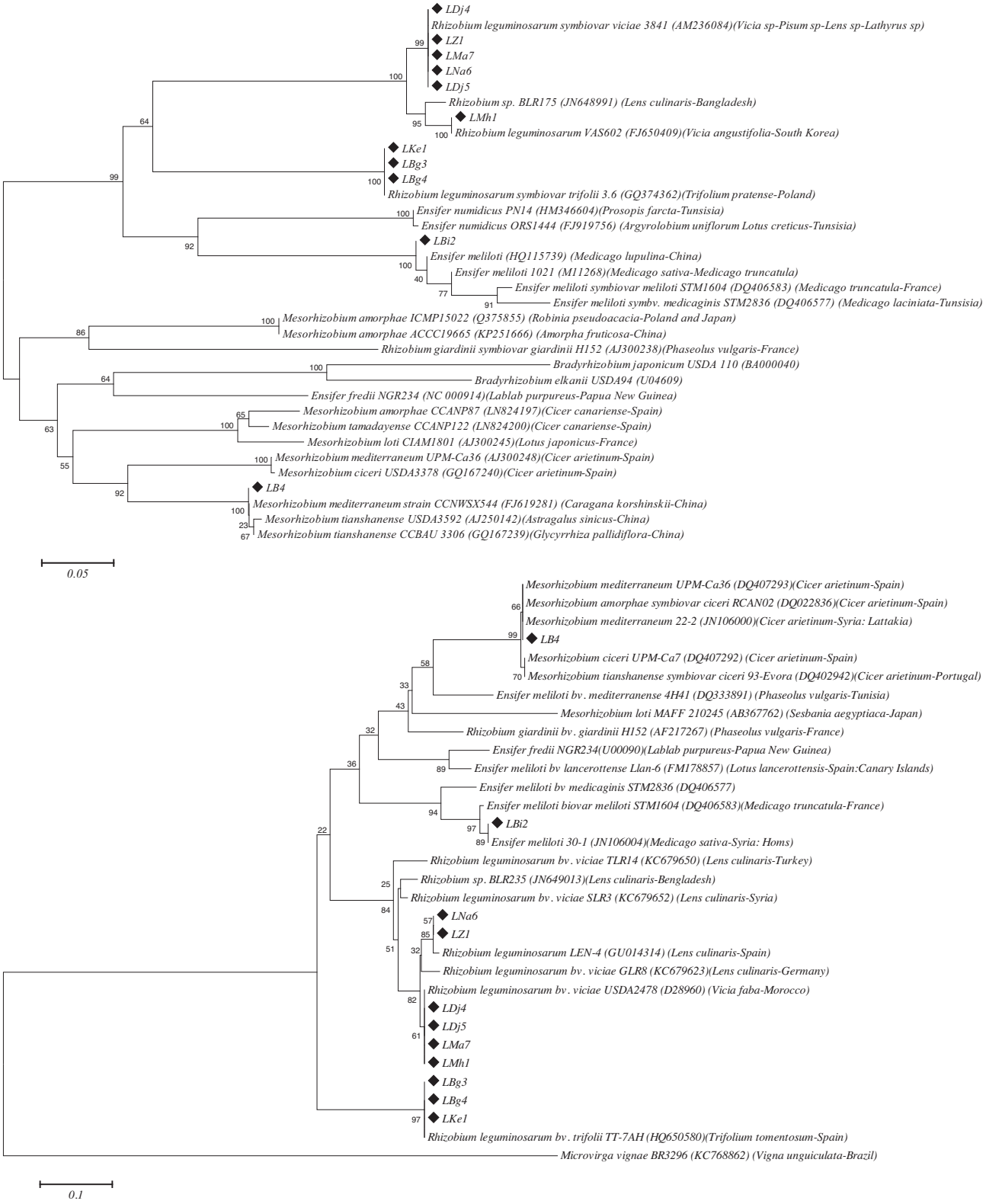

Figure 4. (**A**) Neighbor-joining phylogenetic tree of *nod*A gene sequences of Tunisian *L. culinaris* Medik and reference strains. The novel strains are indicated in bold type and bootstrap values (above 50%) (using 1000 replicates) are indicated at branching points. Bar, 0.1 estimated substitutions. (**B**) Neighbor-joining tree based on *nod*C sequences showing the relationships of the Tunisian *L. culinaris* Medik and reference strains of *Ensifer, Rhizobium* and *Mesorhizobium*. The significance of each branch is indicated by a bootstrap value (above 60%) calculated for 1000 subsets. The scale bar represents the number of nucleotide substitutions per 100 nucleotides.

