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Phylogenetic multilocus sequence analysis of indigenous slow-growing rhizobia nodulating cowpea (*Vigna unguiculata* L.) in Greece

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ABSTRACT

Cowpea (*Vigna unguiculata*) is a promiscuous grain legume, capable of establishing efficient symbiosis with diverse symbiotic bacteria, mainly slow-growing rhizobial species belonging to the genus *Bradyrhizobium*. Although much research has been done on cowpea-nodulating bacteria in various countries around the world, little is known about the genetic and symbiotic diversity of indigenous cowpea rhizobia in European soils. In the present study, the genetic and symbiotic diversity of indigenous rhizobia isolated from field-grown cowpea nodules in three geographically different Greek regions were studied. Forty-five authenticated strains were subjected to a polyphasic approach. ERIC-PCR based fingerprinting analysis grouped the isolates into seven groups and representative strains of each group were further analyzed. The analysis of the *rrs* gene showed that the strains belong to different species of the genus *Bradyrhizobium*. The analysis of the 16S-23S IGS region showed that the strains from each geographic region were characterized by distinct IGS types which may represent novel phylogenetic lineages, closely related to the type species of *Bradyrhizobium pachyrhizi*, *Bradyrhizobium ferriligni* and *Bradyrhizobium liaoningense*. MLSA analysis of three housekeeping genes (*recA*, *glnII*, and *gyrB*) showed the close relatedness of our strains with *B. pachyrhizi* PAC48^T and *B. liaoningense* USDA 3622^T and confirmed that the *B. liaoningense*-related isolate VUEP21 may constitute a novel species within *Bradyrhizobium*. Moreover, symbiotic gene phylogenies, based on *nodC* and *nifH* genes, showed that the *B. pachyrhizi*-related isolates belonged to symbiovar vignae, whereas the *B. liaoningense*-related isolates may represent a novel symbiovar.

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Introduction

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* [1,25,57]. Several studies on cowpea rhizobial diversity performed in Asia, America, Africa, Australia and Europe have isolated strains belonging to the species *Bradyrhizobium elkanii*, *Bradyrhizobium iriomotense*, *Bradyrhizobium japonicum*, *Bradyrhizobium lablabi*, *Bradyrhizobium liaoningense* and *Bradyrhizobium yuanmingense*, uncharacterized *Bradyrhizobium* genospecies, or novel *Bradyrhizobium* lineages [3,6,15,27,39,50–52,55,68,72,75,77,82]. Currently, the genus *Bradyrhizobium* comprises thirty-eight type strains (http://www.bacterio.cict.fr/) and three of them, *Bradyrhizobium kavangense* 14-3^T [18], *Bradyrhizobium manausense* BR 3351^T [53], and *Bradyrhizobium vignae* 7-2^T [17] have been isolated from *Vigna unguiculata* nodules, while type strains isolated from other hosts, such as *Bradyrhizobium daqingense* [65], *Bradyrhizobium huanghuaihaiense* [78], *Bradyrhizobium subterraneum* [16], *Bradyrhizobium paxllaeri*, *Bradyrhizobium icense* [14], *Bradyrhizobium neotropicale* [81], *Bradyrhizobium ottawaense* [73], and *B. yuanmingense* [70] are also capable of nodulating cowpea.

Although rarely, rhizobia belonging in the genera *Sinorhizobium*, *Rhizobium* and *Mesorhizobium* have also been reported to nodulate cowpea under either laboratory [11,22,38,77] or field conditions [21,31,34,68,75,77]. Despite that much research has been done on cowpea-nodulating bacteria in various countries around the world, less information is available for indigenous

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rhizobia nodulating cowpea in Greece and other European countries. Recently, strains isolated from *V. unguiculata* in Spain belonged to the genus *Bradyrhizobium* [6], whereas fast-growing rhizobia of *Ensifer*/*Sinorhizobium* genus were exclusively isolated from cowpea grown in alkaline soils in Greece (Tampakaki et al. unpublished).

In order to gain insight on the genetic diversity of indigenous rhizobia associated with cowpea in Greece, field-grown cowpea nodules were collected from three geographically different regions, named as Epirus, Limnos and Crete. 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 multilocus sequence analysis (MLSA), the sequences of three housekeeping genes were used: *recA* (recombination protein), *glnII* (glutamine synthetase II), and *gyrB* (DNA gyrase B). The taxonomic position at the symbiovar level was determined by the inferred phylogenies based on the symbiotic genes, *nifH* (nitrogenase) and *nodC* (*N*-acetylglucosaminyltransferase). The *nifH* and *nodC* genes have been widely used for the phylogenetic analysis of rhizobia [23,28,37] and the *nodC* is also used to determine the host range and host promiscuity [13,23,33,37,43,46,48,62]. Currently, rhizobia are grouped into symbiovars based mainly on *nodC* gene phylogenetic analyses and on symbiotic characteristics [36,48]. Nodulation tests were performed to assess the ability of the isolates to nodulate their host of origin.

Material and methods

Nodule and soil sampling

Nodules were collected from local cowpea varieties grown in three different geographical regions of Greece, Epirus, Limnos and Crete (Fig. S1). The sampling sites were located in fields without rhizobial inoculation history. Soil samples were collected at the time of nodule collection and their physicochemical characteristics were analyzed at the Agricultural Institute of Kalamata, Greece. The soil characteristics of the sampling sites and their geographical locations are summarized in Table S1.

Isolation of rhizobia

Four nodules per plant were randomly selected from five plants of each region and finally, a total of fourty five 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 [61]. Briefly, the nodules were surface disinfected 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. Sterilized nodules were crushed in a drop of sterile distilled water. The nodule juice was streaked onto yeast-mannitol agar (YMA) plates [7], which was 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 Guimarães et al. [21]. The plants were harvested after six 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 μl containing 100 ng of template DNA, 1× reaction buffer (75 mM Tris–HCl pH 8.8, 20 mM $(NH_4)_2SO_4$, 0.01% Tween 20, 2 mM $MgCl_2$), 0.2 mM dNTPs, 2.5 U GoTaq DNA polymerase (Promega), 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). Conversion, normalization, 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).

