Multilocus sequence analysis of root nodule isolates from *Lotus arabicus* (Senegal), *Lotus creticus*, *Argyrolobium uniflorum* and *Medicago sativa* (Tunisia) and description of *Ensifer numidicus* sp. nov. and *Ensifer garamanticus* sp. nov.

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Nine isolates from *Argyrolobium uniflorum*, *Lotus creticus*, *Medicago sativa* (Tunisia) and *Lotus arabicus* (Senegal) were analysed by multilocus sequence analysis (MLSA) of five housekeeping genes (*recA*, *atpD*, *glnA*, *gltA* and *thrC*), the 16S rRNA gene and the nodulation gene *nodA*. Analysis of the individual and concatenated gene sequences demonstrated that the nine new strains constituted three stable, well-supported (bootstrap and gene sequence similarity values) monophyletic clusters, A, B and C, all belonging to the branch of the genus *Ensifer*, regardless of the phylogenetic reconstruction method used (maximum likelihood, maximum-parsimony, neighbour-joining). The three groups were further characterized by API 100 auxanographic tests, host specificity and *nodA* gene sequence analysis. On the basis of these data, clusters A and C are suggested as representing two novel species within the genus *Ensifer*, for which the names *Ensifer numidicus* sp. nov. (type strain ORS 1407^T=LMG 24690^T=CIP 109850^T) and *Ensifer garamanticus* sp. nov. (type strain ORS 1400^T=LMG 24692^T=CIP 109916^T) are proposed. The cluster B strains were assigned to *Ensifer adhaerens* genomovar A.

INTRODUCTION

Over the past three decades, bacterial taxonomy has been based on a polyphasic approach including both phenotypic and genotypic data with 16S rRNA gene sequence analysis and DNA–DNA hybridization data being considered as the

cornerstones of genotypic characterization for descriptions of novel species (Vandamme *et al.*, 1996; Stackebrandt *et al.*, 2002; Coenye *et al.*, 2005). However, many authors have reported drawbacks with these methods (Ueda *et al.*, 1999; Stackebrandt *et al.*, 2002; Schouls *et al.*, 2003; Stackebrandt,

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Abbreviations: ARDRA, amplified rDNA restriction analysis; ML, maximum-likelihood; MLSA, multilocus sequence analysis; MP, maximum-parsimony; NJ, neighbour-joining.

The GenBank/EMBL/DDBJ accession numbers of the new sequences reported in this paper are provided in Table 1. The GenBank accession numbers for the partial *NodA* gene sequences for *Ensifer adhaerens* strain ORS 529, *Ensifer garamanticus* strain ORS 1400^T, *Ensifer numidicus* strain ORS 1444 and *E. garamanticus* strain STM 354 are FJ919754–FJ919757, respectively.

Supplementary tables listing the primers used for the amplification of the five housekeeping genes (*atpD*, *recA*, *gltA*, *thrC* and *glnA*) and the *nodA* gene and the results of the API 100 galleries are available as supplementary data with the online version of this paper. Additional supplementary figures showing the phylogenetic trees for the five housekeeping genes and a concatenated tree comparing the Tunisian and Senegalese strains with reference strains of the genus *Ensifer* are also available.

2003; van Berkum *et al.*, 2003; Eardly *et al.*, 2005). The ad hoc Committee for the Re-evaluation of the Species Definition in Bacteriology (Stackebrandt *et al.*, 2002; Gevers *et al.*, 2005) suggested the sequence analysis of a set of protein-encoding genes as alternative phylogenetic markers, or multilocus sequence analysis (MLSA). Several recent studies have confirmed that sequences from housekeeping genes can be used for identification at the species level (Wertz *et al.*, 2003; Zeigler, 2003), for evolutionary population genetics and in taxonomy (Stepkowski *et al.*, 2003; Vinuesa *et al.*, 2005a,b; Bailly *et al.*, 2006). The analysis of a small number of carefully selected gene sequences may equal or even surpass the precision of DNA–DNA hybridization for the quantification of genome relatedness and this approach thus has the potential to replace cumbersome DNA–DNA hybridizations (Zeigler, 2003; Martens *et al.*, 2008). Mantelin *et al.* (2006) included MLSA in the description of four novel species of the genus *Phyllobacterium* and Vinuesa *et al.* (2005c) described novel species of the genus *Bradyrhizobium* using phylogenetic analysis of three housekeeping genes, *atpD*, *glnII* and *recA*, combined with other classical genotypic and phenotypic analyses. ‘*Ensifer mexicanus*’ and ‘*Sinorhizobium chiapanecum*’ were described using phenotypic analysis, phylogenies of the *recA*, *gyrA*, *nolR*, *rpoB*, *rrs* and symbiotic genes and confidence intervals of sequence similarity to estimate both inter- and intraspecies variation; all were in correlation with DNA–DNA hybridization data (Lloret *et al.*, 2007; Rincón-Rosales *et al.*, 2009). In addition, MLSA has been applied in diversity studies of the genera *Enterococcus*, *Aeromonas* and *Ensifer* (*Sinorhizobium*) (Naser *et al.*, 2005; Soler *et al.*, 2004; Bailly *et al.*, 2006) and it has been reported as being a highly reproducible and economical method for rapid and reliable species identification.

