Genetic diversity of symbiotic *Bradyrhizobium elkanii* populations recovered from inoculated and non-inoculated *Acacia mangium* field trials in Brazil

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**Abstract**

*Acacia mangium* is a legume tree native to Australasia. Since the eighties, it has been introduced into many tropical countries, especially in a context of industrial plantations. Many field trials have been set up to test the effects of controlled inoculation with selected symbiotic bacteria versus natural colonization with indigenous strains. In the introduction areas, *A. mangium* trees spontaneously nodulate with local and often ineffective bacteria. When inoculated, the persistence of inoculants and possible genetic recombination with local strains remain to be explored. The aim of this study was to describe the genetic diversity of bacteria spontaneously nodulating *A. mangium* in Brazil and to evaluate the persistence of selected strains used as inoculants. Three different sites, several hundred kilometers apart, were studied, with inoculated and non-inoculated plots in two of them. Seventy-nine strains were isolated from nodules and sequenced on three housekeeping genes (*glnII, dnaK* and *recA*) and one symbiotic gene (*nodA*). All but one of the strains belonged to the *Bradyrhizobium elkanii* species. A single case of housekeeping gene transfer was detected among the 79 strains, suggesting an extremely low rate of recombination within *B. elkanii*, whereas the nodulation gene *nodA* was found to be frequently transferred. The fate of the inoculant strains varied depending on the site, with a complete disappearance in one case, and persistence in another. We compared our results with the sister species *Bradyrhizobium japonicum*, both in terms of population genetics and inoculant strain destiny.

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1. Introduction

*Acacia mangium* is a leguminous tree native to northern Australia, Papua New Guinea and Indonesia. It is naturally found in tropical rainforests with a mean rainfall ranging from 1500 to 3000 mm per year. *A. mangium* has been planted widely in the tropics since the early 1980s, first in Indonesia and Malaysia for pulp production, but also for revegetation and rehabilitation purposes. Its main qualities are rapid early growth, good wood quality and its tolerance to different soil pH and composition. In 1998, Turnbull et al. [60] estimated the area planted to *A. mangium* in Asia at 600,000 ha. *A. mangium* belongs to the Mimosoideae tribe, and has the ability to form nitrogen fixing symbiosis with soil bacteria collectively known as rhizobia. It has been found associated with various *Bradyrhizobium* strains in different countries and continents [23,50,55,66], and recently Le Roux et al. [22] suggested that *A. mangium* is preferentially nodulated by *Bradyrhizobium elkanii* species. It has also been found associated, albeit much less frequently, with other species, such as *Rhizobium* sp. [14,65], *Mesorhizobium* sp. [11], and *Ochrobactrum* sp. [39]. In South America, *A. mangium* has been introduced in several countries (Brazil, Colombia, Costa Rica, etc.), and two previous studies identified some isolates nodulating *A. mangium* in Brazil, such as *Bradyrhizobium* sp. [35,36].

Inoculating plants with efficient nitrogen-fixing bacteria is an environment-friendly way of improving the economic and natural growth of legumes, especially in poor or degraded soils. In *A. mangium*, regardless of the symbiotic rhizobial selected, inoculation improves survival in the field by 10% [28]. Galiana et al. [15] reviewed several inoculation field trials in different countries. They showed that inoculation had a positive effect on *A. mangium*
growth, whatever the soil type and the country, and improvement was still detectable after 2–3 years. In addition, the strains did not all display the same effect at the same stage of growth, from the nursery or greenhouse to the field [14]. One main question regarding legume inoculation, either directly in the field, or in the nursery prior to planting/sowing, is not only the survival and persistence of these inoculant strains, but also the possible genetic recombination between inoculant and indigenous strains. In the case of perennial species, the replacement of inoculant strains by local strains over the life span of the tree is also possible. In two A. mangium field trials in Ivory Coast in 1994, Galiana et al. [16] recovered an inoculated strain 42 months after field transplantation, whereas another one disappeared after 19 months. Martin-Laurent et al. [29] and Prin et al. [49] analysed nodules from A. mangium 4 and 6 months after transfer to the field in Singapore and Madagascar respectively. Molecular analyses showed the persistence and the overgrowth of one of the two inoculums to the detriment of the other. In 1990, McLoughlin et al. [30] showed that the success of inoculating soybean with *Bradyrhizobium japonicum* strains in the US was highly variable, and that successful inoculation the first year did not ensure that the inoculated strain would persist in forming nodules in subsequent years. More recently, Mendes et al. [31] showed that even 6 years after its inoculation on soybean in Brazil, the *B. japonicum* serogroup introduced was dominating nodules, occurring in more than 50% of the nodules from treatments where it had never been inoculated.