Table 3. Cross-nodulation test and symbiotic specificity of the lentil LBi_2 and LB_4 strains with *Me. sativa* and *C. arietinum*.

Plant host	Isolates	Nodule number (nodules plant^{-1})	Aerial part dry weight (g plant^{-1})	Relative effectiveness (%)
Medicago sativa	LBi_2	15.16 ±0.03	00.55 ±0.06[a]	73.33 ±01.53[a]
Control − N (T0)		0	00.20 ±0.04[c]	26.67 ±01.67[c]
Control + N (TN)		0	00.75 ±0.08[b]	100 ±0.00[b]
Cicer arietinum	LB_4	14.33 ±0.03	01.08 ±0.43[a]	74.49 ±01.78[a]
Control − N (T0)		0	00.78 ±0.16[c]	53.79 ±01.56[c]
Control + N (TN)		0	01.45 ±0.09[b]	100 ±0.00[b]

Letters on behind of the numbers show statistical differences between isolates at the level of $P < 0.05$ for nodule number. Six replicates were considered for each treatment.

E. numidicus-E. meliloti, respectively (Fig. 4), they are tested for nodulation efficiency, in six replicates, with *Medicago sativa* (*E. numidicus-E. meliloti*) and *Cicer arietinum* (*M. mediterraneum*) host plants. Our results reported that LBi_2 strain was able to induce nodules on *Me. sativa*, with an average of 15.16 ±0.03 nodules/plant, and the aerial part dry weight reached 0.55 ±0.06 gAPDW/plant, demonstrating that the LBi_2 tested strain was symbiotically effective (relative effectiveness = 73.33 ±01.53 ≥ 70%) (Table 3). Similarly, the LB_4 strain was able to induce nodules on *C. arietinum*, with an average of 14.33 ±0.03 nodules/plant, and the aerial part dry weight reached 01.08 ±0.06 gAPDW/plant, demonstrating that the LB_4 tested strain was symbiotically effective (relative effectiveness = 74.49 ±01.78 ≥ 70%) (Table 3).

Phenotypic characterization

Phenotypically, all isolates are fast growers, and acid producers. The growth of the most of isolates was inhibited at pH = 4, only LDj_4 was able to resist, and optimized at pH from 7 to 10 (Table 4). More than 79% of the 142 isolates grew well at NaCl concentrations of 3% with five strains (LZ_1, LDj_4, LDj_5, LMh_1, LB_4) tolerated 4% NaCl and 92% of the isolates showed increased tolerance to high temperature (40°C) and more than 87% of isolates were able to grow at 15°C (Table 4). More than 48% and 87% of strains were resistant to 100 μg ml^{-1} of ampicillin and nalidixic acid, respectively, and most of isolates were sensitive to streptomycin (Table 4).

DISCUSSION

As little work has been done on *Lens culinaris* Medik symbionts, 142 lentil root nodule bacteria were isolated from different localities across Tunisia and characterized by a polyphasic approach (Vandamme *et al.* 1996). Nodulation test showed that all isolates were able to re-induce nodules with lentil within 7 weeks, and RFLP-PCR pattern analysis defined three different patterns, supporting that our strains were genomically related to different species (Laguerre *et al.* 1994; Rashid *et al.* 2009).