PCR amplifications and sequencing

Total template DNA was extracted from each isolate using the GenElute™ Bacterial Genomic DNA Kit (Sigma) according to manufacter'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), and *gyrB* (DNA gyrase B) 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 μM dNTPs (Promega), 2.5 U GoTaq DNA polymerase (Promega), and GoTaq polymerase buffer in a final reaction volume of 50 μl. 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 rDNA 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). For pairwise distance matrixes, the multiple alignments were performed using the algorithm CLUSTAL Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) provided by the European Bioinformatics Institute (EMBL-EBI). For phylogenetic analyses, 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 [56]. Phylogenetic trees were constructed using the neighbor-joining (NJ) and Maximum likelihood (ML) methods in MEGA 6.0 software package. For ML analyses, the gene sequences were appropriately trimmed 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 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 S3.

Results and discussion

Isolation of root nodule bacteria and soil characteristics

A total of 45 bacterial strains were isolated from nodules of local cowpea varieties grown in three different geographical regions of Greece, namely Epirus, Crete and Limnos (Fig. S1). Strains were named "VU", representing the host *V. unguiculata* (VU) followed by either "EP" or "CR" or "LI" for strains isolated from Epirus, Crete or Limnos, respectively. All strains were able to form effective pink-red coloured nodules (Nod+/Fix+) on their host of origin and harbored the typical morphophysiological properties of *Bradyrhizobium* strains, namely slow growth and alkaline production on yeast-mannitol agar (YMA) plates. Soil characterization showed that the soil samples from all regions were acid to neutral, with pH in the range 5.8–7.5 (Table S1).

Genetic diversity of isolates with ERIC-fingerprinting analysis

The genetic heterogeneity of isolates was assessed by ERIC-PCR analysis. A dendrogram was constructed based on the UPGMA algorithm by analyzing the similarity between different ERIC-PCR fingerprinting patterns (Fig. 1). Clustering analysis of the ERIC-PCR fingerprints resulted in seven clusters at a 85% similarity level (Fig. 1). All clusters had unique ERIC-PCR fingerprints indicating that they were not clones. The cut-off value was chosen arbitrar-

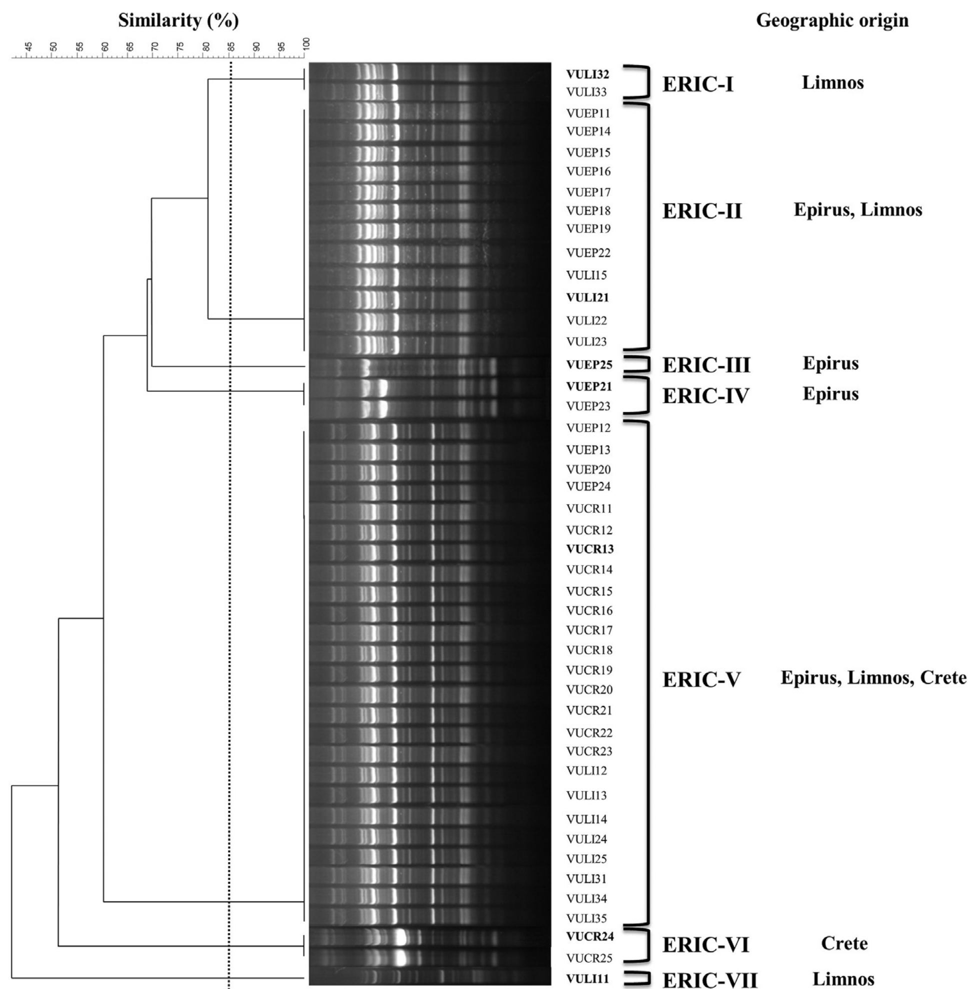

Fig. 1. Dendogram of isolates from field-grown *V. unguiculata* nodules based on ERIC-PCR fingerprinting analysis using the UPGMA algorithm and the Dice coefficient. The dotted line indicates the similarity level dividing the strains into clusters. The representative strains of the clusters for which the 16S rRNA gene and IGS were analyzed are shown in boldface.

ily to define clusters of similar ERIC fingerprints though it has also been used for bradyrhizobia [40].