The genera *Sinorhizobium* and *Ensifer* were recently recognized as forming a single phylogenetic clade (Balkwill, 2005; Willems *et al.*, 2003) and are now united and all species of the genus *Sinorhizobium* have been transferred to the genus *Ensifer*, in line with rule 38 of the Bacteriological Code (Young, 2003; Judicial Commission, 2008). The genus *Ensifer* currently includes eleven species (Wang *et al.*, 2002; Wei *et al.*, 2002; Young, 2003; Toledo *et al.* 2003). Two further species, ‘*Ensifer mexicanus*’ and ‘*Sinorhizobium chiapanecum*’, have been described but the names have not yet been validly published (Lloret *et al.* 2007; Rincón-Rosales *et al.*, 2009). Martens *et al.* (2007, 2008) recently demonstrated that the discriminative power of MLSA for species identification and delineation is higher than 16S rRNA gene sequence analysis and DNA–DNA hybridization within the genus *Ensifer*.

In this study, MLSA was performed to complete the characterization of nine root nodule strains isolated from several legumes in Tunisia (*Argyrolobium uniflorum*, *Lotus creticus*, *Medicago sativa*) and Senegal (*Lotus arabicus*). *Argyrolobium uniflorum* (Tribe Genisteae, Family Fabaceae) is an indigenous herb legume of the Mediterranean basin and is a pastoral and forage legume that is widely distributed in arid and semi-arid regions of Tunisia. This plant plays an

important role in the maintenance of soil fertility, soil coverage and dune stability (Ferchichi, 1996). *Lotus arabicus* L. (tribe Loteae, family Fabaceae), syn. *Lotus mossamedensis* Welw. ex Baker, is a spontaneous annual herb legume of coastal dunes in Senegal. *Medicago sativa* is the most widely cultivated species of lucerne in the world and its symbiotic rhizobial strains have been extensively studied. Six of the isolates have previously been analysed by amplified rDNA restriction analysis (ARDRA) using seven enzymes, total cell protein SDS-PAGE analysis and 16S rRNA gene sequencing (Zakhia *et al.*, 2004) and results suggested their separate positions in the genus *Ensifer* (pro synonym. *Sinorhizobium*). According to the conclusions of Martens *et al.* (2007, 2008), the strains were subjected to MLSA using five housekeeping genes, the most discriminative for taxonomic purposes of the ten housekeeping genes evaluated in the genus *Ensifer*. Genes were analysed individually and as a concatenation using only the congruent genes estimated by the incongruence-length difference (ILD) test (Farris *et al.*, 1995). Trees were constructed using three algorithmic methods for comparison. The characterization was completed by auxanographic tests, DNA–DNA hybridizations, host specificity investigations and sequence analysis of the symbiotic *nodA* gene for biovar description.

METHODS

Bacterial strains and culture media. The strains investigated are listed in Table 1. All strains were grown on yeast-mannitol agar (YMA) (Zakhia *et al.*, 2004) at 28 °C. Isolates were stored at –80 °C in 50 % (v/v) glycerol.

Host specificity. The isolates were tested for nodulation on their original host plant. The seeds from *Medicago sativa* and *Lotus arabicus* were sterilized in 3 % (w/v) calcium hypochlorite and scarified by immersion in 96 % (v/v) sulphuric acid for 20 min. The seeds were then washed five times with sterile water and placed in 1 % (w/v) agar–water at 24–25 °C for germination. After 3–4 days, the seedlings were transferred to agar slant tubes (Bertrand, 1997) for root nodulation trials (5 plants per strain). Plants were grown under continuous light (20 W m^{–2}) at 24 °C and inoculated with 1 ml exponential growth phase YM bacterial suspension. Roots were observed for nodule formation during the first 4 weeks after inoculation. The infectivity of strains isolated from *Argyrolobium uniflorum* has been previously reported by Zakhia (2004).

Genomic DNA isolation. Bacterial DNA was prepared as described by Zakhia *et al.* (2004) or alternatively by the alkaline lysis method (Baele *et al.*, 2000).

DNA amplification. The 16S rRNA gene, internal fragments of five housekeeping genes (*atpD*, *recA*, *gltA*, *thrC* and *glnA*) and the *nodA* gene were amplified using primers listed in Supplementary Table S1 (available in IJSEM Online). PCR amplification of the *atpD*, *recA*, *gltA*, *thrC* and *glnA* genes was performed as described by Martens *et al.* (2007) with the exception that the total volume of the reaction mixture was 25 µl. PCR amplification of the 16S rRNA gene was performed according to Zakhia *et al.* (2004) and *nodA* gene amplification was conducted as described by Zakhia *et al.* (2006).

Gene sequencing and phylogenetic data analysis. The PCR products of the different genes were purified and sequenced as previously

Table 1. GenBank accession numbers for new sequence data

NA, Not amplified.