By 16S rRNA gene sequencing, the new strains are grouped on the phylogram in the *Ensifer*, *Rhizobium* and *Mesorhizobium* genera, as are many other indigenous legume symbionts from Tunisia (Zakhia *et al.* 2004; Rejili *et al.* 2012, 2014). Out of the 11 representatives RFLP-PCR strains, two strains (LMh_1 and LMa_7) shared 100% 16S rRNA sequence similarity with *Rhizobium leguminosarum* sv. *viciae* STDF-Egypt19, recovered from Egyptian faba bean nodules (Abd-Alla *et al.* 2014). *Rhizobium leguminosarum* sv. *viciae* is the specific microsymbiont of the legumes of the tribe *vicieae* (Jordan 1984; Rivas *et al.* 2009); accordingly, its isolation from tunisian lentils is not surprising. In Egypt, Zahran *et al.* (2013) reported that *L. esculentus* is nodulated by *R. leguminosarum* which was similarly recovered from *L. culinaris* nodules in Algeria (Riah *et al.* 2014). Seven isolates (LNa_6, LDj_4, LDj_5, LBg_3, LBg_4, LZ_1 and LKe_1) were closely related to *R. leguminosarum* sv. *trifolii* (sequence similarity values 100%), which was confined to clovers (*Trifolium*) species (Marek-Kozaczuk *et al.* 2013; Kumar *et al.* 2015; Shamseldin *et al.* 2014). From three different geographical origins (Turkey, Syria and Germany) and different localities across Bangladesh country, Rashid *et al.* (2012) reported that *L. culinaris* nodulating rhizobia were closely related to *R. leguminosarum* but separate from the Bangladeshi isolates. Our results also reported that LB_4 strain had, interestingly, 16S rRNA gene sequence that was identical to *Mesorhizobium amorphae* CCNWGS0123, and the phylogeny of LBi_2 is not well resolved, distributed between *Ensifer numidicus* and *E. meliloti*. The 16S rDNA gene sequences of these two strains are closely identical with 10 nucleotides differences along 1450 pb. These observations imply that the rRNA genes of rhizobia may occasionally undergo possible horizontal gene transfer or recombination, variable mutation rates and simple stochastic variation (Eardly *et al.* 2005; Gevers, Cohan and Lawrence 2005; Vinuesa *et al.* 2005), and may not always accurately reflect prokaryotic phylogeny (Konstantinidis, Ramette and Tiedje 2006; von Mering *et al.* 2007; Martens *et al.* 2008; Wu *et al.* 2011). The concatenation of housekeeping *gln*A, *rec*A, *dna*K and 16S *rRNA* genes sequences showed that five strains were affliated to *R. leguminosarum* sv. *trifolii* and four remaining strains to *R. leguminosarum* sv.*viciae*. Surprisingly, the strain LBi_2 belonged perfectly to *E. numidicus* which was first isolated from *Argyrolobium uniflorum* and *Lotus creticus* Tunisian legumes (Merabet *et al.* 2010) and very recently from *Acacia tortilis* subsp. *raddiana* (Fterich *et al.* 2012) under the same Tunisian soils where recovered our strains, while the strain LB_4 shared 100% similarity with *M. loti*, never previously described as symbionts of lentil. In fact, the 16S rRNA genes alignment of *M. amorphae* CCNWGS0123 and *M. loti* ATCC33669 reported 25 nucleotides differences along 1490 nucleotides, confirming the high 16S rRNA gene sequence identity of *Mesorhizobium* species (Gao *et al.* 2004; Kwon *et al.* 2005), and therefore, the comparison of the concatenated housekeeping *gln*A, *rec*A, *dna*K and 16S *rRNA* genes sequences provides, as we report in this study, a fast tool to assess the LB_4 strain delineation. Otherwise based on the branch separation of the 16S rRNA gene phylogeny of *M. amorphae* CCNWGS0123 and *M. loti* ATCC33669 with 92% bootstrap support value, the strain LB_4 could be assigned to the two reference strains *M. amorphae* and *M. loti*. *Mesorhizobium amorphae* was defined as symbiotic bacteria of two different legumes (*Amorpha fruticosa:* Wang, Martínez-Romero and Martínez-Romero 1999; Wang *et al.* 2002

Table 4. Phenotypic characteristics of the 142 isolates classified by PCR–RFLP clusters and 16S rRNA gene sequencing.

Characteristics	Cluster 1 *R. leguminosarum*	Cluster 2 *M. amorphae*	Cluster 3 *E. meliloti*
Number of isolates	123	09	10
Growth at pH			
4	– (60)	+ (02)	– (10)
6	+ (123)	+ (09)	+ (10)
8	+ (123)	+ (09)	+ (10)
10	+ (88)	+ (07)	+ (10)
NaCl tolerance			
2%	+ (123)	+ (09)	+ (10)
3%	+ (123)	+ (09)	+ (10)
4%	+ (122)	+ (08)	–
Growth at temperature			
15°C	+ (123)	+ (09)	+ (09)
35°C	+ (123)	+ (09)	+ (10)
40°C	+ (121)	+ (09)	+ (10)
Antibiotic resistance			
Ampicillin (100 μg ml^{-1})	+ (122)	+ (09)	+ (09)
Streptomycin (100 μg ml^{-1})	– (40)	+ (08)	– (09)
Nalidixic acid (100 μg ml^{-1})	+ (118)	+ (08)	+ (10)

The numbers in parentheses indicate the number of positive isolates of the total number of isolates tested + positive growth, – no growth. R, Rhizobium; M, Mesorhizobium; E, Ensifer.

