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Ensifer fredii symbiovar vachelliae nodulates endemic *Vachellia gummifera* in semiarid Moroccan areas

Omar Bouhnik[a], Salma ElFaik[a], Soufiane Alami[a], Chouhra Talbi[a], Hanane Lamin[a], Hanaa Abdelmoumen[a], Germán Tortosa Muñoz[b], Eulogio J. Bedmar[b], Mustapha Missbah El Idrissi[a,*]

[a] *Centre de Biotechnologie Végétale et Microbienne, Biodiversité et Environnement, Faculty of sciences, Mohammed V University, 4, Ibn Battouta Avenue, Rabat, Morocco*
[b] *Departamento de Microbiología del Suelo y Sistemas Simbióticos Estación Experimental del Zaidín, CSIC Apartado Postal 419, 18080 Granada, Spain*

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ABSTRACT

The purpose of this work was to study the genetic diversity of the nodule-forming bacteria associated with native populations of *Vachellia gummifera* growing wild in Morocco. The nearly complete 16S rRNA gene sequences from three selected strains, following ARDRA and REP-PCR results, revealed they were members of the genus *Ensifer* and the sequencing of the housekeeping genes *recA*, *gyrB*, *dnaK* and *rpoB*, and their concatenated phylogenetic analysis, showed that the 3 strains belong to the species *E. fredii*. Based on the *nodC* and *nodA* phylogenies, the 3 strains clearly diverged from the type and other reference strains of *E. fredii* and formed a clearly separated cluster. The strains AGA1, AGA2 and AGB23 did not form nodules on *Glycine max, Phaseolus vulgaris* and *Medicago truncatula*, and effectively nodulated *V. gummifera*, *Acacia cyanophylla*, *Prosopis chilensis* and *Leucaena leucocephala*. Based on similarities of the *nodC* and *nodA* symbiotic genes and differences in the host range, the strains isolated from Moroccan endemic *V. gummifera* may form a different symbiovar within *Ensifer* species, for which the name "vachelliae" is proposed.

Introduction

Acacias are legume trees which are well adapted to desert climate characteristics such as salinity, temperature and drought [5]; also, most of them have the ability to regenerate, stabilize and fertilize soils via N_2-fixation and mycorrhizal symbioses [18,20]. Additionally, they constitute a source of wood, fodder, medicine and gum, reasons why acacias are widely used in reforestation programs [3]. The old genus *Acacia* was disintegrated in five genera: *Acacia*, *Senegalia*, *Acaciella*, *Mariosousa* and, based on molecular data, some African acacias were renamed *Vachellia* [28]. *Vachellia* species have been shown to improve soil fertility by increasing the nitrogen status of the soil organic matter [26] and to be more efficient than other N_2- and non-N_2-fixing trees in reestablishing C, N and P cycling processes during forest restoration [57].

V. gummifera is an endemic widespread acacia in the Haouz, Rehamna, Tadla and Chaouia plains of Morocco [30], whose species-area has declined drastically as a result of overgrazing, wrong agricultural practices, desertification and natural difficulties for seed germination.

The slow-growing *Bradyrhizobium* species are the main microsymbionts of Australian acacias [46], but *Rhizobium*, *Ensifer*, *Mesorhizobium*, *Burkholderia*, *Phyllobacterium* and *Devosia* have also been found in the nodules [23]. American acacias are nodulated essentially by *Ensifer* [33,52], although nodulation by *Rhizobium* and *Mesorhizobium* has also been reported [45]. *Vachellia* species in Africa are nodulated by *Mesorhizobium* [12,40], *Ensifer* [2,4,13,15–17,22,24,31,39,48] and, to a lesser extent, by *Rhizobium* [37,39] and *Bradyrhizobium* [19,37,40].

Based on *rrs* sequence analyses, Khbaya et al. [24] first reported that rhizobia isolated from *V. gummifera*, *V. raddiana*, *V. cyanophylla* and *V. horrida* growing in soils of the Moroccan Sahara were closely related to *E. meliloti* and *E. fredii*. More recently, the presence of rhizobia in the Merzouga Desert (Morocco) was detected during metagenomic studies carried out by Gommeaux et al. [21], and Sakrouhi et al. [50] reported that all of the rhizobial bacteria recovered from the sand dunes of Merzouga were identified as members of genus *Ensifer*. Those strains were isolated from nodules of *V. tor-*

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[*] Corresponding author.
E-mail address: missbah49@yahoo.fr (M. Missbah El Idrissi).

