Research Article

Received: 23 September 2016 Revised: 10 February 2017 Accepted article published: 21 February 2017 Published online in Wiley Online Library:

(wileyonlinelibrary.com) DOI 10.1002/jsfa.8281

# A novel symbiovar (aegeanense) of the genus *Ensifer* nodulates *Vigna unguiculata*

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## Abstract

**BACKGROUND: Cowpea (*Vigna unguiculata*) forms nitrogen-fixing root nodules with diverse symbiotic bacteria, mainly slow-growing rhizobial species belonging to the genus *Bradyrhizobium*, although a few studies have reported the isolation of fast-growing rhizobia under laboratory and field conditions. Although much research has been done on cowpea-nodulating bacteria in various countries around the world, very limited information is available on cowpea rhizobia in European soils. The aim of this study was to study the genetic and phenotypic diversity of indigenous cowpea-nodulating rhizobia in Greece.**

**RESULTS: The genetic diversity of indigenous rhizobia associated with cowpea was investigated through a polyphasic approach. ERIC-PCR based fingerprinting analysis grouped the isolates into three groups. Based on the analysis of the 16S rRNA genes, IGS and on the concatenation of six housekeeping genes (*recA*, *glnII*, *gyrB*, *truA*, *thrA* and *SMc00019*), rhizobial isolates were classified within the species *Ensifer fredii*. However, symbiotic gene phylogenies, based on *nodC*, *nifH* and *rhcRST* genes, showed that the *Ensifer* isolates are markedly diverged from type and reference strains of *E. fredii* and formed one clearly separate cluster. The *E. fredii* strains were able to nodulate and fix nitrogen in cowpea but not in soybean and common bean.**

**CONCLUSION: The present study showed that cowpea is nodulated under field conditions by fast-growing rhizobia belonging to the species *E. fredii*. Based on the phylogenies, similarity levels of symbiotic genes and the host range, the *Ensifer* isolates may constitute a new symbiovar for which the name 'aegeanense' is proposed.**


*Supporting information may be found in the online version of this article.*

**Keywords:** cowpea; *Ensifer*/*Sinorhizobium*; phylogeny; symbiovar; MLSA; T3SS

## INTRODUCTION

Cowpea [*Vigna unguiculata* subsp. *unguiculata* (L.) Walp] is an important legume crop grown in the tropical and sub-tropical regions of Africa, Asia, southern United States, Central and South America as well as in southern Europe.[1] It is now widely accepted that this crop originated in Africa, more likely in West or Central Africa, around 3000 BC.[2] Later, it was spread to Asia, possibly to India around 1500 BC and from there was moved to China and other south-east Asian countries. The earliest evidence for cowpea cultivation in Europe dates back to 300 BC by the Greeks and around 100 AD by the Romans as mentioned by Theophrastos and Pliny.[3] Cowpea is assumed to have reached in Greece either from India or directly from Central Africa via Egypt.[4] Much later, the cowpea was introduced from Africa to America in the 17th century by both the Spanish explorers and the African slaves.

*V. unguiculata* is known by a variety of names worldwide, but the most popular is cowpea. In Greece, it is referred as 'mavromatiko' (black-eyed bean), 'ampelofasoulo' (vine bean), 'psilofasoulo' (small bean), 'velonaki' (needle bean), and 'arapofasoulo' (black bean).[5] Cowpea is considered as being multi-functional crop since several parts of cowpea plants are used for human consumption and animal feeding.[6] The high nutritional value of cowpea renders it a valuable food in both the developing and developed countries. Compared to other legumes, it has greater agronomic importance because of its tolerance to a wide range of soil pH, high temperatures and drought stress.[7] Moreover, its ability to establish nitrogen fixing symbiosis with rhizobial species constitutes the basis for its use as an intercrop in various cropping systems.[8]

Cowpea forms nitrogen-fixing root nodules with diverse symbiotic bacteria and thus it is considered as being promiscuous. It is mainly nodulated by slow-growing bacteria which constitute a heterogeneous group of rhizobia called as 'cowpea miscellany' belonging to the genus *Bradyrhizobium*.[9] Several studies on cowpea rhizobial diversity have been performed in Asia, America, Africa and Australia.[10–14]

In most studies, the cowpea-nodulating bacteria have been classified as *Bradyrhizobium* spp. However, fast-growing rhizobia have also been reported to nodulate cowpea under laboratory

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conditions,[14–16] while few studies have reported the isolation of fast-growing rhizobia under field conditions.[13,14,17,18] Although much research has been performed on cowpea-nodulating bacteria in various countries around the world, very scant information is available on cowpea-nodulating rhizobia from Europe. Two recent studies showed that strains isolated from *Vigna unguiculata* in Spain and Greece belong to the genus *Bradyrhizobium*.[19,20] However, very little information is available regarding the rhizobia associated with cowpea in Greece and other countries.

In order to gain insight on the genetic diversity of indigenous rhizobia associated with cowpea in Greece, we collected field-grown cowpea nodules from two Aegean islands, named as Evia and Karpathos. The diversity of the rhizobial isolates was assessed by applying the enterobacterial repetitive intergenic consensus (ERIC) fingerprinting technique. Representative isolates were subjected to a detailed polyphasic taxonomic study, in order to identify and determine their taxonomic position at species and symbiovar levels. The identification at the genus/species level was based on the phylogenetic analyses of 16S rRNA genes and the 16S–23S rRNA intergenic spacer region (IGS). For multi-locus sequence analysis (MLSA), the sequences of six housekeeping genes were used: *recA* (recombination protein), *glnII* (glutamine synthetase II), *gyrB* (DNA gyrase B), *SMc00019* (conserved hypothetical protein), *truA* (RNA pseudouridine synthase A), and *thrA* (homoserine dehydrogenase). The taxonomic position at the symbiovar level was determined by the inferred phylogenies based on the symbiotic genes, *nifH* (nitrogenase), *nodC* (*N*-acetylglucosaminyltransferase), and *rhcRST* (conserved type III secretion proteins). The *nifH* and *nodC* genes have been widely used for the phylogenetic analysis of rhizobia[21,22] and the *nodC* is also used to determine the host range and host promiscuity.[22–27] Currently, rhizobia are grouped into symbiovars based mainly on *nodC* gene phylogenetic analyses and on symbiotic characteristics.[26,28] The *rchRST* genes encoding conserved components of the type III secretion system (T3SS) have also been used in phylogenetic analyses of rhizobia[29] as well as other core type III secretion genes, like *rhcC2* and *rhcJ*.[30,31] The rhizobial T3SSs contribute in nodulation in a manner depending on the host legume though they are not present in all rhizobia.[32] Genes for rhizobial T3SS are found in symbiotic islands located either on the chromosome or on symbiotic plasmids (pSym). The genes are highly conserved in *Rhizobiaceae* and display phylogenies congruent to those of 16S rRNA and IGS in rhizobia harbouring T3SS supporting an ancient origin.[29] Previously, a phylogenetic analysis divided the rhizobial T3SSs into three groups, Rhc-I, II and III,[33] which differ in their operon organisation, gene content and functionality.[32] The reference *E. fredii* strains, namely NGR234, HH103 and USDA257, contain two T3SSs belonging to Rhc-I (functional) and Rhc-II (a yet unknown role) with the latter to be restricted so far only in *Ensifer* species.[32] The Rhc-I T3SSs are located on the symbiotic plasmids pSymA, pNGR234a and pSfrHH103d of *E. fredii* USDA257, NGR234 and HH103, respectively.[34–36] In contrast, the Rhc-II T3SSs are located on the chromosome of *E. fredii* USDA257 and on plasmids pNGR234b and pSfrHH103e of NGR234 and HH103, respectively. Due to the location of T3SSs, either on chromosome or on symbiotic plasmids, and their role in legume symbiosis,[32] we included the *rhcRST* loci of both T3SS groups (Rhc-I and Rhc-II), found in *Ensifer fredii* strains, in our phylogenetic analyses in order to fine tune the symbiotic diversity of our isolates. Nodulation tests were performed to assess the ability of the isolates to nodulate their original hosts as well as soybean and common bean. The ability of the isolates to resist antibiotics and tolerate acidic and salinity conditions as well as high temperatures was also analysed.