Strain	SSU	<i>recA</i>	<i>glnA</i>	<i>atpD</i>	<i>thrC</i>	<i>gltA</i>	Source
<i>E. numidicus</i> sp. nov.							
ORS 1444 (=LMG 24691=CIP 109850 ^T)	AY500253	AM946578	AM946562	AM946549	AM946586	AM946596	<i>Argyrobium uniflorum</i> , Tunisia, Zakhia <i>et al.</i> (2004)
ORS 1410 (=LMG 24736=CIP 109858)	AM946566	AM946577	AM946561	AM946550	NA	AM946595	<i>Lotus creticus</i> , Tunisia, Zakhia <i>et al.</i> (2004)
ORS 1407 ^T (=LMG 24690 ^T =CIP 109850 ^T)	AY500254	AM946576	AM946560	AM946551	AM946585	AM946594	<i>Argyrobium uniflorum</i> , Tunisia, Zakhia <i>et al.</i> (2004)
<i>E. garamanticus</i> sp. nov.							
ORS 1400 ^T (=LMG 24692 ^T =CIP 109916 ^T)	AY500255	AM946573	AM946557	AM946546	AM946582	AM946591	<i>Argyrobium uniflorum</i> , Tunisia, Zakhia <i>et al.</i> (2004)
ORS 1401 (=LMG 24693=CIP 109848)	AM946567	AM946574	AM946558	AM946547	AM946583	AM946592	<i>Argyrobium uniflorum</i> , Tunisia, Zakhia <i>et al.</i> (2004)
STM 354 (=LMG 24694=CIP 109849)	AM946568	AM946575	AM946559	AM946548	AM946584	AM946593	<i>Medicago sativa</i> , Tunisia, Zakhia <i>et al.</i> (2004)
<i>E. adhaerens</i> genomovar A							
ORS 2154	AM946569	AM946579	AM946565	AM946553	AM946587	AM946598	<i>Lotus arabicus</i> , Senegal, this study
ORS 2133	AM946570	AM946580	AM946563	AM946554	AM946588	AM946597	<i>Lotus arabicus</i> , Senegal, this study
ORS 529	AM946571	AM946581	AM946564	AM946555	AM946589	AM946599	<i>Lotus arabicus</i> , Senegal, this study

described (Martens *et al.*, 2007) using the primers detailed in Supplementary Table S1 (available in IJSEM Online). Consensus sequences were constructed using the Autoassembler software (Applied Biosystems). Interstrain sequence similarity (S) values were determined applying pairwise sequence alignments using BioNumerics 4.6. software. The nucleotide sequences of partial *atpD*, *recA*, *gltA*, *glnA*, *thrC* and small subunit rRNA (SSU) were independently aligned using the CLUSTAL_X program, version 1.8 (Thompson *et al.*, 1997). Neighbour-joining (NJ), maximum-parsimony (MP) and maximum-likelihood (ML) trees were constructed using PAUP version 4.0b10 (Swofford, 2002). Reference sequences of species of the genus *Ensifer* were included for comparison (GenBank accession nos are listed in Martens *et al.*, 2008). NJ analyses (Saitou & Nei, 1987) were performed using the Kimura 2 correction (Kimura, 1980) and 1000 bootstrap replications; MP analyses were performed using the heuristic search option. For ML analyses, the optimal models of nucleotide substitution were estimated using the program MODELTEST 3.7 (Posada & Crandall, 1998), using Akaike's Information Criterion (AIC) (Posada & Buckley, 2004). The MP trees were used as starting trees for the heuristic search procedure. Bootstrap analyses were performed using 1000 replications of heuristic searches for MP and 100 replications for ML. The ILD test (Farris *et al.*, 1995), implemented in PAUP* and using 1250 replicates, was used to assess incongruence between datasets. A phylogenetic tree was constructed with the concatenated sequences of the congruent housekeeping genes.

DNA–DNA hybridization. DNA was prepared according to a slightly modified procedure of Marmur (1961) as described previously (Willems *et al.*, 2001). Hybridizations were carried out using the microplate method in which unlabelled DNA, non-covalently bound to the microplate, was hybridized with biotinylated probe DNA (Ezaki *et al.*, 1989; Goris *et al.*, 1998; Willems *et al.*, 2001). Hybridizations were performed at 45 °C in 2 × SSC and 50 % formamide.

Phenotypic tests. Microscopic observations, growth at various temperatures and pH values, and tolerance to NaCl were performed as previously described (de Lajudie *et al.*, 1994; Mahdhi *et al.*, 2008).

API 100 galleries (bioMérieux) were used to test the utilization of carbon sources (carbohydrates, amino acids, organic acids) for bacterial growth. Inoculants were obtained from 36 h YMA slant cultures. After inoculation, the galleries were incubated at 30 °C and the results were determined after 1, 2, 4 and 7 days. The results of the auxanographic tests were scored as described previously (Kersters & De Ley, 1984). Jaccard similarity coefficients were calculated and an UPGMA analysis was performed using BioNumerics 4.6.

RESULTS

Nodulation tests

All of the strains studied induced efficient nodulation on their hosts of isolation. This has already been reported by Zakhia (2004) for strains ORS 1400^T, ORS 1401, ORS 1407^T, ORS 1444 (*Argyrobium uniflorum*) and for strain ORS 1410 (*Medicago sativa*). In this study, strains ORS 2154, ORS 2133 and ORS 529, isolated from *Lotus arabicus*, were tested and were effective on their plant of isolation.

16S rRNA gene sequence analysis

Nearly full-length 16S rRNA gene sequences (1340 bp) were determined for strains ORS 1410, ORS 2133, ORS 529, ORS 2154, ORS 1401 and STM 354. The 16S rRNA gene phylogenetic trees constructed using three methods (ML, MP, NJ) resulted in the same groupings. Therefore, only the resulting ML tree is shown in Fig. 1. All isolates studied were placed unambiguously within the class *Alphaproteobacteria* and within the clade of the reference species of the genus *Ensifer*. The isolates were subdivided

Fig. 1. Phylogenetic reconstruction based on individual analysis of the 16S rRNA gene. Analyses were conducted using the ML method. Bootstrap values >75 (using 100 replicates) are indicated at branching points. Bar, 0.1 % estimated substitutions.

into three clusters (clusters A, B and C) supported by high bootstrap values and displaying high intra-cluster sequence similarity.

