



***Rhizobium acaciae* sp. nov., a new nitrogen-fixing symbiovar isolated from root nodules of *Acacia saligna* in Tunisia**

Jihed Hsouna, Houda Ilahi, Jia-Cheng Han, Takwa Gritli, Walid Ellouze, Xiao Xia Zhang, Maroua Mansouri, Praveen Rahi, Mustapha Missbah El Idrissi, Mouad Lamrabet, et al.

► To cite this version:

Jihed Hsouna, Houda Ilahi, Jia-Cheng Han, Takwa Gritli, Walid Ellouze, et al.. *Rhizobium acaciae* sp. nov., a new nitrogen-fixing symbiovar isolated from root nodules of *Acacia saligna* in Tunisia. *International Journal of Systematic and Evolutionary Microbiology*, 2023, 73, pp.art. 005900. <10.1099/ijsem.0.005900>. <hal-04111329>

HAL Id: hal-04111329

<https://hal.inrae.fr/hal-04111329v1>

Submitted on 31 May 2023

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



HAL Authorization

Rhizobium acaciae sp. nov., a new nitrogen-fixing symbiovar isolated from root nodules of *Acacia saligna* in Tunisia

Jihed Hsouna¹, Houda Ilahi¹, Jia-Cheng Han², Takwa Gritli¹, Walid Ellouze³, Xiao Xia Zhang², Maroua Mansouri¹, Praveen Rahi⁴, Mustapha Missbah El Idrissi⁵, Mouad Lamrabet⁵, M'hamed Oubla⁶, Pierre Emmanuel Courty⁷, Daniel Wipf⁷, James T. Tambong^{8,*} and Bacem Mnasri^{1,*}

Abstract

Three bacterial strains, 1AS11^T, 1AS12 and 1AS13, members of the new symbiovar *salignae* and isolated from root nodules of *Acacia saligna* grown in Tunisia, were characterized using a polyphasic approach. All three strains were assigned to the *Rhizobium leguminosarum* complex on the basis of *rrs* gene analysis. Phylogenetic analysis based on 1734 nucleotides of four concatenated housekeeping genes (*recA*, *atpD*, *glnII* and *gyrB*) showed that the three strains were distinct from known rhizobia species of the *R. leguminosarum* complex and clustered as a separate clade within this complex. Phylogenomic analysis of 92 up-to-date bacterial core genes confirmed the unique clade. The digital DNA–DNA hybridization and BLAST-based average nucleotide identity values for the three strains and phylogenetically related *Rhizobium* species ranged from 35.9 to 60.0% and 87.16 to 94.58%, which were lower than the 70 and 96% species delineation thresholds, respectively. The G+C contents of the strains were 60.82–60.92mol% and the major fatty acids (>4%) were summed feature 8 (57.81%; C_{18:1} ω7c) and C_{18:1} ω7c 11-methyl (13.24%). Strains 1AS11^T, 1AS12 and 1AS13 could also be differentiated from their closest described species (*Rhizobium indicum*, *Rhizobium laguerreae* and *Rhizobium changzhense*) by phenotypic and physiological properties as well as fatty acid content. Based on the phylogenetic, genomic, physiological, genotypic and chemotaxonomic data presented in this study, strains 1AS11^T, 1AS12 and 1AS13 represent a new species within the genus *Rhizobium* and we propose the name *Rhizobium acaciae* sp. nov. The type strain is 1AS11^T (=DSM 113913^T=ACCC 62388^T).

INTRODUCTION

Acacia is a multipurpose, fast-growing tree species of the family *Fabaceae*. This genus comprises over 1350 species that are distributed in the warm regions of the world, including Australia, the Americas, Africa and Asia [1]. *Acacia saligna* (Labill.) Wendl is an invasive, fast-growing woody tree that has been introduced to the Mediterranean coast in the Maghreb area including Tunisia for many different purposes, including revegetation of arid regions [2]. These plants enrich soil nitrogen in symbiotic association with various genera of rhizobia such as *Mesorhizobium*, *Rhizobium*, *Ensifer* and preferentially *Bradyrhizobium* [3]. In a recent study, Hsouna *et al.* [4] reported that *Acacia saligna* plants grown in Tunisian soils are promiscuous and were nodulated by fast

Author affiliations: ¹Laboratory of Legumes and Sustainable Agrosystems, Centre of Biotechnology of Borj-Cédria, BP 901 Hammam-lif 2050, Tunisia; ²Agricultural Cultural Collection of China, Institute of Agricultural Resources and Regional Planning, Chinese Academy of Agricultural Sciences, Beijing 100080, PR China; ³Agriculture and Agri-Food Canada, 4902 Victoria Avenue North, Vineland Station, Ontario, L0R 2E0, Canada; ⁴Institut Pasteur, Université Paris Cité, Biological Resource Center of Institut Pasteur (CRBIP), Paris, France; ⁵Faculty of Sciences, Centre de Biotechnologies Végétale et Microbienne, Biodiversité et Environnement, Mohammed V University in Rabat, Rabat, Morocco; ⁶Faculty of Science, Materials and Nanomaterials for Photovoltaic Conversion and Electrochemical Storage, Mohammed V University in Rabat, Rabat, Morocco; ⁷Agroécologie, Institut Agro Dijon, CNRS, Univ. Bourgogne, INRAE, Univ. Bourgogne Franche-Comté, F-21000 Dijon, France; ⁸Agriculture and Agri-Food Canada, 960 Carling Avenue, Ottawa, Ontario, K1A 0C6, Canada.

***Correspondence:** Bacem Mnasri, mnbacemm@yahoo.com; James T. Tambong, james.tambong@agr.gc.ca

Keywords: *Acacia saligna*; nodules; *Rhizobium acaciae*; *Rhizobium leguminosarum* complex.

Abbreviations: ANI, Average Nucleotide Identity; dDDH, Digital DNA–DNA hybridization; YMA, Yeast Mannitol Agar.

The 16S rRNA, *gyrB*, *atpD*, *recA* and *glnII* sequences of strains 1AS11^T, 1AS12 and 1AS13 have been deposited in the GenBank under the accession numbers OM392037, OP782378, OP782379 (16 rRNA), OM429456, OM429455, OM429457 (*gyrB*), OM429388, OM429386, OM429385 (*atpD*), OM429391, OM429392, OM429393 (*recA*), OM429438, OM429440 and OM429442 (*glnII*). The draft genome sequences of 1AS11^T, 1AS12 and 1AS13 were deposited under the accession numbers JAPCZM000000000, JAPCZN000000000 and JAPCZO000000000, respectively. The Illumina NovaSeq 6000 raw reads are deposited in the Sequence Read Archive under bioproject number PRJNA893020.