Beyond the question of the persistence of a given inoculant strain in soil, its genomic stability and integrity, i.e. whether the strain introduced remains genetically isolated in soil, or whether gene exchanges occur with indigenous strains, is also relevant. One strain in soil, its genomic stability and integrity, i.e. whether the inoculated strain would persist in forming nodules in subsoil, through seed migration from a neighbouring indigenous *A. mangium* seedlings had not been inoculated prior to planting. At two, inoculation had been carried out in the greenhouse with two selected *Bradyrhizobium* strains, and seedlings were planted in nearby fields. The genetic diversity of all strains sampled from the fields (both inoculated and non-inoculated) was analysed for three housekeeping genes, recA, dnaK and glnII, selected for their phylogenetic congruence in the *Bradyrhizobium* genus [42] and one symbiotic nodulation gene, nodA, to test for possible horizontal transfer of the symbiotic island within and between local and inoculated bradyrhizobia.

### 2. Materials and methods

#### 2.1. Site descriptions

Samples of *A. mangium* nodules were directly taken from 5 fields located at three sites in Brazil (Table 1). At Itatinga, we took samples from two experimental fields (2 km apart), each with a monoculture of *A. mangium* surrounding by *Eucalyptus* species. In one field, *A. mangium* seedlings had been planted 11 years earlier without any inoculation. In the other one, seedlings had been inoculated 6 years earlier in the nursery with the two rhizobia strains BR3609 (SEMIA6387) and BR6009 (SEMIA6404). These two strains were originally isolated in Porto Trombetas, from *Acacia auriculiformis* (Mimosoideae) and *Lonchocarpus costatus* (Papilionoideae) respectively [12], and were recommended by Embrapa Agrobiologia as efficient with *A. mangium*. These strains were subsequently described as belonging to the *B. elkanii* species [4,32].

The second site, Porto Trombetas, is exploited as a bauxite mine in the State of Pará. Two fields were sampled around the industrial site: the first was planted with *A. mangium* in a rehabilitated bauxite tailings tank (SP-1). This rehabilitation process was performed in a two-step procedure. In 1998, seeds of leguminous trees and shrubs, pre-inoculated with N-fixing bacteria and mycorrhizae (VAM fungi), were hydroseeded (i.e. seeds sown by distribution in a stream of water propelled through a hose) over the tailings with the addition of fertilizer [13]. In the second phase in 1999, after substrate consolidation, *A. mangium* seedlings were planted after inoculation in the nursery using the two selected strains BR3609 and BR6009. In the second field (1.8 km away from the first), spontaneous colonization of *A. mangium* occurred on subsoil, through seed migration from a neighbouring *A. mangium* reforested area. In both field trials, nodules were collected from 1 to 2-year-old plants. These trees were second generation trees in the inoculated plot.

The last site was at Seropedica, a 15 year-old experimental field trial planted with non-inoculated *A. mangium* trees. We randomly sampled from 6 to 10 individual trees in each field, collecting as many nodules as possible from each (Table 1).

