Diversity and phylogeny of symbiotic bacteria nodulating common bean (*Phaseolus vulgaris* L) in Côte d'Ivoire

ABSTRACT

Phaseolus vulgaris L. (common bean) is an agriculturally important legume that benefits from a symbiosis with bacteria belonging to the genus *Rhizobium*. Growing interest in the use of rhizobia as biofertilizers has led to the identification of a large number of rhizobia strains and studies of their diversity. Although much research has been carried out on rhizobia, there is little data on the diversity of rhizobia associated with common bean in Côte d'Ivoire. This study assessed the species diversity of common bean nodulating bacteria in ivorian soils. This diversity was assessed based on 16S rRNA gene sequencing. Ten high-performance bacterial isolates extracted from common bean nodules were used for genetic analysis. The 16S rRNA gene sequences of the native isolates were closely affiliated with members of the genera *Rhizobium*, *Bradyrhizobium, Allorhizobium* and *Sinorhizobium* demonstrating the presence of a diversity of native bean nodule bacteria. This study also reports for the first time the presence of *Allorhizobium taibaishanense* in common bean nodules. These results constitute an important step in the development of an effective microbial inoculum and sustainable food production.

Keywords: Diversity, rhizobia, common bean, sequencing, 16S rRNA, phylogeny.

1. INTRODUCTION

The common bean, its scientific name Phaseolus vulgaris, is a very important legume in the human diet thanks to its richness in proteins, minerals and vitamins [1]. This legume establishes a symbiotic link with rhizobial bacteria for the fixation of atmospheric nitrogen. This allows it to grow in nitrogen-deficient soils [2]. Native to South and Mesoamerica, the plant is currently widely cultivated in tropical and temperate regions around the world [3].

Previously, the rhizobia capable of nodulating *P. vulgaris* were all attributed to a single species named *Rhizobium leguminosarum* [4]. In recent years, the taxonomy of these rhizobia has evolved significantly due to progressive discoveries of greater rhizobial diversity which correlates with the development of new technologies. To date, seven species of rhizobia have been identified as major microsymbionts of common bean. These are *Rhizobium etli* [5], *Rhizobium leguminosarum*, *Rhizobium tropici* [6], *Rhizobium gallicum*,

Rhizobium giardinii [7], *Rhizobium phaseoli* [8] and *Sinorhizobium meliloti* [9]. All these species have been discovered in America as well as in Europe and Africa [10, 11, 12].

Although agronomic information on beans in Africa is legion, studies of the specific diversity of rhizobia which nodulate this plant are rare. Indeed, with the exception of the research by Diouf *et al.* [13] carried out in Senegal, very little information is available on bean rhizobia in West Africa, particularly in Côte d'Ivoire. However, beans are a promising legume for local Ivorian consumption. In addition, the country's statistics could not provide any data on national bean production because this crop would be considered marginal. It is practiced by small producers, particularly women in rural areas [14]. In addition, producers use traditional varieties with low yields without fertilizer to grow beans [15].Faced with all these constraints, inoculation technology using rhizobacteria associated with common beans could constitute an alternative to the use of agrochemical products to increase its production and enhance its cultivation. Therefore, the characterization of rhizobia related to common bean in Ivorian soils constitutes one of the important steps towards the development of an inoculant with the potential to improve the production of this crop in Côte d'Ivoire. It is in this context that this study takes place, the aim of which is to identify the species of local bacteria which nodulate common beans in Ivorian soils.

2. MATERIAL AND METHODS

2.1. Bio-collection

The biological material is composed of bacterial isolates. The latter were isolated from the roots of common bean grown in soil samples collected in different agroecological zones of Côte d'Ivoire. The isolates used in this study were found to be the most efficient on common bean production during field evaluation. These are isolates RPC109, RPC114, RPC115, RPC208, RPC304, RPC404, RPC410, RPC430, RPC431 and RPC509.

2.2. Molecular characterization of isolates

2.2.1. DNA extraction

The extraction of genomic DNA from bacterial isolates was carried out using the phenol-chloroform method applied to a bacterial culture in YEM medium [16]. After bacterial lysis, the samples were treated in a mixture of 500 µl of phenol/chloroform/isoamyl alcohol in a ratio of 25:24:1 and then centrifuged for 10 minutes at 12,000 rpm. The DNA pellet was obtained by adding sodium acetate solution and absolute ethanol. The resulting DNA extract was suspended in 100 µl of TE solution and stored at - 20°C. The concentration and purity of the extracted DNA were determined at 260 and 280 nm using the Nanodrop Spectrophotometer.

