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Phylogenetic diversity of rhizobia nodulating *Phaseolus vulgaris* in Croatia and definition of the symbiovar phaseoli within the species *Rhizobium pisi*

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ABSTRACT

Phaseolus vulgaris is a legume indigenous to America which is currently cultivated in Europe including countries located at the Southeast of this continent, such as Croatia, where several local landraces are cultivated, most of them of Andean origin. In this work we identify at species and symbiovar levels several fast-growing strains able to form effective symbiosis with *P. vulgaris* in different Croatian soils. The identification at species level based on MALDI-TOF MS and core gene sequence analysis showed that most of these strains belong to the species *R. leguminosarum*, *R. hidalgonense* and *R. pisi*. In addition, several strains belong to putative new species phylogenetically close to *R. ecuadorense* and *R. sophoriradicis*. All Croatian strains belong to the symbiovar phaseoli and harbour the α and γ *nodC* alleles typical for American strains of this symbiovar. Nevertheless, most of Croatian strains harboured the γ *nodC* gene allele supporting its Andean origin since it is also dominant in other European countries, where Andean cultivars of *P. vulgaris* are traditionally cultivated, as occurs in Spain. The only strains harbouring the α *nodC* allele belong to *R. hidalgonense* and *R. pisi*, this last only containing the symbiovars viciae and trifolii to date. This is the first report about the presence in Europe of the species *R. hidalgonense*, the nodulation of *P. vulgaris* by *R. pisi* and the existence of the symbiovar phaseoli within this species. These results significantly increase the knowledge of the biogeography of *Rhizobium-P. vulgaris* symbiosis.

Introduction

Phaseolus vulgaris L. (common bean) is a legume indigenous to Central and South American countries which establishes symbiosis in different geographical locations with fast-growing rhizobia from several symbiovars and species mainly belonging to genus *Rhizobium* [24,28,49]. Although *P. vulgaris* is nodulated in American countries by symbiovars such as gallicum and tropici [8,27,33,44] and by unnamed symbiovars, in the American distribution centers of this legume, the symbiovar phaseoli is the predominant endosymbiont of *P. vulgaris* and harbours genes that seem to be exclusive for the nodulation of this legume [4,46]. This symbiovar is linked to American strains isolated from *P. vulgaris* nodules such as *Rhizobium phaseoli* [10], *Rhizobium etli* [43], *Rhizobium hidalgonense* [50,53], *Rhizobium acidisoli* [37,50]. For other american species isolated from *P. vulgaris* nodules such as *Rhizobium ecuadorense* [32] and *Rhizobium esperanzae* [7] it has been recently showed that their type strains also carry the symbiovar phaseoli [13]. In Europe this symbiovar has been found in the species *Rhizobium gallicum* and *Rhizobium giardinii* (currently *Pararhizobium giardinii*) in France [4], *Rhizobium lusitanum* in Portugal [46,47] and *Rhizobium leguminosarum* in Spain [14,26].

The presence of the *nodC* gene alleles typical for the American strains of symbiovar phaseoli in European countries suggests that

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it was dispersed worldwide together with the common bean seeds [14,26]. These seeds were introduced in Europe from Spain and Portugal being distributed to other European countries where this legume is currently cultivated as occurrs in the case of Croatia, where local landraces, which are cultivated by small-scale farmers, mostly are of Andean origin [6]. In fact, as has been previously reported [10], the Andean germplasm was mostly transported to Spain and Portugal and from them to other countries of Europe and Africa, being the Andean germplasm predominant in these areas [16]. The local landraces currently cultivated in Croatia are able to form effective nodules in the soils of this country [29], but the identity of species and symbiovars inducing the formation of these nodules in *P. vulgaris* remains unknown.

Therefore the first aim of this work was the identification of species nodulating *P. vulgaris* in different locations at Croatia by using MALDI-TOF MS, a technique which allows the differentiation of rhizobial species even if they have identical 16S rRNA gene, as occurs with the species *Rhizobium leguminosarum* and *Rhizobium laguerreae* both able to nodulate *P. vulgaris* [13], and by the analysis of different core genes. The second aim was the identification at symbiovar level of the strains isolated in Croatia, which is currently based on the *nodC* gene analysis [28]. Finally, the the analysis of the phylogenetic relationships of the *nodC* gene harboured by strains nodulating *P. vulgaris* in Croatia and in other countries from Europe and America was also performed in order to improve the knowledge about the biography of *P. vulgaris*-rhizobia symbiosis.