## EXPERIMENTAL

### Nodule and soil sampling

Nodules were collected from local cowpea varieties grown in Karpathos and Evia, two Aegean islands of Greece, in July 2014. The sampling sites were located in fields without rhizobial inoculation history. Soil samples were collected at the time of nodule collection and their physico-chemical characteristics were analysed at the Agricultural Institute of Kalamata, Greece. The soil characteristics of the sampling sites and their geographical locations are summarised in Table S1 (in the supporting information).

### Isolation of rhizobia

Five nodules per plant were randomly selected from five plants of each variety and finally, a total of 36 rhizobial strains were isolated. One isolate was retained from each nodule. Standard routine laboratory techniques were applied for the isolation of strains from the nodules as described by Vincent.[37] Briefly, the nodules were surface sterilised by immersion in 95% ethanol for 30 s and then in 3–5% (v/v) solution of sodium hypochlorite for 2–4 min and were washed several times with sterile $ddH_2O$. Sterilised nodules were crushed in a drop of sterile distilled water. The nodule juice was streaked onto yeast–mannitol agar (YMA) plates,[38] which were incubated at 28 °C for 7–10 days. Single colonies were subsequently purified by repeated streaking on YMA medium supplemented with Congo red until pure cultures of the isolates were obtained. Cultures of pure isolates were maintained in 20% glycerol–YMA broth at −80 °C. The nodulation capability of each isolate was tested by inoculating seedlings of the corresponding cowpea cultivars grown in a greenhouse. Six weeks after inoculation, one nodule per plant was excised and rhizobia were re-isolated as described above. Authenticated isolates were stored as above at −80 °C for long-term storage.

### Nodulation tests

The nodulation capacity of isolates was tested on their original hosts in glasshouse pot experiments as described by Azarias Guimarães *et al.*[39] In addition, representative isolates were tested for their capacity to nodulate cowpea, common bean, and soybean. The strains *Bradyrhizobium diazoefficiens* USDA 110 and *Rhizobium tropici* CIAT 899 were used as positive controls for nodulation of soybean and common bean, respectively. The plants were harvested after 6 weeks to record nodulation. Rhizobia were re-isolated from nodules and their identity was confirmed by ERIC-PCR fingerprinting. Nodulation capacity was recorded as positive (Nod+) when nodules were present and negative (Nod−) if were absent. Nitrogen fixation was considered effective when nodules were pink (Fix+) and ineffective if nodules were white (Fix−).

### ERIC fingerprinting

ERIC-PCR fingerprint analysis was performed by using enterobacterial repetitive intergenic consensus primers ERIC 1R and ERIC 2 (Table S2). PCR reactions were carried out in a final volume of 25 $\mu$L containing 100 ng of template DNA, 1× reaction buffer (75 mmol $L^{-1}$ Tris–HCl pH 8.8, 20 mmol $L^{-1}$ $(NH_4)_2SO_4$, 0.01% Tween 20, 2 mmol $L^{-1}$ $MgCl_2$), 0.2 mmol $L^{-1}$ dNTPs, 2.5 U GoTaq DNA polymerase (Promega, Madison, WI, USA), and 50 pmol of each primer. The PCR conditions were: initial denaturation at

  

94 °C for 7 min, 30 cycles of denaturation at 94 °C for 1 min, annealing at 52 °C for 1 min, and extension at 65 °C for 8 min. PCR reactions were terminated by a final extension at 65 °C for 16 min. All PCR products were separated by electrophoresis in 2% agarose containing 0.5 μg $mL^{-1}$ ethidium bromide at 60 V for 3.0 h. The gels were scanned with the GelDoc system (Bio-Rad, Hercules, CA, USA). Conversion, normalisation, and further analysis were performed using the Dice coefficient and the unweighted pair group method with arithmetic mean (UPGMA) cluster analysis with Bionumerics software version 6.1 (Applied Maths, Sint-Martens-Latem, Belgium).

### Phenotypic characterisation

The ability of representative rhizobial isolates to grow under stress conditions (salinity, high temperatures, acidity/alkalinity and antibiotics) was assessed (Table S3). The salt tolerance of the isolates was tested on YMA medium supplied with 0, 0.1%, 0.25% and 0.5%, (w/v) NaCl, according to Moschetti *et al.*[41] The pH tolerance was examined on YMA medium adjusted to a pH range from 4.5 to 10.[40] Temperature tolerance was examined by growing the rhizobial isolates on YMA medium and incubating at 15, 20, 30 and 37 °C.[41] Bacterial growth was examined following 2–3 days of incubation at 30 °C. The intrinsinc sensitivity of the isolates to different antibiotics and concentrations was also assessed by the agar disc-diffusion method[42] and the evaluation was made according to Willems *et al.*[43] The isolates were considered as resistant (R) for diameter inhibition zone 6–8 mm, intermediate (I) for diameter 9–12 mm, and sensitive (S) for diameter >12 mm. The following antibiotics were used (μg per disc): ampicillin (15), carbenicillin (15), chloramphenicol (15) ciprofloxacin (5), gentamicin (30), kanamycin (15), nalidixic acid (30), neomycin (15), penicillin (15), rifamycin (15), spectinomycin (15), streptomycin (15), and tetracycline (15).

### PCR amplifications and sequencing

Total template DNA was extracted from each isolate using the GenElute™ Bacterial Genomic DNA Kit (Sigma, St Louis, MO, USA) according to the manufacturer's instructions. Fragments of genes located either within or outside the symbiosis island were selected for amplification. The DNA fragments of 16S rRNA, IGS (16S–23S rRNA intergenic spacer region), *recA* (DNA recombination protein), *glnII* (glutamine synthetase II), *gyrB* (DNA gyrase B), *SMc00019* (conserved hypothetical protein), *truA* (RNA pseudouridine synthase A), *thrA* (homoserine dehydrogenase), *nifH* (nitrogenase), *nodC* (*N*-acetylglucosaminyltransferase), and *rhcRST* (conserved type III secretion proteins) were amplified by PCR, using the primer pairs described in Table S2. Primers taken from the literature or designed in the present study were slightly modified in such a way to include at their 5′ ends either T7 or SP6 primer sequence to facilitate direct sequencing of the amplicons. Each PCR mixture contained the following: approximately 50 ng genomic DNA, 20 pmol each primer, 200 $\mu$M dNTPs (Promega), 2.5 U GoTaq DNA polymerase (Promega), and GoTaq polymerase buffer in a final reaction volume of 50 $\mu$L. The PCR conditions for the amplification of each gene fragment are described in Table S2. PCR products from the above mentioned gene were purified using the GenElute™ Gel Extraction Kit (Sigma). Purified 16S rRNA fragments were cloned into pGEM-T-easy vector (Promega) and ligated plasmids were then transformed into *Escherichia coli* DH5a cells. Plasmids were purified using GenElute™ Plasmid Miniprep Kit (Sigma) and then sequenced with standard primers. Purified DNA fragments of the rest amplicons were directly sequenced using the standard primers attached in the corresponding primer sequences. All PCR products were commercially sequenced by CEMIA (cemia.eu), Greece.