#### 2.2. Isolation of bacterial isolates

Two nodules per sampled tree, chosen at random, were surface sterilised in 35% hydrogen peroxide for 4–6 min depending on the size of the nodules, rinsed and then crushed in sterile water. The

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### Table 1

Sites sampled.

<table>
<thead>
<tr>
<th>Geographical origin</th>
<th>Latitude</th>
<th>Longitude</th>
<th>Use of inoculant strain</th>
<th>Type of soil</th>
<th>Age of trees</th>
<th>Number of trees</th>
<th>Number of isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Porto Trombetas</td>
<td>01°41'59&quot;</td>
<td>56°24'54&quot;</td>
<td>No</td>
<td>Subsoil</td>
<td>1–2 years</td>
<td>8</td>
<td>16</td>
</tr>
<tr>
<td>Itatinga</td>
<td>01°41'59&quot;</td>
<td>56°25'22&quot;</td>
<td>Yes&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Tailings tank</td>
<td>1–2 years</td>
<td>10</td>
<td>18</td>
</tr>
<tr>
<td>Seropédica</td>
<td>23°02'23&quot;</td>
<td>48°38'39&quot;</td>
<td>No</td>
<td>Experimental field</td>
<td>11 years</td>
<td>6</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>23°02'23&quot;</td>
<td>48°38'39&quot;</td>
<td>Yes&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Experimental field</td>
<td>6 years</td>
<td>10</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>22°45'12&quot;</td>
<td>43°40'12&quot;</td>
<td>No</td>
<td>Experimental field</td>
<td>15 years</td>
<td>9</td>
<td>13</td>
</tr>
</tbody>
</table>

<sup>a</sup> Inoculated by *Bradyrhizobium* strains BR3609 and BR6009.
nodule contents were spread onto YMA Petri dishes [62] supplemented with bromothymol blue as pH indicator. After purification by several subculturings of isolated colonies, isolates were grown and stored at −80 °C in YEM broth supplemented with 20% mineral oil to reduce bacterial proliferation.

2.3. Plant Renodulation Assay (Koch’s postulate)

All isolates were tested to confirm their capacity to nodulate A. mangium in individual monoclonic culture tubes [61]. A. mangium seeds (lot 19297, CSIRO Australian Tree Seed Centre) were dipped into boiling water for 2 min, surface-sterilised for 5 min in 35% hydrogen peroxide, rinsed in sterile distilled water for 2 min and germinated on 10 g l−1 (w/v) water agar Petri dishes for 3 days at 28 °C in the dark. Plants were transferred in CYG Germination Pouches (Mega International, United States) to sterile individual tubes containing 10 ml of a nitrogen-free solution [6]. After a few days, healthy plants were inoculated with 500 μl of a five-day-old YEM liquid bacterial culture. Nodulation results were visually checked after 30 days.

2.4. DNA extraction, amplification and sequencing

Total genomic DNA of each isolate was extracted as described in [10]. Partial dnaK (heat shock chaperone protein) and glnII (glutamine synthetase II) genes were amplified using primers TSDnaK4, TSDnaK2, TSglnII and TSglnFr as described by [56]. The recA (DNA recombination protein) gene fragment was amplified by primers TS2recAf (5′-GCCCTGCGTATCGTCGAAGG-3′) and TS2recAr (5′-CGATCTGCTGATGAAAGTACCC-3′), with an annealing temperature of 59 °C, modified from [56]. The partial nodA (acyltransferase) gene was amplified for all strains, using primers nodA1F or nodAf.brad and primer nodAr.brad [8,9].

