Vigna unguiculata is nodulated in Spain by endosymbionts of Genistaeae legumes and by a new symbiovar (vignae) of genus Bradyrhizobium.

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Abstract

*Vigna unguiculata* was introduced in Europe from Africa where it has its distribution center and where the slow growing rhizobia nodulating this legume have not yet been studied. Previous studies based on *rrs* gene and ITS region analyses showed that *B. yuanmingense* and *B. elkanii* nodulated *V. unguiculata* in Africa, two species that were not found in this study. Using the same phylogenetic markers we showed that in Spain *V. unguiculata*, a legume from Tribe Phaseolae, is nodulated by two species of group I, *B. cytisi* and *B. canariense*, which are common endosymbionts of Genisteae in both Europe and Africa. These two species have not been found up to date in *V. unguiculata* nodules in its African distribution centers. All strains from *Bradyrhizobium* group I isolated in Spain belong to the symbiovar genistearum found at present only in Genisteae legumes in both Africa and Europe. *V. unguiculata* is also nodulated in Spain by a strain from the group II of *Bradyrhizobium* belonging to a novel symbiovar (vignae). Some African *V. unguiculata*- nodulating strains also belong to this symbiovar proposed here.

**Key words:** *Bradyrhizobium, Vigna unguiculata, symbiovar, phylogeny, Spain*
Introduction

The species *Vigna unguiculata* (cowpea) from the Tribe Phaseolae is indigenous to Africa where the Transvaal region is considered its evolutionary center since the oldest varieties of this legume were found in this region [20]. *V. unguiculata* takes part of human diet in African countries [12] because it has high contents in protein (23%), carbohydrates (56%) and fiber (4%) that can fulfill the human essential amino acid requirements complemented with cereals [11]. Moreover, this legume has great agronomic interest due to its resistance to acidity, dryness and high temperatures [5, 8] and by the establishment of nitrogen-fixing symbiosis that allow its use in intercrops with cereals, mainly maize, in the African countries [9, 14]. In these African countries this legume establishes nitrogen-fixing symbioses with several slow growing strains from genus *Bradyrhizobium* being *B. yuanmingense* and *B. elkanii* the main species identified in cowpea nodules [16, 25, 36]. *V. unguiculata* was introduced from Northern Africa in the South of Europe where this legume is currently cultivated in Mediterranean regions such as Extremadura (Spain), a warm region with acidic soils suitable for the cropping of this legume and where it is very appreciated by the consumers. Nevertheless, despite the interest of this legume for intercropping of rotation with non-legumes overall in European Mediterranean countries there are no data about the rhizobia establishing nitrogen-fixing symbiosis with this legume in Europe.

This work is the first one carried out in an European country that aimed the identification of slow growing strains nodulating *V. unguiculata* as well as the analysis of their phylogenetic relationships with those nodulating this legume in African countries. Surprisingly, the results of this work showed the nodulation of *V. unguiculata*, a legume that belongs to the Tribe Phaseolae, by endosymbionts of legumes from Tribe Genisteae that have not been found in African countries. In addition, we detected a phylogenetic lineage belonging to the *Bradyrhizobium* group II scarcely present in Europe whose *nodC* gene correspond to a novel symbiovar within this genus also present in Africa for which we propose the name vignae.

Materials and methods

Strains and nodulation experiments

Plants of *V. unguiculata* were used as trap plants in a soil from Extremadura in Spain. The rhizobial strains were isolated from the nodules according to the method of Vincent [42]. In order to confirm the nodulation capacity of the strains, infectivity tests were conducted in a
growth chamber with controlled conditions using sterile vermiculite as substrate. *Vigna unguiculata* were surface disinfected and seedlings were inoculated as described by Ramírez-Bahena *et al.* [27].

**RAPD fingerprinting**

RAPD patterns were obtained as previously described [30] using the primer M13 (5’-GAGGGTTGCGGTCT –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 Pearson coefficient with Bionumerics version 4.0 software (Applied Maths, Austin, TX).