Material and methods

Sampling and rhizobial strains isolation

Soil samples for isolation of indigenous rhizobial strains were taken from 27 different locations in Northeast Croatia (Fig. S1). Soil samples were taken from family farms producing common bean at least two years before sampling. Trapping host method was employed to obtain 45 isolates of indigenous bean symbionts by using two local landraces, Tresnjevac and Slavonski Zeleni, in the different soils (Table S1). Isolation of strains was performed at the beginning of flowering, from inside reddish nodules, using yeast mannitol agar (YMA) according to Vincent [52] and reinfection experiments were carried out on P. vulgaris as it was previously described [26].

MALDI-TOF MS performing and data analysis

The sample preparation and the MALDI-TOF MS performing was carried out as previously published [12] using a matrix of saturated solution of α-HCCA (Bruker Daltonics, Germany) in 50% acetonitrile and 2.5% trifluoracetic acid. As indicate by the manufacturer we used biomass amounts between 5–100 mg to obtain the spectra and the calibration masses were the Bruker Bacterial Test Standards (BTS) which were as follows (masses as averages): RL36, 4365.3 Da; RS22, 5096.8 Da; RL34, 5381.4 Da; RL33meth, 6255.4 Da; RL29, 7274.5 Da; RS19, 10300.1 Da; RNase A, 13683.2 Da and myoglobin, 16952.3 Da.

MALDI-TOF MS identifications were classified using the score values proposed by the manufacturer: a score value between 2.3 and 3.00 indicated species identification; a score value between 2.0 and 2.299 indicated secure genus identification and probable species identification, a score value between 1.7 and 1.999 indicated probable genus identification, and a score value <1.7 indicated no reliable identification.

The database used was the one generated after the addition of several type strains of recently described species from the phylogenetic group of *R. leguminosarum-R. etli* [13] to the initial database [12]. Cluster analysis was performed based on comparison of strain-specific main spectra created as described above. The dendrogram was constructed by the statistical toolbox of Matlab 7.1 (MathWorks Inc., USA) integrated in the MALDI Biotyper 3.0 software. The parameter settings were: 'Distance Measure = Correlation' and 'Linkage = Average'. The linkage function is normalized according to the distance between 0 (perfect match) and 1000 (no match).

RAPD fingerprinting

RAPD patterns were obtained as previously described [35] using the primer M13 (5′-GAGGGTGGCGGTTCT-3′) and the GoTaq Flexi DNA polymerase (Promega). PCR conditions were: preheating at 95 °C for 9 min; 35 cycles of denaturing at 95 °C for 1 min; annealing at 45 °C for 1 min and extension at 75 °C for 2 min, and a final extension at 72 °C for 7 min. 10 μl of each PCR products were electrophoresed on 1.5% (w/v) agarose gel in TBE buffer (100 mM Tris, 83 mM boric acid, 1 mM EDTA, pH 8.5) at 6 V/cm, stained in a solution containing 0.5 g/ml ethidium bromide, and photographed under UV light. Standard VI (Roche, USA) was used as a size marker. A dendrogram was constructed based on the matrix generated using UPGMA method and the Jaccard's coefficient with Bionumerics version 4.0 software (Applied Maths, Austin, TX).

Phylogenetic analyses of rrs, atpD, recA *and* nodC *genes*

The amplification and sequencing of *rrs*, *recA* and *atpD*, and *nodC* genes were carried out as indicated by Rivas et al. [34], Gaunt et al. [15] and Laguerre et al. [23], respectively. The sequence of the *rrs* gene for the type strain of *R. acidisoli* was obtained in this study because that available in Genbank is too short. The genes were sequenced in the Sequencing DNA service (NUCLEUS) from Salamanca University (Spain). The sequence of the *rrs* gene from the type strain of *R. esperanzae* was obtained joining manually fragments of this gene contained in three different contigs available in Genbank (see Fig. 1). The sequences obtained were compared with those from the GenBank using the BLASTN program [2]. The obtained sequences and those of related bacteria retrieved from GenBank were aligned using the Clustal W program [45]. The phylogenetic distances were calculated according to Kimura's two-parameter model [20]. The phylogenetic trees were inferred using the neighbour joining model [39] and MEGA 7.09 [21] was used for all the phylogenetic analyses.