PCR amplification was performed with a Thermo Electron PxA2 Thermal Cycler in a 25 μl total volume. The reaction mixture contained each deoxynucleotide triphosphate (100 μM), primers (0.4 μM each), MgCl2 (1.5 mM), 0.5 U of Taq DNA polymerase (Promega, Charbonnières, France), the buffer supplied with the enzyme and 50 ng of genomic DNA. The PCR protocol was as follows: initial denaturation (94 °C for 5 min); 35 denaturation cycles (30 s at 94 °C), annealing (30 s at primer specific hybridization temperature), and extension (40 s at 72 °C), and final extension (3 mn at 72 °C). Amplification products were visualized on 1% agarose TAE gel stained with ethidium bromide. PCR fragments were sequenced by subcontracting to Genoscreen, France, using ABI chemistry on ABI3730 sequencers.

2.5. Nucleotide sequence analyses

Multiple nucleotide sequence alignments for the four loci were performed with ClustalX [21] and manually corrected using GeneDoc [40]. A single sequence data file, including one sequence of each different allele obtained, was built using MOTHUR [53]. The number of sequences (n), number of different alleles (R), length of sequence (L), number of polymorphic sites (S) and nucleotide diversity (π) were computed at each locus using DnaSP [24].

Each locus was analysed for intragenic recombination. We explored this issue using several alternative methods implemented in RDP3 [19], using RDP [26], Chimaera [48], Geneconv [44], MaxChi [54], BOOTSCAN/RECSAaN [27] and 3Seq [5]. A first analysis run was performed using each alignment individually. Only recombination events for which at least two methods gave a positive result were kept for further analyses (using phylogenetic tree comparisons) to firmly assess their accuracy (as per [64]).

Recombination between loci was tested taking two approaches. Firstly, a concatenated data file including all four loci was created and subjected to methods implemented in RDP3 [19], similarly to the previous search for intragenic recombination. Secondly, incongruences between phylogenetic trees obtained from each locus separately were tested with the Shimodeira–Hasegawa test implemented in PAUP4 [59]. Maximum likelihood trees were obtained for each locus separately using PAUP4. The best molecular evolution model for each dataset was chosen using Modeltest [47], following the log ratio test. One hundred bootstrap replicates were used to estimate node reliability. We also included several sequences obtained from GenBank in the phylogeny. We included the best hit for each different allele blasted on the databank, but also sequences of each described Bradyrhizobium species and genospecies, whenever available. We also reconstructed a phylogeny with the concatenated dataset of the three core genes (recA, dnaK and glnII). This analysis also included sequences retrieved from four full genomes already sequenced, Bradyrhizobium japonicum USDA110, Bradyrhizobium sp. ORS278, Bradyrhizobium sp. Bta11 and the closely related species Rhodopseudomonas palustris, strain CGA009. We also included several species that had been typed for the same three loci. We thus included sequences from B. elkanii, B. canariense, B. yuanmingense and B. liaoningense in the phylogenetic tree.

2.6. Nucleotide sequence accession numbers

The sequence accession numbers for the four gene fragments of the two inoculant strains and 79 isolates are listed in supplementary material (Table S1). Sequence alignments are available upon request.

3. Results

3.1. Genetic diversity of isolates and recovery of inoculant strains

From 10 to 22 isolates were obtained from each site (Table 1). We obtained a total number of 79 isolates renodulating their original host, A. mangium, confirming their symbiotic status. Although not firmly assessed using statistical tests, all plant–isolate combinations were visually checked as being effective for nitrogen fixation (plants were always greener and bigger than the non-nodulated negative control). The 81 strains in the study (79 isolates + the two BR3609 and BR6009 inoculant strains) were all sequenced for the four loci (no missing data).

3.2. Nucleotide diversity

The sequence lengths obtained ranged from 487 (nodA) to 662 bp (dnaK) (Table 2). The number of different alleles was rather similar between loci, from 19 (dnaK and glnII) to 25 (nodA), the latter being the most polymorphic with the shortest sequences. These differences were reflected in the nucleotide diversity index (π) which was 7.5 higher for nodA than for dnaK. Of the four alignment matrices obtained, only nodA displayed indels, one 3 bp and one 6 bp long. All three other loci lacked indels in the alignment obtained, even when we included other sequences from the databank.
Fig. 1. Maximum likelihood recA phylogeny. The two strains used as inoculants are in bold. One sequence per different allele was included in the dataset. Sequences from other species or genospecies were retrieved from GenBank. Bootstrap values were estimated based on 100 replicates. Only values higher than 75% have been kept in the figure. Isolates shaded in grey were sampled from non-inoculated plots. Isolates in brackets shared the same allele but with a different multilocus haplotype. (PT: Porto Trombetas, It: Itatinga, Ser: Seropédica).