**Analysis of rrs, atpD and nodC genes and 16S-23S intergenic spacer (ITS)**

The *rrs* was amplified and sequenced according to Rivas *et al.* [28], the ITS as described by Peix *et al.* [22]. The *nodC* gene was amplified with the primers and conditions described by Laguerre *et al.* [17], except for the strain VUPME 10 whose *nodC* genes were amplified and sequenced with the following primers nodCBradyF (5’ CGCAAGGCGCAGWTCGC 3’) and nodCBradyR (5’ GGKGTGVAGCGMGAAGCCG 3’). PCR amplifications were performed with a REDExtract-N-Amp PCR Kit (Sigma) or GoTaq Flexi DNA polymerase kit (Promega) or DreamTaq Green DNA polymerase (Thermo) following the manufacturers’ instructions. The bands corresponding to the different genes were purified either directly from the gel by room temperature centrifugation using a DNA gel extraction device (Millipore Co., USA) for 10 min at 5000 g or by elution of the excised band and filtration through silicagel columns using the Qiaquick DNA Gel Extraction Kit (Qiagen, Germany) in all cases following the manufacturers’ instructions. In the case of *nodC* gene from strain VUPME10, The amplified product obtained in the previous section (ca. 800 bp) was cloned into the vector pJet1.2/blunt Cloning Vector using the CloneJET PCR Cloning Kit (Thermo) according to manufacturer’s instructions for the Sticky-End Cloning Protocol. Transformation Protocol was performed using Promega instructions for competent cells JM109 (Promega). The analysis of the
recombinant clones was carried out following the protocol for colony screening by PCR following the cloning kit manufacturer's instructions. The sequence reaction was performed on an ABI PRISM 3100 sequencer using a BigDye terminator v3.1 cycle sequencing kit (Applied Biosystems Inc., USA) as supplied by the manufacturer. The sequences obtained were compared to those held in GenBank by using the BLASTN program [1]. They were aligned by using Clustal W software [40]. Distances calculated according to Kimura's two-parameter model [15] were used to infer phylogenetic trees with the neighbour-joining and maximum likelihood methods [6, 33] with MEGA5 software [39]. Confidence values for nodes in the trees were generated by bootstrap analysis using 1000 permutations of the data sets.

Results and discussion

RAPD fingerprinting analysis

We isolated 40 slow growing strains on YMA plates with typical morphology of genus *Bradyrhizobium* that were able to nodulate this host forming effective nodules (pink red colour). These isolates were analysed by RAPD fingerprinting that allows the differentiation among strains of the same *Bradyrhizobium* species [7, 26]. This technique provides an estimation of the genetic diversity showing that strains with about 75% identity belong to the same species [26]. Our isolates were this way distributed into nine groups of RAPD with similarity percentages lower than this value (Fig. 1), from which we selected representative strains for gene sequence analysis.

Analysis of rrs gene and 16S-23S ITS region

The strains isolated from *V. unguiculata* nodules in Africa belong to genus *Bradyrhizobium* and have been mainly identified on the basis of their rrs gene and ITS region [16, 25, 36] that allowed their placement into the groups I and II of *Bradyrhizobium* proposed by Menna *et al.* [19]. The results of the analysis of these phylogenetic markers showed that our strains mostly belong to the group I with a single strain clustering into group II (Figs. 2 and 3, S1 and S2). Within group I, the isolates cluster in different phylogenetic lineages some of them branching with previously described species such as that represented by the strain VUPME29, (RAPD group III) which was identified as *B. canariense* since it has rrs gene and ITS sequences identical to those of its type strain BTA-1T.
The strains VUPME04, VUPME82 and VUPME26 representing the RAPD groups VI, VII and VIII, respectively, were identified as *B. cytisi* since they have *rrs* gene and ITS sequences almost identical to those of its type strain CTAW11T. The strain VUPME50 representing the RAPD group VII has *rrs* gene and ITS region 100% identical to that of strain BGA-1 currently classified into *B. japonicum*. The strains VUPMI37, presenting the RAPD type IV and VUPMI11 representing the RAPD group IX, formed a cluster related to *B. canariense* with more than 99% and 96.8% identity in the *rrs* gene and ITS region, respectively. The strain VUPMI33, presenting the RAPD type I, formed independent branches in the phylogenetic trees of both *rrs* gene and ITS region, being close to *B. japonicum* USDA 6T with 97.5% identity in this last region. Finally, within the group II we only found a strain, VUPME10 showing the RAPD type II, closely related to *B. pachyrhizi* PAC48T with identities higher than 99% in the *rrs* gene and 97% in the ITS region.