Five supplementary figures are available with the online version of this article.

growing organisms that could constitute putative new species within the genus *Rhizobium*. The 100 bacterial strains obtained in the study of Hsouna *et al.* [4] were characterized into three distinct ribotypes based on *rrs* PCR-RFLP analysis. Sequence analyses of *rrs* and four housekeeping genes (*recA*, *atpD*, *gyrB* and *glnII*) assigned 30 isolates to four putative new lineages of the genera *Rhizobium* and *Bradyrhizobium* and a single isolate to *Sinorhizobium meliloti*. One of these new lineages consisted of 10 isolates, including the representative strains 1AS11^T, 1AS12 and 1AS13. Phylogenetic analysis of 1734-bp concatenated sequences of *recA*, *atpD*, *glnII* and *gyrB* clustered strains 1AS11^T, 1AS12 and 1AS13 in a distinct clade within the *R. leguminosarum* complex (Rlc). The closest described species were *R. laguerreae* and *R. indicum* [4]. Interestingly, based on the divergence of *nodA/nodC* sequences and host range nodulation patterns used in previous study for symbiovar delineation [5–7], strains 1AS11^T, 1AS12 and 1AS13 were classified as a new symbiovar named *saligna* [4]. The three strains effectively nodulated *A. saligna*, *A. salicina* and *Leucaena leucocephala*, while they failed to nodulate *Glycine max*, *Phaseolus vulgaris* and *Retama raetam*.

In the present study, the taxonomic status of strains 1AS11^T, 1AS12 and 1AS13 was investigated using a polyphasic approach including genome-based analyses.

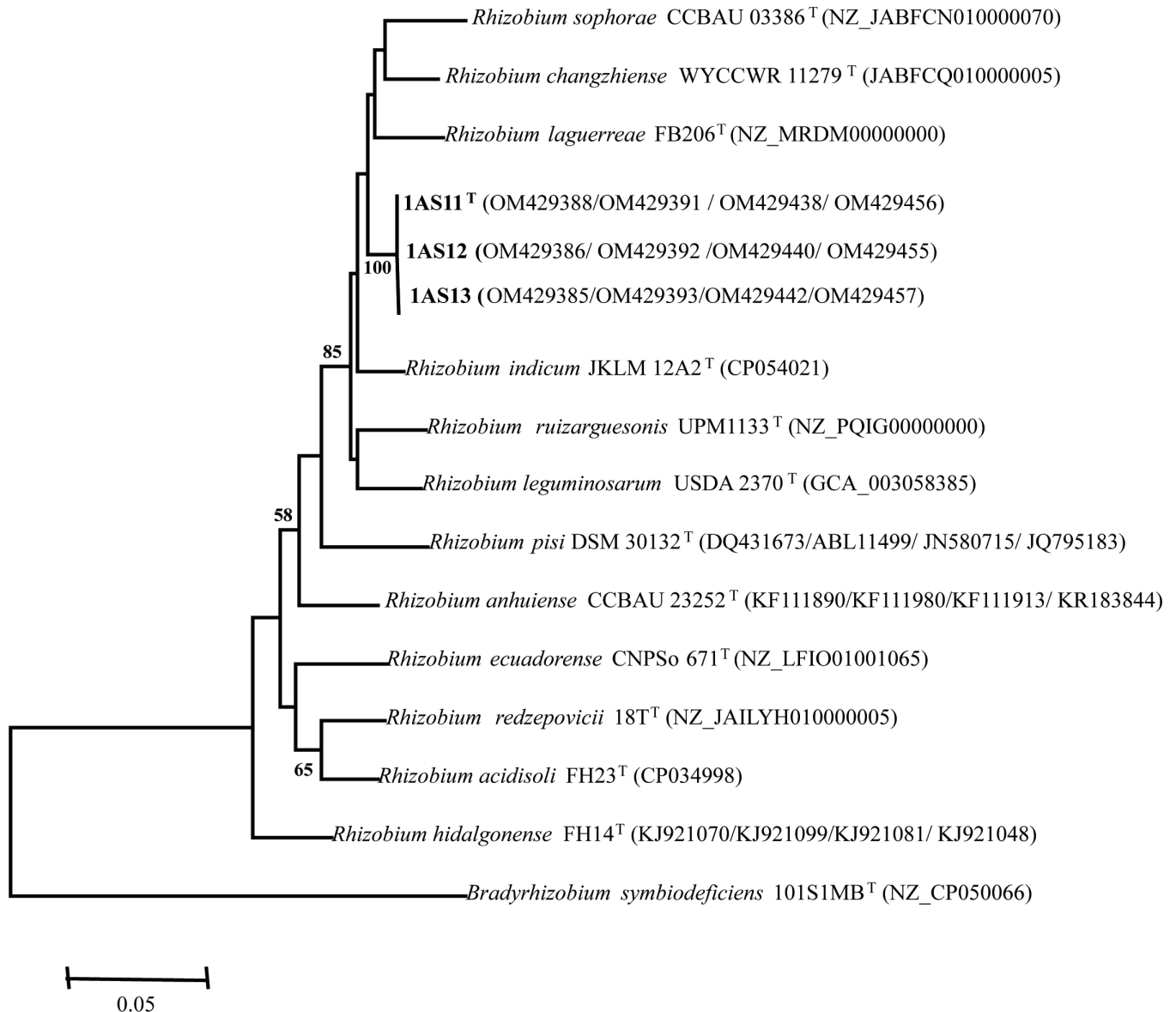


Fig. 1. Maximum-Likelihood-based phylogenetic analysis of *recA-atpD-gyrB-glnII* concatenated gene sequences (1734 nucleotides). The three novel strains are in bold. Bootstrap values ≥ 50 are indicated for each node (1000 replicates). After species name, the strain designation is given followed by the NCBI accession number of the sequence used. The scale indicates the number of substitutions per site.