Results and discussion

Isolated strains

The 45 strains analysed in this study were isolated from effective *P. vulgaris* nodules (pink colour) in different soils from Northeast Croatia (Table S1, Fig. S1). From them, 16 strains were isolated from acid soils with pH ranging from 4.9 to 6.1, 15 strains from neutral soils with pH ranging from 6.5 to 7.7 and 14 strains from basic soils with pH ranging from 7.9 to 8.0 (Table 1). The isolated strains were able to reinfect *P. vulgaris* forming typical effective nodules in its roots (data not shown).

MALDI-TOF MS analysis

MALDI-TOF MS is a reliable method for bacterial identification that allows the differentiation among species from family *Rhizobiaceae* [12] even those belonging to phylogenetically closely related species such as *R. laguerreae* and *R. leguminosarum* which contain strains able to nodulate *P. vulgaris* [13]. Therefore, we used this technique to identify the strains isolated from Croatian soils and the obtained results showed that all of them belong to genus *Rhizobium* matching with score values higher than 2.0 with the type

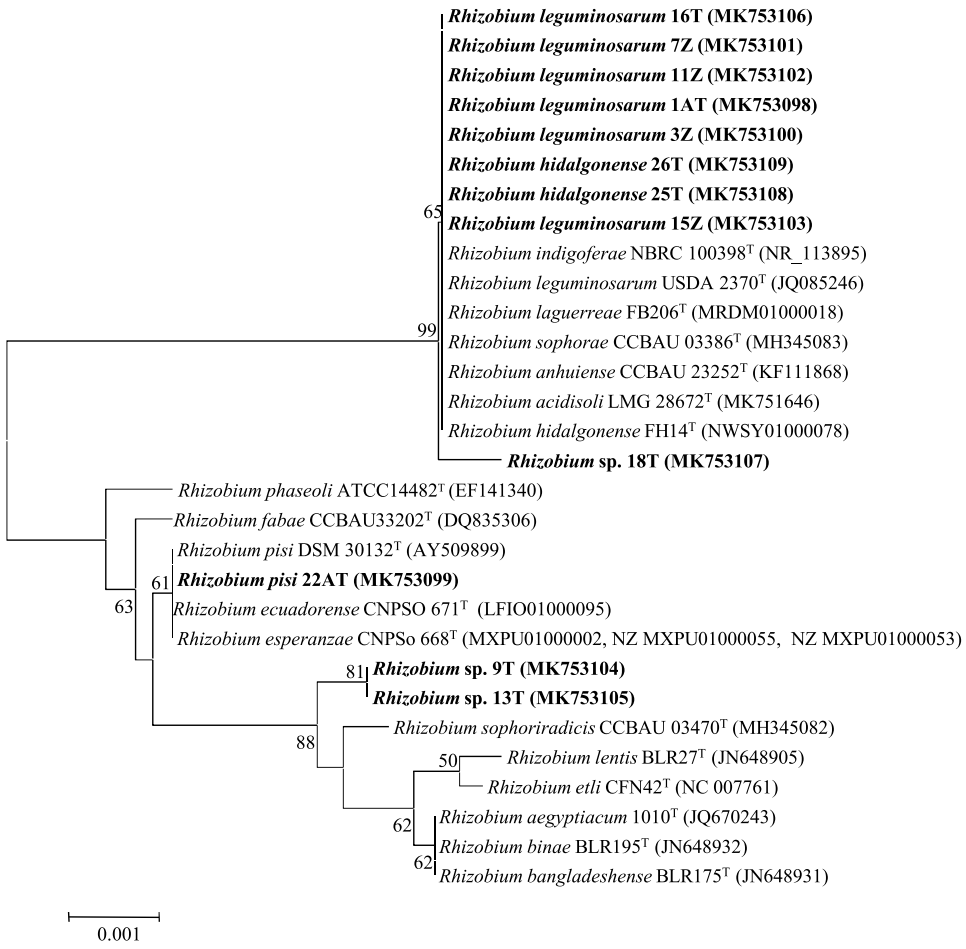

Fig. 1. Neighbour-joining phylogenetic rooted tree based on *rrs* gene sequences (1350 nt) showing the taxonomic location of representative strains from different groups of RAPD within the genus *Rhizobium*. Bootstrap values calculated for 1000 replications are indicated. Bar, 1 nt substitution per 1000 nt. Accession numbers from Genbank are given in brackets.

strains of *R. leguminosarum*, *R. pisi*, *R. sophoriradicis*, *R. hidalgonense* and *R. ecuadorense* (Table 1). From these species, only *R. leguminosarum* has been previously reported as *P. vulgaris* endosymbiont in European countries to date [14,17,23,26].