### Phylogenetic analyses

The sequences of *rrs* genes were compared with those of bacterial type strains using the EzTaxon-e server (http://eztaxon-e.ezbiocloud.net). Sequence similarity searches were performed at the National Center for Biotechnology Information (NCBI) server using BLASTN (http://www.ncbi.nlm.nih.gov/blast) and sequences from closely related type strains as listed on the List of Prokaryotic Names with Standing in Nomenclature (LPSN) (www.bacterio.net) and reference strains were retrieved for phylogenetic analyses from the GenBank/EMBL database (http://www.ebi.ac.uk/Tools/sss/fasta/nucleotide.html). The partial gene sequences obtained in this study, together with sequences retrieved from GenBank were aligned using the CLUSTAL W software in the MEGA 6.0 software package.[44] Phylogenetic trees were constructed using the neighbour-joining (NJ) and maximum likelihood (ML) methods in MEGA 6.0 software package. For NJ trees of single and concatenated sequences, the distances were computed using the Kimura 2-parameter or the Tamura-3 parameter method, with the pairwise deletion option and 1000 replications were used. For ML analyses, the gene sequences were trimmed to the same length and were concatenated. The best-fit models of nucleotide substitution were determined in MEGA 6 and the most appropriate were selected for the construction of ML trees as referred in the figure legends. The ML trees were bootstrapped using 1000 replications. The percentage similarity of the genes was estimated using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/).

### Nucleotide sequence accession numbers

All sequences from cowpea isolates were deposited in the GenBank database and the accession numbers are listed in Table S4.

## RESULTS

### Isolation of root nodule bacteria and soil characteristics

A total of 36 bacterial strains were isolated from nodules of local cowpea varieties grown in Karpathos and Evia islands of Greece. Strains were named 'VU', representing the host *Vigna unguiculata* (VU) followed by either 'KA' or 'EV' for strains isolated from Karpathos or Evia, respectively. All isolates were fast growing, acid-producing bacteria that formed effective pink–red coloured nodules on their host of origin. Soil characterisation showed that all the soil samples were alkaline, with pH range 7.9 to 8.1 (Table S1).

### Genetic diversity of isolates with ERIC fingerprinting analysis

To gain an insight into the genetic heterogeneity of the isolates, ERIC-PCR analysis was performed. Clustering analysis of the ERIC-PCR products resulted in three main clusters at a 65% level of similarity (Fig. 1). This cut-off value was chosen arbitrarily to define clusters of similar ERIC fingerprints though it has also been used for rhizobia.[45] Although, the *Ensifer* strains shared identical 16S RNA gene sequences, they were classified by ERIC fingerprinting into three distinct ERIC groups (I–III). In some cases, isolates obtained from nodules of the same plant displayed two to three distinct ERIC-DNA profiles. ERIC cluster I included nine isolates (VUKA2, VUKA7, VUKA21, VUEV1, VUEV4, VUEV6, VUEV12, VUEV16,

  

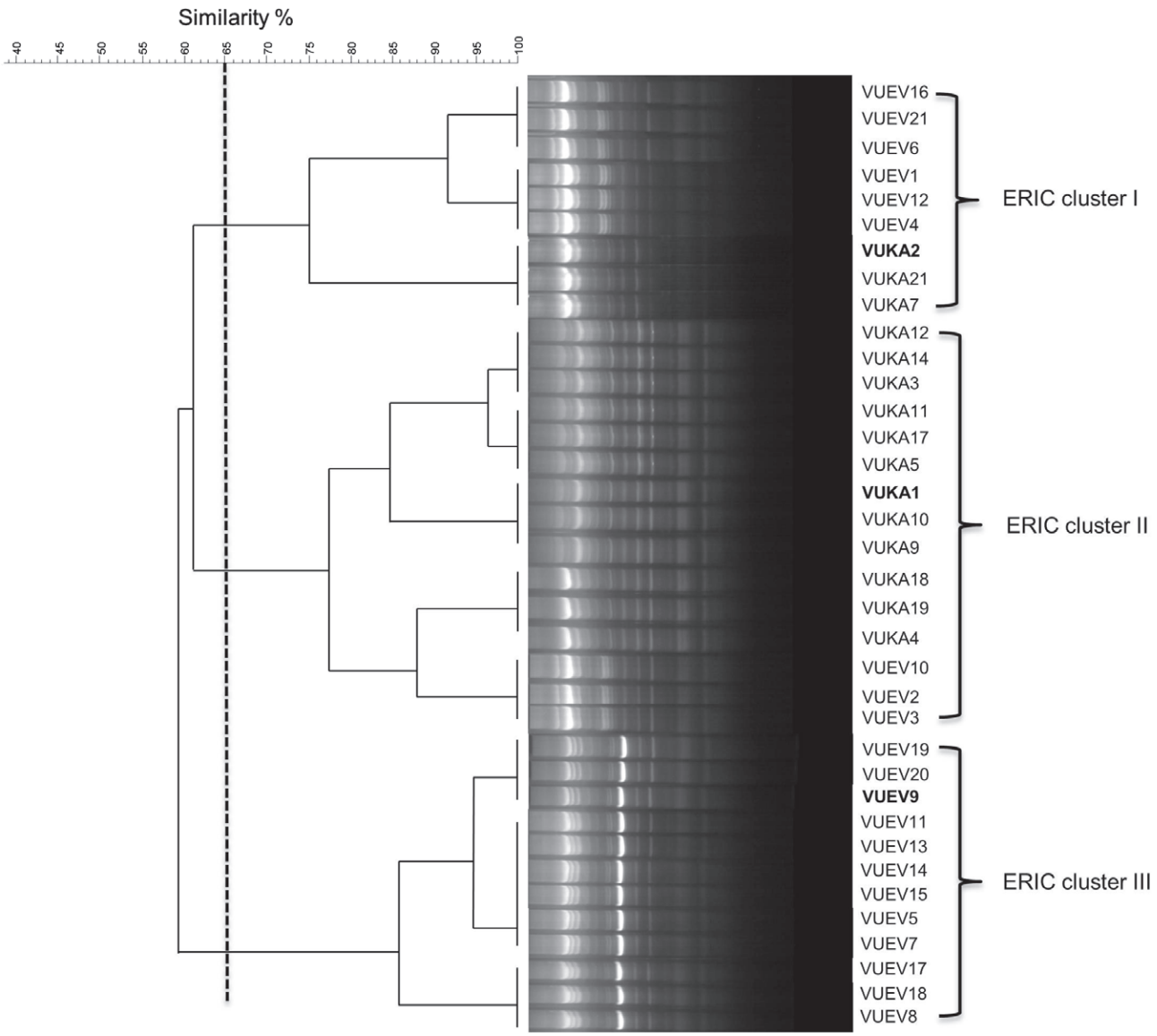


**Figure 1.** Dendogram of isolates from field-grown *V. unguiculata* nodules based on ERIC-PCR fingerprinting analysis using the UPGMA algorithm and the Dice coefficient. The dotted line indicates the similarity level dividing the strains into clusters. The representative strains of the clusters analysed in this study are shown in boldface.

VUEV21), ERIC cluster II comprised 15 isolates (VUKA1, VUKA3, VUKA4, VUKA5, VUKA9, VUKA10, VUKA11, VUKA12, VUKA14, VUKA17, VUKA18, VUKA19, VUEV3, VUEV2, VUEV10), and ERIC cluster III involved 12 isolates (VUEV5, VUEV7, VUEV8, VUEV9, VUEV11, VUEV13, VUEV14, VUEV15, VUEV17, VUEV18, VUEV19, VUEV20). Clusters I and II contained strains from both islands, while cluster III included strains only from Evia (Fig. 1). Therefore, the prevalence of *Ensifer* isolates and the lack of *Bradyrhizobium* strains in both islands might be related to soil alkalinity whereas the presence of cluster III only in Evia island might be due to either diverse soil characteristics and/or different cowpea varieties used.