Table 1. Basic statistics of whole-genome sequences of the three strains of *Rhizobium acaciae* sp. nov, sequenced using Illumina NovaSeq 6000 technology

Description	1AS11 ^T	1AS12	1AS13
Number of short reads (151 bp length)	33112583	33112583	33112583
Average coverage depth	1213.9×	1314.8×	1226.6×
Contig count	84	79	112
Largest contig (bp)	761366	1110614	761366
Total length (bp)	8011864	7494653	8015410
Contigs N ₅₀ (bp)	247715	270518	247715
Contigs L ₅₀	12	9	12
Protein-encoding genes with functional assignment	5037	4743	5036
Total protein-coding sequences (CDSs)	7714	7127	7758
misc_RNA	75	67	75
Number of rRNA	3	3	3
Number of tRNA	48	50	48
Number of tmRNA	1	1	1
G+C content (mol%)	60.82	60.92	60.82

Table 2. Digital DNA–DNA hybridization (dDDH), average nucleotide identity (ANI) and BLAST-based average nucleotide identity (ANiB) values among *Rhizobium acaciae* sp. nov. AS11^T (type strain) and related *Rhizobium* species

<i>Rhizobium</i> species	dDDH	ANI	ANiB
<i>Rhizobium acaciae</i> sp. nov. 1AS11 ^T (JAPCZO01)	100	100	100
<i>Rhizobium acaciae</i> sp. nov. 1AS13 (JAPCZM01)	100 (0)	99.99	99.97
<i>Rhizobium acaciae</i> sp. nov. 1AS12 (JAPCZN01)	90.7 (0.0114)	98.84	98.72
<i>Rhizobium indicum</i> JKLM 12A2 ^T (CP054021)	60.0 (0.0515)	94.95	94.58
<i>Rhizobium laguerreae</i> FB 206 ^T (MRDM01)	56.1 (0.0588)	94.08	93.19
<i>Rhizobium changzhiense</i> WYCCWR 11279 ^T (JABFCQ01)	53.8 (0.0634)	93.68	93.08
<i>Rhizobium sophorae</i> CCBAU 03386 ^T (JABFCN01)	53.2 (0.0645)	93.59	92.50
<i>Rhizobium ruizarguesonis</i> UPM 1133T (PQIG01)	52.7 (0.0656)	93.52	92.27
<i>Rhizobium leguminosarum</i> DSM 106839 ^T (JACHFX01)	49.1 (0.0737)	92.67	91.34
<i>Rhizobium indigoferae</i> CCBAU 71042 (JABFCO01)	49.0 (0.0740)	92.66	91.57
<i>Rhizobium trifolii</i> DSM 30141 ^T (JACJHV01)	49.2 (0.0736)	92.60	91.48
<i>Rhizobium anhuiense</i> CGMCC 112621 ^T (BMFI01)	42.5 (0.0915)	91.07	89.97
<i>Rhizobium acidisoli</i> FH23 ^T (CP034998)	37.3 (0.1090)	89.50	88.79
<i>Rhizobium redzepovicii</i> 18T ^T (JAILYH01)	36.5 (0.1123)	88.91	87.60
<i>Rhizobium hidalgenense</i> FH14 ^T (LODW01)	35.9 (0.1146)	88.68	87.16

ISOLATION AND ECOLOGY

The three strains were isolated from root nodules of *A. saligna* grown in the locality of Borj Cedria in Tunisia [4]. Nodules were surface sterilized and bacteria were extracted following the standard procedures of Vincent [8]. Pure single colonies of the bacterial cultures were obtained by repetitive streaking on yeast–mannitol agar (YMA) as described previously [4]. Aliquots of the purified single colonies were preserved in 15% (v/v) glycerol suspensions at −80 °C. The nodulation and nitrogen-fixation performance of the three novel strains was tested on various legume species.