ERIC cluster I included 2 isolates (VULI32 and VULI33), ERIC cluster II comprised 12 isolates (VULI15, VULI21, VULI22, VULI23, VUCR11, VUCR14, VUCR15, VUCR16, VUCR17, VUCR18, VUCR19, VUCR22), ERIC cluster III involved a single isolate (VUEP25), ERIC cluster IV comprised two isolates (VUEP21 and VUEP23), ERIC cluster V involved 25 isolates (VULI12-VULI14, VULI24-VULI25, VULI31, VULI34–VULI35, VUEP12–VUEP13, VUEP20, VUEP24, VUCR11–VUCR23), ERIC cluster VI involved two isolates (VUCR24 and VUCR25), and ERIC cluster VII involved a single isolate (VULI11). The isolates in each ERIC-cluster shared identical fingerprints indicating that they might be clones. Noteworthy, isolates obtained from plants at different sampling sites displayed identical or very similar ERIC patterns. The cluster V was observed in strains isolated from all regions and the cluster II was found in strains from Epirus and Limnos. However, clusters consisted of one or two isolates (VI and VII) were found only in one region and they may represent local rhizobial genotypes being adapted either to the specific local variety or to the local conditions (Fig. 1). Our results indicated that some genotypes, like those in cluster II and V, might be more competitive in nodulation of cowpea at various locations than those belonging to the rest of clusters. In some cases, isolates obtained from nodules of the same plant displayed two to three distinct ERIC-DNA profiles. For example, clusters II, V, and VII were found in nodules of the same plant. The genetic diversity of the isolates may not be related with their geographic origin but more likely to soil characteristics. Consistent to that, the cowpea-nodulating rhizobia isolated from other regions in Greece having alkaline soils were exclusively fast-growing rhizobia (Tampakaki et al. unpublished). Therefore, the isolates of the current study represent indigenous rhizobia probably adapted to the specific environmental conditions of the sampling sites.

Phylogenetic analysis of the 16S rRNA gene and the 16S-23S IGS regions

According to the ERIC grouping results, seven isolates (VULI11, VULI21, VULI13, VULI32, VUEP25, VUEP21, and VUCR24) representing the different ERIC clusters and originating from different geographic regions were chosen for *rrs* and IGS sequence analyses. Nearly full-length *rrs* gene sequences (1432 bp) were determined for all representative isolates and a region of 1237 bp was considered for the alignment. The 16S rRNA gene phylogenetic tree showed that all isolates were members of the family Bradyrhizobiaceae of the Alphaproteobacteria and were part of the genus *Bradyrhizobium* (Fig. 2). Previously, it was shown that *Bradyrhizobium* species are divided into two well-supported phylogenetic lineages, designated I (represented by *B. japonicum*) and II (represented by *B. elkanii*), based on phylogenetic analyses of *rrs* gene and 16S–23S rRNA IGS [32]. The representative isolates of ERIC clusters I, II, V, VI and VII displayed identical *rrs* gene sequences and clustered in *Bradyrhizobium* lineage II. Their closest type strains were *B. elkanii* USDA 76^T, *B. tropiciagri* SEMIA 6148^T and *Bradyrhizobium pachyrhizi* PAC48^T, with 100%, 99.9% and 99.9% identity, respectively. The *rrs* sequences of the isolates representing clusters III and IV were identical and were closely related to that of *B. japonicum* USDA 6^T (99.9%) and *B. liaoningense* USDA 3622^T (99.8%) which fall within *Bradyrhizobium* lineage I. Interestingly, the same similarity levels were obtained by comparing the isolates VUEP21 and VUEP25 with *Bradyrhizobium* sp. VUPME50, *Bradyrhizobium* sp. VUPMI11 and *Bradyrhizobium* sp. VUPMI37 (Fig. 2 and Table S4) isolated from cowpea in Spain [6].

However, the 16S rRNA phylogeny is known to lack discriminative power since several *Bradyrhizobium* species have *rrs* gene identities higher than 99% [5,32,59,62,63,66,67]. Previous studies have shown that the IGS region of *Bradyrhizobium* strains displays high sequence variation and it is considered as a useful marker for differentiation of closely related *Bradyrhizobium* species that usually display similarities lower than 95.5% [32,45,66,67].

To better clarify the taxonomic position of our isolates, their IGS regions were PCR amplified and directly sequenced. For phylogenetic analysis, nearly full-length IGS regions were used. The length of the nucleotide alignments was determined by the availability of IGS sequences in the GenBank. Therefore, the sequences were trimmed appropriately so that only their aligned parts (749 bp for VULI11, VULI13, VUEP21, and VUEP25, 753 bp for VULI21 and VULI32, and 853 bp for VUCR24) were included in the analysis. Phylogenetic trees generated either by the Neighbor-Joining or the Maximum Likelihood (ML) method gave similar results and separated the isolates in four clusters (ML tree is shown in Fig. 3). Each cluster was characterized by a distinct IGS-type. The IGS-type 1 was found among the isolates of ERIC-groups III (VUEP25) and IV (VUEP21), the IGS-type 2 was present in the isolate of ERIC-group VI (VUCR24), the IGS type 3 was identified among the isolates of ERIC-groups V (VULI13) and VII (VULI11), and the IGS-type 4 was found among the isolates of ERIC-groups I (VULI32) and II (VULI21). The IGS sequences within each IGS-type were nearly identical (99.9–100%) and of the same size, whereas the similarities and the length of the sequences varied among the IGS types. Moreover, the isolates originating from the three geographic regions had distinct IGS types with similarity values in the range of 73–90%, suggesting that they may belong to different genospecies. The isolates of the IGS type 3 (VULI11 and VULI13) formed a cluster together with the recently described species *B. ferriligni* CCBAU 51502^T, isolated from *Erythrophleum fordii* nodules in China [71] and they showed 98.5% similarity (Fig. 3 and Table S4). Although, the isolates of the IGS type 4 (VULI21 and VULI32) showed 98.7% similarity to *B. ferriligni* CCBAU 51502^T, they formed a slightly distinct cluster (Fig. 3 and Table S4). The latter isolates had IGS sequences with an insertion of 11 bp that was absent in isolates of IGS type 4. Congruently with *rrs* phylogeny, the isolate VUCR24 formed an independent branch which was closely related to *B. pachyrhizi* PAC48^T with 95.9% similarity (Fig. 3 and Table S4). The IGS region of VUCR24 (IGS type 3) had an insertion of 76 bp located between the two tRNAs, which was absent in closely related species. Lastly, the IGS sequences of the VUEP21 and VUEP25 (IGS type 1) were closely related to *B. liaoningense* USDA 3622^T with 96.1% and 96.2% similarity values, respectively (Table S4). Moreover, the IGS regions of the strains VUEP21 and VUEP25 were longer compared to *B. liaoningense* USDA 3622^T due to an insertion of about 15 bp near to the end of their IGS regions.