### Phylogenetic analysis of the 16S rRNA gene and IGS

According to the ERIC grouping results, three isolates (VUKA1, VUKA2 and VUEV9) representing the main ERIC clusters were chosen for 16S and IGS sequence analysis. Nearly full-length 16S rRNA gene sequences (1383 bp) were determined for all representative isolates. The 16S rRNA gene phylogenetic tree showed that all isolates were members of the family *Rhizobiaceae* of the Alphaproteobacteria (Fig. 2A). All isolates displayed identical 16S rRNA gene sequences which were also identical to that of *Ensifer fredii* USDA257, supported by a bootstrap value of 73% (Fig. 2A). However, the 16S rRNA phylogeny can not provide adequate differentiation for species identification of the isolates since different type species can harbour identical or nearly identical 16S rRNA gene sequences (e.g. *E. fredii* and *E. americanus*), as previously reported.[46] Contrarily, 16S–23S IGS sequence analysis displays high discriminative power among closely related strains.[47] In the present study, all isolates produced one band of the same size in PCR amplification of the IGS regions. For phylogenetic analysis, the full-length IGS regions including part (~10 nt) of their corresponding 16S and 23S sequences were used, while the gaps in the IGS alignment were not penalised, as previously suggested.[48] Phylogenetic analysis of the IGS sequences clustered the VUKA1, VUKA2 and VUEV9 isolates together with *Ensifer fredii* USDA257

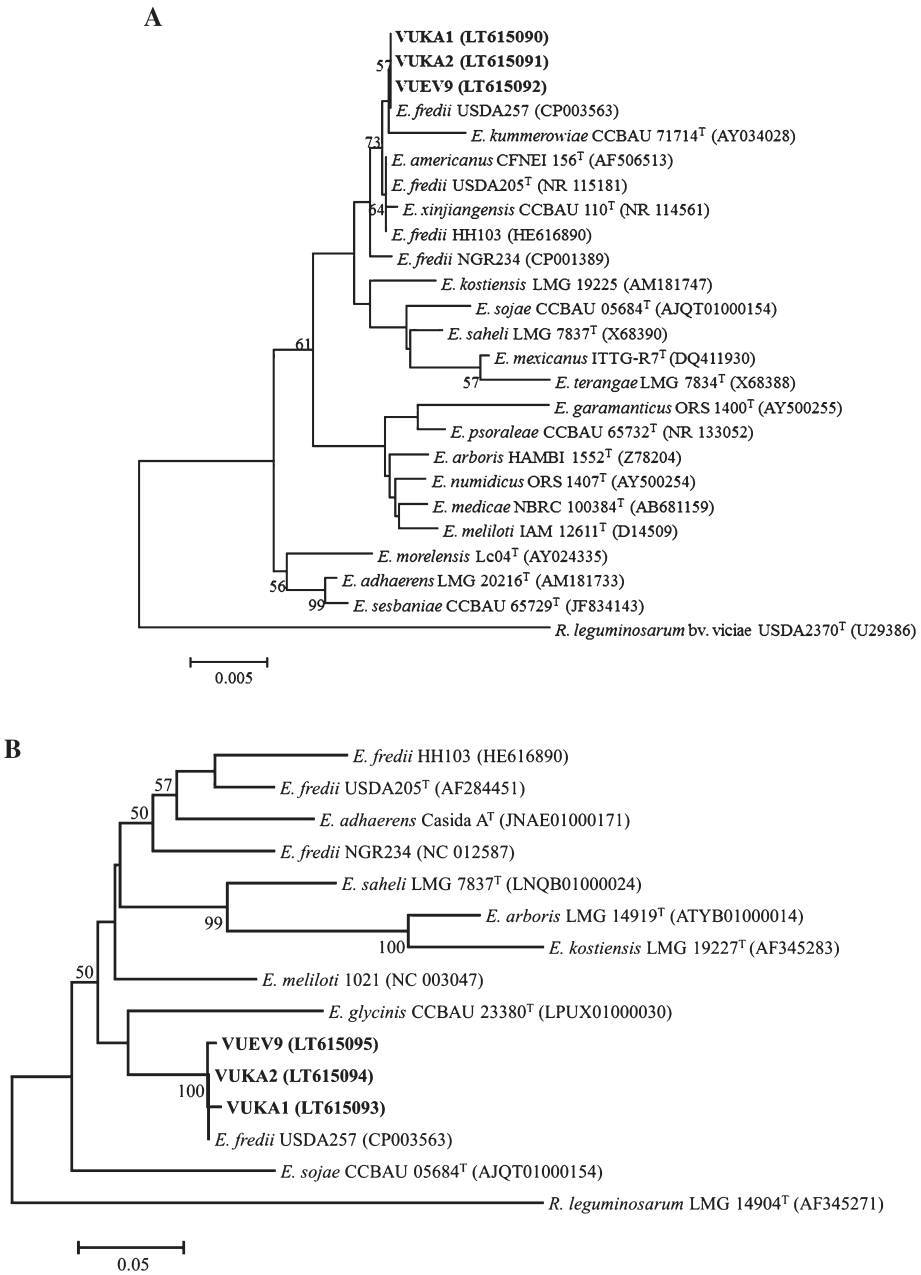


**Figure 2.** Neighbour-joining phylogenetic tree based on nearly complete 16S rRNA gene sequences (A) and on the 16S–23S IGS regions (B) of strains from nodules of *V. unguiculata* from this study and of reference/type strains. Representative strains isolated from *V. unguiculata* nodules from Karpathos and Evia are designated VUKA and VUEV, respectively, and are shown in boldface. Type strains are indicated by superscript 'T'. GenBank accession numbers of the sequences are indicated within parentheses. *Rhizobium leguminosarum* bv. viciae was included as an outgroup. Bootstrap values (greater than 50%) were calculated for 1000 replications and are shown at the nodes. The scale bar shows the number of nucleotide substitutions per site. The evolutionary distances were computed using the Kimura-2 parameter model and the evolutionary analysis was conducted in MEGA 6. The genus names are abbreviated as follows: E., *Ensifer*; R., *Rhizobium*.

(Fig. 2B). The full-length IGS sequences (1166 bp) of the isolates VUKA1, VUKA2 and VUEV9 were nearly identical (99.1–99.3%) and were highly similar (99.5–99.6%) to *E. fredii* USDA257, while they displayed lower similarities, varied from 75.3% to 78.6%, with the other reference *E. fredii* strains. The neighbour-joining (NJ) IGS tree revealed similar relationships between our isolates and type strains comparable to those of the 16S rRNA gene NJ tree. These results clearly demonstrated that the representative isolates from both Karpathos and Evia belong to *Ensifer* genus.