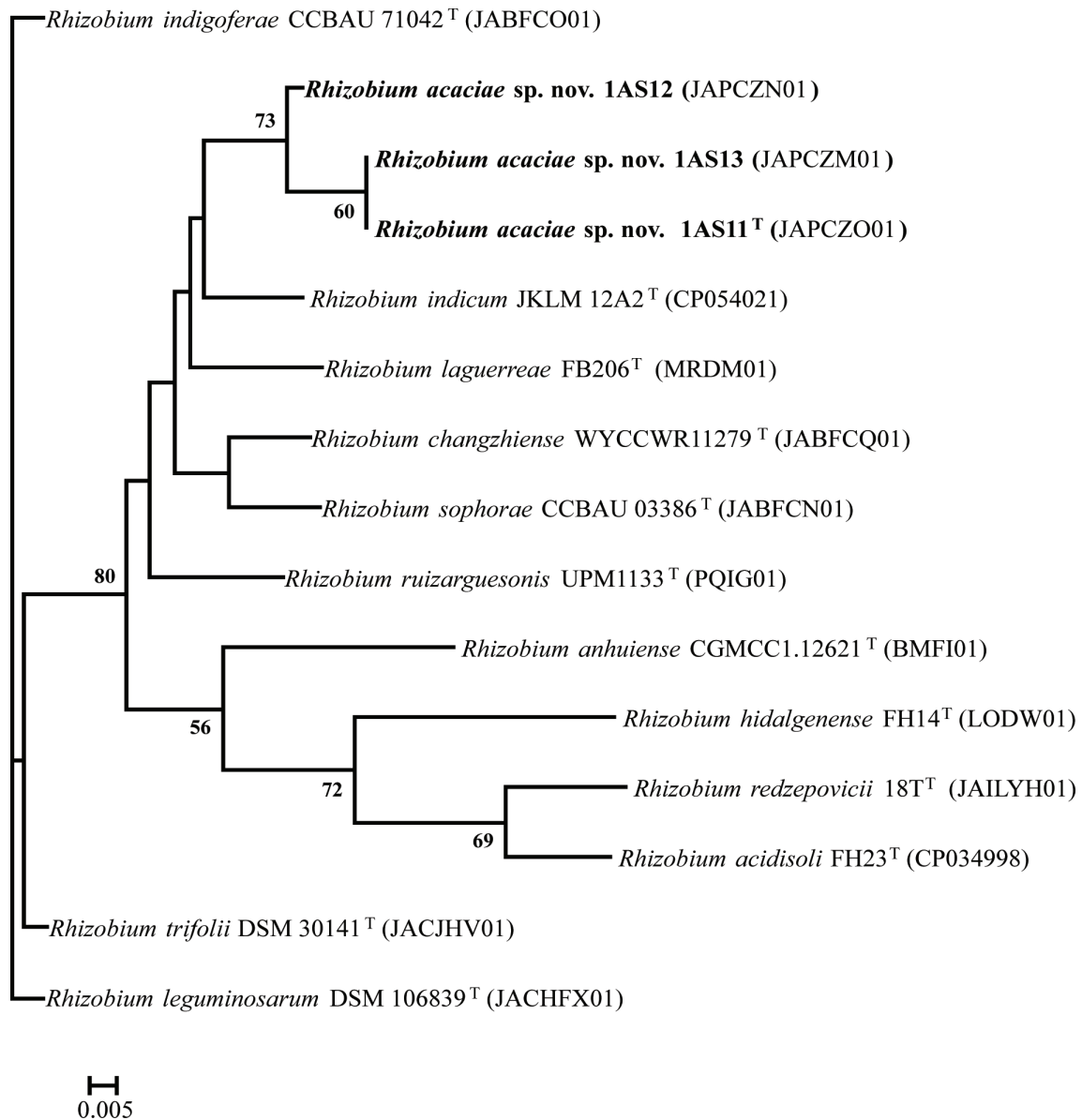


Fig. 2. Unrooted maximum-likelihood phylogenetic tree based on UBCGs (concatenated alignment of 92 core genes) inferred using RAxML-ng with the GTR +CAT model. Percentage bootstrap values are given at branching points. Bar, 0.005 substitution per position.

***rrs*, *recA*, *atpD*, *gyrB* AND *glnII* PHYLOGENY**

Bacterial DNA was extracted from three isolates and used as template for 50 µl PCR reactions [4]. The *rrs* gene were sequenced using the primers fD1 and rD1 [9]. Amplification and sequencing of *recA*, *atpD*, *glnII* and *gyrB* genes were carried out according to the methods of Hsouna et al. [4]. PCR-amplified products were purified from agarose gels using the EZ-10 spin column DNA gel extraction Minipreps Kit (Bio Basic Canada Inc.) following the manufacturer's instructions. Purified DNA fragments were sequenced at the Centre of Biotechnology, Borj-Cédria, Tunisia. The sequence similarities in the DNA databases were examined using the BLAST program (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). The *rrs* gene sequences were compared with NCBI reference sequences. Sequences were aligned using the ClustalW2 software (www.ebi.ac.uk/Tools/clustalw2/). Maximum-likelihood (ML) phylogenetic trees were inferred using the MEGA7 [10] using the Kimura two-parameter substitution model [11] with the pairwise deletion option [12]. Bootstrap analysis was based on 1000 re-samplings and values >50% are shown on the phylogenetic trees.

In the phylogenetic tree based on partial *rrs* gene sequences (1215 nt), all three new strains grouped together and showed 100% similarity to *R. laguerreae*, *R. changzhiense*, *R. anhuiense*, *R. leguminosarum*, *R. ruizarguesonis*, *R. acidisoli*, *R. hidalgonense*, *R. indicum*, *R. sophorae* and *R. redzepovicii* type strains. Except for *R. anhuiense*, *R. acidisoli* and *R. hidalgonense*, these species