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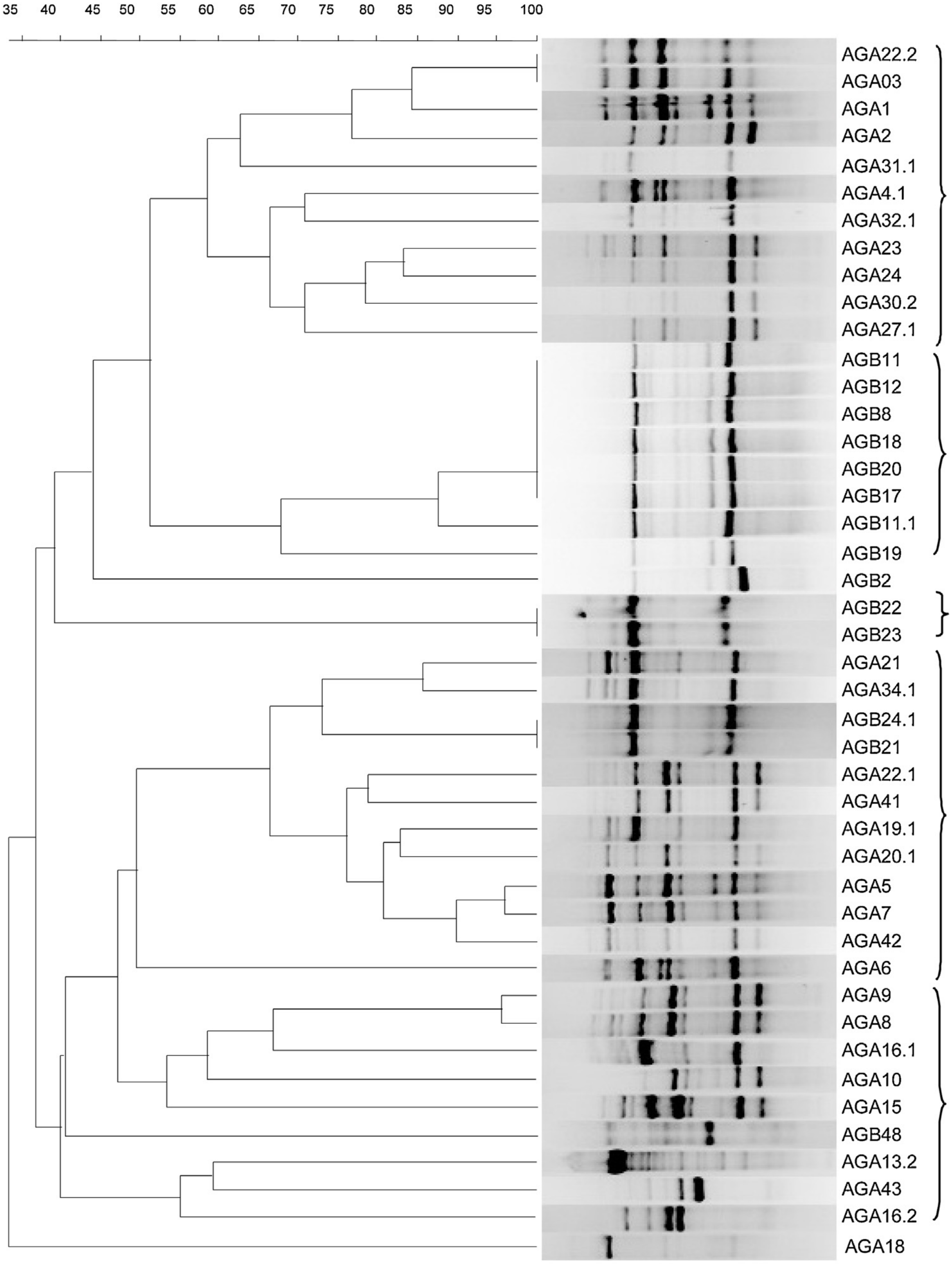

Fig. 1. Cluster analysis of REP-PCR genomic fingerprints patterns from *V. gummifera* isolates using the Dice product moment correlation coefficient and UPGMA method.

tilis and *V. gummifera* and were phylogenetically related to *Ensifer* strains obtained from *Tephrosia purpurea* in the Thar Desert (India) and *Phaseolus filiformis* in the sand beach of Baja California (Mexico), for which the name *E. aridi* was proposed [32].