### Multi-locus sequence analysis of housekeeping genes

In order to define a more robust phylogenetic position of the *Ensifer* isolates, phylogenetic trees based on the concatenated sequences of the six housekeeping genes *truA*, *thrA*, *SMc00019*, *gyrB*, *recA* and *glnII* were constructed. Sequences of the corresponding housekeeping genes from type and reference strains were retrieved from the Genbank and were trimmed appropriately. The sequence availability in the GenBank determined both the number of type strains/taxa included in our analysis as well as the length of the nucleotide alignments for the

  

six housekeeping genes. Consequently, the length of the alignments used were 339 bp, 750 bp, 336 bp, 657 bp, 522 bp and 516 bp for *truA*, *thrA*, *SMc00019*, *gyrB*, *recA* and *glnII*, respectively (Table S5). Aligned sequences from the selected genes were concatenated and 3120 bp positions were obtained (Table S5). The housekeeping genes *gyrB*, *recA* and *glnII* have been used widely in phylogenetic studies of *Ensifer* strains,[49–52] while, recently, the core genes *truA*, *thrA* and *SMc00019* were demonstrated to produce phylogenies congruent with the reference species tree of rhizobia.[31,53,54] The discriminatory power of the selected gene sequences used for the *Ensifer* taxa was estimated by the number of parsimony-informative sites contained in each gene (Table S5). In our analysis, *SMc00019* had the greatest percentage of parsimony-informative characters (31.5%), of the positions used, followed by *truA* (31.5%), *gyrB* (30.3%), *glnII* (29.2%), *thrA* (26.7%) and *recA* (23.5%). The concatenated sequence of *truA–thrA–SMc00019* was more parsimony-informative (28.6%) than that of *gyrB–recA–glnII* (26.4%). These results were consistent with previous studies that evaluated the use of these genes as highly reliable markers for species discrimination within rhizobia.[46,50,54]

In the individual NJ phylogenetic trees for all housekeeping gene sequences analysed, the *Ensifer* isolates formed a distinct group, closely related to *E. fredii* USDA257 supported by 100% bootstrap value (data not shown). No significant variation in tree topologies was observed for all genes tested. The maximum likelihood (ML) phylogenetic tree of *Ensifer* strains, based on six housekeeping genes, was constructed and was congruent with those of the individual housekeeping genes, but grouping was more robust and confident (Fig. 3A). The ML trees based on combined sequences of *truA + thrA + SMc00019* and *gyrB + recA + glnII* also revealed congruent topologies (Fig. 3B and C). In all analyses, the *Ensifer* isolates were placed along with *E. fredii* USDA257 on well-supported separate clades (100%) in the ML trees based either on the six or three concatenated gene sequences. Although this phylogeny was consistent with the 16S rRNA gene analysis the concatenated gene analysis provided a better phylogenetic resolution and support of the *Ensifer* isolates. Based on pairwise comparisons of the six core genes (Table S6) the *Ensifer* isolates displayed highest sequence similarity to *E. fredii* USDA257 (99.6%), while they displayed lower similarities to the strains *E. fredii* USDA205$^T$ (93.8%), *E. fredii* HH103 (93.6%) and *E. fredii* NGR234 (92.6%).

### Phylogenetic analysis of symbiotic genes

The symbiotic plasmid-borne genes *nodC*, *nifH* and *rhcRST-I* for the representative *Ensifer* isolates were amplified and partially sequenced (726 bp, 702 bp and 1168 bp, respectively). The *nodC*-, *nifH*- and *rhcRST*-I based phylogenies placed the *E. fredii* strains into three distinct well-supported clusters as presented in Figs 4 and 5, respectively. The cluster I contained our *Ensifer* isolates (VUKA1, VUKA2 and VUEV9), the cluster II consisted of two strains, *Ensifer* sp. PC2 and *Ensifer* sp. TW10, and the cluster III harboured *E. fredii* type/reference strains. *Ensifer* sp. PC2 and *Ensifer* sp. TW10 have been isolated from *Prosopis cineraria* (wild tree legume of *Mimosoideae*) and *Tephrosia wallichii* (perennial shrub of *Papilionoideae*), respectively, in India.[55] On the basis of the 16S-based phylogeny[56,57] and also in agreement with our MLSA analyses of core genes (Fig. 3), the *Ensifer* strains PC2 and TW10 were distantly related to *E. fredii* strains, whereas they were closely related to *E. kostiensis* LMG 19227$^T$, *E. terangae* LMG 7834$^T$ and *E. sojae* CCBAU 05684$^T$. However, based on symbiotic gene phylogenies these strains are closely related to *Ensifer fredii* strains.

Pairwise *nodC* gene sequence similarities ranged from 98% to 100% within each cluster, while the similarities between the clusters I and II varied from 97.15% to 97.29% and between the clusters I and III varied from 95.01% to 95.44% (Table S7). Similarly, the *Ensifer* isolates shared identical *nifH* gene sequences and displayed similarities of 96.89–97.11% to PC2 and TW10 strains and 96.67–96.89% to *E. fredii* type/reference strains (Table S7). The similarity levels of *rhcRST*-I sequences of our isolates were 96.17% with cluster II and varied from 92.26% to 92.35% with cluster III (data not shown). Partial *rhcRST*-II gene sequences (1251 bp) were also obtained from the representative *Ensifer* isolates to contrast *rhcRST*-I phylogeny. As shown in Fig. 5, the *rchRST* II-based phylogeny was congruent with that of core genes (Fig. 3) and the *rhcRST*-II gene sequences of our *Ensifer* isolates displayed strong similarities (98.31–98.4%) with those of *E. fredii* USDA257 (data not shown), indicating the close relationship of their chromosomal backgrounds. Similar topologies and high bootstrap values were obtained in the NJ and ML phylogenetic trees based on amino acid sequences deduced from *nodC*, *nifH and rhcRST* sequences (data not shown).

### Phenotypic characterisation

The ability of representative rhizobial isolates (VUKA1, VUKA2 and VUEV9) to grow under stress conditions (salinity, high temperatures, acidity/alkalinity and antibiotics) was assessed. The phenotypic characteristics of representative cowpea isolates are listed in Table S3. All the rhizobial isolates were sensitive to salinity and temperature and grew well at up to 0.25% NaCl and 30 °C, respectively. Regarding their tolerance to acidic and alkaline conditions, all the isolates grew well on alkaline media (pH range 7.0–10.0). The *Ensifer* isolates showed resistance in chloramphenicol, nalidixic acid, neomycin, while they displayed intermediate resistance in kanamycin, and spectinomycin. All isolates were sensitive to ampicillin, ciprofloxacin, gentamycin, penicillin, rifamycin and tetracycline. Moreover, nodulation tests performed on cowpea, soybean and common bean showed that the *Ensifer* isolates were able to nodulate and fix nitrogen in cowpea but not in soybean and common bean, as the corresponding positive control strains did (Table S3).

## DISCUSSION

In the current study, 36 cowpea nodulating bacteria were isolated from two islands of the Aegean Sea in Greece and their phylogenetic classification is reported. ERIC fingerprinting analysis grouped the isolates with 65% similarity level into three groups. Phylogenetic analysis based on 16S rRNA assigned the isolates into the genus *Ensifer*. However, 16S rRNA phylogeny has low discriminatory power at the species level within a genus, including rhizobia.[47] Therefore, for species definition, representative strains from each ERIC group were subjected to a multi-locus sequence analysis based on 16S–23S IGS regions, six housekeeping and three symbiotic genes. All isolates were identified as fast-growing bacteria and were defined as representing *E. fredii* strains.

