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Genetic diversity of rhizobial populations recovered from three *Lotus* species cultivated in the infra-arid Tunisian soils

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Abstract

Eighty-three bacterial strains isolated from root nodules of *Lotus creticus*, *L. pusillus*, and *L. arabicus* grown in infra-arid Tunisian soils were characterized using a polyphasic approach including phenotypic analysis, rep-PCR and PCR-RFLP analyses of the 16S rRNA gene. Phenotypically, all isolates are fast growers the majority of which grow at a pH of between 5.5 and 9. Most of the tested isolates tolerate NaCl concentrations from 1.39% to 3.48%. By rep-PCR fingerprinting, the genomic similarity varied from 30% to 98%. All tested isolates were clustered into 32 rep-PCR clusters at the similarity level of 80%. The genomic divergence of strains revealed by rep/PCR analysis appeared to be very important since a molecular polymorphism delimiting symbionts for each species of *Lotus* was identified. With the high-resolution of rep-PCR profiles of the isolates obtained using Pearson's/UPGMA analysis, the isolates were resolved into 60 different profiling groups to undergo 16S ARDRA analyses. The analysis of all restriction fragments from each strain based on the UPGMA algorithm from the combined patterns showed that *Lotus* isolates are very diverse and that they were affiliated to *Sinorhizobium*, *Rhizobium*, and *Mesorhizobium* genera.

Keywords: *Lotus* sp.; rep-PCR fingerprinting; PCR-RFLP 16S rRNA; Rhizobia; Arid habitat

1. Introduction

Lotus sp. is a large (150 spp.), cosmopolitan genus that occupies two major centres of diversity, the Mediterranean region (including portions of Europe, Africa, and Western Asia) and Western North America [1]. It includes annual and perennial plants with strong branched taproots [2]. It is one of about ten genera within the tribe Loteae and is the only genus in the tribe with an intercontinental distribution [3,4]. Species of the genus *Lotus* are increasingly utilized in pastures throughout the world because of their high productivity over a wide range of soils [5]. In addition, the interest in *Lotus* genus plants over the last decade has increased as greater emphasis is being placed on reducing nitrogen (N) and phosphorus (P) inputs into farming systems and lowering cattle stocking rates to reduce environmental pollution and land degradation [5]. This genus includes plant species that are adapted to a wide range of habitats from marine environments to high altitudes, and from sandy to heavy saline soils [6–9]. In this context, indigenous legumes of arid regions of Tunisia such as *L. creticus*, *L. pusillus*, and *L. arabicus* because of their nitrogen-fixing symbiosis with legume-nodulating bacteria (LNB), collectively called rhizobia, contribute to soil fertility by enhancing soil nitrogen content and organic matter. In previous work, the natural nodulation resource of these three legumes prospected from different sites in the arid

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climate of Tunisia was investigated [10]. Results showed that nodules presented globular shapes, and *L. creticus* presented the highest number of nodules followed by *L. pusillus* and *L. arabicus*.

Rhizobia that nodulate *Lotus* species include both fast-growing *Mesorhizobium loti* [11] and slow-growing *Bradyrhizobium* sp. [12]. Zakhia et al. found that strains isolated from *L. creticus* grown in the infra-arid region of Tunisia were *Rhizobium* genus species [13]. Although the taxonomic characterization of the nodulating bacteria associated to *L. creticus* has been examined [13], the *L. pusillus* and *L. arabicus* microsymbionts have not been considered to the best of our knowledge. In the present work, the taxonomic diversity of 83 rhizobial bacteria isolated from root nodules of *L. creticus*, *L. pusillus*, and *L. arabicus* sampled in the infra-arid zone of South Tunisia was investigated using phenotypic and genomic approaches including generation time, salt and pH tolerance, rep-PCR and PCR-RFLP of the 16S rRNA gene.

2. Materials and methods

2.1. Rhizobial strains

All the strains used in this study are listed in Table 1. The 83 rhizobial isolates from the *L. creticus*, *L. pusillus*, and *L. arabicus* plants were obtained from root nodules of plants growing in various geographic regions of the infra-arid region of Tunisia. Rhizobial strains were isolated on yeast mannitol agar (YMA) or TY medium from root nodules of field-grown plants and purified by repeated streaking [14]. All the bacteria were kept in 20% (v/v) glycerol at −20 °C and cultured in YMA medium at 28 °C.