The number of legumes not yet analyzed for their nitrogen fixing abilities and their association with rhizobia is still very high which means that the number of rhizobial species and symbiovars is still underestimated and will increase [53]. Rogel et al. [47] proposed the term of symbiovar to group the strains within a species that are able to establish symbiosis with a specific legume and symbiovars are defined on the basis of *nodA* and mainly *nodC* symbiotic genes phylogenies [53]. Legumes are nodulated by different symbiovars in the genera *Rhizobium*, *Ensifer*, *Mesorhizobium*, *Neorhizobium*, *Pararhizobium* and *Bradyrhizobium*. Actually, there are sixteen symbiovars described in the genus *Ensifer* [47,51,53].

Because the diversity of bacteria nodulating acacias in the semi-arid region of Morocco is largely unknown, here we aimed to the identification of the microsymbionts associated with the endemic wild-grown *V. gummifera*. To this purpose, ninety four bacteria were isolated from the root nodules of *V. gummifera* growing in soils from the Haha-Essaouira and Bounaga regions. The strains were grouped using REP-PCR and ARDRA methodologies and identified by sequencing the 16S rRNA, *recA*, *gyrB*, *dnaK* and *rpoB* genes. PCR amplification was also used to look for the symbiotic *nodA* and *nodC* genes, and they were studied by sequencing and phylogenies. The phylogenetic analysis of the symbiotic *nod* genes and plant inoculation tests showed *V. gummifera* was nodulated by *E. fredii*, of which strains AGB2, AGB23 and AGA1 clustered out the 3 symbiovars, fredii, mediterranense and aegeanense already reported in that species [38,51], and for which the name vachelliae is proposed.