2.2.2. PCR amplification of 16S rRNA gene

The 16S rRNA region of rhizobia isolates was amplified using primers FD1 (5-AGAGTTTGATCCTGGCTCAG-3) and RD1 (5-AAGGAGGTGATCCAGCC-3) as previously described by Weisburg *et al.* [16]. The PCR reaction was carried out in a total volume of 25 µL containing 12.5 µL of MasterMix, 1 µL of each of the primers, 9.5 µL of water and 1 µL of template DNA extract. The resulting mixtures were incubated in a thermal cycler under the

following conditions: initial denaturation at 95°C for 3 min followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, extension at 72 °C for 2 min and a final extension at 72 °C for 3 min. The fragments of the 16S rDNA gene were revealed by horizontal electrophoresis on a 1% (w/v) agarose gel stained with BET (100 mg/mL) in TAE buffer. The migration was done for 45 min at 100 volts and finally the bands were visualized under ultraviolet light and photographed using a transilluminator.

2.2.3. Sequencing and analysis of the 16S rRNA gene

The PCR products were transferred to Inqaba Biotech (Pretoria, South Africa) for purification and sequencing by the automated Sanger method. The 16S rRNA sequences of the amplicons were determined using the ABI 3730 sequencer (Applied Biosystems, USA).

2.2.4. Analysis of sequencing data

2.2.4.1. The 16 S rRNA Sequences assignment for species rhizobia identification

An *in silico* analysis of ribosomal DNA sequences was carried out to identify common bean rhizobium species. To do this, the Forward and reverse sequences of the 16S rRNA gene were aligned with reference sequences from the GenBank database using the clustal X program and the BLAST tool (Basic Local Alignment Search Tool; http://www.ncbi .nih.gov).

2.2.4.2. Phylogenic analysis

First of all, a multiple alignment of the sequences from this study and the reference sequences from Genbank was carried out using the clustal W program. The evolutionary history of the isolates was presented in the form of a dendrogram using the maximum likelihood method based on the Tamura-Nei model [18]. The tree with the highest log likelihood (bootstrap values ≥ 50%) was displayed. It was drawn to scale, with branch lengths measured in number of substitutions per site. The proportion of sites where at least one unambiguous base is present in at least one sequence for each descendant clade is shown next to each internal node in the tree. The analysis focused on 18 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Non-coding. All positions containing gaps and missing data have been eliminated. There were a total of 133 positions in the final dataset. This evolutionary or phylogenetic analysis of rhizobia species was carried out in MEGA version 11 software [19].

3. RESULTS

3.1. Molecular characteristic of rhizobia isolates

PCR amplification of the genomic region of the 16S rRNA of the 10 bacteria isolated from the nodules of common bean (*Phaseolus vulgaris* L.) generated, on agarose gel, a single DNA band of approximately 1500 bp (Figure 1).

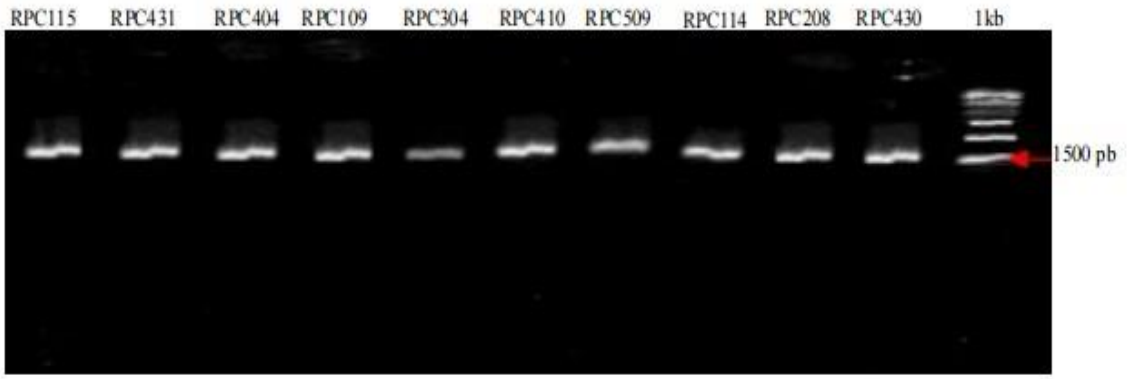

Figure 1. Gel electrophoresis image of PCR-amplified 16S rRNA gene of 10 bacterial isolates on 1.4% agarose gel