RAPD patterns analysis

RAPD fingerprinting is a technique commonly used to analyse the intraspecific diversity in genus *Rhizobium* [10,41,46,48,51] and to group strains for their posterior identification by gene sequencing [51]. After the mathematical analysis of the obtained RAPD patterns we found the existence of 12 groups or branches with similarity lower than 70% (Table 1, Fig. S2). These results revealed the high diversity of the isolated strains allowing us the selection of representative ones for gene analyses.

rrs *gene analysis*

Although the *rrs* gene analysis continued to be the basis of bacterial classification, species with identical or very closely related *rrs* genes have been recently described in different rhizobial genera, as occurs in the genus *Rhizobium* [13,51]. Particularly within the phylogenetic group containing the type species of this genus, *R. leguminosarum*, several recently described species have identical *rrs* genes (Fig. 1). The results obtained in this study showed that most of Croatian strains have identical *rrs* gene sequences as the type strains of *R. leguminosarum*, *R. indigoferae*, *R. laguerreae*, *R. anhuiense*, *R. sophorae*, *R. acidisoli* and *R. hidalgonense* and one strain, 18T, has a different nucleotide in its *rrs* gene with respect to these species (Fig. 1). The strain 22AT has a *rrs* gene whose sequence is identical to that of *R. pisi*, *R. ecuadorense* and *R. esperanzae* and finally, the *rrs* gene of the strain 9T has two different nucleotides with respect to the type strain of *R. sophoriradicis*, its closest related species (Fig. 1). The results of *rrs* gene analysis agree with those of MALDI-TOF MS, but this gene does not allow the differentiation among some *Rhizobium* species from the groups to which belong the Croatian strains and therefore, the identification of these strains through genomic approaches should be performed by analysing other more variable genes [13].

Analysis of recA *and* atpD *genes*

The differentiation among *Rhizobium* species with identical *rrs* genes can be carried out by analysing *recA* and *atpD* genes, which are sequenced for all *R. leguminosarum* related species and have been widely used for the identification of *P. vulgaris* nodulating strains [10,11,13,14,26,38,46] and therefore these two genes were used in this study in order to confirm the identification of the Croatian strains (Fig. 2). The obtained results showed that the representative strains of RAPD groups III and IV clustered with the type strain of *R. hidalgonense* and belonged to this species since they showed similarity values higher than 99% in both *recA* and *atpD* genes. The representative strain of RAPD group II showed 100% similarity with the type strain of *R. pisi* in *recA*and *atpD* genes, and both clustered