According to Willems et al. [67], two bradyrhizobial strains with less than 95.5% IGS sequence similarity are considered to belong in different genospecies [32,45,67]. This genospecies threshold has recently been re-evaluated to 97% since more newly described *Bradyrhizobium* type species have shown higher similarities in their IGS region sequences [19]. Indeed, in our pairwise sequence comparisons, highest IGS sequence similarities displayed between the pairs of *B. lupini* USDA3051^T and *B. canariense* BTA-1^T (98.25%), *B. lablabi* CCBAU23086^T and *B. paxllaeri* LMTR 21^T (98.02%), *B. embrapense* SEMIA 6208^T and *B. viridifuturi* SEMIA 690^T (97.54%). Therefore, the high IGS sequence similarity (98.5–98.7%) of strains VULI11, VULI13, VULI21, and VULI32 to *B. ferriligni* CCBAU 51502^T suggested they may belong to the same genospecies. However, the absence of an insertion in the IGS of VULI11, VULI13 implied that they may constitute a novel lineage. Moreover, the isolates VUCR24 and VUEP21 displayed highest IGS similarity values ~96% compared with those of currently described *Bradyrhizobium* type species, *B. pachyrhizi* and *B. liaoningense*, respectively (Table S4), suggesting that they may represent two putative novel lineages. Noteworthy, the isolates VUCR24 and VUEP21 harbored insertions

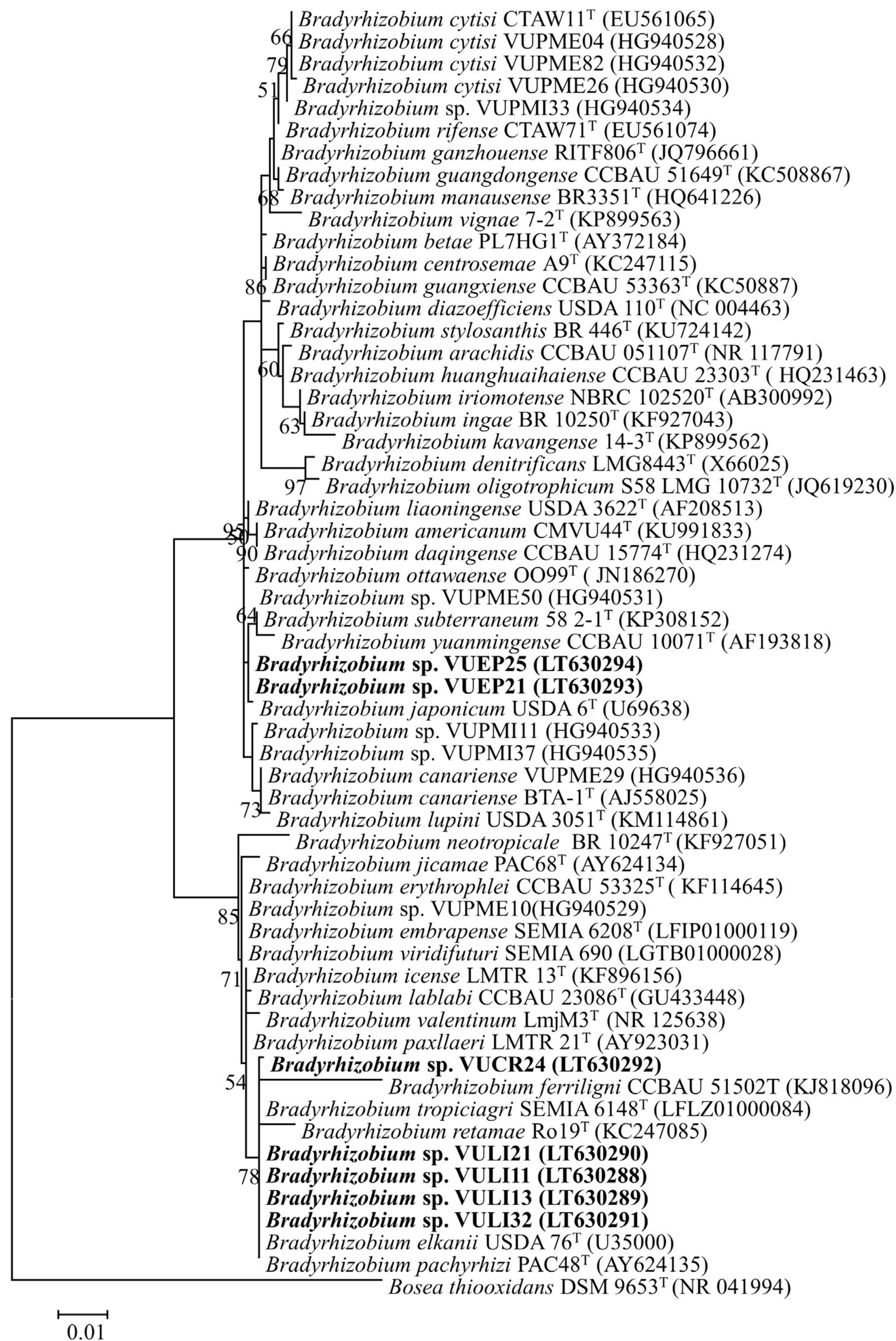

Fig. 2. Maximum likelihood phylogenetic tree based on nearly complete 16S rRNA gene sequences showing taxonomic relationships of the strains representing the different ERIC groups. Strains isolated in the present study are shown in boldface and type strains are indicated by superscript "T". GenBank accession numbers of the sequences are indicated within parentheses. Bootstrap values (greater than 50%) were calculated for 500 replications and are shown at the nodes. The scale bar shows the number of nucleotide substitutions per site. Phylogenetic analysis was conducted in MEGA 6 using the Tamura 3-parameter model plus Gamma rate distribution (T92 + G).

in their IGS sequences, which were absent from their closest relatives, *B. pachyrhizi* and *B. liaoningense*, respectively.