Cluster A (bootstrap value 97 %) included isolates ORS 1444, ORS 1410 and ORS 1407 and formed a homogeneous group (sequence similarity values 100 %) that was equally distant to *Ensifer arboris*, *Ensifer medicae* and *Ensifer meliloti* (99.7 % sequence similarity).

Cluster B strains (ORS 529, ORS 2154 and ORS 2133) also shared identical 16S rRNA gene sequences and formed a clade grouping together with the genomovars of *Ensifer adhaerens* (≥ 99.6 –99.8 % sequence similarity) and reference strains of *Sinorhizobium morelense* (99.0–99.2 % sequence similarity), although low bootstrap values were displayed for this group (35 %).

Strains ORS 1400^T, ORS 1401 and STM 354 formed a homogeneous group (Cluster C, 100 % interstrain gene sequence similarity values) supported by a bootstrap value of 85 %, and clustered in the vicinity of *Ensifer terangae* (48 % bootstrap value and 98.8 % sequence similarity). The reference strain of '*E. mexicanus*' LMG 23932, which was described recently (Lloret *et al.*, 2007), was related to these taxa. It was not included in our analyses, but formed a distinct subclade, sharing sequence similarity values of 97.8–98.8 % with clusters A, B and C and 99.5 % with strains of *E. terangae*.

Sequence analysis of individual genes

The five housekeeping gene fragments examined in this study had different lengths: *glnA* (977 bp), *gltA* (681 bp), *recA* (550 bp), *atpD* (461 bp), *thrC* (636 bp). Within each group

A, B and C, the three strains of each group had identical sequences for all genes tested (except for ORS 1410 of group A for which the *thrC* gene could not be amplified). Sequence analysis of the five individual housekeeping genes resulted in different tree topologies (see Supplementary Fig. S1. in IJSEM Online). However, the same three separate clusters A, B and C were revealed with high bootstrap support for all clusters in all gene analyses, including those of the 16S rRNA genes. These clusters occupied different positions relative to the reference species depending on the gene considered.

In the *atpD* gene tree topology, *Agrobacterium tumefaciens* grouped with *S. morelense*, the three genomovars of *E. adhaerens* and all strains from cluster B (100 % bootstrap), although this cluster had low bootstrap support (32 %). A second cluster (bootstrap value 43 %) contained all other species of the genus *Ensifer* and clusters A and C (both clusters 100 % bootstrap value).

In the case of the *gltA* gene, all strains of the genus *Ensifer*, together with clusters A, B and C, formed a single separate but poorly supported clade (bootstrap value 38 %). Within this clade, all strains from cluster C formed a significant subclade with *E. terangae* (100 % bootstrap). Another well-supported subgroup (bootstrap value 98 %) was formed by all strains from cluster B and *E. adhaerens* genomovar A.

In the *glnA* gene analysis, all strains of the genus *Ensifer* (and clusters A, B and C) were grouped together with high bootstrap support (75 %) and the same two significant subclusters that appeared in the *gltA* gene tree topology were found (bootstrap values 100 and 95 % for cluster C/*E. terangae* and cluster B/*E. adhaerens* genomovar A, respectively).

In the *thrC* gene tree, all strains of the genus *Ensifer* together with clusters A, B and C, formed a single, separate but less supported clade (bootstrap value 48 %), in which the same two subclades were again distinguished (bootstrap values 100 and 98 % for cluster C/*E. terangae* and cluster B/*E. adhaerens* genomovar A, respectively). In the final single gene tree, the *recA* tree, all *Ensifer* strains and clusters A, B and C again formed a separate, but poorly supported, clade (bootstrap value 27 %). A well-supported subgroup (bootstrap value 95 %) was formed by all strains from cluster B and *E. adhaerens* genomovar A.

Thus, cluster A formed a separate, well-supported group (bootstrap value 100 %) in all single gene phylogenies with identical sequences for the three strains (100 %). Cluster A was located at different positions when compared with the reference species of the genus *Ensifer* and no significant clusterings were apparent. The highest observed sequence similarities between cluster A strains and the reference species ranged between 86.9 % with *Ensifer saheili* and *Ensifer kostiensis* for the *thrC* gene and 94.3 % with reference strains *Ensifer meliloti* and *Ensifer medicae* for the *atpD* gene.

Cluster B grouped with *E. adhaerens*, more specifically *E. adhaerens* genomovar A, with high bootstrap values in all housekeeping gene analyses, except for the *atpD* gene. Sequence similarities with *E. adhaerens* genomovar A

strains ranged between 96.4 % for *thrC* to 98.4 % for the *recA* gene. With both other genomovars, the range was lower at 90.5 % (*gltA*) to 95.3 % (*glnA*).