Our results contrast with those obtained in Africa, where *V. unguiculata* was distributed to Europe, since we did not find in our work the species *B. yuanmingense* from group I and *B. elkanii* from group II reported in *V. unguiculata* nodules in different African countries (Figs. 2 and 3, S1 and S2), including the considered distribution centers of this legume [25, 36]. Interestingly, the strains isolated from *V. unguiculata* in Spain were identified as *B. cytisi* and *B. canariense*, species not found up to date in *V. unguiculata* nodules in Africa despite *B. cytisi* was firstly isolated in this continent [4] and *B. canariense* was found in *Lupinus* nodules in South Africa [37]. These two species are common endosymbionts of Genisteae legumes being *B. canariense* the main endosymbiont of Genisteae in Europe [2, 35, 38, 41] where this species is probably indigenous [37]. Only a common cluster was found both in Europe (reported in this work) and in Africa [36], which is the cluster of strain BGA-1, branching in *Bradyrhizobium* group I (see Figs. 2 and 3). This strain is currently classified as *B. japonicum* although it formed a lineage within this species with only 97% identity with respect to the type strain USDA 6T in the ITS region. As occurs with *B. cytisi* and *B. canariense*, this lineage is commonly found in Europe and Africa in Genisteae legumes [13, 35, 37, 38, 41]. Within group II the lineage found in Spain has not been found up to date in African countries (figures 2 and 3). It is remarkable that strains from this group have been scarcely found in European soils with only some strains nodulating *Lupinus mariae-josephi* [34] and one strain of the recently described species, *B. retamae* [7].
Therefore, the results of the analysis of core markers showed that *V. unguiculata* strains isolated in Spain mostly belong to species that were not found up to date in its nodules in the African countries. It is therefore an interesting finding the fact that the species *B. canariense* and *B. cytisi* nodulate this legume in Spain because they are common endosymbionts of Genistae legumes and *V. unguiculata* belongs to the Tribe Phaseolae. Nevertheless, since the ability to nodulate legumes is dependent of their promiscuity degree as well as to the host range of rhizobia, the most interesting point is to analyze the symbiovars to which the strains isolated in this study belong.

*Analysis of nodC gene*

The *nodC* gene is a phylogenetic marker related to the host range of rhizobia and promiscuity level of legumes [10, 24, 26, 29, 31, 32], and the analysis of its sequences has been recently proposed to be considered a minimal standard for definition of symbiovars in rhizobia [21, 32]. This gene has been used to delineate the symbiovars defined within genus *Bradyrhizobium*, which currently are genistearum, glycinearum, retamae and sierranevadense [2, 7, 43]. Although it has not been established a cutoff value to differentiate these symbiovars, the available data suggest to consider it around 90% similarity since values higher than this percentage are presented by strains from symbiovar glycinearum (Figs. 4 and S3).

According to the results, all strains from *Bradyrhizobium* group I nodulating *V. unguiculata* in this study belong to symbiovar genistearum (Figs. 4 and S3) that is the common endosymbiont of Genisteae legumes in Europe [2, 38, 41] and Africa [3, 37]. However this symbiovar was not previously found in *V. unguiculata* in Africa. In fact African *V. unguiculata* strains were reported to cluster within a clade different from that occupied by strains isolated from Genisteae legumes according to the *nodA* gene analysis [36]. The available *nodC* gene sequences of African strains belonging to *Bradyrhizobium* group I placed them into two clusters (Figs. 4 and S3), one of them also containing the type strain of *B. arachidis* and other corresponding to the symbiovar glycinearum, a common endosymbiont of the Phaseolae *Glycine max* (soybean), to which also belong the type strains of *B. japonicum* and *B. daqingense* nodulating this legume and *B. huanghuaihainense* that nodulated soybean and *V. unguiculata* [44]. Therefore, this is the first report about the ability of the symbiovar genistearum, common endosymbiont of Genisteae legumes, to nodulate the Phaseolae legume...
*V. unguiculata* in Europe, proposed geographical origin of strains nodulating Genisteae legumes [37].

