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Culture-independent assessment of the diazotrophic *Bradyrhizobium* communities in the Pampa and Atlantic Forest Biomes localities in southern Brazil

Joanna Banasiewicz [a], Bruno B. Lisboa [b], Pedro B. da Costa [c], Gilson Schlindwein [b], Stephanus N. Venter [d], Emma T. Steenkamp [d], Luciano K. Vargas [b], Luciane M.P. Passaglia [c], Tomasz Stępkowski [a,*]

[a] Department of Biochemistry and Microbiology, Institute of Biology, Warsaw University of Life Sciences (SGGW), Nowoursynowska 159, 02-776 Warsaw, Poland
[b] Fundação Estadual de Pesquisa Agropecuária (FEPAGRO), Rua Gonçalves Dias 570, 90130-060 Porto Alegre, RS, Brazil
[c] Departamento de Genética and Programa de Pós-graduação em Genética e Biologia Molecular, Instituto de Biociências, Universidade Federal do Rio Grande do Sul. Av. Bento Gonçalves, 9500, Caixa Postal 15.053, 91501-970 Porto Alegre, RS, Brazil
[d] Department of Biochemistry, Genetics and Microbiology, Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria 0002, South Africa

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ABSTRACT

The isolation of rhizobial strains from the root and stem nodules remains a commonly used method despite its limitations as it enables the identification of mainly dominant symbiotic groups within rhizobial communities. To overcome these limitations, we used genus-specific *nifD* primers in a culture-independent assessment of *Bradyrhizobium* communities inhabiting soils in southern Brazil. The majority of *nifD* sequences were generated from DNA isolated from tropical-lowland pasture soils, although some soil samples originated from the Campos de Cima da Serra volcanic plateau. In the nifD tree, all the bradyrhizobial sequences comprised 38 clades, including 18 new clades. The sequences generated in this study were resolved into 22 clades and 21 singletons. The *nifD* bradyrhizobial assemblage contained *Azorhizobium* and α-proteobacterial methylotrophic genera, suggesting that these genera may have acquired their *nif* loci from *Bradyrhizobium* donors. The most common in the lowland pasture soils subclade III.3D branch comprises the isolates of mainly an American origin. On the other hand, subclade III.4, which was earlier detected in Brazil among *Bradyrhizobium* isolates nodulating native lupins, appears more common in the Campos de Cima da Serra soils. The second-largest group, Clade XXXVIII, has not yet been reported in culture-dependent studies, while another common group called Clade I represents a symbiovar predominating in Australia. The identification of the diverse *nifD* Clade I haplotypes in the tropical-lowland pastures infested by Australian *Acacia* spp implies that the introduction of these legumes to southern Brazil has resulted in the dissemination of their bradyrhizobial symbionts.
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Introduction

The emergence of new molecular and bioinformatic methods in the last 20 years has contributed to breakthroughs in our understanding of the complexity of microbial communities. One of the most remarkable achievements was the discovery of multiple new phyla of mostly uncultivable Bacteria and Archaea, which was followed by the elucidation of their metabolic properties [10,12,34,58]. These new discoveries have revealed the complex functional interdependencies among bacterial and archaeal taxa, shedding light on the causes of their unculturability [47]. In most cases, these advances were catalyzed by increased access to next-generation sequencing technologies. Recent genome-resolved metagenomics has dramatically expanded our understanding of microbial diversity [33,73,84]. However, mass-scale sequencing of PCR amplicons (also referred to as metabarcoding) remains valuable for assessing the taxonomic composition of microbial communities from diverse habitats, for instance, when using the 16S rRNA gene as marker Semenov et al. [65]. By employing protein-coding gene markers such as those involved in biogeochemical cycles (e.g., nitrogen and sulfur cycles), metabarcoding studies can focus on organisms critical to these processes [9]. The use of protein-coding genes as markers for metabarcoding studies also enables the evaluation of microbial community structure at much finer scales of taxonomic resolution than is usually possible with 16S rRNA data alone [63].

* Corresponding author.
E-mail address: sttommic@yahoo.co.uk (T. Stępkowski).

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Despite the progress achieved in the metabarcoding analyses of microbial communities from diverse habitats, much remains to be learned about microbes associated with plants, including bacteria (collectively termed rhizobia) involved in symbiotic association with Leguminosae (Fabaceae). Rhizobia have an enormous ecologic impact delivering the majority of accessible nitrogen in terrestrial habitats [79]. Notably, although the application of new research technologies led to the discovery of novel taxonomic lineages, nearly all knowledge concerning the diversity of rhizobia comes from studies of root or stem-nodule isolates [68,77]. Culture-dependent approaches have several limitations because the rhizobial strains isolated from nodules may belong to dominant taxa, representing only a tiny proportion of the diversity within the local community. Therefore, there is a growing conviction that the elucidation of rhizobium community structure requires the application of alternative methods, especially the methods exploiting novel molecular and bioinformatic technologies [33,54].