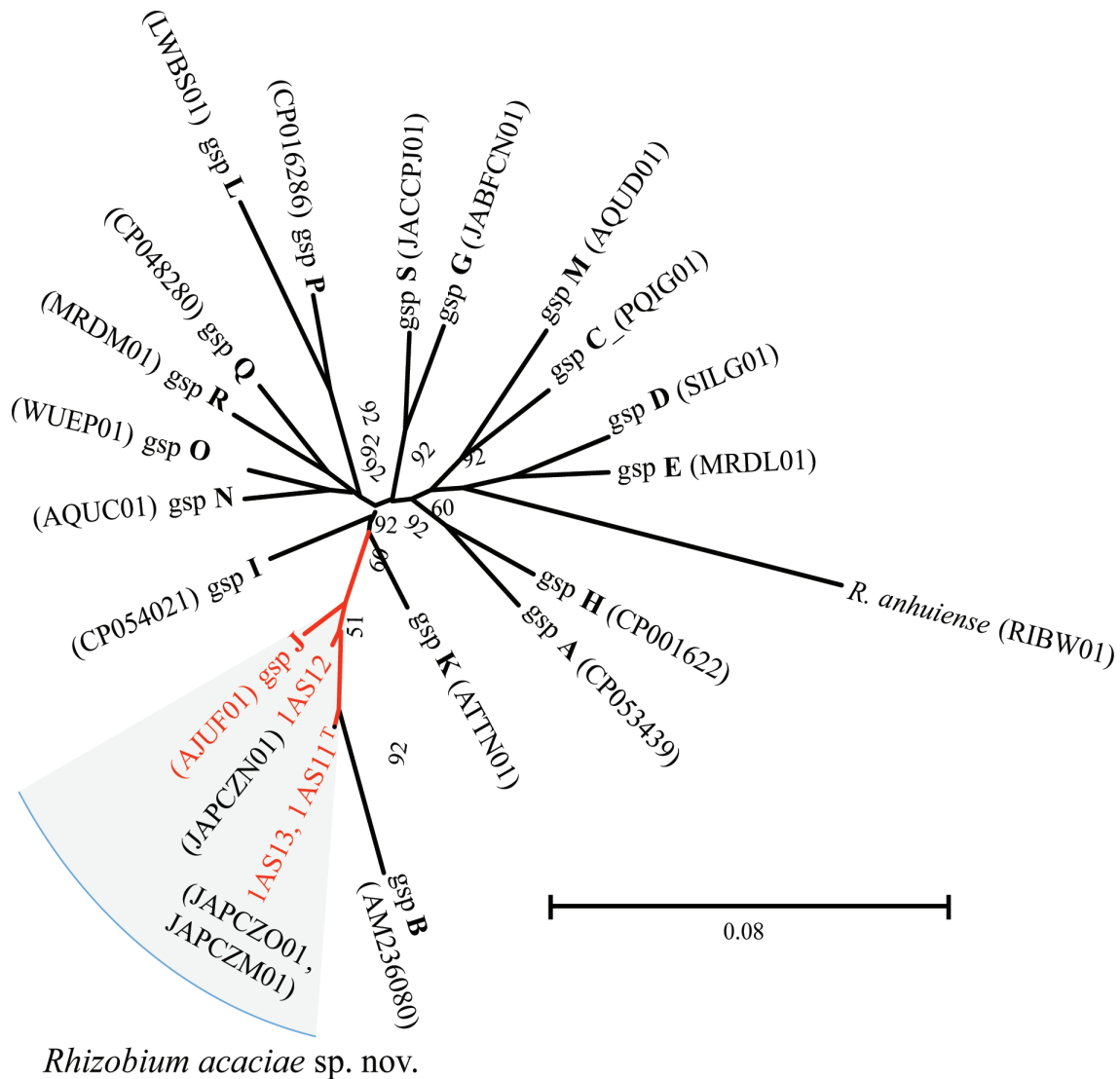


Fig. 3. Maximum-likelihood phylogenetic tree based on UBCGs (concatenated alignment of 92 core genes) of strains of *Rhizobium acaciae* sp. nov. and representatives of the 18 genomespecies proposed by Young *et al.* [13]. Tree was inferred using RAxML-ng with the GTR +CAT model. Percentage bootstrap values are given at branching points. Strains of *R. acaciae* sp. nov. are affiliated with genomespecies j. Letters in bold denote the genomespecies proposed by Young *et al.* [13].

were recently named to belong to the Rlc [13]. Fig. S1 (available in the online version of this article) shows an ML phylogenetic tree with the three strains of the proposed *R. acaciae* sp. nov. clustering with the type strains of the Rlc at a bootstrap value of 76%. This suggests that the three strains of *R. acaciae* sp. nov. belong to the Rlc. Neighbour-joining and minimum-evolution algorithms were also used to reconstruct phylogenetic relationships and showed similar topologies (Figs S2 and S3). Given that, *rrs* phylogenies have been reported to show low-resolution power among closely related species [14, 15], multilocus sequence analysis was performed using four housekeeping genes (*recA*, *atpD*, *gyrB* and *glnII*). Based on *recA-atpD-gyrB-glnII* concatenated sequences (1734bp), the three novel strains were divergent from strains of type strains of previously described *Rhizobium* species of the Rlc group and the closest described species with were *R. indicum*, *R. laguerrea* and *R. changzhiense*. Based on the phylogenetic analysis of *recA-atpD-gyrB-glnII*, the three strains of *R. acaciae* sp. nov. clustered in a distinct clade (bootstrap of 100%), an indication that these strains are unique (Fig. 1).

GENOMIC RELATEDNESS AND PHYLOGENOMICS

Draft whole-genome sequences of the three new strains were generated using Illumina NovaSeq 6000 technology (G  nome Qu  bec, Montreal, Canada). The quality of the raw reads (2  150bp) was checked using the FastQC algorithm [16]. *De novo* assembly was

Table 3. Phenotypic results on Biolog GENIII MicroPlates

Strains: 1AS11^T; 2, 1AS12; 3, 1AS13; 4, *Rhizobium indicum* JKLM 12A2^T; 5, *Rhizobium laguerreae* FB206^T; 6, *Rhizobium changzhiense* WYCCWR 11279^T.
 +, Positive; −, negative; w, weakly positive.

<i>Rhizobium acaciae</i> sp. nov.						
Biolog substrate	1	2	3	4	5	6
Trehalose	+	+	+	w	−	−
Sucrose	−	−	−	+	−	−
Turanose	+	+	+	w	−	−
Raffinose	+	+	+	−	−	−
Lactose	+	+	+	−	−	−
Melibiose	+	+	+	−	+	−
Methyl β-D-glucoside	+	+	+	−	+	+
N-Acetyl-D-glucosamine	+	+	+	−	+	+
N-Acetyl-β-D-mannosamine	+	+	+	−	+	−
N-Acetyl-D-galactosamine	−	−	−	−	+	−
N-Acetyl neuraminic acid	−	−	−	−	−	+
α-D-Glucose	+	+	+	−	−	−
D-Mannose	+	+	+	−	−	−
D-Galactose	+	+	+	−	+	+
D-Fucose	+	+	+	−	+	+
L-Rhamnose	+	+	+	−	−	+
D-Arabitol	+	+	+	−	−	+
myo-Inositol	+	+	+	−	−	+
Glycerol	+	+	+	−	+	+
D-Fructose-6-PO ₄	−	−	−	+	+	−
L-Glutamic acid	+	+	+	−	+	+
L-Histidine	+	+	+	−	−	+
L-Pyroglutamic acid	+	+	+	−	+	−
D-Galacturonic acid	−	−	−	+	+	−
D-Gluconic acid	+	+	+	−	+	+
D-Glucuronic acid	−	−	−	+	+	−
Mucic acid	−	−	−	−	−	+
Quinic acid	−	−	−	−	−	+
Methyl pyruvate	+	+	+	−	−	−
L-Lactic acid	−	−	−	−	−	+
D-Malic acid	−	−	−	−	−	+
L-Malic acid	−	−	−	−	+	w
Tween 40	−	−	−	−	w	+
α-Hydroxy-butyric acid	−	−	−	−	−	+
β-Hydroxy-D,L butyric acid	−	−	−	−	+	+

Continued

Table 3. Continued

<i>Rhizobium acaciae</i> sp. nov.						
Biolog substrate	1	2	3	4	5	6
α -Keto-butyric acid	–	–	–	–	–	+
Acetoacetic acid	–	–	–	–	–	+
Propionic acid	–	–	–	–	–	+
Acetic acid	–	–	–	w	–	+
Formic acid	–	–	–	–	–	+
Chemical sensitivity						
pH 6	+	+	+	–	–	–
pH 5	+	+	+	–	–	–
NaCl 1%	–	–	–	–	+	–
Antibiotic resistance						
Rifamycin	–	–	–	–	–	+
Lincomycin	–	–	–	–	–	+
Kanamycin	+	+	+	NA	–	–
Streptomycin	+	+	+	NA	–	–
API ZYM						
Alkaline phosphomonoesterase	–	–	–	–	–	w
Esterase	–	–	–	NA	–	w
Cystinyl-arylamidase	w	w	w	NA	+	+
<i>N</i> -Acetyl- β -glucosaminase	–	–	–	NA	–	+
α -Mannosidase	w	w	w	NA	+	+
β -Fucosidase	w	w	w	NA	+	+