Combination of the four loci resulted in 28 different genotypes (Table S1). Fifteen were only represented by a single isolate, whereas others were represented by 2–8 isolates. The main genotype in terms of frequency was haplotype 1 (identical to strain BR6009, 20 isolates) which was used for inoculation at Itatinga. Of the two strains used for inoculation in the nursery, only the latter was recovered from our samples, at high frequency in the inoculated plot in Itatinga (82%, 18 out of 22 isolates), but also twice from the non-inoculated plot at the same site (2 out of 10 isolates). This strain was never recovered from the inoculated field trial at Porto Trombetas, and strain BR3609 was never detected at all among the 79 isolates.

3.3. Recombination and lateral gene transfer

None of the four loci, taken individually, revealed intragenic recombination (data not shown). We first combined the three housekeeping genes loci in a single alignment to detect recombination between loci. Only a single unambiguous recombination could be detected between one of the three core genes and others, involving a recA transfer. The two strains BR3648 and BR3656 shared the same alleles for both dnaK and glnII but their recA allele differed for 22 different nucleotide sites, resulting in a completely different phylogenetic position (see Fig. 1). When including nodA in the analyses, several examples of lateral transfer were revealed. Eleven nodA alleles clustered in the same well-supported clade, whereas they were dispersed in several clades based on the three other loci. The nodulation locus, and possibly the cluster of genes involved in nodulation specificity, had undergone a much higher rate of recombination than the other loci, even though nodulation genes have always been found located on the chromosome in the *Bradyrizhobium* strains [18].

3.4. Phylogenetic analyses

The four alignments were used separately to reconstruct maximum likelihood phylogenies (see recA and nodA in Figs. 1 and 2 respectively, dnaK and glnII in supplementary material Figs. S1 and S2). The three core gene phylogenies clustered all strains but one (BR10109) within the *B. elkanii* clade, although not with high boot-
B. yuanmingense to pachyrhizi species [51]. The strain BR10109 appeared to be related biolum genospecies (IX, X, VII and XI), or the recently described but not in the from any previously described of the strains fell in the same clade as a B. elkanii retrieved from GenBank, isolates appeared to be related to various strap values. Depending on the loci and sequences that could be retrieved from GenBank, isolates appeared to be related to various B. elkanii strains (USDA76, USDA94, LMG6147), several Bradyrhizobium genospecies (IX, X, VII and XI), or the recently described B. pachyrhizi species [51]. The strain BR10109 appeared to be related to B. yuanmingense/B. liaoningense in the two dnaK trees, but not in the nodA tree, where it was close to B. betae.

The nodA phylogeny clustered strains in three different clades. Strain BR10109 was strongly supported in the same clade as the photosynthetic ORS285 strain. However, this may have been an effect of a Long Branch Attraction artefact. Fourteen haplotypes fell in a single clade together (and 100% bootstrap value) with two strains isolated from Acacia albida in Senegal (ORS130) and Mucuna aterrima in Brazil (CPAC-M9). This clade appeared as the sister clade of a B. japonicum/B. yuanmingense clade. It is worth noting that none of the strains fell in the same clade as a nodA sequence retrieved from any previously described B. elkanii strain. The third group of nodA haplotypes clustered in a clade with no described species, with only one sequence retrieved from GenBank, amplified from Bradyrhizobium sp. CH2493, and one strain isolated from Lupinus paraguariensis, also in Brazil.