2.2. Nodulation test

A nodulation test was performed for the representative rep-PCR isolates. The *Lotus* sp. of the seeds, collected from wild plants, were surface-sterilized in 98% sulphuric acid for 3 min, washed extensively with sterile distilled water and suspended for 5 min in 0.5% sodium hypochlorite and germinated in petri dishes using 0.8% agar. Germinated seedlings were aseptically transferred to agar plates with 70 ml of nitrogen-free nutrient solution described by Rigaud and Puppo [15]. Inoculation was performed in an aseptic room for each host plant automatically after transfer with 20 μl of the appropriate isolates of rhizobial broth culture containing approximately 10^9 cells/ml. A nodulation test of *L. arabicus* isolates was performed on *L. pusillus* and *L. creticus* seeds. Plants not inoculated were included. Ten plants were routinely tested with each isolate. Forty days later, plants were examined for root nodulation.

2.3. Physiological analysis

2.3.1. Growth rate

Analysis of the growth rate was assessed as described by Mahdhi et al. [16]. Isolates were cultured in Erlenmeyer flasks containing 50 ml of YEM medium and incubated at 28 °C on a rotatory shaker (180 r/min). Growth was checked by measuring the optical density at 600 nm every 2 h. The generation time of each isolate was deduced from the exponential phase of the growth curves.

2.3.2. Salt tolerance

Determination of NaCl tolerance was assessed on YEM agar plates containing 1.39%, 2.09%, 2.79%, and 3.48% NaCl concentrations.

2.3.3. pH tolerance

Tolerance to extreme pH was tested on YMA medium set at different pH levels, using the buffers MES (14 mM) (Sigma) for pH ranging between 5.5 and 6.7, Tris–HCl (12.5 mM) for pH = 8 and NaOH for pH = 9.

2.4. rep-PCR fingerprinting

Total DNA extracted by the standard method of Sambrook et al. [17] was used as templates. For rep-PCR, the primers used are REP1R-I (5′-IIIICGICGICATCIGGC-3′) and REP2-I (5′-ICGICTTATCIGGCCTAC-3′) [18]. PCRs were carried out basically in 25 μl of mixture solution containing about 5 μl (≈20 ng) of template DNA, 3 μl of Polymerase (Taq) buffer (10×), 3 μl of DMSO (100%), 3 μl of deoxynucleoside triphosphate (1.25 mM), 4 U of Taq DNA polymerase (Sigma, Spain), 5 μl of $MgCl_2$ (7 mM), 1 μl for each primer (50 pmol), and 4.2 μl of Water milliQ. The following PCR program was used: initial denaturation at 95 °C for 6 min; 30 cycles at 94 °C for 1 min, 40 °C for 1 min and 65 °C for 8 min; and final extension at 65 °C for 16 min. Amplified DNA was separated in 1.2% (w/v) agarose gels, stained with ethidium bromide (10 mg/ml) and photographed using UV light (260 nm). Comparative analysis of electrophoretic REP 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 [19]. Gel normalization, background substraction and zone definition were performed as previously described [20]. Index of diversity (H', [21]) was estimated based on the number of isolates belonging to each group of profiles in rep-PCR, considering a 80% similarity in the cluster analysis [22,23].

2.5. PCR-RFLP of 16S rRNA gene or 16S ARDRA

16S rDNA PCR amplification was carried out with primers fD1 and rD1 [24] as described by Herrera-Cervera et al. [25]. Aliquots of 8–10 μl of the amplified 16S rDNA were digested with the restriction endonucleases HinfI and MspI provided by Fermentas and the ARDRA patterns were resolved on 3% (w/v) agarose gels (Pronadisa, Spain) during 2 h at 80 mV. Analysis of the restriction fragments and the construction of the dendrogram were

Table 1
Lotus species isolates and reference strains 16S used in this study, their geographic origin and genotypic characterization by rep-PCR and ARDRA 16S.