performed using Unicycler version 0.4.8 [17] and assessed using QUAST [18] as implemented in PATRIC 3.6.12 [19, 20]. Contigs of length <300 bp were discarded. Where required, assembled genome sequences were annotated using Prokka version 1.12 [21]. Table 1 shows the basic statistics of the sequenced draft genomes. For each of the strains, 33 112 583 raw reads were generated. After quality checks, the reads used in the assembling processed gave an average coverage depth of 1213.9 $\times$, 1314.8 $\times$ and 1226.6 $\times$ with contig counts of 84, 79 and 112, for strains 1AS11^T, 1AS12 and 1AS13, respectively. The completeness of the genomes was 100% with coarse and fine consistencies of 99.8–99.9% and 96.8–96.9%, respectively, with a contamination rate of 0.5% (CheckM algorithm) [22]. The largest contigs were 761366, 1110614 and 761366 bases and had N_{50} values of 247715, 270518 and 247715 bases for strains 1AS11^T, 1AS12 and 1AS13. The total lengths of the draft genomes were 8.01, 7.49 and 8.02 Mb, respectively, with G+C contents of 60.82–60.92 mol% (Table 1). Annotation reports of the genome sequences identified a total of 7714, 7127 and 7758 protein-coding sequences as well as 48 or 50 tRNAs, three rRNAs and one tmRNA (Table 1). The generated new draft whole-genome sequences were used to confirm the uniqueness of these strains relative to closest related *Rhizobium* species.

Genome-sequence-based digital DNA–DNA hybridization (dDDH; GGDC 3.1 [23]), BLAST-based average nucleotide identity (ANIb; JSpecies [24]) and ANI (FastANI [25]) values were computed and compared to 12 validly published close *Rhizobium* species to validate the taxonomic position of strains. The three strains (1AS11^T, 1AS12 and 1AS13) exhibited dDDH, ANIb and ANI values significantly above the species-level cut-offs of 70 and 96%, validating that these three belong to the same *Rhizobium* species (Table 2). For example, strain 1AS11^T showed dDDH, ANI and ANIb values of 100, 99.99 and 99.97% with strain 1AS13 (Table 2). Strain 1AS12 exhibited lower dDDH, ANIb and ANI values with 1AS11^T but still significantly above the species-level cut-off (Table 2). The type strain of the proposed *R. acaciae* sp. nov, strain 1AS11^T, had lower relatedness with the 12 closest species with values significantly below the cut-off values of 70 and 96% for dDDH and ANIb and ANI, respectively (Table 2). *R. indicum* JKLM 12A2^T showed the highest dDDH of 60.0% (cut-off, 70%) and ANIb and ANI values of 94.58 and 94.95% (cut-off, 96.00%) with strain 1AS11^T followed by *R. laguerreae* FB206^T with values of 56.10, 93.19 and 94.08% (Table 2), all below the respective

Table 4. Fatty acid composition (%) of 1AS11^T and its related *Rhizobium* type strains

Strains: 1, 1AS11^T; 2, *Rhizobium indicum* JKLM 12A2^T; 3, *Rhizobium laguerreae* FB206^T; 4, *Rhizobium changzhense* WYCCWR 11279^T. –, Not detected.

Fatty acid	1	2	4	4
C _{15:0} 3OH	0.27	–	–	–
C _{17:0}	0.78	–	–	–
C _{17:1} ω8c	0.26	–	–	–
C _{17:1} ω6c	0.1	–	–	–
C _{17:0} 3OH	0.16	–	–	–
C _{17:1} anteisoω9c	0.32	–	–	–
C _{18:1} ω9c	0.6	–	–	0.5
C _{18:1} ω7c 11-methyl	13.24	1.8	1.9	–
C _{20:1} ω7c	0.45	–	–	–
C _{20:2} ω6,9c	0.17	–	–	–
Summed feature 8*	57.81	68	69.3	74.9

*Summed feature 8: C_{18:1} ω7c.

cut-off species delineation threshold. These data are consistent with the results of the *recA-atpD-gyrB-glnII* phylogenetic analysis, suggesting that strains 1AS11^T, 1AS12 and 1AS13 constituted a novel species in the genus *Rhizobium*. The dDDH, ANIb and ANI results were corroborated by the phylogenomics tree [26, 27] inferred using GBDP-derived intergenomic distances and computed on the Type (Strain) Genome Server (TyGS [28, 29]) (Fig. S4). Also, the uniqueness of strains 1AS11^T, 1AS12 and 1AS13 was validated by phylogenetic analysis of a concatenated alignment of 92 core genes as implemented in the up-to-date bacterial core gene (UBCG) tool [30] (Fig. 2). Both TyGS and UBCG pipelines generated phylogenomic trees that showed strains 1AS11^T, 1AS12 and 1AS13 clustering uniquely from the closest known species within the genus *Rhizobium* (Fig. S4; Fig. 2). Based on these genome data and analyses, these new strains of the genus *Rhizobium* constitute a new species.

In addition, the whole genome sequences generated in this study were used to locate the proposed new species within the 18 genomospecies (gsp) groupings according to Young *et al.* [13]. The UBCG tool was used to implement an ML phylogenomics tree of strains 1AS11^T, 1AS12 and 1AS13 and the proposed representatives of the genomospecies of Young *et al.* [13]. Fig. 3 shows an unrooted tree of the *R. acaciae* strains clustering closely with the representative strain of gsp J. The gsp J was represented by two strains WSM1455 (AJUF01) and WSM1481 (AQUM01) and these strains were classified as unique [13] but deposited in GenBank as *Rhizobium leguminosarum* bv. viciae. Interestingly, a taxonomy check using GenBank's algorithm flagged these two strains as “inconclusive” to be classified as *R. leguminosarum*. These two unique strains, WSM1455 (AJUF01) and WSM1481 (AQUM01), exhibited dDDH and ANI values of 83.10 and 98.07% with the type strain of the proposed *R. acaciae* 1AS11^T. These values are significantly above the species-level cut-off of 70 and 96%, respectively, suggesting that strains WSM1455 and WSM1481 are genetically valid members of the proposed new species, *R. acaciae* sp. nov. Both WSM1481 and WSM1455 were isolated from roots of *Vicia faba* plants in the Greek island of Mykonos (GOLD database; <https://gold.jgi.doe.gov/organism?id=Go0007354>). This suggests that members of the proposed new species, *R. acaciae* sp. nov., might have preference for plant species of the family *Fabaceae*, from cultivated legumes (broad beans) to small wild trees, e.g. golden wreath wattle (*Acacia saligna*).