3.2. Affiliation of 16S rRNA gene sequences of bean bacterial isolates

Analysis of the 16S rRNA sequences of the isolates in this study shows that they identify with several species of bacteria (Table 1). Thus, isolate RPC109 from the locality of Djébonoua shares a similarity of 99.47% with the species *Allorhizobium taibaishanense* whose accession number is MG851723.1. The isolate RPC115 from the locality of Gagnoa is close to the species *Rhizobium tropici*, the sequence of which is accessible at number MG852217.1 with an identity percentage of 99.29%. The rhizobia RPC114, RPC208, RPC410 and RPC509 isolated from the soils of the locality of Ferkéssédougou, identify respectively with the species *Rhizobium pusense* (MW513398.1; 97.80%), *Rhizobium sp*. (KT387838.1; 95.27%), *Rhizobium leguminosarum* (KY940047.1; 96.40%) and *Sinorhizobium sp*. (EF173322.1; 83.03%). Isolate RPC430 from the Yamoussoukro locality aligns with the species *Bradyrhizobium japonicum* (KY940048.1) at 96.46% identity and with the species *Rhizobium leguminosarum* (KY940047.1) at 96.39% identity. identify. As for isolate RPC431, it identifies with the species *Rhizobium phaseoli* (KF638348.1) with a similarity rate of 98.10%. The two isolates RPC304 and RPC404 from the locality of Botro identify with the species *Allorhizobium taibaishanense* (MG851723.1) with identity percentages of 97.71 and 99.65%, respectively.

In total, the analysis of the 16S RNA sequences of the isolates revealed diversity in this bacterial pool composed of four bacterial genera: *Rhizobium, Bradyrhizobium, Allorhizobium* and *Sinorhizobium*. The profile of this diversity is dominated by the Rhizobium genus, representing alone 50% of the bacterial genera identified in this study, followed by the *Allorhizobium* genus with a frequency of 30% (Figure 2). The genera *Bradyrhizobium* and *Sinorhizobium* are the least represented with a frequency of 10% (Figure 2). In the genus *Rhizobium* we find species such as *Rhizobium pusense*, *Rhizobium leguminosarum*, *Rhizobium phaseoli* and *Rhizobium tropici*.

Table 1. Characteristics of the coding sequences of the 16s region of rhizobia strains

Isolates	Collection site	Sequence length	Reference organism	Accession number	Accession length	Identity percentage	E-Valeur
RPC109	Djébonoua	1174	*Allorhizobium taibaishanense*	MG851723.1	568	99.47%	0.0
RPC114	Ferkéssédougou	1222	*Rhizobium pusense*	MW513398.1	1299	97.80%	0.0
RPC115	Gagnoa	1198	*Rhizobium tropici*	MG851722.1	567	99.29%	0.0
RPC208	Ferkéssédougou	1191	*Rhizobium sp.*	KT387838.1	1401	95.27%	0.0
RPC304	Botro	1226	*Allorhizobium taibaishanense*	MG851723.1	568	97.71%	0.0
RPC404	Botro	1173	*Allorhizobium taibaishanense*	MG851723.1	568	99.65%	0.0
RPC410	Ferkéssédougou	1197	*Rhizobium leguminosarum*	KY940047.1	844	96.40%	0.0
RPC430	Yamoussoukro	1306	*Bradyrhizobium japonicum*	KY940048.1	832	96.46%	0.0
RPC431	Yamoussoukro	1222	*Rhizobium phaseoli*	KF638348.1	1495	98.10%	0.0
RPC509	Ferkéssédougou	1250	*Sinorhizobium sp.*	EF173322.1	1464	83.03%	$2,10^{-32}$