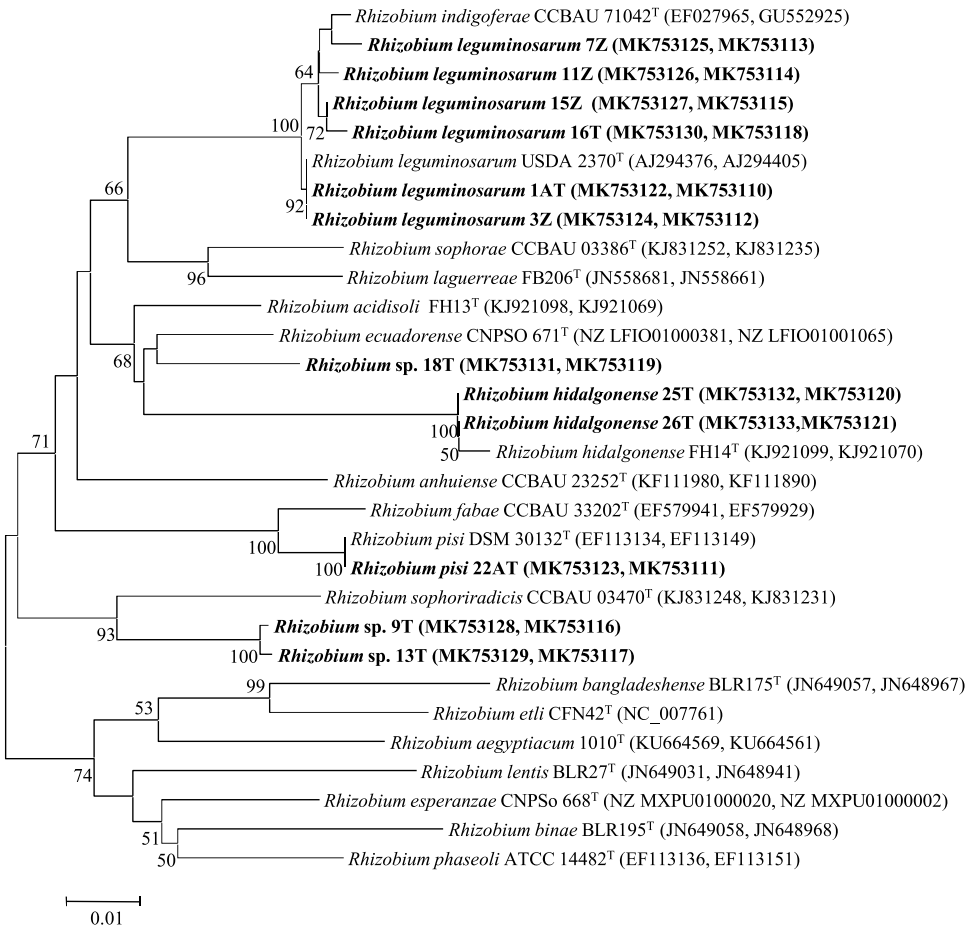

Fig. 2. Neighbour-joining phylogenetic tree based on *recA* and *atpD* concatenated gene sequences (733 nt) showing the position of representative strains from each group within genus *Rhizobium*. Bootstrap values calculated for 1000 replications are indicated. Bar, 1 nt substitution per 100 nt. Accession numbers from Genbank are given in brackets.

with the type strain of *R. fabae*. The representative strains of RAPD groups VII and IX showed 100% similarity in their *recA* and *atpD* genes with respect to the type strain of *R. leguminosarum* and clustered with the strains from groups VIII, X, XI and XII which showed similarities higher than 98% in *recA* and *atpD* genes with respect to the type strains of both *R. leguminosarum* and *R. indigoferae*. These two type strains also showed similarities higher than 98% in these genes and they are probably synonyms [9]. The representative strain of RAPD group I clustered with the type strain of *R. ecuadorense* showing similarity values near to 97% in *recA* and *atpD* genes. The representative strains of RAPD groups V and VI clustered with the type strain of *R. sophoriradicis* showing similarity values near to 98% and 93% in *recA* and *atpD* genes, respectively.

These results confirmed those from MALDI-TOF MS analysis allowing the identification of 18 Croatian strains as *R. leguminosarum*, 10 as *R. hidalgonense* and 2 as *R. pisi*. The remaining strains probably belong to two undescribed species or subspecies, in which 12 Croatian strains belong to a species related with *R. sophoriradicis*, and the 3 remaining ones belong to a group related to *R. ecuadorense*. Among the species identified in the present study, only the type strains of *R. hidalgonense* and *R. ecuadorense* were originally isolated from *P. vulgaris* nodules [32,52].

Analysis of the nodC *gene*

The differentiation of symbiovars within species of genus *Rhizobium* is commonly carried out through *nodC* gene analysis [28]. Based on the analysis of this gene the symbiovar phaseoli is clearly distinguishable from other symbiovars including viciae and trifolii, which are sometimes found within a single species, as occurs in the case of *R. leguminosarum* [14]. The symbiovar phaseoli has been found in strains of several *Rhizobium* species isolated from *P. vulgaris* nodules in the American distribution centers of this legume, including *R. phaseoli*, *R. etli*, *R. hidalgonense*, *R. acidisoli*, *R. esperanzae* and *R. ecuadorense*. This symbiovar is worldwide distributed and it has been found in strains of *R. leguminosarum* and *R. lusitanum* isolated from *P. vulgaris* nodules in European countries as Spain, France and Portugal (Fig. 3). All strains isolated in Croatia in this study belong to the symbiovar phaseoli and they cluster in two groups containing strains harbouring the α or γ *nodC* alleles of *P. vulgaris*-nodulating American strains [1]. Most Croatian strains carry the γ *nodC* allele which is dominant in European countries [14,26]. Nevertheless, the strain 26T from *R. hidalgonense* harboured the *nodC* α allele, which was also harboured by the type strain of this species FH14^T. This allele is also harboured by the strain 22AT of *R. pisi*, a species which to date only contains the symbiovars viciae and trifolii [25].