Our study provides evidence for the first time that strains assigned to *E. fredii* are indigenous in Greece and are able to nodulate local cowpea varieties in alkaline soils under field conditions. The occurrence of *E. fredii* strains in field-grown cowpea nodules suggested that they were real microsymbionts of *V. unguiculata* under alkaline conditions. Furthermore, the lack of isolation of *Bradyrhizobium* spp. that are common microsymbionts of cowpea,

  

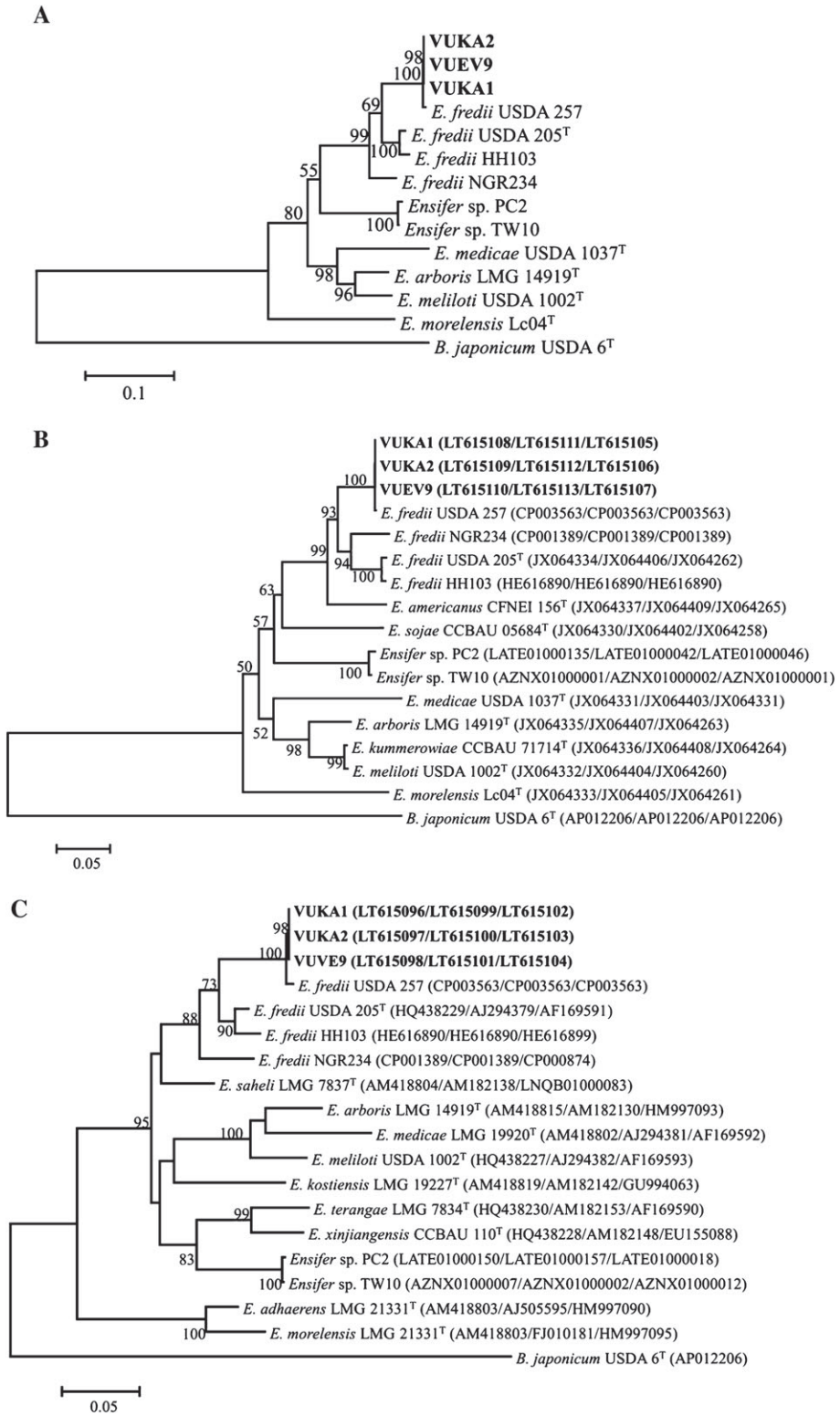


**Figure 3.** Maximum likelihood phylogeny tree based on concatenated sequences of (A) *truA*, *thrA*, *SMc00019*, *gyrB*, *recA* and *glnII* (3120 nt), (B) *truA*, *thrA* and *SMc00019* (1425 nt) and (C) *gyrB*, *recA* and *glnII* (1695 nt). Bootstrap values (greater than 50%) were calculated for 1000 replications and are shown at the nodes. Type strains are indicated by superscript 'T'. Isolates obtained in the present study are shown in boldface. GenBank accession numbers of the sequences are indicated within parentheses. Sequences marked with asterisks were retrieved from the Microscope database (https://www.genoscope.cns.fr/agc/microscope). *Bradyrhizobium japonicum* USDA $6^T$ was included as an outgroup. The scale bar shows the number of nucleotide substitutions per site. Phylogenetic analyses were conducted in MEGA 6 using the maximum likelihood algorithm with the General Time Reversible model plus Gamma rate distribution (GTR + G). The genus names are abbreviated as follows: E., *Ensifer*; B., *Bradyrhizobium*.

  