Isolates or strains	Plant host	Geographic origin	rep-PCR cluster	16S rDNA type	PCR-RFLP cluster
New isolates					
LAC113	*Lotus creticus*	Msarref	4	Nt	Nt
LAC231	*L. creticus*	OuedDkouk	16	6	2
LAC232	*L. creticus*		10	6	2
LAC234	*L. creticus*		5	6	2
LAC241	*L. creticus*		8	8	2
LAC243	*L. creticus*		1	21	8
LAC244	*L. creticus*		5	Nt	Nt
LAC247	*L. creticus*		23	Nt	Nt
LAC249	*L. creticus*		16	8	2
LAC2410	*L. creticus*		5	6	2
LAC2411	*L. creticus*		1	13	4
LAC332	*L. creticus*	Rsifat	13	21	8
LAC441	*L. creticus*	Elgrabat	1	7	2
LAC512	*L. creticus*	Elkestil	6	21	8
LAC513	*L. creticus*		1	19	7
LAC522	*L. creticus*		11	Nt	Nt
LAC532	*L. creticus*		1	Nt	8
LAC533	*L. creticus*		14	1	1
LAC551	*L. creticus*		1	Nt	Nt
LAC552	*L. creticus*		21	Nt	Nt
LAC553	*L. creticus*		10	1	1
LAC555	*L. creticus*		3	Nt	Nt
LAC562	*L. creticus*		24	8	2
LAC5102	*L. creticus*		11	5	2
LAC5112	*L. creticus*		32	11	4
LAC611	*L. creticus*	Nefta	12	Nt	Nt
LAC733	*L. creticus*	Dhiba	6	17	6
LAC741	*L. creticus*		25	3	1
LAC742	*L. creticus*		3	24	8
LAC751	*L. creticus*		4	2	1
LAC753	*L. creticus*		1	16	5
LAC754	*L. creticus*		13	Nt	Nt
LAC755	*L. creticus*		14	2	1
LAC7510	*L. creticus*		4	14	5
LAC7511	*L. creticus*		31	20	7
LAC765	*L. creticus*		6	18	6
LAC771	*L. creticus*		1	4	2
LAC772	*L. creticus*		28	4	2
LAC811	*L. creticus*	Fjé	11	21	8
LAC812	*L. creticus*		17	7	2
LAC813	*L. creticus*		4	22	8
LAC814	*L. creticus*		11	23	8
LAC821	*L. creticus*		3	23	8
LAC822	*L. creticus*		25	23	8
LAC831	*L. creticus*		22	9	3
LAC833	*L. creticus*		20	23	8
LAC834	*L. creticus*		12	Nt	Nt
LAC841	*L. creticus*		20	23	8
LAC845	*L. creticus*		4	Nt	Nt
LPS114	*Lotus pusillus*	Msarref	26	21	8
LPS134	*L. pusillus*		8	23	8
LPS161	*L. pusillus*		2	Nt	Nt
LPS162	*L. pusillus*		11	Nt	Nt
LPS163	*L. pusillus*		14	Nt	Nt
LPS164	*L. pusillus*		9	11	4
LPS246	*L. pusillus*	OuedDkouk	4	8	2
LPS342	*L. pusillus*	Rsifat	19	Nt	Nt
LPS431	*L. pusillus*	Elgrabat	3	8	2
LPS512	*L. pusillus*	Mdou	29	8	2
LPS514	*L. pusillus*		9	15	5
LPS522	*L. pusillus*		11	Nt	Nt
LPS532	*L. pusillus*		27	25	8
LPS541	*L. pusillus*		11	7	2
LPS542	*L. pusillus*		23	21	8
LPS543	*L. pusillus*		16	13	4
LPS545	*L. pusillus*		9	Nt	Nt
LPS631	*L. pusillus*	Dar Dhaoui	20	Nt	Nt
LPS651	*L. pusillus*		2	11	4
LPS652	*L. pusillus*		11	Nt	Nt
LPS661	*L. pusillus*		11	12	4
LPS662	*L. pusillus*		13	Nt	Nt
LPS663	*L. pusillus*		2	Nt	Nt
LPS664	*L. pusillus*		1	11	4
LPS712	*L. pusillus*	Elhamma	28	Nt	Nt
LPS713	*L. pusillus*		11	21	8
LPS715	*L. pusillus*		11	26	9
LPS741	*L. pusillus*		15	10	3
LPS811	*L. pusillus*	Elkestil	10	21	8
LPS822	*L. pusillus*		18	14	5
LPS911	*L. pusillus*	Bouhedma Park	7	23	8
LBS122	*Lotus arabicus*	Bouhedma Park	29	11	4
LBS131	*L. arabicus*		30	21	8
LBS132	*L. arabicus*		29	Nt	Nt
Strain reference					
Mesorhizobium loti R7A	*Lotus corniculatus*	Ezz, Spain	NT	19	7
Sinorhizobium meliloti 1021	*Medicago sativa*	Ezz, Spain	NT	21	8
Rhizobium galegae	*Galegae orientalis*	Ezz, Spain	NT	sp	1
Rhizobium etli CFN42	*Phaesolus vulgaris*	Ezz, Spain	NT	sp	2
Mesorhizobium ciceri	*Cicer arietenum*	Ezz, Spain	NT	sp	6

Ezz, Estaciòn Experimental del Zaidìn, CSIC, Granada, Spain; NT, not tested; sp, separate.

performed by InfoQuest FP from Bio-Rad as described for rep-PCR.