Comparison of the IGS sequences generated in this study to those available in GenBank showed that the VULI11 and VULI13 shared >99% similarity with an uncultured *Bradyrhizobium* sp. clone 2006/115 [39] found in *V. unguiculata* nodules in South Africa, *Bradyrhizobium* sp. KO19A isolated from root nodules of a wild legume in Japan. The IGS of strains VULI21 and VULI32 were highly similar (>99%) to *Bradyrhizobium* sp. BR 3262, which is used as commercial inoculant for *V. unguiculata* in Brazil, *Bradyrhizobium* sp. SEMIA 6160 isolated from *Albizia lebbeck* in Brazil [32], *B. elkanii* S3-28 isolated from nodules of *Pueraria phaseoloides* in Cameroon, *B. elkanii* USDA 33 and USDA 94 isolated from soybean nodules [60], and *Bradyrhizobium* sp. AD1T2-3-1 isolated from nodules of *Vigna subterranea* in Angola [19]. Notably, the IGS of all the above strains had the insertion of 11 bp found in our isolates VULI21 and

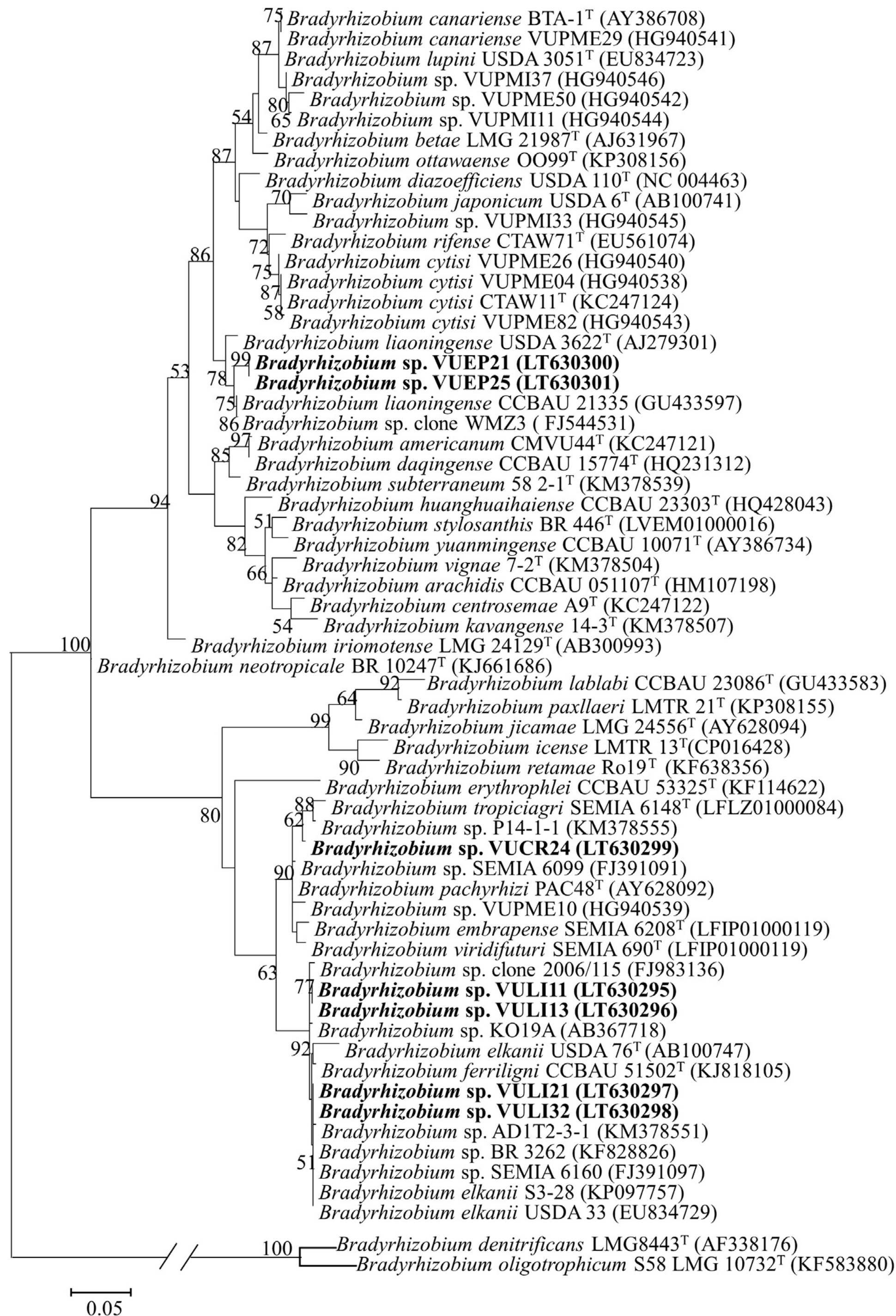

Fig. 3. Maximum likelihood phylogenetic tree based on 16S–23S IGS sequences showing taxonomic relationships of the strains representing the different ERIC groups. Strains isolated in the present study are shown in boldface and type strains are indicated by superscript "T". GenBank accession numbers of the sequences are indicated within parentheses. Bootstrap values (greater than 50%) were calculated for 500 replications and are shown at the nodes. The scale bar shows the number of nucleotide substitutions per site. Phylogenetic analysis was conducted in MEGA 6 using the Tamura 3-parameter model plus Gamma rate distribution (T92 + G).

VULI32. Moreover, the IGS of VUCR24 displayed highest similarity (96.7%) to that of *Bradyrhizobium* sp. P14-1-1 isolated from nodules of *Vigna subterranea* in Angola [19] which also harbored the 76 bp insertion, found in VUCR24, though with slight differences. Lastly, the VUEP21 and VUEP25 displayed highest similarity (98%) to *B. liaoningense* CCBAU 21335 isolated from *Arachis hypogaea* in China [10] and to an uncultured *Bradyrhizobium* sp. clone WMZ3 found in cowpea nodules in China [74], which also contained the 15 bp insertion, present in VUEP21 and VUEP25 strains.

Multilocus sequence analysis of housekeeping genes

In order to refine the phylogeny of our strains, the MLSA approach was performed on four isolates (VULI11, VULI21, VUCR24 and VUEP21) representing each IGS type. Three housekeeping genes *recA*, *glnII* and *gyrB* used widely in phylogenetic studies of *Bradyrhizobium* strains were selected for MLSA [2–4,32,44,63,79,58,76,80]. 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 three housekeeping genes. Consequently, the lengths of the alignments used were 381 bp, 477 bp, and 525 bp, for *recA*, *glnII*, *gyrB*, respectively (Table S4). Aligned sequences from the selected genes were concatenated and 1383 bp positions were obtained. Since *gyrB* sequences are not available for all currently described type species of *Bradyrhizobium*, the MLSA was also performed by concatenating only the *recA* and *glnII* sequences in order to include all *Bradyrhizobium* type species.