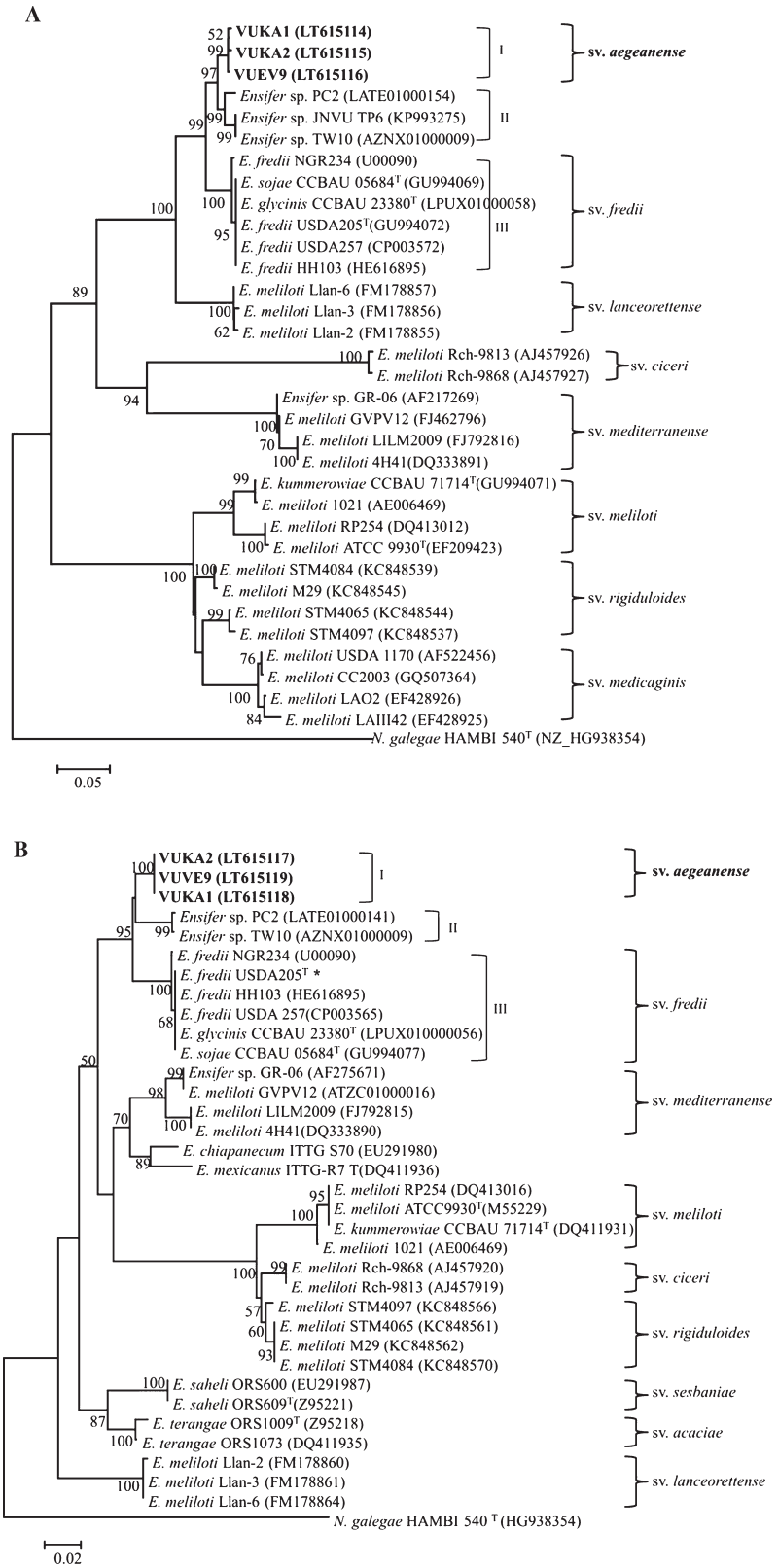


**Figure 4.** Neighbour-joining phylogenetic tree based on 702-bp and 450-bp alignment of the *nodC* (A) and *nifH* (B) nucleotide sequences, respectively. Bootstrap values (greater than 50%) were calculated for 1000 replications and are shown at the nodes. Type strains are indicated by superscript 'T'. Isolates obtained in the present study are shown in boldface. GenBank accession numbers of the sequences are indicated within parentheses. Sequences marked with asterisks were retrieved from the Microscope database (https://www.genoscope.cns.fr/agc/microscope). *Neorhizobium galegae* HAMBI 540$^T$ was included as an outgroup. The scale bar shows the number of nucleotide substitutions per site. Phylogenetic analyses were conducted in MEGA 6 using the Tamura 3-parameter model plus Gamma rate distribution (T92 + G). The genus names are abbreviated as follows: E., *Ensifer*; N., *Neorhizobium*.

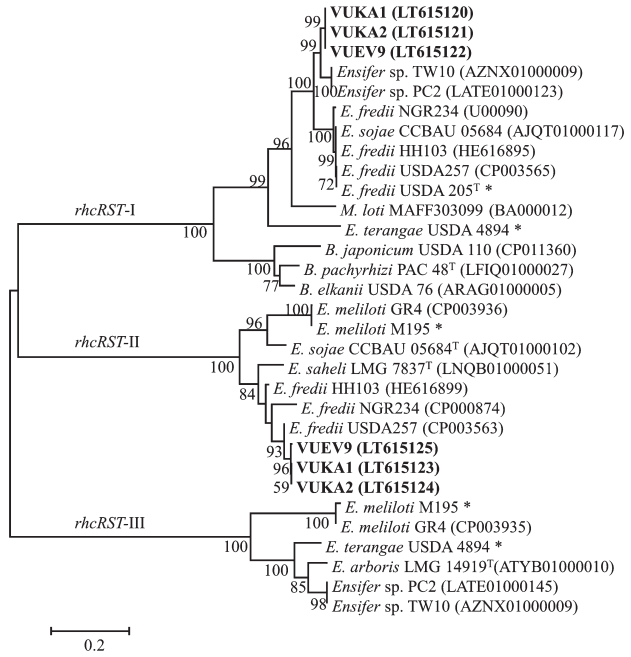


**Figure 5.** Neighbour-joining phylogenetic tree based on the alignment of the *rhcRST*-I (1150 bp), *rhcRST*-I (1127 bp) and *rhcRST*-III (1115 bp) nucleotide sequences. Bootstrap values (greater than 50%) were calculated for 1000 replications and are shown at the nodes. Type strains are indicated by superscript 'T'. Isolates obtained in the present study are shown in boldface. GenBank accession numbers of the sequences are indicated within parentheses. Sequences marked with asterisks were retrieved from the Microscope database (https://www.genoscope.cns.fr/agc/microscope). The scale bar shows the number of nucleotide substitutions per site. Phylogenetic analyses were conducted in MEGA 6 using the Tamura 3-parameter model plus Gamma rate distribution (T92 + G). The genus names are abbreviated as follows: B., *Bradyrhizobium*; E., *Ensifer*; M., *Mesorhizobium*.

could be attributed either to the absence of bradyrhizobia from the sampling sites or to the out-competition of bradyrhizobia from *E. fredii* strains under alkaline soil conditions. The latter is in agreement with a previous study that showed the high competitiveness of *E. fredii* HH103 against *B. japonicum* USDA110 for nodulating soybeans in alkaline soils.[58]

Multi-locus sequence analysis (MLSA) is widely used in bacterial species definition providing a high resolution of phylogenetic relatedness at the inter- and intra-specific level.[59–61] The chosen housekeeping genes were among the best-performing genes for differentiation and description novel species within *Ensifer*.[46,50,54,62] According to Konstantinidis *et al.*[63] the average nucleotide identity (ANI) of at least three core genes (3300 bp) can produce a phylogeny of closely related organisms more congruent to the whole-genome-based phylogeny. Based on studies from diverse bacteria, thresholds of ANI, 94–96%, have been proposed as a gold standard for species delineation, corresponding closely to 70% DNA–DNA hybridisation (DDH).[64] However, these values can be differentiated depending on the species. An ANI value of 97.3% has been previously proposed as the cut-off for species delineation in *Ensifer*.[46] In our analysis, the ANI values, based on six core genes, between the *Ensifer* isolates and the *E. fredii* USDA257 were 99.3% indicating their close relatedness. However, the *Ensifer* isolates displayed ANI values of 92.1–93.5% with the type strain *E. fredii* USDA205 and other *E. fredii* reference strains, which are lower than that considered for delineation of most bacterial species. In addition to having lower ANI values from the threshold, the *Ensifer* isolates and the *E. fredii* USDA257 formed a highly supported monophyletic group within the *E. fredii* clade both in the individual and in the combined phylogenetic trees of all core genes tested. In view of these findings, we assume that our *Ensifer* isolates along with the *E. fredii* USDA257 may represent a new lineage or even a putative new species distinct from the type strain *E. fredii* USDA205. Several lines of evidence suggest that the species status of *E. fredii* strains should be revised. Based on whole-genome fingerprinting methods, a previous study showed the separation of defined strains of *E. fredii*, such as NGR234, HH103 and USDA257, into three clusters, at a similarity level of 62%.[44] More recently, Tian *et al.*[53] reported that the *E. fredii* NGR234 may represent a species other than *E. fredii* based on whole genome alignments (ANI < 93%). Indeed, the whole genome-based ANI values (gANI), as we calculated them by the algorithm implemented in the Integrated Microbial Genomes (IMG) database,[65] between the three reference *E. fredii* strains, NGR234, HH103 and USDA257, ranged from approximately 89.5% to 92.5%, which were lower than the threshold of species delineation (94%) implying that they may represent three distinct species. In our analysis, the ANI values of the three reference *E. fredii* strains (92.4–94.1%) showed, albeit slightly higher, strong correlation to those based on their gANI (89.5–92.5%), thereby underpinning the reliability of the inferred phylogenies of our isolates in the present study.