3. Results

3.1. Phenotypic characterization

Phenotypically, all isolates are fast growers (GT < 6 h). Four isolates from *L. creticus* have a generation time of between 5 and 6 h (Table 2). The majority of the isolates are able to grow on buffered media at a pH of between 5.5 and 9 with optimal growth at a pH ranging between 7 and 8 (Table 2). Most of the tested isolates tolerate high NaCl concentrations. Only six isolates are unable to grow in the presence of 3.48% NaCl (Table 2). A nodulation test was performed for 60 representative rep-PCR isolates. Results showed that only 13 isolates failed to nodulate their

Table 2
Results of phenotypic characteristics of *Lotus* sp. microsymbionts.

Characteristics	*L. creticus* isolates	*L. pusillus* isolates	*L. arabicus* isolates
No. of isolates	49	31	3
Growth at pH			
5.5	+(45), −(4)	+(30), −(1)	+(3)
6	+(48), −(1)	+(30), −(1)	+(3)
7	+(49)	+(31)	+(3)
8	+(49)	+(31)	+(3)
9	+(48), −(1)	+(31)	+(3)
Generation time (GT)			
$2 \leqslant GT \leqslant 6$	45	31	3
$5 \leqslant GT \leqslant 6$	4	*	*
NaCl tolerance			
1.39%	+(49)	+(31)	+(3)
2.09%	+(48), −(1)	+(31)	+(3)
2.79%	+(46), −(3)	+(30), −(1)	+(3)
3.48%	+(45), −(4)	+(29), −(2)	+(3)

+, positive growth; and −, no growth; *, no isolates. The number in parentheses indicates the number of isolates from the whole number of isolates.

host plant of origin and they are represented in Fig. 2 by clusters IV and V. The other isolates formed 10–15 nodules per plantlet after 40 days.

3.2. Isolates diversity assessed by rep-PCR genomic fingerprinting

All new isolates produced characteristic PCR banding patterns in rep-PCR fingerprinting. The similarity of the fingerprints varied from 30% to 98%. All isolates showed an abundance of repetitive sequences. The size and the distribution of bands have proved very distinct and unique to each isolate. The size of the bands was between 90 and 3000 bp and their numbers ranged from 1 to 13 bands per profile. But most bands showed a molecular weight of between 100 and 1000 bp. In a cluster analysis, a total of 32 rep-PCR clusters were resolved at 80% similarity (Table 1 and Fig. 1). These results meant that the rep-PCR is powerful to evaluate genomic diversity in bacterial populations; it may be able to differentiate nodule isolates from different *Lotus* species.

3.3. PCR-RFLP of 16S rRNA gene or 16S ARDRA

Sixty isolates representing the different rep-PCR clusters were selected to undergo 16S ARDRA. All tested strains produced a single band of about 1500 bp. This size corresponded well to the expected size of the 16S rDNA genes of most members of the *Rhizobiaceae* [26]. HinfI and MspI endonucleases produced polymorphic restriction patterns (fragments that were less than 90 bp in size were not considered). The analysis revealed three to five different restriction patterns per enzyme. The analysis of all restriction fragments showed the distribution of *Lotus* sp. isolates into

