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Vigna unguiculata is nodulated in Spain by endosymbionts of Genisteae legumes and by a new symbiovar (vignae) of the genus *Bradyrhizobium*

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ABSTRACT

Vigna unguiculata was introduced into Europe from its distribution centre in Africa, and it is currently being cultivated in Mediterranean regions with adequate edapho-climatic conditions where the slow growing rhizobia nodulating this legume have not yet been studied. Previous studies based on *rrs* gene and ITS region analyses have shown that *Bradyrhizobium yuanmingense* and *B. elkanii* nodulated *V. unguiculata* in Africa, but these two species were not found in this study. Using the same phylogenetic markers it was shown that *V. unguiculata*, a legume from the tribe Phaseolae, was nodulated in Spain by two species of group I, *B. cytisi* and *B. canariense*, which are common endosymbionts of Genisteae in both Europe and Africa. These species have not been found to date in *V. unguiculata* nodules in its African distribution centres. All strains from *Bradyrhizobium* group I isolated in Spain belonged to the symbiovar genistearum, which is found at present only in Genisteae legumes in both Africa and Europe. *V. unguiculata* was also nodulated in Spain by a strain from *Bradyrhizobium* group II that belonged to a novel symbiovar (vignae). Some African *V. unguiculata*-nodulating strains also belonged to this proposed new symbiovar.

Introduction

The species *Vigna unguiculata* (cowpea) from the tribe Phaseolae is indigenous to Africa where the Transvaal region is considered to be its evolutionary centre since the oldest varieties of this legume have been found there [20]. *V. unguiculata* forms part of the human diet in African countries [12] because it has high contents of protein (23%), carbohydrates (56%) and fibre (4%) that can fulfil the human essential amino acid requirements when complemented with cereals [11]. Moreover, this legume has great agronomic interest due to its resistance to acidity, dryness and high temperatures [5,8], as well as the establishment of nitrogen-fixing symbiosis that allows its use as an intercrop with cereals, mainly maize, in certain African countries [9,14]. In these African countries, this legume establishes nitrogen-fixing symbioses with several slow-growing strains from the genus *Bradyrhizobium*, with *B. yuanmingense* and *B. elkanii* being the main species identified in cowpea nodules [16,25,36].

V. unguiculata was introduced from Northern Africa into Southern 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 consumers. Nevertheless, despite the interest of this legume for intercropping and rotation with non-legumes, there are generally no data from European Mediterranean countries concerning the rhizobia that establish nitrogen-fixing symbiosis with it.

Therefore, this is the first study carried out in a European country that aimed to identify the slow-growing strains nodulating *V. unguiculata*, as well as analyse their phylogenetic relationships with strains nodulating this legume in African countries. Surprisingly, the results showed nodulation of *V. unguiculata* by endosymbionts of legumes from the tribe Genisteae that have not been found in African countries. In addition, a phylogenetic lineage was detected that belonged to *Bradyrhizobium* group II, which is scarcely present

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in Europe, whose *nodC* gene corresponded to a novel symbiovar within this genus that was also present in Africa, and 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 under controlled conditions using sterile vermiculite as the 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′-GAGGGTGGCGGTTCT-3′) and the GoTaq Flexi DNA polymerase (Promega). PCR conditions were: preheating at 95 °C for 5 min; 35 cycles of denaturing at 95 °C for 1 min; annealing at 45 °C for 1 min and extension at 72 °C for 2 min, with a final extension at 72 °C for 7 min. Ten μL of each PCR product were electrophoresed in 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^{-1} 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 the UPGMA method and the Pearson coefficient with Bionumerics version 4.0 software (Applied Maths, Austin, TX).

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

The *rrs* gene was amplified and sequenced according to Rivas et al. [28], and 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 primers nodCBradyF (5′-CGCAAGGCGCAGWTCGC-3′) and nodCBradyR (5′-GGKGTGVAGCGMGAAGCCG-3′). PCR amplifications were performed with the REDExtract-N-Amp PCR kit (Sigma) or the GoTaq® Flexi DNA Polymerase kit (Promega) or DreamTaq™ Green DNA Polymerase (Thermo) by 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 silica gel columns using the QIAquick® DNA Gel Extraction Kit (Qiagen, Germany), following the manufacturers' instructions. In the case of the *nodC* gene from strain VUPME10, the amplified product obtained in the previous section (ca. 800 bp) was cloned into the pJet1.2/blunt Cloning Vector using the CloneJET™ PCR Cloning Kit (Thermo), according to the manufacturer's instructions for the Sticky-End Cloning Protocol. The Transformation Protocol was performed using the 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], and they were aligned 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] in the 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

A total of 40 slow-growing strains with typical morphology of the genus *Bradyrhizobium* were isolated on yeast-mannitol agar (YMA) plates, and they were able to nodulate the host since they formed effective pink-red coloured nodules. These isolates were analysed by RAPD fingerprinting that allows the differentiation between strains of the same *Bradyrhizobium* species [7,26]. This technique provides an estimation of the genetic diversity and shows that strains with approximately 75% identity belong to the same species [26]. As a result of the analysis, the isolates were distributed into nine RAPD groups with similarity percentages lower than 75% (Fig. 1), from which representative strains were selected for gene sequence analysis.

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

The strains isolated from *V. unguiculata* nodules in Africa belong to the genus *Bradyrhizobium* and have been identified mainly on the basis of their *rrs* gene and ITS region [16,25,36] which has allowed their placement into the *Bradyrhizobium* groups I and II proposed by Menna et al. [19]. The results of the analysis of these phylogenetic markers showed that the strains in the current study mostly belonged to group I but with a single strain clustering in group II (Figs. 2 and 3, S1 and S2).

Within group I, the isolates clustered in different phylogenetic lineages and some of them branched with previously described species, such as that represented by strain VUPME29, (RAPD group III) which was identified as *B. canariense* because it had *rrs* gene and ITS sequences identical to those of its type strain BTA-1^T.

Strains VUPME04, VUPME82 and VUPME26 representing RAPD groups V, VI and VIII, respectively, were identified as *B. cytisi* since they had *rrs* gene and ITS sequences almost identical to those of the type strain CTAW11^T.

Strain VUPME50, representing RAPD group VII, had the *rrs* gene and ITS region 100% identical to those of strain BGA-1, which is currently classified into *B. japonicum*.

Strains VUPMI37, representing RAPD type IV, and VUPMI11, representing RAPD group IX, formed a cluster related to *B. canariense* with more than 99% and 96.8% identity of the *rrs* gene and ITS region, respectively.

Strain VUPMI33, representing RAPD type I, formed independent branches in the phylogenetic trees of both the *rrs* gene and ITS region, but was close to *B. japonicum* USDA 6^T with 97.5% identity in this last region.

Finally, within group II, only a single strain, VUPME10, was found showing RAPD type II, which was closely related to *B. pachyrhizi* PAC48^T with identities higher than 99% in the *rrs* gene and 97% in the ITS region.

The results contrasted with those obtained in Africa, from where *V. unguiculata* was distributed to Europe, since we did not find the species *B. yuanmingense* from group I and *B. elkanii* from group II, which have been reported in *V. unguiculata*