Strains ORS 1400^T, ORS 1401 and STM 354 formed the monophyletic Cluster C in all gene phylogenies (bootstrap value ranging from 98 to 100 %; sequence similarity values 100 % for all genes). This cluster displayed sequence similarities with its closest neighbour *E. terangae* in the range of 92.2 % (for *recA*) to 96.4 % (for *gltA*). Cluster C together with *E. terangae* was also related to the '*E. mexicanus*' reference strain LMG 23932 in the analysis of three housekeeping genes (*glnA*, *gltA* and *recA*) (not included in Supplementary Fig. S1). The sequence similarities between Cluster C strains and '*E. mexicanus*' ranged from 92.2 % for *recA*, over 93.7 % for *gltA*, to 95.8 % for *glnA*. Between *E. terangae* and '*E. mexicanus*' somewhat comparable interspecies sequence similarity values amounted to 96.6 % for *recA*, 93.5 % for *gltA* and 96.2 % for *glnA*. This indicated that '*E. mexicanus*', *E. terangae* and the isolates from Cluster C all represented distinct genomic species.

Sequence analysis of concatenated housekeeping genes

The *atpD* gene was found not to be congruent with the other genes ($P < 0.01$) while all other housekeeping genes showed compatible phylogenetic signals (data not shown). A tree was constructed from the concatenated sequences of the four congruent genes *recA*, *glnA*, *gltA*, and *thrC* ($P > 0.01$) estimated by the ILD test (Farris *et al.*, 1995). The ILD test values ranged from 0.0120 for *thrC* and *recA* to 0.6288 for *thrC* and *glnA*. In view of the ILD test results, the aligned sequences for *recA*, *thrC*, *gltA* and *glnA* were concatenated and an alignment of 2704 nucleotides was obtained (comprising 1486 invariable sites, 237 variable but parsimony uninformative sites and 981 parsimony informative sites).

The combined analysis showed a significant cluster (bootstrap value 97 %), comprising all strains of the genus *Ensifer* together with clusters A, B and C. The cluster was subdivided into two closely related subclusters. One well-supported subcluster (bootstrap value 100 %) includes *S. morelense*, the three *E. adhaerens* genomovars and cluster B. As in most single gene analyses, cluster B formed a significant subclade together with *E. adhaerens* genomovar A. The second major subcluster (bootstrap value 99 %) comprised all other strains of the genus *Ensifer* and clusters A and C. As found with most single gene trees, Cluster C formed a significant sub branch with *E. terangae* (bootstrap value 100 %). Cluster A strains ORS 1407^T and ORS 1444 also formed a reliable cluster (bootstrap value 100 %), which was well separated from all other genomic species. Since a *thrC* sequence could not be obtained for strain ORS 1410, this strain could not be included in the concatenated housekeeping gene analysis. As indicated, all mentioned clusters were supported by higher bootstrap values in the concatenated tree than in the single gene trees and are therefore more robust.

Table 2. DNA–DNA hybridization values (%) between species of the genus *Ensifer* and strains of cluster B isolated from *Lotus arabicus* in Senegal

Strain	1	2
1. ORS 529	100	99
2. ORS 2133	99	100
<i>E. adhaerens</i> genomovar A LMG 10007	74	75
<i>E. adhaerens</i> genomovar B R-7457	60	59
<i>E. adhaerens</i> genomovar C LMG 20216 ^T	47	47

DNA–DNA hybridization

To determine to which *E. adhaerens* genomovar Cluster B could be designated, we performed DNA–DNA hybridizations between two strains (ORS 529 and ORS 2133) of this cluster and three *E. adhaerens* reference strains, representing the three different genomovars (Willems *et al.*, 2003) (Table 2). In agreement with the gene sequence analyses, strains ORS 529 and ORS 2133 displayed high DNA–DNA hybridization values (99%), proving that the strains represent the same genomic species. The hybridization values of strains ORS 529 and ORS 2133 with *E. adhaerens* genomovars B and C strains were below 70%, indicating that the two strains from Cluster B are not members of *E. adhaerens* genomovar B or genomovar C. However, DNA–DNA relatedness values between Cluster B strains and the *E. adhaerens* genomovar A reference strain exceeded 70% which confirmed the assumption drawn from MLSA that Cluster B strains are strains of *E. adhaerens* genomovar A.

nodA gene sequencing

The *nodA* gene was sequenced in strains representing both of the different clusters and host plants of isolation: two cluster C strains (ORS 1400^T/*Argyrobium uniflorum* and STM 354/*Medicago sativa*), one cluster B strain ORS 529 (isolated from *Lotus arabicus*, as were the two other strains of the cluster) and two Cluster A strains (ORS 1444/*Argyrobium uniflorum* and ORS 1410/*Lotus creticus*). The *nodA* gene could not be amplified in strain ORS 1407^T. The analysis of *nodA* gene sequences by the NJ method (Fig. 2) shows that strains isolated from *Argyrobium uniflorum*, *Lotus arabicus* and *Medicago sativa* formed a separate cluster. This cluster was grouped with the *nodA* gene of *E. meliloti* and the *E. medicae* branch with high bootstrap support. In this study, the taxonomic position of *nodA* gene sequences of isolates from *Argyrobium uniflorum* is reported for the first time.

Numerical analysis of auxanographic tests

All strains were tested for use of 99 substrates as the sole carbon source for growth, using API 100 galleries (see Supplementary Table S2 in IJSEM Online). Some discriminative features could be observed.

In contrast to their closest phylogenetic neighbours, *E. medicae* LMG 19920^T, *E. meliloti* LMG 6133^T and *E. arboris* LMG 14919^T, strains of Cluster A, did not grow on xylitol or DL-glycerate. Results for other substrates were variable between members of this cluster. About 28 substrates were utilized differently by strains ORS 1444, ORS 1410 and ORS 1407^T and 13 substrates were only metabolized by strain ORS 1407^T (dulcitol, D-lyxose, 1-O-methyl α -D-glucopyranoside, 3-O-methyl D-glucopyranose, D-gluconate, L-histidine, succinate, fumarate, ethanolamine, DL- β -hydroxybutyrate, L-aspartate, L-alanine and propionate).