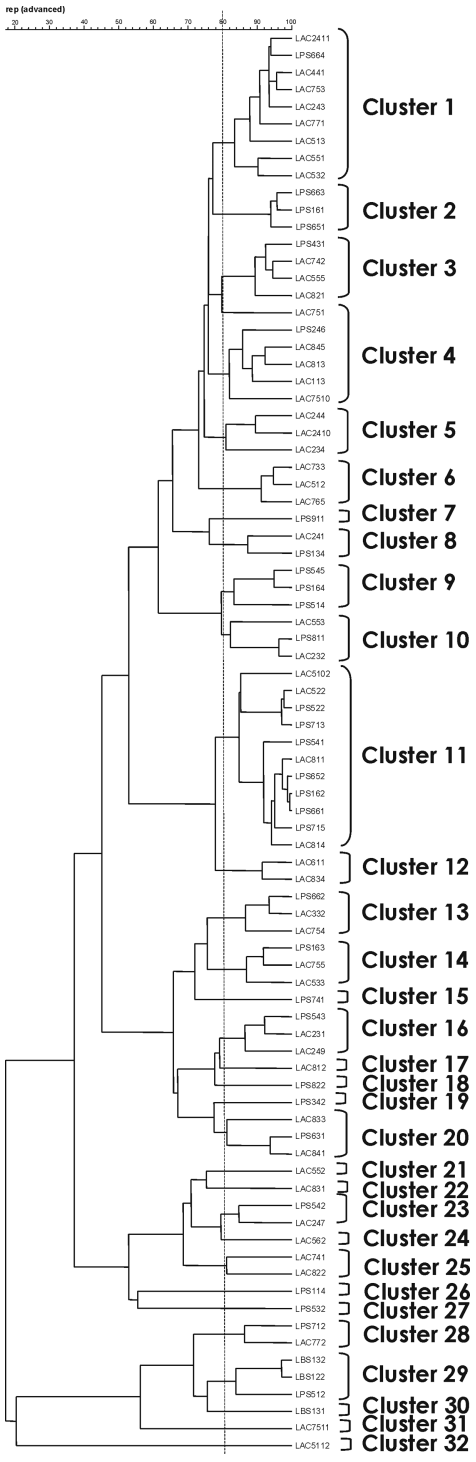

Fig. 1. Dendrogram showing the genetic diversity of rhizobial isolates from the *Lotus* sp. in Tunisia. Clustering analysis based on the rep-PCR fingerprints was performed using the UPGMA method in the GelCompar program InfoQuest FP from Biorad.

26 16S rDNA types (Table 1). Each 16S rDNA type comprises one to ten isolates (Table 1). As shown by Fig. 2, clustering analysis based on the ARDRA fingerprints was performed as a similarity dendrogram, and all strains were delineated into eight clusters. Cluster one contains five new isolates, two of which shared 86% similarity of ARDRA