The Shimodeira–Hasegawa (SH) test implemented in PAUP was performed on all four trees and datasets (Table 3). We only included phylogenies with “our” strains from this study since none of the “reference” strains from the databank was common to all of the four datasets. As expected, the nodA tree was significantly rejected by all three datasets. All single-gene phylogenies obtained with the three core genes were significantly incongruent one against the other, and rejected based on the SH test.

However, as shown in Vinuesa et al. [64], the mean and median congruence levels of trees increase with the number of concatenated partitions used to infer them. We thus concatenated the three core genome loci to build up a single matrix and reconstructed a maximum likelihood tree (Fig. 3). The glnII tree was congruent (not rejected) with the concatenated dataset, whereas both the recA and dnaK trees were significantly rejected (p < 0.002 each). Nevertheless, the concatenated tree obtained strongly clustered all strains but one in the B. elkanii clade, confirming the preponderance of that species in our samples. The clades also usually grouped together

![Fig. 2. Maximum likelihood nodA phylogeny. Sequences from various Bradyrhizobium species were included in the dataset. One sequence obtained in this study per distinct clade was used in a blast search to retrieve the closest related sequence in the databank. Host legume species and country of origin are indicated for GenBank sequences. Isolates shaded in grey were sampled from non-inoculated plots. Isolates in brackets shared the same allele but with a different multilocus haplotype. (PT: Porto Trombetas, It: Itatinga, Ser: Seropédica).](image-url)

**Table 3**

<table>
<thead>
<tr>
<th>Locus</th>
<th>recA</th>
<th>dnaK</th>
<th>glnII</th>
<th>Conc</th>
</tr>
</thead>
<tbody>
<tr>
<td>nodA</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>recA</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.002</td>
</tr>
<tr>
<td>dnaK</td>
<td>0.001</td>
<td>0.002</td>
<td>0.002</td>
<td>0.002</td>
</tr>
<tr>
<td>glnII</td>
<td>0.391</td>
<td>0.391</td>
<td>0.391</td>
<td>0.391</td>
</tr>
</tbody>
</table>

SH test using RELL bootstrap (one-tailed test); number of bootstrap replicates = 1000. The P value indicates the significance of the difference in $-\ln L$ scores (Diff $-\ln L$) for each locus compared individually or with the concatenated (Conc) tree.
4. Discussion

4.1. Diversity of Bradyrhizobium strains associated with A. mangium in Brazil

*A. mangium* was introduced into Brazil <20 years ago, its area of origin being confined to northern Queensland in Australia and Papua New Guinea. At the 3 study sites, seedlings were introduced without any artificial inoculation, forcing *A. mangium* to interact with local strains. *A. mangium* is usually described as a promiscuous nodulating species, able to associate with a wide range of bacteria. Indeed several different genera have been isolated from *A. mangium*, including *B. japonicum*, *B. elkanii*, *Rhizobium*, *Mesorhizobium* and *Ochrobactrum*. In our study, 39 out of the 40 isolates obtained from the non-inoculated field trials were clustered in the *B. elkanii* clade. The 22 strains sampled from inoculated fields (excluding original inoculant strains) were all classified as *B. elkanii*. Le Roux et al. [22] previously suggested such preference, which is not strict symbiotic specificity, however. Indeed, Clapp et al. [11] characterized 26 strains trapped by *A. mangium* in Indonesia, of which 12 were identified as *B. elkanii* and 13 as *B. japonicum*, the last strain isolated being related to *Mesorhizobium loti*. Fremont et al. [14] also isolated both *B. japonicum* and *B. elkanii* strains after trapping with *A. mangium* in Malaysia. Nuswantara et al. [41] isolated 10 strains from Indonesia, all allegedly belonging to *B. elkanii*. The lack (or almost) of *B. japonicum* strains in our samples may, however, have resulted from its scarcity in these soils. Parker and Kennedy [45] showed that the distributions of the two species *B. elkanii* and *B. japonicum* in the northeastern United States, after trapping with 13 native legume species, were not similar. However, several studies have demonstrated that Brazilian soil naturally harbours *B. japonicum* strains [36]. These results (and others) reinforced the question of the virtual absence of *B. japonicum* in our samples. This unbalanced ratio between the two species is all the more surprising in that at one site hydroseeding provided a large cocktail of strains, including *B. japonicum* (de Faria, pers. comm.). Moreover, at least one strain (SEMA 6420) included in that cocktail, originally isolated from *A. mangium* in Brazil, was described as *B. japonicum* based on the 16S sequence [33]. *B. japonicum* might have been eliminated from this soil due to environmental constraints, such as the pH or organic matter content. For instance, Giongo et al. [17] showed that these parameters significantly affected the level of microbial diversity in areas that had been used as commercial