Taxonomy and biogeography remarks

The strains analysed in this study belong to a species complex that contains some species with identical *rrs* genes as occurred in the case of *R. leguminosarum*, *R. indigoferae*, *R. laguerreae*, *R. anhuiense*, *R. sophorae*, *R. hidalgonense* and *R. acidisoli* or in the case of *R. pisi*, *R. ecuadorense* and *R. esperanzae* (Fig. 1). Nevertheless, these species can be differentiated among them because they have

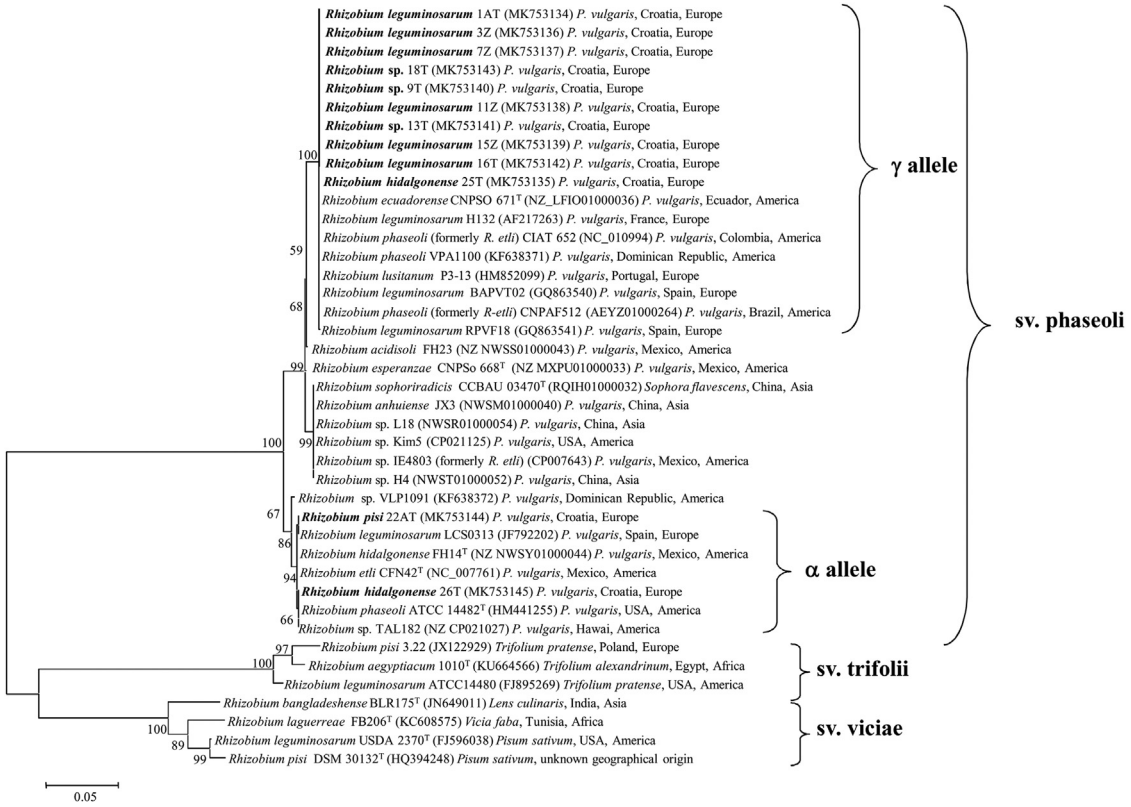

Fig. 3. Neighbour-joining phylogenetic tree based on *nodC* gene sequences (795 nt) showing the position of representative strains from each group within the symbiovars viciae and phaseoli. Bootstrap values calculated for 1000 replications are indicated. Bar, 5 nt substitution per 100 nt. Accession numbers from Genbank are given in brackets.

divergent housekeeping *recA* and *atpD* genes, except in the case of *R. indigoferae* whose type strain is very closely related to that of *R. leguminosarum* (Fig. 2). The high similarity vaues found in the *recA* and *atpD* genes between these two strains suggest that the type strain of *R. indigoferae* should be affiliated to the species *R. leguminosarum*. Nevertheless, the formal reclassification of the type strain of *R. indigoferae* currently available in culture collections must take into account that the *rrs* gene of the original type strain of this species has a sequence very divergent to that of the current available type strain [9]. The reclassification of the current type strain of *R. indigoferae* into *R. leguminosarum* is particularly important since many strains nodulating several cultivated legumes in different countries belong to this species, for instance many strains isolated in this work and in previous studies from nodules of *Pisum* [22], *Vicia* [3,40,51], *Lens* [31], *Phaseolus* [14,26,38], *Lathyrus* [51] and *Trifolium* [30,51].