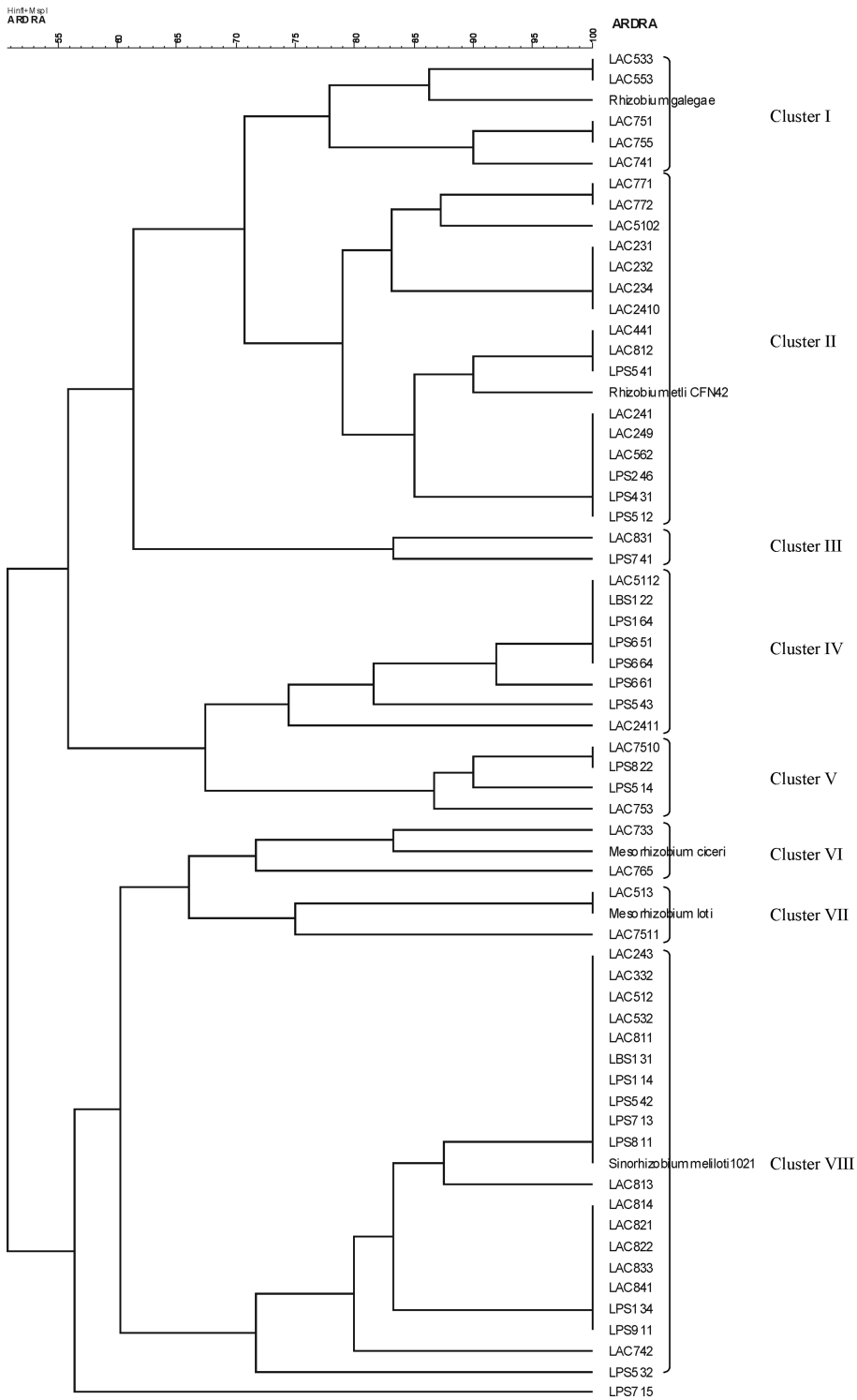

Fig. 2. Dendrogram, based on the 16S ARDRA gene, that shows the phylogenetic relationships among the rhizobial isolates from the *Lotus* sp. in Tunisia. Clustering analysis was performed using the UPGMA method in the GelCompar program InfoQuest FP from Biorad.

patterns with *Rhizobium galegae* type strain. Cluster two contains ten new isolates. Only three of them showed nearly identical ARDRA patterns (90% similarity) with *Rhizobium etli* CFN42. Clusters 3, 4, and 5 consist of two, eight, and four new isolates, respectively. Cluster 6 comprises the *Mesorhizobium ciceri* strain and two new isolates (similarity from 70% to 80%). Cluster 7 encompasses one isolate revealing 100% pattern similarity to *M. loti* R7A. Cluster 8 included 21 isolates, ten of which exhibit identical ARDRA patterns with *Sinorhizobium meliloti* 1021. A separate position was occupied by isolate LPS715.

3.4. Relation between genotypic structure and geographic origin of the isolates

Sampling sites are geographically very close; nevertheless, the REP from *L. creticus* isolates grouped the bacteria isolated from different sampling sites in different branches. Similar results are shown analyzing the REP tree from *L. pusillus* isolates. This was confirmed by statistical analysis that showed no correlation was found between the rep-PCR fingerprinting and the site of isolates origin (χ^2 test: $\chi^2 = 1.615$, ddl $= 9$, $P = 0.996$).

4. Discussion

A collection of 83 isolates was obtained from *L. creticus, L. pusillus*, and *L. arabicus* nodules isolated from 12 diverse geographic southern regions of Tunisia (Table 3) and characterized by a polyphasic approach including phenotypic and genomic analyses [27]. The methods we used were generation time, salt and pH tolerances, rep-PCR fingerprinting and finally, PCR-RFLP of the 16S rRNA gene.

Phenotypically, all *Lotus* sp. isolates are fast growers (generation time <6 h) and are able to grow on buffered media at a pH of between 5.5 and 9 with optimal growth at a pH ranging between 7 and 8 as shown by Jordan [28]. Cooper [29] reported that rhizobial strains which nodulate *Lotus* sp. show marked differences to acidity, with fast-growing *M. loti* [11], being tolerant of pH 4.5 and slow-growing *Bradyrhizobium* sp. (*Lotus* sp.) [28] being sensitive to this pH value. In arid soils of Tunisia, Mahdhi et al. [16,30] reported that bacteria, belonging to genera *Ensifer* and *Rhizobium*, were able to grow at a pH of between 6 and 12 but none could grow at a pH of 4. In addition, most of the tested isolates tolerate NaCl concentrations from 1.39% to 3.48% and approximately 22% of isolates continued to grow well at 3.48% (500 mM). These results corroborate the earlier reports on the *Sinorhizobium* genus strains isolated from wild legumes in Tunisia [13,16,30–32]. Strains of *S. meliloti* ORS665^T and *Mezorhizobium mediterraneum* ORS2739^T tolerate 3% NaCl [16] and bacteria-nodulating *Acacia*, *Prosopis*, and *Leucaena* are revealed to be resistant to 500–850 mM NaCl [33,34].