and *Sophora alopecuroides*: Zhao *et al.* 2010). It is also known that plants have a broad or narrow range of rhizobia by which they can be effectively nodulated (e.g. *Mimosa* sp.: Gehlot *et al.* 2013; *Vachellia jacquemontii:* Sankhla *et al.* 2016), whereas host-range legumes were also mentioned (Broughton and Perret 1999). The presence of bacteria belonging to different genera in the same host plant might be a result of genetic diversification and adaptation of the bacteria to their environment (Fuentes *et al.* 2002; Gehlot *et al.* 2012, 2013). For instance, we report (Table 1) that *R. leguminosarum* sv. *trifolii* and sv. *viciae* have a universal distribution in both low and high EC (salinity) and total carbonate content, whereas the presence of *M. amorphae* and/or *M. loti* is restricted to saharian climate with both low EC (02.41 ms cm^{-1}) and total carbonate content (03.63%). High EC (07.62 ms cm^{-1}) and low total carbonate content (06.84%) are accompanied with the presence of *E. numidicus* in the humid soil of Bizerte. The biogeographical pattern and the genomic results imply that soil directs the symbiosis between lentil and rhizobia in Tunisia, and that rhizobia have to undergo the selection pressures from both host legume and soil conditions, as reported earlier (Zhang *et al.* 2011; Gehlot *et al.* 2012, 2013). Very recently, Sankhla *et al.* (2016) reported that stressful conditions caused by the alkaline soil of the Thar Desert of India have resulted in *V. (Acacia) jacquemontii* being nodulated by diverse and promiscuous *Ensifer* species. Although lentil host genotype was the main factor determining rhizobial diversity, Lemaire *et al.* (2015) reported that ecological factors such as soil acidity and site elevation were positively correlated with genetic variation within *Mesorhizobium*, indicating an interplay of host and environmental factors on the distribution of the semi-arid Fynbos ecosystem in South Africa.

In order to provide complementary information on host nodulation (Mergaert *et al.* 1997), our results reported that three strains (LBg_3, LBg_4 and LKe_1) shared 97%–100% similarity with *R. leguminosarum* sv. *trifolii* isolated from *Trifolium pratense* (*nod*A phylogeny) (Mazur *et al.* 2011; Poland) and *T. tomentosum* (*nod*C phylogeny) (Ruiz-Diez *et al.* 2012; Spain), while *nod*C and *nod*A sequences of other three srains (LDj_4, LDj_5 and LMa_7) are similar to ones of *R. leguminosarum* sv. *viciae* USDA2478 isolated from *Vicia faba* on Morocco (Ueda *et al.* 1995). Although many publications have previously shown that lentil was nodulated by *R. leguminosarum* sv. *viciae* (e.g. Jordan 1984; Rivas *et al.* 2009), Ruiz-Díez *et al.* (2012) reported that, among four strains nodulating *L. culinaris* Medik in semi-arid areas of Central Spain, only two strains were assigned, by 16S rRNA gene sequencing, to *R. leguminosarum* sv. *trifolii* with 85% bootstrap support value. Recently, in arid and semi-arid regions of Algeria, Boukhatem *et al.* (2012) reported that *A. karroo* and *A. seyal* are nodulated by *R. leguminosarum* sv. *trifolii* LMG14904. So, it would now be interesting to perform the cross-nodulation test of our strains on clover species and to clarify the taxonomic status of the species *R. leguminosarum* sv. *trifolii* since a new species *R. pisi* was defined to reclassify a *R. leguminosarum* strain nodulating *Pisum sativum, T. repens* and *Phaseolus vulgaris* (Ramírez-Bahena *et al.* 2008). In the phylogram of *nod*A sequences, the strain LBi_2 had *nod*A gene sequence that was identical to *E. numidicus*-*E. meliloti* cluster. Merabet *et al.* (2010) reported that the *nod*A sequence of *E. numidicus* is most closely related to those of the *E. meliloti* and *E. medicae* clade. Nodulation efficiency of LBi_2 with *Medicago sativa* host plant showed that it was symbiotically effective (Table 3). Interestingly, in the Bizerte location where *E. numidicus* is isolated, different alfalfa species are naturally grown, indicating the *nod*A gene propensity towards lateral gene transfer from a common ancestor to *E. numidicus* being studied (Sullivan *et al.* 1995; Louvrier, Laguerre and Amarger 1996; Paffetti *et al.* 1996; Urtz and Elkan 1996). In addition, Fterich *et al.* (2012) reported that *E. numidicus*, isolated from *A. tortilis* subsp. *raddiana* under Tunisian soils, was able to nodulate *Me. sativa* and *Me. truncatula*. The analysis of the *nod*C sequence of LB_4 symbionts provided results congruent to those of the *nod*A genes and they are almost identical to *nod*C and *nod*A genes of *M. mediterraneum* reference strain. *Mesorhizobium mediterraneum* species was described to specifically nodulate *Cicer arietinum* (chickpea) host plant (Nour *et al.* 1995; Jarvis *et al.* 1997). The LB_4 strain was symbiotically effective on *C. arietinum* (Table 3). The LB_4 strain carry 16S rRNA gene phylogenetically convergent to *M. amorphae* and concatenated sequences to