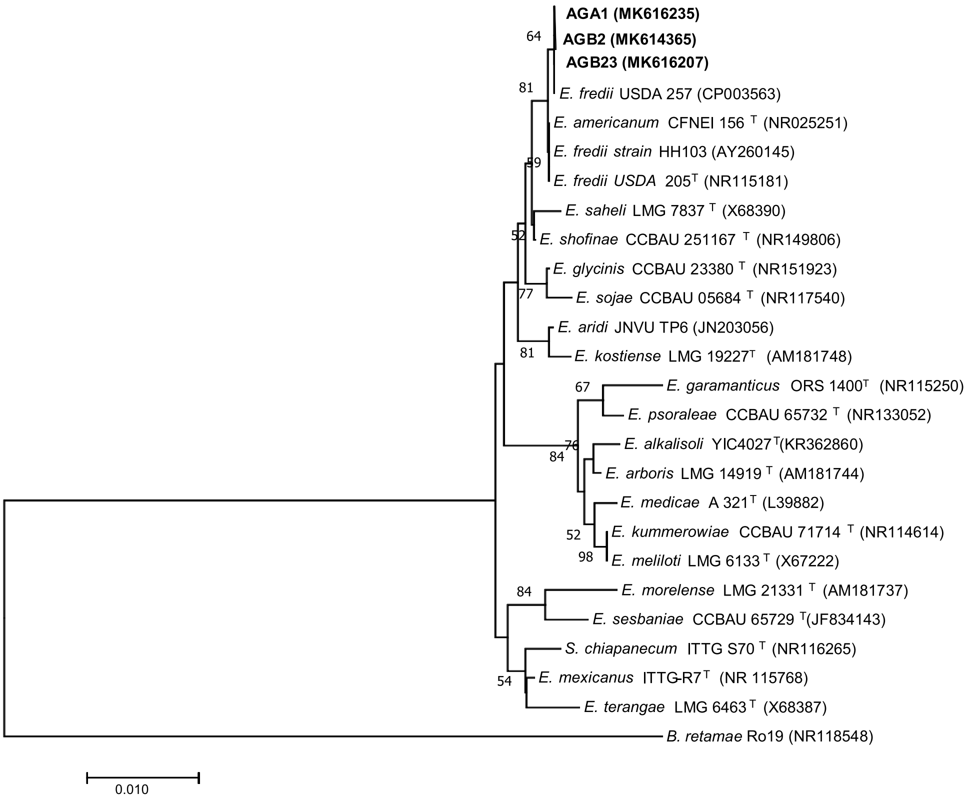

Fig. 2. Neighbor-joining phylogenetic tree based on partial 16S rRNA sequences of strains AGB2, AGB23 and AGA1 isolated from nodules of *V. gummifera* and related species within the genus *Ensifer*. The analysis was based on 1298 nucleotides. Isolates are denoted in bold. Bootstrap values are indicated as percentages derived from 1000 replications. Values lower than 50 are not shown. Bar, 1 nucleotide substitution per 100 nucleotides. The tree is rooted with *B. retamae* Ro19^{T}.

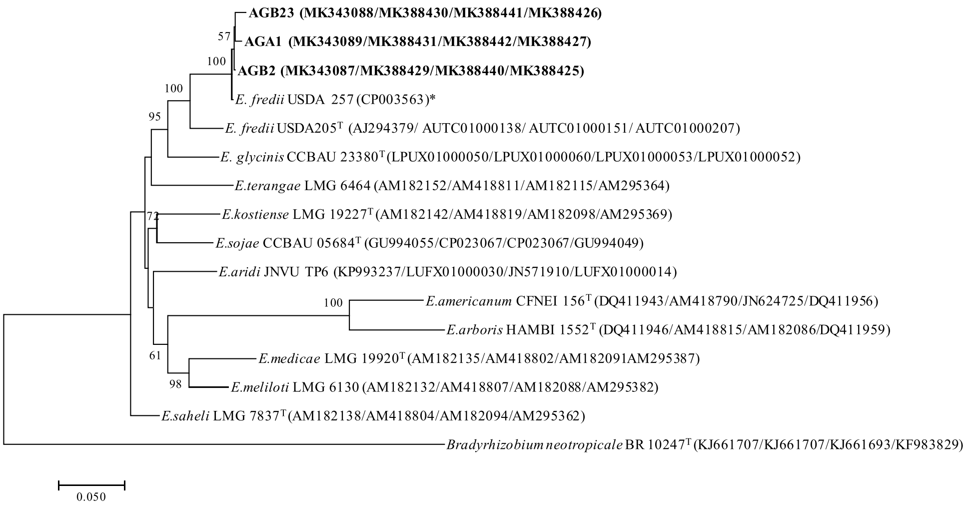

Fig. 3. Neighbor-joining phylogenetic tree based on concatenated *recA* + *gyrB* + *dnaK* + *rpoB* sequences of strains from nodules of wild grown *V. gummifera* and phylogenetically related species within the genus *Ensifer*. The analysis was based on 1376 nucleotides. Isolates are denoted in bold. Bootstrap values are indicated as percentages derived from 1000 replications. Values lower than 50 are not shown. Bar, 1 nucleotide substitution per 100 nucleotides. The tree is rooted with *B. neotropicale* BR10247^{T}.

Materials and methods

Sampling sites, isolation of bacteria from nodules and culture conditions

Soil was taken around the roots of *V. gummifera* grown wild near the localities of Amhach and Bounaga; the former is located (31°16′44.694 N, 9°28′57.263 W) in the Haha-Essaouira region and is best known by its important natural argan (*Argania spinosa*) forest, which is vulnerable and endangered by goat overgrazing; the latter (31°57′57.292 N, 7°56′26.564 W) is situated North to Marrakech, on the road to Casablanca, in an area with a severe climate characterized by low temperatures in winter, hot summers, low and random rainfall and weak soils with few crop-cover. Seeds of *V. gummifera* were scarified using 98% sulfuric acid for 30 min, rinsed thoroughly with sterile water, transferred to Petri dishes containing 0.6% (w/v) agar and allow to germinate under dark conditions at 28 °C. Seedlings (2 per pot) were planted in 2-kg pots containing separately each of the two soils. After growth for 60 days, nodules appeared in the roots were picked up, pooled together and