In the individual ML phylogenetic trees of *recA*, *glnII* and *gyrB*, the *Bradyrhizobium* isolates formed three or four distinct groups (Figs. S2–S4). The isolates VULI11, VULI21, VUCR24 were closely related to *B. pachyrhizi* PAC48^T, *B. ferriligni* CCBAU 51502^T, and *B. elkanii* USDA 76^T, whereas the isolate VUEP21 was closely related to *B. liaoningense* USDA 3622^T and *B. japonicum* USDA 6^T (Figs. S2–S4 and Table S4). Slight differences in tree topologies of the individual ML trees were observed. Incongruence of phylogenetic relationships for *Bradyrhizobium* housekeeping genes in some species has also been reported in previous studies, which may be the result of recombination, migration or lateral gene transfer [24,44,64]. ML phylogenetic analysis based on the concatenated sequences was more robust and confident and showed that isolates VULI11, VULI21, VUCR24 clustered, though in three separate branches, together with *B. pachyrhizi* PAC48^T (Figs. 4 and S5), which was isolated from nodules of *Pachyrhizus erosus* in Costa Rica [42,47]. The ML phylogenetic trees based on the concatenated sequences of either the three or the two genes gave similar results in the NJ (data not shown) and ML trees (Figs. 4 and S5). Based on pairwise comparisons of the three concatenated sequences, the isolates VULI11, VULI21, VUCR24 displayed highest sequence similarities to PAC48^T, 97.5%, 97.9% and 98.2%, respectively (Table S4) suggesting that these isolates may belong to *B. pachyrhizi* PAC48^T. Noteworthy, in our analysis, the pair of *B. paxllaeri* LMTR 21^T and *B. jicamae* LMG 24556^T showed highest sequence similarity, 97.1%. Though, the similarity values of our isolates compared with PAC48^T are slightly higher than 97.1%, it is still possible that our isolates might constitute uncharacterized lineages and further analysis is needed.

On the ML trees of the concatenated gene sequences, the isolate VUEP21 was clustered, though in a separate branch, with *B. liaoningense* USDA 3622^T (Figs. 4 and S5). The isolate VUEP21 showed 94%, 96.4% and 93.3% in *recA*, *glnII* and *gyrB* genes, respectively, and 94.5% in the concatenated gene sequences with respect to its closest relative *B. liaoningense* USDA 3622^T (Table S4), which was isolated from soybean nodules in China [69]. The above similarities values of single core genes were lower to those found among several *Bradyrhizobium* type species [8,9,40,42,45]. Moreover, in our pairwise analysis, 22 pairs of 33 *Bradyrhizobium* type species showed similarity values in the *recA*-*glnII*-*gyrB* concatenated sequences higher than 95%, with the highest values to be presented between the pairs of *B. paxllaeri* LMTR 21^T and *B. jicamae* LMG 24556^T (97.1%), *B. pachyrhizi* PAC48^T and *B. elkanii* USDA 76^T (96.9%), *B. tropiciagri* CNPSo 1112^T and *B. embrapense* CNPSo 2833^T (96.5%), and *B. paxllaeri* LMTR 21^T and *B. lablabi* CCBAU 23086T^T (96.4%).

Although the isolate VUEP21 is closely related to *B. liaoningense*, the similarity values for the single core genes *recA*, *glnII* and *gyrB*, as well as for their concatenated gene sequences were lower than those found in validly described *Bradyrhizobium* species, suggesting that the isolate VUEP21 could represent a putative novel species within *Bradyrhizobium*. These results were also congruent with those obtained by the IGS analysis of VUEP21.

Phylogenetic analysis of symbiotic genes

Currently, the similarities of *nodC* sequences together with the host spectrum of rhizobia are used to define symbiovars in rhizobia [36,48]. To date, eight symbiovars have been described within *Bradyrhizobium*, which are centrosemae, genistearum, glycinearum, phaseolarum, retamae, sierranevadense, tropici, vignae [6,12,20,40,41,62].

Partial sequences of the symbiotic genes *nodC*, and *nifH*, 732 bp, and 714 bp, respectively, for the *Bradyrhizobium* isolates VULI11, VULI21, VUCR24 and VUEP21, were amplified and sequenced. The *nodC*- and *nifH*-based phylogenies placed the *Bradyrhizobium* strains into two distinct well-supported clusters as presented in Fig. 5A and B, respectively. In the *nodC* phylogenetic tree, the strains VULI11, VULI21 and VUCR24 formed a cluster with the strain *Bradyrhizobium* sp. VUPME10 that was isolated from cowpea nodules in Spain and represents the newly described symbiovar vignae [6]. The *nodC* gene sequences of strains VULI11, VULI21 and VUCR24 displayed high sequence identities, 98.7%, 95.3% and 99.2%, respectively, with respect to *Bradyrhizobium* sp. VUPME10, further supporting that these strains belong to the symbiovar vignae. Interestingly, this symbiotic lineage has also been found in cowpea-nodulating strains in Africa, China and Brazil [10,54,77]. The strains from these regions, *Bradyrhizobium* sp. CCBAU 23005, *Bradyrhizobium* sp. CCBAU 51012, *Bradyrhizobium* sp. STM3062, and *B. pachyrhizi* BR 3262, showed more than 95% identity in their *nodC* gene sequences compared to our strains, but those with *nodC* sequences shorter than the aligned ones (630 bp) were not included in the phylogenetic analysis (Fig. 5A). The presence of rhizobial lineages related to the symbiovar vignae in almost all continents indicates that they might have high competitive nodulation capacity against indigenous rhizobia found in different soils. Indeed, the strain *B. pachyrhizi* BR 3262 is used as commercial inoculant for *V. unguiculata* in Brazil [83]. Interestingly, none of the strains isolated in the present study belonged to the symbiovar genistearum which was found in *V. unguiculata* nodules in Spain [6].

The *nifH* phylogeny of our strains was congruent to that of the *nodC* gene suggesting coevolution of these symbiotic genes. Strains VULI11, VULI21 and VUCR24 had more than 99.8% internal identities and formed a highly supported cluster related to *B. pachyrhizi* PAC48^T and *B. elkanii* USDA 76^T, although their identity values were varied between 92.7–92.9%. As *nifH* sequences for the representative strain, *Bradyrhizobium* sp. VUPME10, of the symbiovar vignae is not available in GenBank, other strains (CCBAU 23005 and BR3262) with high similarity (>98%) on their *nodC* sequences with respect to VUPME10 were included in the *nifH* analysis. These strains showed high *nifH* identity values (>99.8%) with respect to strains VULI11, VULI21 and VUCR24 confirming further their symbiotic relatedness and their placement within the sv. vignae. Moreover the congruent phylogeny of concatenated housekeeping and symbiotic gene sequences of strains VULI11, VULI21 and VUCR24, as well as those clustered within symbiovar vignae, underpins the maintenance of symbiotic genes through vertical transmission.