Cluster B strains used putrescine in contrast to the three genomovars (A, B, C) of *E. adhaerens*. Seven substrates [(+)-L-sorbose, dulcitol, D-tagatose, citrate, 5-keto-D-gluconate, D-gluconate and glutarate] were not metabolized by members of Cluster B and were used diversely among the three genomovars of *E. adhaerens*.

Cluster C, which appeared to be close to *E. terangae* in phylogenetic analyses, differed from this species in the test results for ten substrates. These ten substrates were assimilated by *E. terangae* LMG 7834^T and not by any member of Cluster C. However, malonate was metabolized by all strains of Cluster C and not by *E. terangae* LMG 7834^T.

Auxanographic data were analysed numerically (data not shown). Clusters A and C formed two related clusters but were separate from the other species of the genus *Ensifer*. Strains of Cluster B were included in *E. adhaerens* as observed in the genotypic analysis, with strain ORS 2154 close to *Ensifer adhaerens* genomovar B strain R-7457.

DISCUSSION

In this study, we performed MLSA to characterize nine strains from Tunisia and Senegal which had been isolated from the root nodules of several legumes. Previously, six of them had been partially characterized by ARDRA, SDS-PAGE and 16S rRNA gene sequencing (Zakhia *et al.*, 2004). Genes for MLSA were chosen according to previous taxonomic and phylogenetic studies: *recA* (recombinase A) and *atpD* (ATP synthase beta subunit) were used in a phylogenetic study of the genera *Agrobacterium* and *Rhizobium* (Gaunt *et al.*, 2001); *glnA* (glutamine synthase) was used by Turner & Young (2000) and *gltA* (citrate synthase) was used by Hernandez-Lucas *et al.* (2004). In a study evaluating the taxonomic potential of ten housekeeping genes, Martens *et al.* (2007, 2008) found these genes and additionally *thrC* (threonine synthase) useful for identification and for the inference of phylogenetic relationships of species of the genus *Ensifer*. Phylogenetic analyses of the five housekeeping genes were compared with the 16S rRNA gene tree using three algorithmic methods (ML, MP and NJ). We completed the characterization with auxanographic tests and *nodA* gene sequences for biovar determination.

The *nodA* gene is a key gene for the establishment of symbiosis; it is present as a single copy and has a size of

Fig. 2. Neighbour-joining phylogenetic tree of *nodA* gene sequences of Tunisian and Senegalese strains. The novel strains are indicated in bold type and bootstrap values >60% (using 1000 replicates) are indicated at branching points. Bar, 0.05 estimated substitutions.

590–660 bp (Moulin *et al.*, 2004). Except for some photosynthetic species of the genus *Bradyrhizobia* (Giraud *et al.*, 2007), all bacteria described thus far as being capable of establishing a symbiosis with legumes harbour nodulation (*nod*) and nitrogen fixation (*nif*, *fix*) genes. This opens up the possibility of screening for these genes as an alternative to nodulation tests. The encoded $\alpha\beta$ -unsaturated Nod factor protein is a key enzyme for Nod factor synthesis, transferring an acyl chain on a chitin oligomer, resulting in a biologically active NodA molecule. The sequence of the *nodA* gene provides information regarding the symbiotic characteristics of the rhizobium and may be predictive for the type of Nod factor produced and for host specificity (Lortet *et al.*, 1996; Debéllé *et al.*, 2001). As the *nodA* gene is frequently plasmid-borne and has a propensity towards lateral gene transfer, the sequence of the gene is usually indicative of the biovar rather than the species affiliation (Haukka *et al.*, 1998).

All phylogenies based on the three algorithmic methods unambiguously placed the nine root nodule strains within

the Class *Alphaproteobacteria*, in the *Ensifer* clade. The nine strains were subdivided into three monophyletic clusters with a high intra-species sequence similarity of 100% for all gene sequence analyses.

Cluster A formed a separate cluster with variable positions relative to the reference species of the genus *Ensifer* in the phylogenies based on the 16S rRNA gene and all of the housekeeping genes. In the concatenated gene sequence analysis, Cluster A formed a single, separate cluster which was clearly distinguished from all of the other clusters. The sequence similarities between Cluster A strains and strains of related reference species supported this segregation. The gene sequence similarity values between members of Cluster A and the other genotypic clusters ranged between 82 and 94.3% in the different gene analyses (see Supplementary Fig. S1 in IJSEM Online). '*Sinorhizobium chiapanecum*', related to *E. terangae*, was reported during the process of revision of the present work (Rincón-Rosales *et al.*, 2009). The available *recA* and 16S rRNA gene sequences of this species were

retrieved and it was observed that both genes place 'S. chiapanecum', *E. terangae* and 'E. mexicanus' in a cluster [96.3–96.9% (*recA*) and 99.3–99.7% (16S rRNA gene) internal similarity values], away from Cluster A, with interspecies similarity values ranging from 92.2–92.6% (*recA*) and 98.4–98.9% (16S rRNA gene).

These values illustrate a clear gap in sequence similarity levels within Cluster A strains and between Cluster A and other reference strains, corresponding to the inter- and intra-species sequence similarity value gap observed by Martens *et al.* (2007, 2008) in their comparison of MLSA and DNA–DNA hybridization data. This indicates that Cluster A strains are well distinguished from the reference species of the genus *Ensifer* and therefore represent a new genomic species.