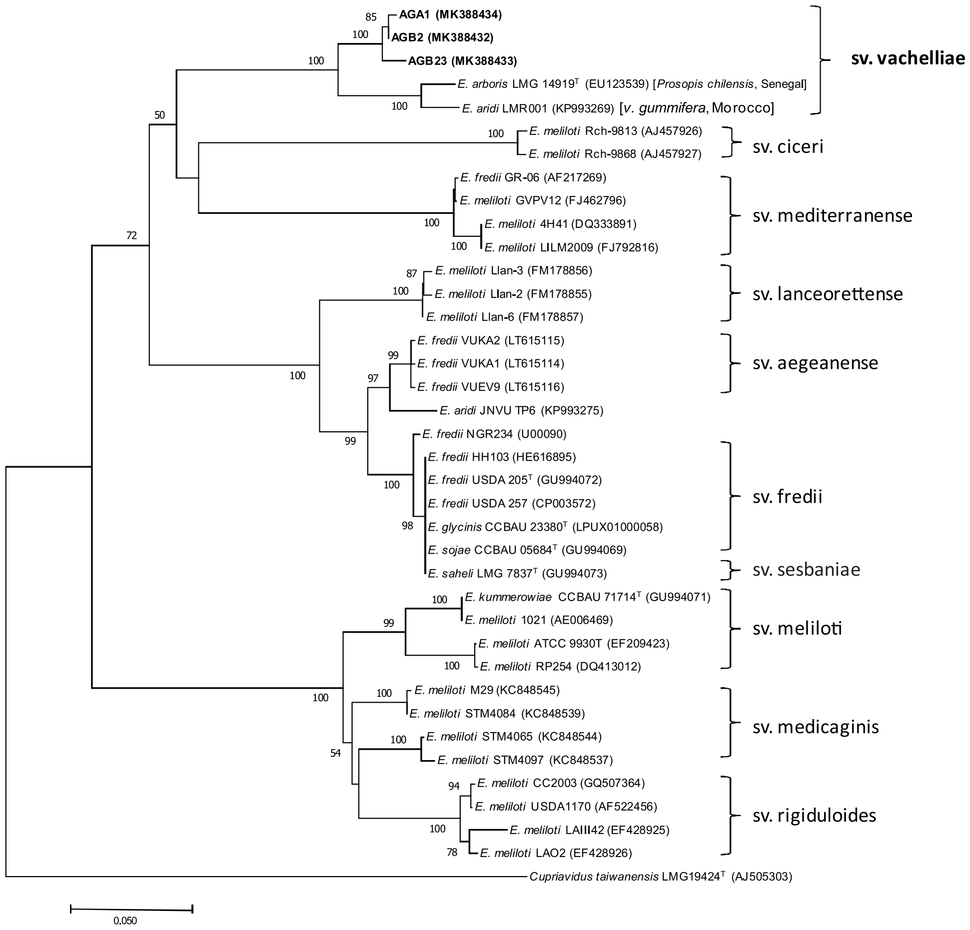

Fig. 4. Neighbor-joining phylogenetic tree based on *nodC* gene sequences of strains AGB2, AGB23 and AGA1 and related species within the genus *Ensifer*. The analysis was based on 605 nucleotides. Isolates are denoted in bold. Bootstrap values are indicated as percentages derived from 1000 replications. Values lower than 50 are not shown. Bar, 5 nucleotide substitution per 100 nucleotides. The original geographical origin of the species is shown in brackets. The tree is rooted with *C. taiwanensis* LMG 19424^T.

surface-sterilized by immersion in ethanol for 30 s, then in 3% calcium hypochlorite for 3 min, and finally rinsed several times with sterile water [42]. The nodules were then placed independently in Petri dishes and crushed. The resulting suspension was streaked on solid yeast extract mannitol (YEM) medium [56] supplemented with Congo red 0.0025% (w/v) and incubated at 28 °C for 10 d. Single colonies were picked and re-streaked several times on the same medium until the colony morphology was homogeneous. After identification, rhizobial strains used in this study were routinely grown on YEM medium.