Table 3
Number of isolates, rep-PCR data and Shannon diversity index of each sampled site.

Sampled sites	Number of isolates	Number of rep-PCR clusters at 80% similarity	Diversity index (H')
Msarref	7	7	2.81
OuedDkouk	11	7	2.66
Rsifat	2	2	1
Elgrabat	2	2	1
Elkestil	14	10	3.18
Nefta	1	1	1
Dhiba	12	9	3.08
Fjé	11	8	2.92
Mdou	8	6	2.50
Dar Dhaoui	7	5	2.24
Elhamma	2	1	1
Bouhedma	4	3	1.5

By genomic analysis, the rep-PCR method, which is suitable for distinguishing strains at the species level and below [35–38], was used for initial grouping of the isolates. Our results showed that for all isolates, the size of the bands was between 90 and 3000 bp and their numbers ranged from one to 13 bands per profile. Strains of *Bradyrhizobium japonicum* presented several bands with size varying between 100 and 5000 bp [39], while *S. meliloti* strains demonstrated few bands with molecular weights between 700 and 4000 bp [36]. In order to quantify the diversity among the isolates for each sampling site, the Shannon–Weaver index (H') was used, maintaining the basis of 80% similarity (Table 3). The results revealed a genetic diversity among these different *Lotus* sp. microsymbionts: Msarref ($H' = 2.81$), OuedDkouk ($H' = 2.66$), Rsifat ($H' = 1$), Elgrabat ($H' = 1$), Elkestil ($H' = 3.18$), Nefta ($H' = 1$), Dhiba ($H' = 3.08$), Fjé ($H' = 2.92$), Mdou ($H' = 2.50$), Dar Dhaoui ($H' = 2.24$), Elhamma ($H' = 1$), and Bouhedma ($H' = 1.5$). Vargas et al. found an H' of 4.3 by studying the genetic diversity of *Acacia mearnsii* nodulating rhizobia in the State of Rio Grande do Sul, Brazil [40]. Andrade et al. found a diversity index of 3.93 analyzing common bean rhizobia in Brazilian acid soils altered by liming [41]. Lõhmus et al. obtained Shannon indexes of 4.63 and 4.56 among cultivable bacterial communities extracted from soil–root interface and rhizosphere bulk soil, respectively [42]. Giongo et al. obtained Shannon indexes varying from 3.95 to 6.17 among bradyrhizobia strains nodulating soybean isolated from South Brazilian fields [43]. The genetic divergence of strains revealed by rep-PCR is very important since a molecular polymorphism delimiting symbionts for each species of *Lotus* was identified. In fact, clusters 5 and 6 (Fig. 1) delimited *L. creticus* symbionts, while clusters 7 and 9 delimited *L. pusillus* isolates which interfered with cluster 29 related to *L. arabicus* (Fig. 1). The specific profiles obtained by this study could be of significant ecological interest in inoculation programs allowing monitoring of the strain in the new environment where it would be introduced.

A subset of 32 representative bacteria of each population previously analyzed by rep-PCR was chosen for 16S rDNA analysis, totaling 60 strains. L'ARDRA or restriction fragment length polymorphism (RFLP) is a rapid procedure for identification and classification of bacteria, as well as for preliminary determination of the systematic relationship [26,44,45]. A dendrogram obtained using the UPGMA analysis clustered those 60 strains into nine ARDRA clusters (Fig. 2). Microsymbionts of *L. pusillus* and *L. arabicus* species recently reported as nodulated [10] were isolated for the first time. It was previously reported that rhizobia-nodulating *Lotus* species included both fast-growing *Rhizobium loti* [46], modified actually as *M. loti* [11], and slow-growing *Bradyrhizobium* sp. [12]. Our results showed that two new isolates nodulating *L. creticus* are closely related to *M. loti* R7A (ARDRA cluster

VII, Fig. 2). We also found and for the first time that *L. creticus* was nodulated by *M. ciceri* (ARDRA cluster VI, Fig. 2).