The auxanographic tests also confirmed that members of Cluster A represented a novel species of the genus *Ensifer*. The *nodA* gene sequence of a representative strain of Cluster A, strain ORS 1444, was examined in order to obtain some information regarding the Nod factor of *Argyrobium uniflorum* microsymbionts. The results showed that this strain grouped in a separate cluster close to the *E. meliloti*/*E. medicae* *nodA* group. In view of our phenotypic and genotypic results and those obtained in previous studies (Zakhia *et al.*, 2004), we propose a novel species of the genus *Ensifer* for the Cluster A strains with the name *Ensifer numidicus* sp. nov. Strain ORS 1407^T is designated as the type strain.

In a parallel and independent work, Mahdhi *et al.* (2008) described root nodule isolates from *Argyrobium uniflorum* in the same region of Tunisia. Among the most effective strains (potential candidates for inoculant production), 13 formed a homogeneous and separate group on the *Ensifer* branch when investigated by 16S rRNA gene PCR-RFLP. Three representative strains of this group were sequenced, and they shared identical nucleotide sequences with strain ORS 1444. This group may thus be considered as members of *E. numidicus* sp. nov.

Cluster B formed a monophyletic group in all phylogenies and grouped separately with *E. adhaerens* genomovar A in the majority of the gene phylogenies. In the 16S rRNA gene tree, Cluster B strains were grouped together with the genomovars of *E. adhaerens* and *S. morelense*. In the single and concatenated housekeeping gene analyses, Cluster B grouped with *E. adhaerens* genomovar A, supported by high bootstrap values. The analysis of the gene sequence similarities placed Cluster B in the gap between intra- and interspecies sequence similarities values, confirming that the members of Cluster B belong to the species *E. adhaerens* but with an uncertain genomovar position. Following the recommendation of Willems *et al.* (2003) for genomovar discrimination, DNA–DNA hybridization experiments were conducted. The results confirmed that members of Cluster B were different from genomovars B and C and belonged to *E. adhaerens* genomovar A. Phenotypic and genotypic analyses clearly indicated that Cluster B grouped

with *E. adhaerens* genomovar A. All strains from Cluster B nodulate the plant from which they were isolated, *Lotus arabis*. This is in contrast to all recognized members of *E. adhaerens* (Casida, 1982; Willems *et al.*, 2003), even though Rogel *et al.* (2001) demonstrated that they may acquire nodulation capacity upon the introduction of symbiotic plasmids.

Cluster C formed a separate monophyletic group in all phylogenies, essentially in the *E. terangae*/*E. mexicanus* clade. A similar grouping was obtained using the four congruent genes (*glnA*, *gltA*, *thrC* and *recA*) in individual gene analyses and in the concatenated gene trees. Sequence similarity values indicated a clear gap between the similarity levels within Cluster C strains and between strains of Cluster C and other reference strains. Values between Cluster C strains and the reference strains were comparable with those at the interspecies level found by Martens *et al.* (2008) in their study comparing MLSA and DNA–DNA hybridization data. This indicated that Cluster C was distinct and formed a separate genospecies. In addition, the numerical analysis of auxanographic results confirmed that Cluster C was separate from all species of the genus *Ensifer*. In contrast to their closest phylogenetic neighbour *E. terangae* LMG 7834^T, Cluster C strains were able to utilize malonate, but not *p*-hydroxybenzoate. Two representative strains of Cluster C, ORS 1400^T and STM 354, isolated from two different legumes, exhibited *nodA* gene sequences which grouped together (bootstrap value 99%) with those from Cluster A and B, in the *E. meliloti* and *E. medicae* branch. The combination of the results obtained previously by Zakhia *et al.* (2004) and in this study by MLSA and additional 16S rRNA and *nodA* gene sequence analyses and auxanographic tests indicate that Cluster C represents a novel genospecies in the *E. terangae*/*E. mexicanus* clade, for which we propose the name *Ensifer garamanticus* sp. nov., with strain ORS 1400^T as the type strain.

Additional DNA–DNA hybridization experiments to establish the separate species status of clusters A and C were not performed due to the clarity of the MLSA evidence. In this study, the highest gene sequence similarity level with other species of the genus *Ensifer* obtained for Cluster A was 94.3% (*atpD*). For Cluster C, this value was 96.4% (*gltA*). In view of the finding that strains of Cluster B, with housekeeping gene sequence similarity levels of 96.4–98.4% with *E. adhaerens* genomovars B and C, produced DNA–DNA relatedness levels below the species threshold (Table 2, 47–60%), even lower DNA–DNA binding between strains that have lower gene sequence similarity levels in housekeeping genes would be expected. This information is also in line with a previous elaborate comparison of housekeeping gene sequences and DNA–DNA hybridization data (Martens *et al.*, 2008) where it was shown that MLSA of selected housekeeping genes can accurately predict relationships between closely related organisms. It is concluded Clusters A and C represent two novel species of the genus *Ensifer* species, for which the names of *Ensifer numidicus* and *Ensifer garamanticus* are proposed, respectively.

Description of *Ensifer numidicus* sp. nov.

Ensifer numidicus (nu.mi'di.cus. L. masc. adj. *numidicus* pertaining to the country of Numidia, Numidian, the Roman denomination of the region in North Africa from which the majority of the organisms were isolated).