DNA isolation, PCR fingerprinting and DNA ribotyping

Genomic DNA was extracted according to Chen and Kuo [10]. The quantity of DNA was assessed using a NanoDrop^{TM} spectrophotometer. Isolated DNAs were stored at −20 °C for subsequent uses. Repetitive extragenic palindromic (REP)-polymerase chain reactions (PCR) were carried out using primers REP1R-I and REP2-I as previously described [11]. The DNA bands in each electrophoretic lane were coded for input into a database including all the strains studied. The Dices's similarity coefficient was used to construct the distance matrix from which a dendrogram was built using the unweighted pair group with arithmetic mean (UPGMA) implemented in the GelCompar II software (Applied Maths). For amplified ribosomal DNA restriction analysis (ARDRA), purified PCR products were separately digested with the endonucleases *Cfo*I, *Msp*I, *Hinf*I and *Hae*III (Promega Corp. Madison WI, USA) according to manusfacturer's instructions. An *in silico* ARDRA with 25 restriction enzymes was also performed using the Silent Mutator program. The 16S rRNA sequences were virtually digested using the enzymes *Acu*I, *Aor*13HI, *Asp*718I, *Bam*HI, *Bci*VI, *Bfu*AI, *Bpm*I, *Bse*RI, *Bsm*I, *Bsp*13I, *Bsp*MI, *Bst*6I, *Bve*I, *Cai*I, *Eam*1104I, *Ear*I, *Eco*147I, *Gsu*I, *Kpn*2I, *Kpn*I, *Nme*AIII, *Pce*I, *Pfo*I, *Pst*NI, StuI. The Restriction Comparator program [7] was used to select the enzymes and the Silent Mutator software [8] to scan the sequences and recognize sites for each restriction enzyme and build a concatenated sequences database. The Statistica software version 7.0 was then used to generate a dendrogram of similarities based on UPGMA.

DNA sequencing and analysis

PCR amplifications of 16S rRNA gene fragments were carried out using the two universal primers fD1 and rD1 as indicated earlier [58]. Partial sequences of *rec*A, *dnaK*, *gyrB* and *rpoB* genes were obtained using primers and conditions described previously [34,35]. The symbiotic *nodA* and *nodC* genes were amplified using primer pairs nodA1f/nodAb1r [9] and nodCFn/nodCI [29], respectively. Amplification products were cleaned using the PCR product purification system of Qiagen and then subjected to cycle sequencing using the same primers as for PCR amplification, with ABI Prism Dye Chemistry, and analyzed with a 3130 x l automatic sequencer at the sequencing facilities of the National Centre for Scientific and Technical Research (CNRST) in Rabat (Morocco). All the obtained sequences were compared with those from GenBank using the BLASTN program [1] and the EzBiocloud Database (https://www.ezbiocloud.net/) [59]. MEGA 7.0 was used for all the phylogenetic