PHYSIOLOGY AND CHEMOTAXONOMY

Colonies of strains 1AS11^T, 1AS12 and 1AS13, as examined on YMA, were typical rhizobial-like translucent, cream-white and convex. The morphology of the bacterial species was examined with an atomic force microscope (FlexAFM Nanosurf AG) operated in the tapping imaging mode in phase contrast, at room temperature, using a CoreAFM Microscope, Nanosurf Isostage 300. Cells were 0.4–0.5×1–2 μm in size (Fig. S5). The phenotypic and physiological features of the novel isolates were determined and compared with their most related type strains. The characteristics tested included the use of sole carbon and nitrogen sources, tolerance to NaCl, pH, resistance to antibiotics and fatty methyl ester content. Biochemical characteristics, enzyme activities and utilization of carbon sources were performed using the API ZYM systems (07584D and 25 200, bioMérieux) and the Biolog GEN III system following the manufacturers' instructions. Antibiotic resistance testing was carried out on Yeast extract Mannitol (YEM) plates supplemented with five concentrations of each antibiotic (5, 10, 50 80 and 100 μg ml⁻¹). The antibiotics used were streptomycin, kanamycin, ampicillin, neomycin, erythromycin, chloramphenicol and tetracycline. Tolerance to NaCl, pH and

temperature was checked in liquid YEM as previously reported [31]. The optimum pH for growth was assessed on YMA medium plates adjusted to pH 4–10 using 1 M HCl (pH 4.0 and 5.0) or 1 M NaOH (pH 7–10 prior to autoclaving).

Carbon substrate characteristics (Table 3) shared by all three strains of *R. acaciae* sp. nov. that differed from at least one of their closest related type strains were the ability to utilize trehalose, turanose, raffinose, lactose, melibiose, methyl β -D-glucoside, *N*-acetyl-D-glucosamine, *N*-acetyl- β -D-mannosamine, α -D-glucose, D-mannose, D-galactose, D-fucose, L-rhamnose, arabinol and *myo*-inositol. In addition, strains of *R. acaciae* were able to use L-glutamic acid, L-histidine and L-pyroglutamic acid. Other characteristics shown by the strains of the novel species that differed from at least one of these closest type strains were their weak positive enzymatic reaction for cystinyl-arylamidase, α -mannosidase and β -fucosidase and negative reactions for *N*-acetyl- β -glucosaminase. *Rhizobium acaciae* sp. nov. strains were resistant to kanamycin (10 μ g ml⁻¹) and streptomycin (10 μ g ml⁻¹) but sensitive to lincomycin and rifamycin. Fatty acid methyl esters were analysed using the Microbial Identification System (MIDI) and the Microbial Identification software package (Sherlock version 6.1; MIDI database, TSA6). The fatty acid composition and abundance varied between 1AS11^T and closely related type strains of *Rhizobium* species (Table 4). Indeed, the amount of C_{18:1} ω 7c 11-methyl in strain 1AS11^T was higher than that in strains FB206^T of *R. laguerreae* and JKLM12A2^T of *R. indicum*. Also, we detected small amounts of C_{15:0} 3OH (0.27%), C_{17:0} (0.78%), C_{17:1} anteiso ω 9c (0.32%), C_{17:1} ω 8c (0.26%), C_{17:1} ω 6c (0.1%), C_{17:0} 3OH (0.16%), C_{20:1} ω 7c (0.45%) and C_{20:2} ω 6,9c (0.17%) that were absent in FB206^T and JKLM12A2^T.

DESCRIPTION OF RHIZOBIUM ACACIAE SP. NOV.

Rhizobium acaciae (a.ca'ci.ae. L. gen. n. *acaciae*, of the plant genus *Acacia* the host plant from which the novel species was isolated).

Cells of *R. acaciae* are Gram-stain-negative, non-spore forming rods. Colony morphology, as examined on YMA, was typical rhizobial-like translucent, cream-white and convex after 2–3 days of incubation at 28°C. All strains grew at 16–37°C (optimum, 28°C) and pH 6–10 (optimum, pH 7). Strains 1AS11^T, 1AS12 and 1AS13 were not able to grow at NaCl concentrations higher than 0.6%. Detailed phenotypic and biochemical data for the *R. acaciae* strains are given in Table 3. The strains can be distinguished from their most closely related *Rhizobium* species by their ability to use trehalose, turanose, raffinose, lactose, melibiose, methyl β -D-glucoside, *N*-acetyl-D-glucosamine, *N*-acetyl- β -D-mannosamine, α -D-glucose, D-mannose, D-galactose, fucose, L-rhamnose, D-arabinol and *myo*-inositol as sole carbon sources; the strains showed weak positive enzymatic reactions for cystinyl-arylamidase, α -mannosidase and β -fucosidase and negative reactions for *N*-acetyl- β -glucosaminase. Strains are resistant to 10 μ g ml⁻¹ kanamycin and 10 μ g ml⁻¹ streptomycin, but sensitive to lincomycin and rifamycin. The major fatty acid of the type strain 1AS11^T is C_{18:1} ω 7c with 57.81% content.

The type strain of *Rhizobium acaciae* sp. nov. is 1AS11^T (=DSM 113913^T=ACCC 62388^T), which was isolated from root nodules of *Acacia saligna* grown in Borj Cedria, a city in Ben Arous governorate, Tunisia. The G+C content of 1AS11^T is 60.82mol% and the genome size is approximately 8.01 Mb.

Funding information

This study was funded by the PHC Maghreb project (34MAG21) and the Tunisian Ministry of Higher Education and Scientific Research. The contribution of James T. Tambong was funded by Agriculture and Agri-Food Canada through projects J-002272 and J-002295.

Acknowledgements

All contributions made by members of the Centre of biotechnology of Borj Cedria are sincerely appreciated.

Conflicts of interest

The authors declare that there are no conflicts of interest.