M. loti, while *nod*C and *nod*A gene sequences showed more than 99% similarity with respect to those from *M. mediterraneum*, the common chickpea nodulating species in Spain and Portugal (Rivas *et al*. 2007). The highly similar *nod*C and *nod*A genes between LB_4 and reference strain evidenced that lateral transfer of the symbiotic genes might have occurred among the lentil-nodulating rhizobia in Tunisia (Sullivan *et al*. 1995; Louvrier, Laguerre and Amarger 1996; Paffetti *et al*. 1996; Urtz and Elkan 1996), since in the Park Bouhedma location where *Mesorhizobium* is isolated, chickpea is historically cultivated. According to the symbiotic gene sequence and the *C. arietinum* host plant, the LB_4 symbiont would be included in the new infrasubspecific division named *M. amorphae* sv. *ciceri* proposed by Rivas *et al*. (2007) to include the strains able to effectively nodulate *C. arietinum*, or to chickpea *M. loti* rhizobia isolated from Portuguese soils as reported by Laranjo *et al*. (2004). The presence of *M. amorphae* sv. *ciceri* and chickpea *M. loti* on Tunisian soils suggests that *L. culinaris* endosymbionts could have been first spread to the Nile from the near east, to Central Europe and then to the Indian Subcontinent and the Mediterranean Basin by the end of Bronze Age together with the legume seeds (Cubero 1984).

By phenotypic and symbiotic efficiency analysis, we reported a great diversity among and within strains as mentioned by Trinick and Hadobas (1990a) for other legume plants. The two strains LZ_1 (*R. leguminosarum*) and LBi_2 (*E. numidicus*) are considered the most effectives with a 92.73 (±0.46%) and 89.09% (±0.75%) dry biomass, respectively. For phenotypic analysis, the majority of the isolates are able to grow at pH between 6 and 8. Previous publications reported that most strains of *Sinorhizobium* and *Rhizobium* species are very acid sensitive (Brockwell, Bottomley and Thies 1995), while Lemaire *et al*. (2015) mentioned that *Mesorhizobium* species are the dominant symbionts of legumes in the acidic soils of the semi-arid Fynbos ecosystem in South Africa. As for salinity and temperature tolerance, our results showed that lentil *R. leguminosarum* (122 strains) and *M. amorphae* (or *M. loti*) (8 strains) strains are able to grow at up to 4% NaCl, while *E. numidicus* lentil strains do not tolerate 4% NaCl concentrations (Table 4), and the majority of them is resistant to high temperature (40°C). These results corroborate our earlier reports on the root nodule bacteria isolated from wild legumes in Tunisia (Rejili *et al*. 2012, 2014) and could be considered a specific adaptation to high temperatures and soil salinity that characterize arid regions, offering them advantages to adapt to different environments for survival and nodulation (Wei *et al*. 2008; Gehlot *et al*. 2012, 2013). Regarding the intrinsic resistance to antibiotics, we reported that lentil new strains were resistant to 100 $\mu g\ ml^{-1}$ of ampicillin and nalidixic acid, and *E. numidicus* strains were sensitive to streptomycin, but Merabet *et al*. (2010) reported that the laters cannot be identified by biochemical and physiological characters alone.

In summary, our study is the first report on the characterization of *L. culinaris* Medik microsymbionts in Tunisia. We evidenced a novel biodiversity among bacteria isolated from *L. culinaris* Medik. We demonstrated that, in Tunisian soils, rhizobia that nodulated lentil belonged almost exclusively to the known *R. leguminosarum*, and to the three species *E. numidicus*, *R. leguminosarum* sv. *trifolii* and *M. amorphae* sv. *cicer* (or *M. loti*), not considered, up to now, as a natural symbiont of lentil. *Ensifer numidicus* has been described as a specific symbiont of the *Argyrolobium-Lotus creticus-Acacia*; *M. amorphae* sv. *ciceri* (or *M. loti*) is specific to *Cicer* species, while *R. leguminosarum* sv. *trifolii* is confined to clovers species. Furthermore, our results reported that these new species were able to nodulate effectively lentil, which indicated that they were fully compatible with the host plant. The degree of specificity between leguminous plants and rhizobia is highly variable. Our results show that the host spectrum of *R. leguminosarum* sv. *trifolii*, *E. numidicus*, *M. amorphae* (or *M. loti*) enlarged to lentil and that *L. culinaris* Medik might be a rather promiscuous host plant.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSEC online.

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