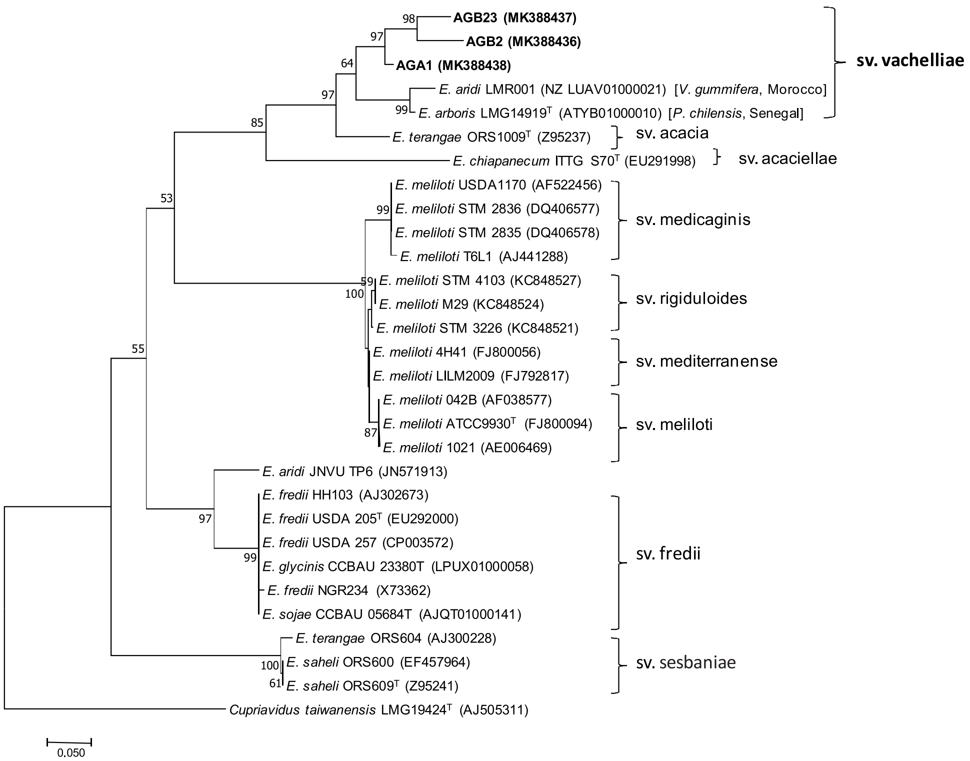

Fig. 5. Neighbor-joining phylogenetic tree based on *nodA* gene sequences of strains AGB2, AGB23 and AGA1 and related species within the genus *Ensifer*. The analysis was based on 472 nucleotides. Isolates are denoted in bold. Bootstrap values are indicated as percentages derived from 1000 replications. Values lower than 50 are not shown. Bar, 5 nucleotide substitution per 100 nucleotides. The geographical origin of the species is shown in brackets. The tree id -rooted with *C. taiwanensis* LMG 19424^T.

analyses [27]. Distances were calculated according to Kimura's two-parameter model [25] and the neighbor-joining algorithm [49] was used to infer the phylogenetic trees. Identity values were calculated by pairwise analysis and gaps were not considered. The accession numbers of the nucleotide sequences used in this study are shown in the figure trees.

Nodulation tests

Selected strains were tested for nodulation on *V. gummifera*, *A. cyanophylla*, *Prosopis chilensis*, *Leucaena leucocephala*, *Phaseolys vulgaris*, *Medicago truncatula* and *Glycine max*. After seed sterilization and germination, the seedlings were transferred into Gibson tubes containing sterile N-free nutrient agar [6], inoculated separately with 1 ml (approximately 10^8 cells/ml) and kept at 26 °C for 60 d under a 16.0/8.0 h light/dark photoperiod. Indirect effectiveness of the nodules for N_2-fixation was estimated by visual assay of red leghemoglobin presence in cross-sections and by the dark green intensity of the leaves compared to uninoculated control plants.

Results and discussion

Here we report on the isolation and identification of rhizobial strains from root nodules of *V. gummifera* grown wild in the argan forest of Amhach and Bounaga in the Essaouira-Haha and Marrakech regions (Morocco), respectively, areas that are characterized mainly by neutral to slightly alkaline weakened soils with high thermal amplitudes and low rainfall.

Out of the 94 strains isolated from root nodules of *V. gummifera*, 44 harbored *nodC* and *nodA* genes which are involved in the synthesis of the rhizobial Nod factor signaling molecule [41]. The absence of nodulation genes in 53% of the isolates was confirmed by their inability to nodulate *V. gummifera* during reinfection tests. The presence of bacteria within the legumes nodules that elicit neither nodulation nor nitrogen fixation is now well recognized but it is not known whether their presence within the nodules is biologically important or not [14,36,43,54].

According to the results, the 44 strains nodulating *V. gummifera* showed 7 different REP-PCR patterns at 55% similarity (Fig. 1). This technique has proven a powerful tool to assess the diversity of bacteria in different ecosystems and to avoid duplication and clonality [11,55]. ARDRA assays using the *Cfo*I, *Msp*I, *Hin*fI and *Hae*III restriction enzymes showed that all the seven strains representing the REP-PCR groups clustered in 1 ribotype (data not shown). Based on the ARDRA and REP-PCR results, the strains AGB2, AGB23 and AGA1 were then chosen at random for subsequent studies. Except for those strains, further characterization of other *V. gummifera*-nodulating strains was not pursued in this study.