In all phylogenetic analyses, our isolates grouped together with *B. pachyrhizi* indicating their close phylogenetic relatedness. Although *B. pachyrhizi* was originally isolated from *Pachyrhizus erosus* in Mexico [42], *B. pachyrhizi*-related bacteria were also isolated in Mexico using cowpea and siratro (*Macroptilium atropurpureum*) as trap plants [35]. The edaphoclimatic conditions of our sampling sites matched to those in Mexico defined by a warm climate and acid soils implying that *B. pachyrhizi*-related bacteria predominate in such regions and thereby accounting for their existence and predominance in acid soils of Greece. Moreover, the high proportion of these isolates in different regions of Greece indicated that they were probably better-suited for cowpea in these fields and were

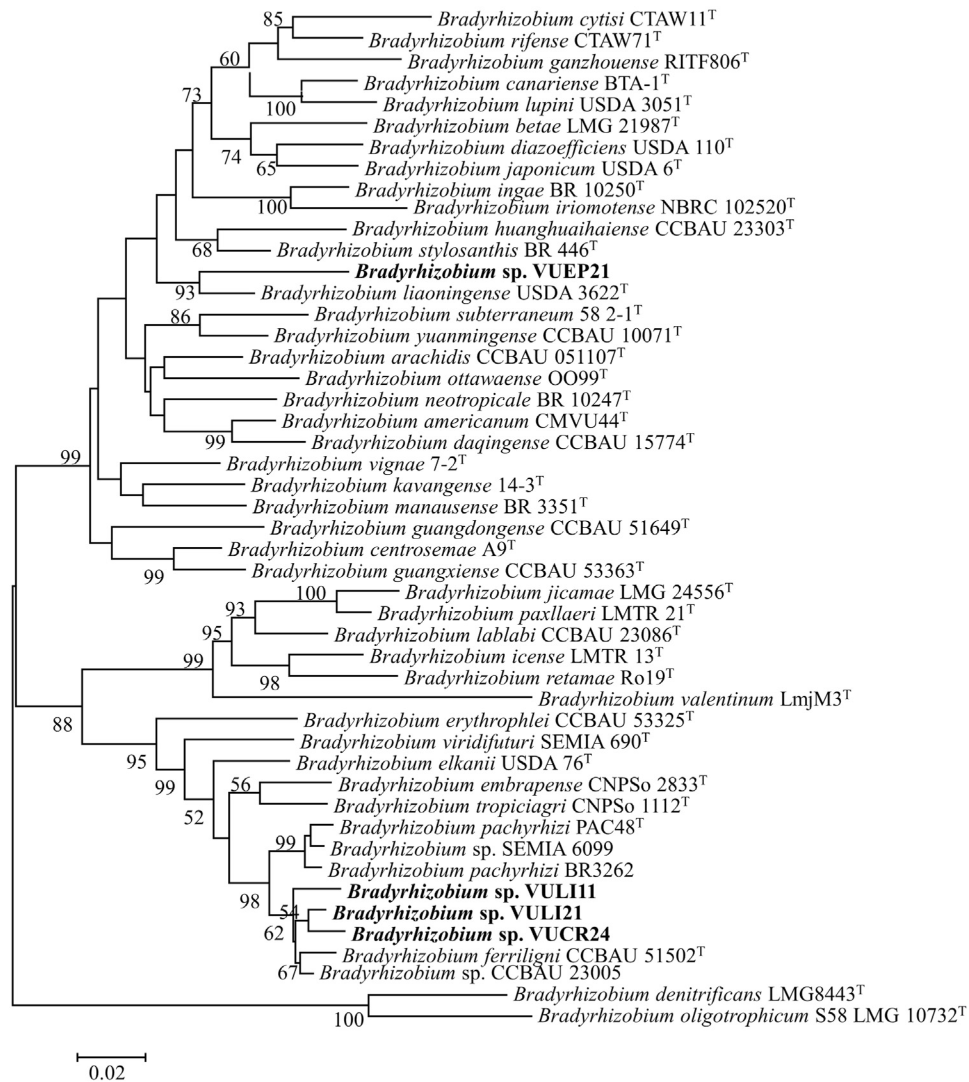

Fig. 4. Maximum likelihood phylogenetic tree based on 858-bp concatenated sequence of *recA* and *glnII* showing taxonomic relationships of the strains representing the different IGS types. Strains isolated in the present study are shown in boldface and type strains are indicated by superscript "T". GenBank accession numbers of the sequences are given in Figs. S2, S3. Bootstrap values (greater than 50%) were calculated for 500 replications and are shown at the nodes. The scale bar shows the number of nucleotide substitutions per site. Phylogenetic analysis was conducted in MEGA 6 using the maximum likelihood algorithm with the General Time Reversible model plus Gamma rate distribution plus invariant site (GTR + G + I).

probably more competitive under given conditions. Contrarily, only fast-growing rhizobia were isolated from alkaline soils in Greece (Tampakaki et al. unpublished). Although various edaphoclimatic parameters can affect the distribution of cowpea rhizobia worldwide, it seems that the soil pHs may have shaped the distribution of cowpea-nodulating bacteria in our sampling sites, which is in line with previous studies showing the major role of pH in geographic distribution of several *Bradyrhizobium* species [26,29,30,49,79]. On the other hand, host genotypes might not influenced bradyrhizobia diversity in our sampling sites since different local plant varieties were used.