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**Fig. 3.** Maximum likelihood phylogeny based on a concatenated dataset including the three loci recA, dnaK and glnII. Each different multilocus haplotype obtained in the study was represented in the dataset. The coding following isolate names gives the site of origin (PT: Porto Trombetas, It: Itatinga, Ser: Seropédica). The two strains in bold were used as inoculants. The external data were taken either from full genome sequences (*R. palustris*, Btai1, ORS278 and USDA110) or from a combination of several single sequences.
crop fields. Whatever the explanation, B. elkanii appears to be better adapted to A. mangium in Brazil, at least in terms of survival and nodulation, and should be chosen as a priority for inoculation.

4.2. Genic diversity, recombination and persistence of the inoculum

Bradyrhizobium is a complex and blurred genus that has been the subject of many taxonomic and phylogenetic studies [52]. Several have focused on phylogenetic congruence between loci on a species or genus level, estimating the rate of lateral gene transfer over the diversification of the various species and genospecies. Nzoué et al. [42], Parker and Kennedy [45] or Batista et al. [3] showed that recombination and gene transfer between and within Bradyrhizobium lineages occurred frequently, to the extent of resulting in statistically incongruent phylogenies. However, all these studies highlighted that the results were dependent upon the loci studied, but also the lineage. Vinuesa et al. [63] suggested frequent homologous recombination within but not across lineages, and detected variable levels of recombination depending on the lineage [64]. Parker and Kennedy [45] estimated that lateral gene transfer altered genealogical relationships of different portions of the genome, with gene transfer between divergent taxa of Bradyrhizobium, from B. elkanii to the B. japonicum group. Minamisawa et al. [34] showed horizontal gene transfer between B. elkanii and B. japonicum strains in soil, suggesting potential horizontal transfer of nod genes between bradyrhizobia and other bacterial populations in the soil environment. In our study, we only detected one case of gene recombination between strains, leading to the replacement of the recA copy in strain BR3648 by another copy from an (unknown) strain. Within-species recombination thus appeared to be extremely limited between these strains. Moreover, none of the B. elkanii strains harboured any B. japonicum housekeeping sequences, suggesting a lack of gene transfer between species. Sampling at various sites might also have influenced our results in creating an artificial significant Linkage Disequilibrium value, due to the admixture of several genetically isolated populations (i.e. equivalent to a Wahlund effect). However, even within each population, the LD was maximum, supporting the lack of recombination (data not shown). The fact that we focused on the B. elkanii species, for which no similar study is yet available, makes it difficult to compare our results with others. Our data showed however that B. elkanii would appear to be less subject to recombination than B. japonicum. The same results were observed in the Sinorhizobium (Ensifer) genus, where Bailly et al. [1] suggested a different rate of recombination between the two sister species S. melloti and S. medicae.