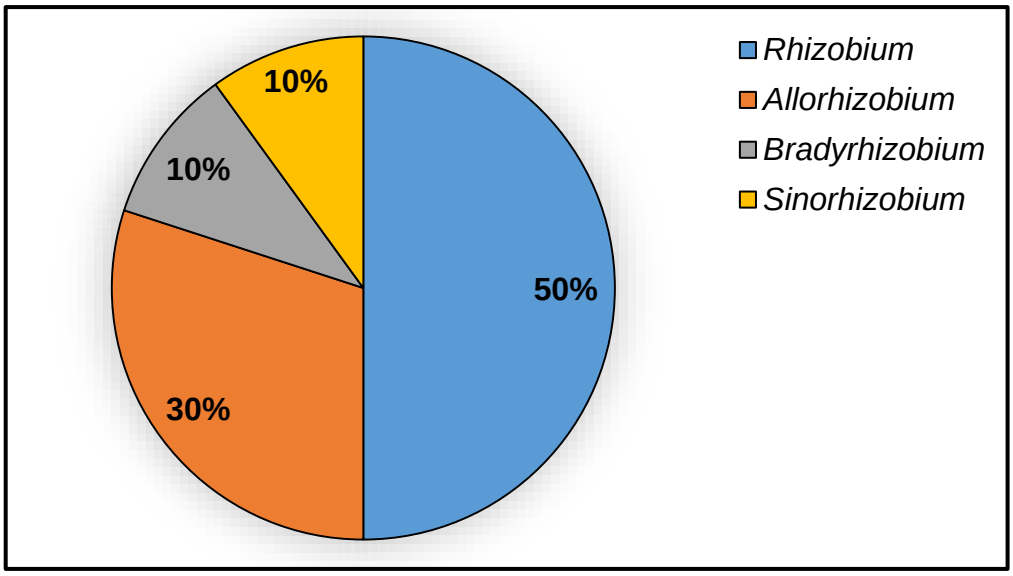

Figure 2. Distribution of genera of rhizobiacea nodulating common bean

3.3. Taxonomic structure of isolates

Phylogenic analysis made it possible to identify five bacterial groups. In these groups, the ten study isolates were closely related to the rhizobia reference strains in GenBank with the exception of RPC509 (Figure 3). Thus in group I, the RPC114 isolate is linked to the reference strain *Rhizobium pusense*. Group II is composed of isolates RPC208 and RPC431 closely related to the reference strains *Rhizobium sp* and *Rhizobium phaseoli* respectively. In group III, a phylogenetic relationship is observed between isolates RPC410 and RPC430 with the reference strains *Rhizobium leguminosarum* and *Bradyrhizobium japonicum*. The isolates RPC109, RPC304 and RPC404 are found in group IV with a sequence similarity of 97.71 and 99.65% to the *Allorhizobium taibaishanense* strain. Finally, group V includes the RPC115 isolate closely related to the reference strain *Rhizobium tropici*