Since *R. leguminosarum* is the main endosymbiont in Europe of legumes such as *Vicia*, *Trifolium* or *Lathyrus* with distribution or domestication centers located in this continent, a possible European origin has been proposed for this species [3,40,51]. *R. leguminosarum* was also the most abundant species in *P. vulgaris* nodules in European soils previously cultivated with this legume such as the cases found in Spain [14,26] and Croatia, where *R. leguminosarum* with 18 strains was the most frequent species in the analysed *P. vulgaris* nodules (Table 1). The presence of *nodC* gene alleles typical of symbiovar phaseoli in these strains supports the hypothesis that they have been acquired from American *P. vulgaris* endosymbionts dispersed worldwide with the seeds of this legume [14,26].

A remarkable finding is the nodulation of *P. vulgaris* in Croatia by strains belonging to the species *R. hidalgonense* that also has *rrs* gene identical to that of the type strain of *R. leguminosarum*, but unlike *R. indigoferae*, *R. hidalgonense* has divergent *recA* and *atpD* genes (Fig. 2). Although the type strain of *R. hidalgonense* was isolated from Mexico, an American origin of this species is doubtful taking into account the abundance of strains from this species in the analysed Croatian soils. The type strain of this species belongs to the symbiovar phaseoli and harbours the α allele of the *nodC* gene, nevertheless the Croatian strains harbour the α or γ alleles of this gene (Fig. 3). These results show that the analysis of more strains of this species isolated in other American and European soils is necessary to explore the geographical origin of *R. hidalgonense*.

It is also remarkable that two putative new *Rhizobium* species are able to nodulate *P. vulgaris* in Croatian soils. One of them was minoritary and contains three strains related to *R. ecuadorense*, a species nodulating *P. vulgaris* originally isolated in Ecuador [32]. The type strain of *R. ecuadorense* and the Croatian strains related to this species belong to the symbiovar phaseoli and harboured the γ *nodC* gene allele (Fig. 3). The scarce number of strains from this putative new species found in Croatian soils could mean that they are indigenous to America, but further studies are necessary in soils from different continents in order to confirm this hypothesis.

Containing 12 strains, the second putative new species was most abundant in the analysed soils. It was related to *R. sophoriradicis*, a species that was isolated from *Sophora flavescens* nodules [18], but whose type strain belongs to the symbiovar phaseoli [38]. Although this strain harboured the γ *nodC* gene allele [38], when a longer fragment of this gene (800 nt) is analysed it can be observed that the *nodC* gene sequence of *R. sophoriradicis* type strain is identical only to those of some Chinese strains nodulating *P. vulgaris* [46] and to those of strains EI5803 and Kim5 isolated from *P. vulgaris* nodules in Mexico [44] and USA [19], respectively (Fig. 3). Therefore, these strains carry a different *nodC* allele that to date has not been found in Europe being linked to strains nodulating different legumes, such as *S. flavescens* and *P. vulgaris*, in the case of China [46]. This could support the hypothesis that common beans were

Table 1
Results of MALDI-TOF MS analysis of strains analysed in this study.