Besides the genetic distinctiveness of the reference *E. fredii* strains, their morphophysiological and symbiotic properties also corroborate the discrimination of the *E. fredii* strains. Initially, the *E. fredii* strains were divided into two subgroups, designated chemovars (cv.) *fredii* and *siensis*.[66–68] The type strains of cv. *fredii* and cv. *siensis* were reported to be the *E. fredii* USDA205 (=ATCC 35423) and the *E. fredii* USDA201, respectively, with the latter to also include the strains USDA194 and USDA257.[67] Although all strains are capable of nodulating cowpea they can be differentiated on the basis of their ability to nodulate soybean. *E. fredii* USDA201 and HH103 are able of nodulating soybean (Asiatic and American varieties), whereas NGR234 is unable and USDA257 nodulates soybean in a cultivar-specific manner.[16,67,69] Taken together, these findings support the revision of the phylogenetic affiliation of *E. fredii* strains. In recent years, an emerging demand towards a re-evaluation of the guidelines used for definition of bacterial and archaeal species is growing due to advances in whole-genome sequencing.[70] Theretofore, the *Ensifer* strains isolated in the present study are considered as *E. fredii* with close relatedness to the lineage specified by USDA257. Although the representative *Ensifer* isolates were clustered into the same group in all phylogenies based both on housekeeping and symbiotic genes analysed, they were still genetically distinct lineages as revealed by the ERIC fingerprinting analysis. This intra-specific genomic variation may due to differences on plasmids, non-essential genes and DNA regions.

In the present study, three symbiotic genes were analysed to determine the symbiotic diversity of our isolates. Individual ML phylogenetic trees, based on *nifH*, *nodC* and *rhcRST-I*, were congruent suggesting co-evolution of all three symbiotic genes and placed the *Ensifer* isolates into well-supported clusters divergent from the other type/reference *E. fredii* strains, USDA205$^T$, USDA257, NGR234 and HH103. Moreover, the similarity levels of *nifH*, *nodC* and *rhcRST*-I between our *E. fredii* strains and the type/reference ones ranged from 96.7% to 96.9%, 95% to 95.4%, and 92.3%, respectively. In contrast, the type and reference *E. fredii* strains harboured highly similar (>99.5%) symbiotic genes (*nodC*, *nifH* and *rhcRST*-I) and were clustered together in all single symbiotic

  

trees except for NGR234, which was placed in a slight divergent branch. The phylogeny based on *rhcRST*-II of the *E. fredii* isolates was congruent with the combined tree of the six housekeeping genes and confirmed further their close genetic relationships to *E. fredii* USDA257. Taken together, our data indicated that the *E. fredii* isolates shared similar chromosomal background with *E. fredii* USDA257, while their symbiotic genes were phylogenetically divergent to those from all other *E. fredii* strains suggesting that they may belong to a new symbiotic lineage.

Several studies have shown that rhizobia with similar chromosomal backgrounds can accommodate different symbiotic genes and vice versa.[21,71–73] Recently, the term symbiovar was coined by Rogel *et al.*[26] to represent a group of bacterial strains of the same species thereby sharing a similar chromosomal background but possessing distinct symbiotic capabilities in host plants, differences in host range and symbiotic gene sequences. Currently, most symbiovars are defined by their host spectrum as well as by the similarities of *nodC* sequences at the molecular level which are related both with the host range of rhizobia and the symbiotic promiscuity of legume hosts.[26,28,74] A symbiovar can be found in different species and a rhizobial species can also encompasses different symbiovars.[21,23,27] The latter is the case of cowpea *Ensifer* rhizobia isolated in the present study, which share highly similar chromosomal backgrounds but harbour different symbiotic genes with *E. fredii* strains. In general, strains belonging to the same symbiovar have high similarity levels of *nodC* (>98%), though some symbiovars can accommodate strains with similarity levels close to 95%, as exemplified by the sv. *meliloti*, *medicaciginis* and sv. *rigiduloides*.[75–78] This similarity value has previously been proposed as cut-off for symbiovar discrimination within *Ensifer,* which led to the suggestion that the current sv. *rigiduloides* should be split into two symbiovars.[77] So far, nine symbiovars have been described within the *Ensifer* genus and two of them include *E. fredii* strains.[26] The sv. *fredii* (formerly bv. *fredii*) defined by Scholla and Elkan[67] contains *E. fredii* strains nodulating soybean and the sv. *mediterranense* for *E. fredii* strains that do not nodulate soybean but fix nitrogen with common bean, and it is also present in *E. meliloti* strains.[24] The similarity levels of *nodC* sequences within each symbiovar are more than 98%. Our *E. fredii* strains displayed highest *nodC* similarities (about 95%) to strains of sv. *fredii*. However, unlike the latter, our strains were not able to nodulate common bean and soybean. Therefore, considering a 95% similarity value for *nodC* as a cut-off for symbiovar delineation within *Ensifer*, together with the differences in the host range compared to the strains of sv. *fredii*, the *E. fredii* strains isolated in the present study may represent a new symbiovar within *Ensifer* for which the name '*aegeanense*' is proposed since it was found in islands of the Aegean Sea in Greece. This symbiovar was clearly distinct from the other symbiovars described so far within *Ensifer* on the basis of divergence in *nodC* sequences and host range, as proposed by Rogel *et al.*[26]

Remarkably, phylogenies based on the three symbiotic gene loci placed the *E. fredii* isolates close to the strains *Ensifer* sp. PC2 and *Ensifer* sp. TW10, albeit in separate groups, supported by high bootstrap values. These strains were isolated from perennial legumes in India and their genomes were recently sequenced.[56,57] Compared to our strains, they showed *nodC* similarity values of about 97% and roughly similar host ranges, namely PC2 and TW10 were able to nodulate cowpea but not common bean. However, no information is still available for their nodulation ability on soybean. Furthermore, *Ensifer* sp. JNVU TP6 also isolated from wild legume (*Tephrosia purpurea*) in the Indian Thar desert[55] displayed highly similar *nifH* and *nodC* sequences to *Ensifer* sp. TW10 and clustered together in the corresponding trees.[79] Taken together, these data show that our isolates are closely related in symbiotic loci with strains phylogenetically divergent to *E. fredii* strains. The incongruence between the phylogenies based on symbiotic and housekeeping genes is indicative of lateral transfer of symbiotic genes, a phenomenon that plays a significant role in the evolution of rhizobia.[80] Therefore, the symbiotic genes of our isolates and those of the desert strains were likely acquired by lateral gene transfer from an unknown yet common ancestor and then they diverged to fulfil specific adaptations to different legume niches.[26] Hence, we assume that PC2, TW10 and JNVU TP6 may either fall within the sv. *aegeanense* harbouring phylogenetically divergent *nodC* alleles or constitute an as yet undescribed symbiovar. Analogous cases to the first possible scenario have been reported in sv. *phaseoli*, which carries two different *nodC* alleles with 97% identity,[81] and in sv. *meliloti*,[77] which is divided into three groups harbouring *nodC* alleles with identities of about 96–97%. However, the aforementioned strains may constitute a different symbiovar although the present data do not allow the assignment of these strains into the same or different symbiovars. The host range of the above strains needs to be studied more extensively and the cut-off values of *nodC* similarities for delineation different symbiovars should be estimated more precisely. In the future, the analysis of more *nodC* sequences, the inclusion of more symbiotic-related determinants and the detailed description of the host range in each symbiovar would result in the definition of criteria circumscribing the symbiovar concept and will lead to a more precise assignment of rhizobial strains within current or new symbiovars.

## CONCLUSION

Fast-growing rhizobia were isolated and phylogenetically classified for the first time as cowpea-nodulating rhizobia in alkaline soils in Greece. The isolates were classified as *E. fredii* and may constitute a new symbiovar for which the name sv. *aegeanense* is proposed. These findings further confirm the promiscuity of cowpea and extend our knowledge regarding the diversity, distribution and evolution of cowpea-nodulating rhizobia in European soils.

## ACKNOWLEDGEMENTS

We thank Dr Penelope J. Bebeli and Dr Ricos Thanopoulos for assistance with the collection of cowpea nodules. The present work was supported by the EUROLEGUME research project funded by the 7th Research Framework Programme of the European Union (613781). We also thank the anonymous reviewers for a critical reading of the manuscript.

## SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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