On the other hand, the single strain belonging to the group II of *Bradyrhizobium*, VUPME 10, clustered with other species of this group such as *B. pachyrhizi* and *B. elkanii* but forming a divergent new lineage that presented less than 89% identity with respect to the remaining symbiovars of genus *Bradyrhizobium* (Figs. 4 and S3). This new lineage is also present in *V. unguiculata* nodules in Africa constituting a novel symbiovar of *Bradyrhizobium* able to nodulate *Vigna*, for which we propose the name vignae.

All our results confirm those obtained after the *nodA* gene analysis in African strains showing that *V. unguiculata* is a very promiscuous legume able to establish symbiosis with slow growing rhizobia belonging to different clades [36]. This is a common finding not only in *V. unguiculata* [18, 44], but also in other Phaseolae legumes nodulated by *Bradyrhizobium* such as soybean or *Pachyrhizus* that are nodulated by several *nodC* lineages (Figs. 4 and S3). This promiscuity allows the Phaseolae legume *V. unguiculata* to nodulate with the symbiovar genistearum of *Bradyrhizobium* which is the common endosymbiont of Genisteae legumes in Europe [2, 38, 41]. The presence of the new symbiovar vignae in both African and European soils and the complete identity of the *nodC* gene from the strains isolated in these two continents indicate that they have the same origin suggesting the dispersion of this symbiovar with *V. unguiculata* seeds from Africa to Europe, as reported for other rhizobial endosymbionts and their hosts’ seeds [23]. This hypothesis should be further confirmed when more isolates from this new symbiovar are available in African and European soils.

In summary, our results based on the analysis of the core phylogenetic markers showed that: (i) *V. unguiculata* is nodulated by two species not previously found in this legume in Africa, *B. canariense* and *B. cytisi*, only found up to date in nodules of Genisteae legumes; (ii) *B. cytisi* is reported for the first time in this work to be present in Europe; (iii) The analysis of the symbiotic *nodC* gene confirmed the high promiscuity of *V. unguiculata* able to nodulate with several symbiovars including the symbiovar genisteearum, found in this work by first time in *V. unguiculata* nodules in Europe, and by a new symbiovar (vignae) present in both Africa and Europe.

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References


nodulation genes that belong to the large pantropical clade common in Africa. Mol. Phylogenet. Evol. 48, 1131-1144.


Figure legends

Figure 1. Dendrogram obtained for the strains of *Vigna unguiculata* using Pearson’s coefficient and UPGMA analysis of the RAPD profiles.

Figure 2. Neighbour-joining phylogenetic rooted tree based on *rrs* gene sequences (1485 nt) showing the taxonomic affiliation of the strains representative of the different RAPD groups. Bootstrap values calculated for 1000 replications are indicated. Bar, 1 nt substitution per 100 nt. Genbank Accession numbers are given in brackets.

Figure 3. Neighbour-joining phylogenetic rooted tree based on 16S-23S rRNA internal transcribed spacer (ITS) sequences (810 nt) showing the taxonomic affiliation of the strains representative of the different RAPD groups. Bootstrap values calculated for 1000 replications are indicated. Bar, 1 nt substitution per 100 nt. Genbank Accession numbers are given in brackets.

Figure 4. Neighbour-joining phylogenetic tree based on *nodC* gene sequences (400 nt) showing the position of representative strains from different RAPD groups. Bootstrap values calculated for 1000 replications are indicated. Bar, 5 nt substitution per 100 nt. Genbank Accession numbers are given in brackets.
Figure 3
Figure S1. Maximum likelihood phylogenetic rooted tree based on *rrs* gene sequences (1485 nt) showing the taxonomic affiliation of the strains representative of the different RAPD groups. Bootstrap values calculated for 1000 replications are indicated. Bar, 1 nt substitution per 100 nt. Genbank Accession numbers are given in brackets.
Figure S2. Maximum likelihood phylogenetic rooted tree based on 16S-23S rRNA internal transcribed spacer (ITS) sequences (810 nt) showing the taxonomic affiliation of the strains representative of the different RAPD groups. Bootstrap values calculated for 1000 replications are indicated. Bar, 2 nt substitution per 100 nt. Genbank Accession numbers are given in brackets.
Figure S3. Maximum likelihood phylogenetic tree based on nodC gene sequences (400 nt) showing the position of representative strains from different RAPD groups. Bootstrap values calculated for 1000 replications are indicated. Bar, 5 nt substitution per 100 nt. Genbank Accession numbers are given in brackets.