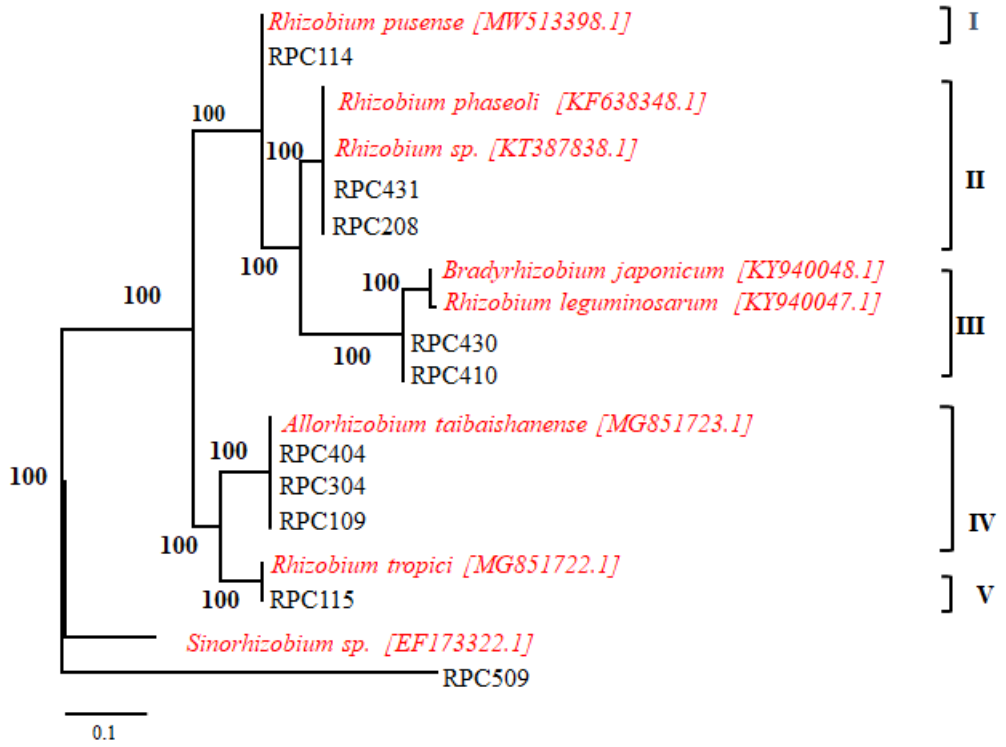

Figure 3. Maximum likelihood phylogenetic tree deduced from 16S rDNA gene sequences of local rhizobia isolated from common bean.

4. DISCUSSION

Amplification of partial 16S rRNA gene sequences of the isolates using specific primers generated a single band of approximately 1,500 base pairs. This is generally the approximate fragment size of that target gene that is commonly used for bacterial identification [20].

Analysis of the 16S rRNA sequences of isolates from the different agroecological regions of Côte d'Ivoire showed that the symbiotic bacteria of common bean are very diverse. The profile of this diversity is dominated by species belonging to the genus *Rhizobium*. This result is corroborated by several authors who in their work showed that the symbiont bacteria isolated from *P. vulgaris* nodules on different continents mainly belong to the genus Rhizobium [21, 22, 23, 24]. This is because unlike other *Phaseolus* species which associate with *Bradyrhizobium*, the common bean shows a clear preference for the genus *Rhizobium* [10].

The genus *Rhizobium* was represented by four species of bacteria which are *Rhizobium pusense, Rhizobium leguminosarum, Rhizobium phaseoli* and *Rhizobium tropici*. These species were previously isolated from common bean nodules by Gunnabo *et al*. [25] in Ethiopia; Torrez-Gutiérrez *et al*. [26] in Ecuador and by Gununu *et al*. [27] in Eswatini. These species are considered to be the predominant species isolated from bean nodules in the Andean region, their native area [25, 28]. The spread of *P. vulgaris* microsymbionts to other continents would have been made thanks to its grains [27] and would suggest its possible mode of migration in its American [10], European centers of diversification [29], African [21, 25] and Asian [30].

This study also revealed that in addition to the genus *Rhizobium*, there are other phylogenetically related bacterial genera that could nodulate common bean. Indeed, the study noted the presence of species belonging to the genera *Bradyrhizobium, Allorhizobium* and *Sinorhizobium*. These results indicate a strong nodulation promiscuity of *P. vulgaris* with various nitrogen-fixing bacteria in the soils of Côte d'Ivoire. They corroborate the studies carried out by Shamseldin & Velázquez [24] which reveal the promiscuity of the common bean.

Just like the species Rhizobium sullae, mycrosymbiont of Hedysarum coronarium L., discovered for the first time in common bean by Soares *et al* [31], *Allorhizobium taibaishanense* would be isolated and identified for the first time in this plant through this study. *Allorhizobium taibaishanense* is a recently discovered species in *Kummerowia stratia* and described by Yao *et al*. [32]. The discovery of this new species of bacteria in common bean could be explained by the evolutionary nature of rhizobia due to a transfer of symbiotic plasmids between species.

Based on the symbiotic efficiency results, all rhizobium strains showed bean nodulation potential. However, the phylogenetic structure showed that these strains were not closely related. This would suggest that the rhizobia strains in the study are not restricted to one phylogenetic group [33].

5. CONCLUSION

The results of the study noted the presence of a wide diversity of bacteria which nodulate beans in Ivory Coast. These isolates were divided into four genera including *Rhizobium, Bradyrhizobium, Allorhizobium* and *Sinorhizobium*. Some of the bacterial species discovered in this study, notably *Allorhizobium taibaishanense*, had never previously been described as endosymbionts of *P. vulgaris*. In view of their agronomic and symbiotic performances, these isolates should be subjected to more in-depth investigations under different environmental conditions. Such research will provide a better understanding of the symbiosis between *P. vulgaris* and rhizobial bacteria. They will also make it possible to determine whether these promising strains can be used as effective commercial inoculants, thus contributing to the sustainability of cropping systems.

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