Short, aerobic, Gram-negative, non-spore-forming rods. Strains grow on yeast-mannitol medium, on which they form white slightly mucous colonies after 3 days at 28 °C. At the molecular level, this species can be differentiated by phylogenetic analysis based on several housekeeping genes (*recA*, *glnA*, *gltA*, *thrC* and *atpD*) and 16S rRNA gene sequencing. Its phylogenetic neighbours are *E. medicae*, *E. meliloti* and *E. arboris*. Detailed phenotypic features for strains of the species are given in Supplementary Table S2. Can be distinguished from *E. medicae*, *E. meliloti* and *E. arboris* by the differential use of a combination of growth substrates. Does not grow on xylitol or DL-glycerate. Results for other substrates were variable between members of this cluster (Cluster A). In the API 100 gallery, about 28 substrates are used differently by strains ORS 1444, ORS 1410 and ORS 1407^T. The type strain metabolizes only 13 substrates: dulcitol, D-lyxose, 1-O-methyl α -D-glucopyranoside, 3-O-methyl-D-glucopyranose, D-gluconate, L-histidine, succinate, fumarate, ethanolamine, DL- β -hydroxybutyrate, L-aspartate, L-alanine and propionate. Strains cannot be identified by biochemical and physiological characters alone. Strains are able to nodulate *Argyrobium uniflorum* (ORS 1444 and ORS 1407^T) and *Lotus creticus* (ORS 1410). The sequence of the nodulation gene *nodA* differs from those of other species of the genus *Ensifer* but is most closely related to those of the *E. meliloti* and *E. medicae* clade.

The type strain is ORS 1407^T (=LMG 24690^T=CIP 109850^T). The DNA G+C content of the type strain is 62.8 mol% (HPLC).

A group of 13 *Sinorhizobium* sp. strains reported by Mahdhi *et al.* (2008), AB1, AB3, AB5, AB6, AB10, AM1, AM2, AM3, AS1, AS2, AS3, AS4, AS5, may be considered as belonging to *Ensifer numidicus* and are included as members of this species. These strains were isolated independently from *Argyrobium uniflorum* in South Tunisia. The strains form a homogeneous group by PCR-RFLP of the 16S rRNA gene. Three representative strains of this group STM 4034 (=AB1), STM 4036 (=AB3), STM 4039 (=AS1), share identical 16S rRNA gene sequences with strains of *E. numidicus* as isolated in this study. The three latter strains form the most effective symbiosis with *Argyrobium uniflorum* and strain STM 4036 is the most efficient. The additional strains tolerate pH values of 6–9, 1–2% (w/v) NaCl and grow at 40 °C, but not at 45 °C. They are sensitive to ($\mu\text{g ml}^{-1}$) ampicillin (100) and streptomycin (100), but resistant to kanamycin (100) and nalidixic acid (100).

Description of *Ensifer garamanticus* sp. nov.

Ensifer garamanticus (ga.ra.man'ti.cus. L. masc. adj. *garamanticus*, pertaining to Garamante, Garamantian, the

country of Garamantes, from which the strains were isolated).

Short, aerobic, Gram-negative, non-spore-forming rods. The strains form white and slightly mucous colonies on YMA after 48–72 h incubation at 28 °C.

Strains efficiently nodulate *Argyrobium uniflorum* (ORS 1400^T, ORS 1401) and *Medicago sativa* (STM 354). Can be distinguished from other species by phylogenetic analysis based on several housekeeping (*recA*, *glnA*, *gltA*, *thrC* and *atpD*) genes and 16S rRNA gene sequencing. Malonate is used by all strains for growth, but is not used by the closest phylogenetic neighbour *E. terangaie*. Detailed phenotypic features for all strains are given in Supplementary Table S2. In the API 100 galleries, ten substrates are used for growth by *E. terangaie* LMG 7834^T but not by any member of *Ensifer garamanticus* (Cluster C). Malonate is metabolized by all strains and not by *E. terangaie* LMG 7834^T.

The type strain is ORS 1400^T (=LMG 24692^T=CIP 109916^T). The DNA G+C content of the type strain is 62.4% (HPLC).

The group formed by *Sinorhizobium* sp. strains STM 4015, STM 4016, STM 4027, STM 4031, STM 4032, isolated from *Genista saharae* in South Tunisia by Mahdhi *et al.* (2007), share identical 16S rRNA gene sequence with the type strain of *E. garamanticus*. They may be thus be considered as members of this species. These strains generally tolerate high temperatures (40 °C), high pH values (7–12) and high NaCl concentrations (1–4%, w/v) for growth. They nodulate their plant of isolation, so that *Genista saharae* may also be considered as a potential host plant of *Ensifer garamanticus*.

ACKNOWLEDGEMENTS

The authors are grateful to F. Boukhatem, B. Dreyfus, A. Galiana, L. Moulin, G. Béna, B. Hamed Bey, H. Ameziane and L. Bouchentouf for helpful discussions. C. M., F. Z., A. B. and A. S. are indebted to the Département Soutien-Formation (DSF) of the Institut de Recherche pour le Développement (IRD), France, for a PhD fellowship and ESCD grants and M. Mosbah for the AUF grant. A. W. is grateful to the Fund for Scientific Research – Flanders for a postdoctoral fellowship. This work was supported by the Commission of the European Communities RT-program contract BACDIVERS QLRT-2001-02097.

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