The nearly complete sequence of the 16S rRNA genes of the strains AGB2, AGB23 and AGA1 showed that the tree strains were members of the genus *Ensifer* of the family *Rhizobiaceae* of the Alphaproteobacteria The phylogenetic tree (Fig. 2) inferred from the concatenated 16S rRNA gene sequences revealed the three strains had 99.86–99.92% similarities with *E. fredii* USDA 205^T, 99.93%–100% with *E. fredii* USDA 257 and 99.86–99.92% with *E. americanum* CFNEI 157^T (Fig. 2). An *in silico* ARDRA analysis using 25 different restriction enzymes also revealed that the strains were closer to *E. fredii* USDA 257 than to *E. fredii* USDA 205^T and *E. americanum* CFNEI 156 T (Supplementary Fig. S1). Amplification of the *recA*, *gyrB*, *dnaK* and *rpoB* genes yielded single bands of approximately 0.6, 0.7, 0.3 and 1.0 kb, respectively (data not shown), and their sequencing and single neighbor joining phylogenetic analysis (Supplementary material Figs. S2–S5) also revealed a closer affiliation with *E. fredii* USDA 257. A concatenated phylogenetic tree based on *recA* + *gyrB* + *dnaK* + *rpoB* sequences (Fig. 3) gave similar results to those obtained for the single phylogenetic tree of each gene with similarity values of 99.42%, 98.84%, and 99.20%

between *E. fredii* USDA 257 and the strains AGB2, AGB23 and AGA1, respectively. Lowe similarity values, 94.33%, 93.90 and 93.90% were obtained, respectively, after pairwise comparisons with *E. fredii* USDA 205^T.

The phylogenies of the symbiotic *nodC* and *nodA* genes of AGB2, AGB23 and AGA1 were somewhat inconsistent with those of the core genes used in this study. In this sense, a neighbor joining phylogenic tree (Fig. 4) showing the relationships between the partial *nodC* sequences from strains AGB2, AGB23 and AGA1 and the *nodC* sequences of other validly described *Ensifer* species revealed they had 92.60–93.50% similarity with *E. aridi* LMR001^T isolated from *V. gummifera* growing in the sand dunes of Merzouga (Morocco) and 92.04–92.98% similarity with *E. arboris* LMG 14919^T recovered from nodules of *P. chilensis* in Senegal. Also, the phylogenetic tree based on *nodA* sequences (Fig. 5).grouped the three strains with *E. aridi* LMR001^T with similarity values ranging from 84.47–89.85%.

Currently, the symbiovars fredii, mediterranense and aegeanense [38,51,53], which nodulate *G. max*, *P. vulgaris* and *V. unguiculata*, respectively, have been described within *E. fredii*. Our results show that *nodC* (Fig. 4) and *nodA* (Fig. 5) sequences of strains AGB2, AGB23 and AGA1 clustered apart from those of the abovementioned symbiovars. Except for the symbiovar aegeanense *nodA* gene whose sequence has not been reported, the *nodA* sequences of AGB2, AGB23 and AGA1 were assembled in a group with *Ensifer* species nodulating *Mimoseae* species. This group is close to *E. teranga* strains ORS 1073 and ORS 1009^T, isolated from *Senegalia* species form the acacia symbiovar.

The determination of the host range of the strains AGB2, AGB23 and AGA1 showed that they nodulated *V. gummifera*, *A. cyanophylla*, *P. chilensis* and *L. leucocephala* but not *M. truncatula*, *P. vulgaris* and *G. max*. Considering a 95% similarity value for *nodC* as a cut-off for symbiovar delineation within *Ensifer* [44,51], on the basis of divergence in *nodA*/*nodC* sequences and host range as proposed by Rogel et al. [47], the *E. fredii* strains AGB2, AGB23 and AGA1 isolated in this study may represent a new symbiovar for which the name “vachelliae” is proposed since it was isolated from root nodules of *V. gummifera*. It is now known that *V. gummifera* in Morocco can be nodulated by two different species of *Ensifer*, maybe depending on the origin of isolation. In the desert part of the country, *E. aridi* has been reported as the nodule-forming rhizobial bacteria [32]; whereas *E. fredii* could be the main microsymbiont in mildest semiarid areas of the Haha-Essaouira and Marrakech regions. In the phylogenetic trees of *nodC* and *nodA*, the strains AGB2, AGB23 and AGA1 grouped together with *E. aridi* LMR001 and *E. arboris* LMG14919^T, this leading to the suggestion that they may belong to the same symbiovar “vachelliae”.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.syapm.2019.06.004.

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