The strain VUEP21 formed a separate highly supported cluster, both in the *nodC* and *nifH* phylogenetic trees, and its closest relative was the species *B. centrosemae* A9^T which is representative of the newly described symbiovar centrosemae [41]. The *nifH* gene of VUEP21 had 94.6% identity with respect to the strain A9^T and less than 88.2% with strains of sv. glycinearum (Table S4). The *nodC* gene of strain VUEP21 showed 90.4% identity compared to that of strain A9^T and less than 81.7% to strains belonging to sv. glycinearum. Taking into account that the identities of *nodC* gene sequences between strains, within each symbiovar described so far in *Bradyrhizobium*, are higher than 92%, the strain VUEP21 could be considered to represent a novel symbiovar within *Bradyrhizobium*. Remarkably, *nodC* sequences with more than 92% identity with respect to VUEP21 were not found in the GenBank. The strain VUEP21 was distantly related to *B. centrosemae* A9^T in the phylogenies of 16S, IGS, *recA* and *glnII*. Since *gyrB* sequences were not available for the strain A9^T, it was not included in the phylogeny of *gyrB* and that of concatenated housekeeping genes. In contrast, the phylogeny of 16S, IGS and that of concatenated housekeeping genes placed, albeit in separate branches, the strain VUEP21 close to *B. liaoningense* USDA 3622^T, which belongs to symbiovar glycinearum. The incongruence between the phylogenies based on symbiotic and housekeeping genes is indicative of lateral transfer of symbiotic genes. Therefore, the symbiotic genes of VUEP21 were likely acquired through lateral gene transfer and were differentiated in the course of evolution.

Moreover, in our nodulation tests, all *B. liaoningense*-related isolates formed effective nodules with their host of origin. In a previous study, *B. liaoningense*^{T} failed to nodulate local cowpea varieties [19]. The divergence of their symbiotic genes may account

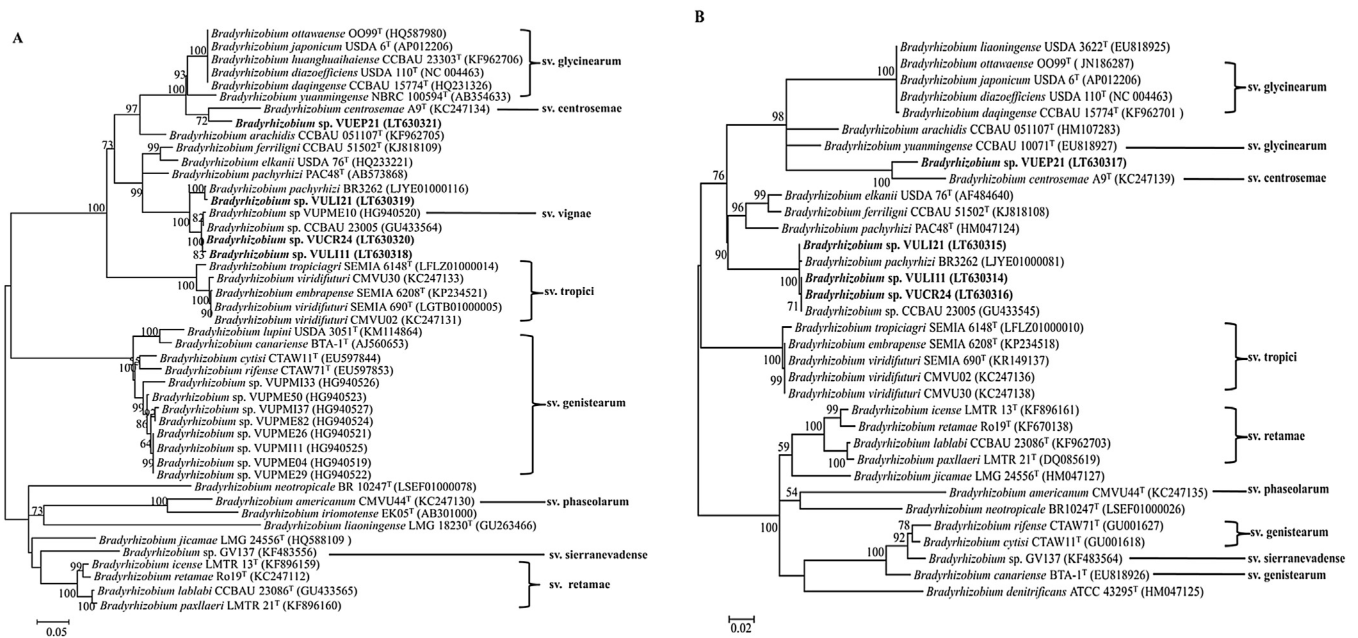

Fig. 5. Maximum likelihood phylogenetic trees based on 630-bp and 645-bp alignment of the *nodC* (A) and *nifH* (B) nucleotide sequences, respectively. The taxonomic relationships of the strains representing the different IGS types are shown. Strains isolated in the present study are shown in boldface and type strains are indicated by superscript "T". GenBank accession numbers of the sequences are indicated within parentheses. 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. Phylogenetic analysis was conducted in MEGA 6 using the Tamura 3-parameter model plus Gamma rate distribution plus invariant site (T92 + G + I) and the Tamura 3-parameter model plus invariant site (T92 + I) for the *nodC* and *nifH* nucleotide sequences, respectively.

for the differences observed in their host specificities. Besides, the *B. liaoningense*-related isolates were phylogenetically more related, with respect to *nodC* and *nifH*, to *Bradyrhizobium* type species which are known to nodulate cowpea, such as *B. daqingense* [65], *B. huanghuaihaense* [78] and *B. yuanmingense* [70]. However, no data are available regarding the nodulation ability of *B. centrosemae* A9^T on cowpea. Lastly, the rare reprensentation of *B. liaoningense*-related isolates in our survey might be due either to the neutral pH of their sampling site or to their low competitiveness in the local environmental conditions. Altogether, our results show that the strain VUEP21 could represent not only a putative new species but also a novel symbiovar within *Bradyrhizobium*.

In conclusion, the present study provides the first analysis on the phylogenetic diversity of indigenous cowpea-nodulating bradyrhizobia in Greece. Studies investigating indigenous rhizobia, all over the world, in fields without rhizobial inoculation history are of great importance for selecting novel strains adapted to the local environmental conditions. Such strains often exhibit a better performance in similar habitats and thus they are more preferable for inoculant formulations. In the current study, putative novel lineages of *Bradyrhizobium* have been isolated and phylogenetically classified. Their close phylogenetic relationships with strains used as inoculants render them worthy for further investigation as inoculants in fields with similar edapho-climatic conditions. Future studies on DNA–DNA relatedness, phenotypic and symbiotic properties are needed to demonstrate whether they represent novel species and/or symbiovars.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.syapm.2017.01.001.

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