Although only a single lateral core gene transfer event could be detected in our samples, nodulation gene transfer conversely occurred extremely frequently. Nodulation genes are known to lie behind symbiotic specificity, and several studies have shown that nod gene transfers are frequent between rhizobial lineages, even between alpha and beta proteobacteria [9]. Whilst nearly all our strains clustered in the same monophyletic group (in the three core genes phylogeny), nodA alleles clustered in two divergent clades (plus one single clade allele). It was striking to find that none of the nodA sequences fell closely related to the two B. elkanii strains we included in the phylogeny, even though they were closely related to this species in terms of core gene phylogeny. Nodulation gene transfers have been well described in the Mesorhizobium model [57,58]. Parker et al. [46] showed nitrogenase nifD gene transfer between Bradyrhizobium lineages, and Barcellos et al. [2] showed that symbiotic genes were horizontally transferred from a B. japonicum inoculant strain to indigenous E. fredii and B. elkanii.

At the two sites where A. mangium seedlings were introduced after nursery inoculation, none of the local strains recovered acquired symbiotic genes from the inoculants. Inoculation was carried out in 1999 and 2003 at the Porto Trombetas and Itatinga sites respectively, theoretically leaving enough time for recombination and gene transfer to take place. Several studies have shown that, outside its native area, A. mangium inoculated with native Australian Bradyrhizobium strains generally gives excellent growth responses compared to the local spontaneous bacterial strains [15], although A. mangium promiscuity results in non-inoculated plants being extremely easily colonized by local bacteria in field trials [49]. In our case, the local strains might have been as competitive and efficient as the inoculant strains, at least in terms of nodulation, thereby not favouring symbiotic gene acquisition by local strains.

4.3. Artificial inoculation and persistence of the inoculum

At two of the three sites tested, A. mangium seedlings were inoculated in the nursery prior to their introduction. We obtained contrasting results on the persistence of those inoculant strains in the soil, with a high frequency of re-isolation of one inoculant strain at Itatinga, but a complete lack at Porto Trombetas. Two major parameters differed between the two sites. Firstly, at Porto Trombetas, the stressful environmental conditions (a bauxite tailing tank), coupled with the initial hydroseeding including a combination of various legume species and rhizobial strains, created a highly competitive environment, where introduced strains would appear to have been eliminated. Secondly, we took samples directly from the inoculated trees in the Itatinga field, whereas at Porto Trombetas we collected nodules from 1 to 2-year-old second generation trees. The latter trees must have reacquired their symbiotic bacteria, since there was no vertical transmission in the rhizobial symbiotic association, thus going through the competition between strains present in the soils. Nonetheless, this is possible, since we isolated the inoculant strain twice in nodules sampled in the non-inoculated field trial at Itatinga, proving that the inoculated strain was able, after short-distance migration, to settle in a new environment and colonize the roots of local A. mangium. The same “invasion” was also observed in Madagascar [49].

The question of the persistence of the inoculant in soil is crucial for both ecological and agronomic reasons. Whether the introduced strain remains in the soil, and whether it has an impact on the local indigenous microbial population are important questions that have been addressed several times for soybean. Although Brutti et al. [7] confirmed that B. japonicum strains could be recovered 5 years after introduction in an arable soil in Argentina, Obaton et al. [43] showed that this stability could be extremely variable between sites, suggesting that the problem of competition could be solved by repeated inoculation. Loureiro et al. [25] showed that the introduction of bradyrhizobial strains in a soil that was originally devoid of nodulating and effective rhizobia had an impact on local rhizobial diversity, possibly through acquisition of the ability to nodulate soybean for local strains. The influence of environmental factors was also highlighted by Hungria and Vargas [20] in terms of nodulation limitation, but also in the selection of stress tolerant inoculants strains. Our results, even though based on B. elkanii when all other studies have focused on B. japonicum, confirmed that the stability and persistence of an inoculated strain is unpredictable. A combination of many factors, including competition for both nodulation of hosts and adaptation to environmental factors, needs to be considered to estimate the survival of strains in soil.

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Appendix A. Supplementary data

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