Strains	Organism (best match)	Score values	RAPD group
Acid soils (pH 4.89–6.46)			
7T	*R. leguminosarum* USDA 2370^T	2.250	VII
7Z	*R. leguminosarum* USDA 2370^T	2.000	VIII
8T	*R. leguminosarum* USDA 2370^T	2.150	XI
8Z	*R. ecuadorense* LMG 27578^T	2.204	I
12T	*R. ecuadorense* LMG 27578^T	2.041	I
12Z	*R. leguminosarum* USDA 2370^T	2.153	XI
14Z	*R. hidalgonense* LMG 29288^T	2.557	IV
16T	*R. leguminosarum* USDA 2370^T	2.085	X
17T	*R. leguminosarum* USDA 2370^T	2.000	IX
17Z	*R. leguminosarum* USDA 2370^T	2.023	IX
22AT	*R. pisi* DSM 30132^T	2.261	II
22AZ	*R. hidalgonense* LMG 29288^T	2.563	III
22BT	*R. hidalgonense* LMG 29288^T	2.469	IV
22BZ	*R. pisi* DSM 30132^T	2.254	II
23T	*R. leguminosarum* USDA 2370^T	2.234	IX
23Z	*R. hidalgonense* LMG 29288^T	2.523	III
Neutral soils (6.57–7.70)			
2T	*R. leguminosarum* USDA 2370^T	2.151	IX
2Z	*R. leguminosarum* USDA 2370^T	2.002	XI
3T	*R. leguminosarum* USDA 2370^T	2.270	VII
3Z	*R. leguminosarum* USDA 2370^T	2.097	IX
13T	*R. sophoriradicis* LMG 27898^T	2.269	V
13Z	*R. leguminosarum* USDA 2370^T	2.138	IX
15Z	*R. leguminosarum* USDA 2370^T	2.000	XII
20T	*R. hidalgonense* LMG 29288^T	2.577	IV
20Z	*R. hidalgonense* LMG 29288^T	2.584	IV
21T	*R. sophoriradicis* LMG 27898^T	2.236	V
21Z	*R. leguminosarum* USDA 2370^T	2.170	IX
26T	*R. hidalgonense* LMG 29288^T	2.544	III
26Z	*R. hidalgonense* LMG 29288^T	2.566	III
27T	*R. leguminosarum* USDA 2370^T	2.018	IX
27Z	*R. hidalgonense* LMG 29288^T	2.485	III
Basic soils (7.56–8.01)			
1AT	*R. leguminosarum* USDA 2370^T	2.118	VII
1BT	*R. sophoriradicis* LMG 27898^T	2.150	V
1BZ	*R. sophoriradicis* LMG 27898^T	2.175	V
5T	*R. sophoriradicis* LMG 27898^T	2.099	V
5Z	*R. sophoriradicis* LMG 27898^T	2.145	V
9T	*R. sophoriradicis* LMG 27898^T	2.031	VI
11T	*R. sophoriradicis* LMG 27898^T	2.176	V
11Z	*R. leguminosarum* USDA 2370^T	2.041	XI
18T	*R. ecuadorense* LMG 27578^T	2.163	I
18Z	*R. sophoriradicis* LMG 27898^T	2.209	V
19T	*R. sophoriradicis* LMG 27898^T	2.242	V
19Z	*R. sophoriradicis* LMG 27898^T	2.201	V
25T	*R. hidalgonense* LMG 29288^T	2.442	IV
25Z	*R. sophoriradicis* LMG 27898^T	2.232	V

directly introduced from Latin America into China, which can be considered a secondary diversity center of *P. vulgaris* [54].

It is remarkable that the γ *nodC* gene allele was the most abundant among the Croatian strains supporting its Andean origin [10] because it is dominant in European countries where Andean cultivars of *P. vulgaris* are traditionally cultivated, as occurs in Spain and Croatia [5,14,26,29]. Nevertheless, two strains isolated in Croatia harboured the α allele which is carried out by the type strains of *R. etli* and *R. phaseoli* isolated in North America [42].

Nevertheless, the most interesting finding of this study was the nodulation of *P. vulgaris* in Croatian soils by *R. pisi*, a species which has not been previously reported in *P. vulgaris* nodules. The type strain of this species presented similarity values of *recA* and *atpD* genes near to 98% with respect to that of *R. fabae* indicating that the taxonomic status should be revised [36]. Moreover, the Croatian strain 22AT of *R. pisi* belongs to the symbiovar phaseoli and carries the α *nodC* gene allele that has been found in the strain *R. hidalgonense* 26 T isolated in Croatia and in the type strains of several American species nodulating *P. vulgaris* (Fig. 3). The species *R. pisi* has been scarcely reported in legume nodules worldwide and to date only contained the symbiovars viciae and trifolii [25] being this study the first report about the existence of the symbiovar phaseoli within this species.

In summary, the Croatian *P. vulgaris*-nodulating strains analyzed in this study belong to the species *R. leguminosarum*, *R. hidalgonense*, *R. pisi* and to two putative new species of genus *Rhizobium*. All these strains belong to the symbiovar phaseoli and most of them harboured the γ allele of *nodC* gene. It is the first time to identify *R. hidalgonense* in European soils and its strains carry the α or γ *nodC* gene alleles. The species *R. pisi*, found in *P. vulgaris* nodules in this study, carries the α *nodC* gene allele typical of the symbiovar phaseoli, which is reported for the first time in this species, which to date only contains the symbiovars viciae and trifolii. Therefore, the results of this study constitute new relevant information for the biogeography on *Rhizobium-P. vulgaris* symbiosis, since they show that the symbiovar phaseoli is widely distributed in European countries linked to very diverse *Rhizobium* species.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.syapm.2019.126019.

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