References

- Adiamo OQ, Netzel ME, Hoffman LC, Sultanbawa Y. Acacia seed proteins: low or high quality? a comprehensive review. *Compr Rev Food Sci Food Saf* 2020;19:21–43.
- Swelim DM, Nassef MA, Elkhathib EI. Survival and shelf life of leguminous trees rhizobia as affected by sterilization, culture dilution and maltose and trace elements enriched carrier. *J Appl Sci Res* 2010;1366–1372.
- Amrani S, Noureddine N-E, Bhatnagar T, Argandoña M, Nieto JJ, et al. Phenotypic and genotypic characterization of rhizobia associated with *Acacia saligna* (Labill.) Wendl. in nurseries from Algeria. *Syst Appl Microbiol* 2010;33:44–51.
- Hsouna J, Grittli T, Ilahi H, Ellouze W, Mansouri M, et al. Genotypic and symbiotic diversity studies of rhizobia nodulating *Acacia saligna* in Tunisia reveal two novel symbiovars within the *Rhizobium leguminosarum* complex and *Bradyrhizobium*. *Syst Appl Microbiol* 2022;45:126343.
- Rincón-Rosales R, Lloret L, Ponce E, Martínez-Romero E. Rhizobia with different symbiotic efficiencies nodulate *Acaciella angustissima* in Mexico, including *Sinorhizobium chiapanecum* sp. nov. which has common symbiotic genes with *Sinorhizobium mexicanum*. *FEMS Microbiol Ecol* 2009;67:103–117.
- Tampakaki AP, Fotiadis CT, Ntatsi G, Savvas D. A novel symbiovar (aegeanense) of the genus *Ensifer* nodulates *Vigna unguiculata*. *J Sci Food Agric* 2017;97:4314–4325.
- Rogel MA, Ormeño-Orrillo E, Martínez Romero E. Symbiovars in rhizobia reflect bacterial adaptation to legumes. *Syst Appl Microbiol* 2011;34:96–104.
- Vincent JM. *A Manual for the Practical Study of the Root-nodule Bacteria*. Oxford and Edinburgh: Blackwell Scientific; 1970.
- Weisburg WG, Barns SM, Pelletier DA, Lane DJ. 16S ribosomal DNA amplification for phylogenetic study. *J Bacteriol* 1991;173:697–703.

10. Kumar S, Stecher G, Tamura K. MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. *Mol Biol Evol* 2016;33:1870–1874.
11. Kimura MA. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol* 1980;16:111–120.
12. Gevers D, Cohan FM, Lawrence JG, Spratt BG, Coenye T, et al. Opinion: re-evaluating prokaryotic species. *Nat Rev Microbiol* 2005;3:733–739.
13. Young JPW, Moeskjær S, Afonin A, Rahi P, Maluk M, et al. Defining the *Rhizobium leguminosarum* species complex. *Genes* 2020;12:111.
14. Mulet M, Lalucat J, García-Valdés E. DNA sequence-based analysis of the *Pseudomonas* species. *Environ Microbiol* 2010;12:1513–1530.
15. González AJ, Cleenwerck I, De Vos P, Fernández-Sanz AM. *Pseudomonas asturiensis* sp. nov., isolated from soybean and weeds. *Syst Appl Microbiol* 2013;36:320–324.
16. Andrews S. A quality control tool for high throughput sequence data; 2010. <https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>
17. Wick RR, Judd LM, Gorrie CL, Holt KE. Unicycler: resolving bacterial genome assemblies from short and long sequencing reads. *PLoS Comput Biol* 2017;13:e1005595.
18. Gurevich A, Saveliev V, Vyahhi N, Tesler G. QUAST: quality assessment tool for genome assemblies. *Bioinformatics* 2013;29:1072–1075.
19. Wattam AR, Abraham D, Dalay O, Disz TL, Driscoll T, et al. PATRIC, the bacterial bioinformatics database and analysis resource. *Nucleic Acids Res* 2014;42:D581–91.
20. Davis JJ, Wattam AR, Aziz RK, Brettin T, Butler R, et al. The PATRIC bioinformatics resource center: expanding data and analysis capabilities. *Nucleic Acids Res* 2020;48:D606–D612.
21. Seemann T. Prokka: rapid prokaryotic genome annotation. *Bioinformatics* 2014;30:2068–2069.
22. Parks DH, Imelfort M, Skennerton CT, Hugenholtz P, Tyson GW. CheckM: assessing the quality of microbial genomes recovered from isolates, single cells, and metagenomes. *Genome Res* 2015;25:1043–1055.
23. Meier-Kolthoff JP, Auch AF, Klenk H-P, Göker M. Genome sequence-based species delimitation with confidence intervals and improved distance functions. *BMC Bioinformatics* 2013;14:60.
24. Richter M, Rosselló-Móra R, Oliver Glöckner F, Peplies J. JSpeciesWS: a web server for prokaryotic species circumscription based on pairwise genome comparison. *Bioinformatics* 2016;32:929–931.
25. Jain C, Rodriguez-R LM, Phillippy AM, Konstantinidis KT, Aluru S. High throughput ANI analysis of 90K prokaryotic genomes reveals clear species boundaries. *Nat Commun* 2018;9:5114.
26. Farris JS. Estimating phylogenetic trees from distance matrices. *The American Naturalist* 1972;106:645–668.
27. Lefort V, Desper R, Gascuel O. FastME 2.0: a comprehensive, accurate, and fast distance-based phylogeny inference program. *Mol Biol Evol* 2015;32:2798–2800.
28. Meier-Kolthoff JP, Carbasse JS, Peinado-Olarte RL, Göker M. TYGS and LPSN: a database tandem for fast and reliable genome-based classification and nomenclature of prokaryotes. *Nucleic Acids Research* 2022;50:D801–D807.
29. Meier-Kolthoff JP, Göker M. TYGS is an automated high-throughput platform for state-of-the-art genome-based taxonomy. *Nat Commun* 2019;10:2182.
30. Na S-I, Kim YO, Yoon S-H, Ha S, Baek I, et al. UBCG: Up-to-date bacterial core gene set and pipeline for phylogenomic tree reconstruction. *J Microbiol* 2018;56:280–285.
31. Mnasri B, Mrabet M, Laguerre G, Elarbi M, Ridha A. Salt-tolerant rhizobia isolated from a Tunisian oasis that are highly effective for symbiotic N₂-fixation with *Phaseolus vulgaris* constitute a novel biovar (bv. mediterranense) of *Sinorhizobium meliloti*. *Arch Microbiol* 2007;187:79–85.

Five reasons to publish your next article with a Microbiology Society journal

1. When you submit to our journals, you are supporting Society activities for your community.
2. Experience a fair, transparent process and critical, constructive review.
3. If you are at a Publish and Read institution, you'll enjoy the benefits of Open Access across our journal portfolio.
4. Author feedback says our Editors are 'thorough and fair' and 'patient and caring'.
5. Increase your reach and impact and share your research more widely.

Find out more and submit your article at microbiologyresearch.org.