As shown by l'ARDRA cluster I (Fig. 2), five isolates, microsymbionts of *L. creticus*, are closed to *R. galegae* type strain. Similar results were found by Zakhia et al. [13] for strains isolated from *L. creticus* grown in the infra-arid region of Tunisia. The presence of *R. galegae* in the nodules of some Mediterranean legumes was strongly demonstrated [47–49]. ARDRA/PCR analysis showed also that for the first time 14 isolates, microsymbionts of *L. creticus*, and two isolates, microsymbionts of *L. pusillus* (cluster II, Fig. 2) are closely similar to *R. etli* CFN42. *R. etli* is the predominant *Phaesolus vulgaris*-nodulating species in Mexico, Colombia, and Argentina [50–53]. *R. etli* bv. phaseoli is found in regions where common bean has been introduced, such as Spain, France, Austria, Senegal, Gambia, and Tunisia [25,54–58]. The results presented in the cluster VIII included 21 isolates, 10 of which share nearly identical ARDRA patterns with *S. meliloti* 1021. One isolate related to *L. arabicus* was included into this cluster (Fig. 2). To the best of our knowledge, results have been reported on the *Sinorhizobium* genus as microsymbionts of the *Lotus* sp. However, Mahdhi et al. and Zribi et al. [16,30,32,59] reported recently, in Tunisian arid soils, the presence of *Sinorhizobium* genus for legume-nodulating bacteria (LNB). Similar results were found by Zakhia et al. [13,60] for LNB in arid regions of Tunisia and by Mahdhi et al. and Jebara et al. [31,61] for rhizobia-nodulating medic legumes. No correlation was found between genetic diversity of both the LNB and *Lotus* isolates and their geographic distribution. Khbaya et al. reported that distribution of rhizobia is independent of the site of origin [62]. The acquisition of genetic materials from several ancestral forms coupled with the strong anthropogenic factor in present distributions makes it very difficult to assess the role of geographical isolation in rhizobial evolution.

Our results showed that some isolates belonging to LNB failed to nodulate their host of origin. The same results were reported by Zakhia et al. [13] considering that mediterranean plants are recalcitrant in the point of nodulation. The hosts are very specific in their requirements; since the used seeds come from a different host compared with the original isolations, this explanation can confirm the nodulation failures of the isolates. The non-nodulation *in vitro* of the *Lotus* sp. by some isolates may also indicate that these legumes are not the natural hosts of these strains. It would now be interesting to demonstrate their capacities to induce nodule development on other wild legumes grown on the same Tunisian soils. It is also important to investigate the existence of *nod* and *nif* genes in these strains by southern hybridization before the status of these strains as nodule symbionts could be considered.

Our results showed that Tunisian *Lotus* rhizobia belong to *Sinorhizobium*, *Rhizobium*, and *Mesorhizobium* species: *L. creticus* was nodulated by three rhizobium genera, *L. pusillus* only by two genera (*Sinorhizobium* and *Rhizobium*) and *L. arabicus* only by *Sinorhizobium* genus nodule bacteria. Jarvis et al. reported that rhizobia-nodulating *Lotus* species belong to fast-growing *R. loti*, modified actually as *M. loti* [11] and Jordan [12] also mentioned that slow-growing *Bradyrhizobium* sp. nodulate *Lotus* species. No *Bradyrhizobium* strains were recovered in our results. These findings confirm those reported by Mahdhi et al. and Zribi et al. [16,30,32,59] with wild LNB in Tunisia. However, Zakhia et al. [13] mentioned that among 69 strains isolated from wild legumes in the infra-arid zone of Tunisia, only two strains were assigned to the *Bradyrhizobium* genus. The large diversity of rhizobia-nodulating *Lotus* species may indicate that *Lotus* sp. accept many different microsymbionts and indicate narrow host specificity.

In summary, our study is the first report on the characterization of *L. pusillus* and *L. arabicus* microsymbionts in Tunisia. We evidenced a novel biodiversity among bacteria isolated from the *Lotus* species. Three genera, *Sinorhizobium*, *Rhizobium*, and *Mesorhizobium* were represented among the *Lotus* isolates, most of which were related to previously described LNB in Tunisian soils. *L. creticus* nodulated by several genomspecies representing one, two or three genera can be qualified as promiscuous.

From an applied perspective, the leguminous species selected for this study are suitable for revegetation and soil-restoration projects in the arid soils of Tunisia. In addition, the inoculation of seeds and seedlings with appropriate native rhizobia resistant to salinity and acidity would guarantee root nodulation, enhance plant performance, and reintroduce these micro-organisms in the soil.

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