All known rhizobia are classified in 18 circumscribed genera belonging to α and β-Proteobacteria [20]. *Bradyrhizobium* is probably the most divergent and cosmopolitan genus among all these genera due to the broadest range of nodulated Fabaceae tribes [5,45,49]. Until recently, this taxon has been perceived as mainly involved in the nitrogen-fixing symbiosis with Fabaceae plants [8,68,72]. For this reason, the majority of the published works have focused on legume-nodulating isolates even though a growing number of reports describe non-nodulating or even non-diazotrophic *Bradyrhizobium* isolates [13,19,30,35,48,50,64,77]. The existence of a diverse group of *Bradyrhizobium* species is also supported by the fact they often represent the dominant bacterial group in many habitats, regardless of the occurrence of potential legume hosts [22,23,36].

In this work, we aimed to use a culture-independent method for elucidating the diazotrophic portion of the *Bradyrhizobium* communities in soils collected in the Rio Grande do Sul, in southern Brazil. We specifically targeted areas representing remnants of the Pampa Biome and Araucaria moist forest (Atlantic Forest Biome), both of which are highly endangered ecosystems in Brazil due to the intensive agricultural exploitation and the introduction of exotic plant species [40,56,57,62]. To achieve our aim, we used metabarcoding based on *Bradyrhizobium*-specific primers for the generation of *nifD* amplicons from DNA extracted from soil samples. What is worth mentioning, this project followed the phylogenetic characterization of *Bradyrhizobium* isolates recovered from soil samples collected in the same areas as the current study. In this culture-dependent study, the bradyrhizobia were isolated from root nodules of several Fabaceae species used as legume traps [6]. The application of the two different approaches allowed us to evaluate their effectiveness and limitations.

Materials and methods

Description of sampling sites

Twenty soil samples were collected at six different sites (A-F) in the Rio Grande do Sul – Brazil's southernmost state (Fig. 1). With two exceptions (sample B-16–57 and F-16–46), soil samples were taken from the rhizosphere (10–20 cm depth) of the Fabaceae species (see Table 1, where information concerning sampling sites is included). Most of our soil samples (14) were collected at Viamão, in tropical pasture representing plant communities typical for the Crystalline Shield in the Rio Grande do Sul. This Pampa Biome plant community is characterized by the predominance of herbaceous plants belonging to the Gramineae, Asteraceae, and Fabaceae (Leguminosae) [62]. The dominant vegetation comprises the grasses *Andropogon lateralis* and *Sorghastrum albescens* [3,66]. At Viamão, soil samples were collected in February 2016 and 2017 at three sites (A, B, and C; each comprising an area 3 m × 3 m). Site A comprised part of a gently sloping hill. Site B was situated somewhat higher, near a granite outcrop, while *Acacia mearnsii* trees infested site C. The red clay Ultisol soils in these areas are acidic and highly weathered. They are also depleted in calcium, magnesium, and potassium.

From Sites D, E and F, soil samples were collected in February of 2016. Sites E and F were located in the Campos de Cima da Serra, a basaltic plateau rising to 900–1200 m.a.s.l. The plateau was originally covered by the Araucaria Moist Forest, much of which has been turned into pasture. The Campos de Cima da Serra is also inhabited by some temperate legumes, both native (*Lupinus* spp.) and exotic (*Ulex europaeus*) [16]. The typical soils associated with the plateau are unfertile and acidic [3]. Sample D-16–1, collected in February of 2016, was originated from the rhizosphere of *Chamaecrista falcata* growing in fertile Mollisol soil (Table 1).

Isolation of bacteria from root nodules

Rhizobial strains were isolated from two soil samples (A-16-53 and B-16-58) collected at Viamão using *Macroptilium atropurpureum* and *Crotalaria tweediana* as a plant-trap. The rhizobia obtained using this approach were used in all subsequent analyses, particularly the molecular experiments where they served as positive controls. For the purposes of trapping, seeds were immersed in 96% alcohol for 30 s and sodium hypochlorite solution (3.5% w/v) for 5 min for surface disinfection. Afterward, they were washed five times with sterile distilled water. The seeds were then placed in Petri dishes with sterile distilled water, and after seven days, the seedlings were transferred to 2 L pots filled with sterilized vermiculite and 0,05 kg of soil sample and cultivated for three months. Non-inoculated control plants were sown in sterilized vermiculate to verify the aseptic conditions of the experiment. Ten-times diluted, nitrogen-free Jensen medium was used for watering the growing plants [78]. Disinfection of nodules and bacteria isolation was performed as described by Beukes et al. [8]. The nodule isolates were grown at 28 °C on a modified yeast extract-mannitol agar (HPC) medium containing mannitol, succinate, galactose, and glycerol as carbon sources [6]. The plates were incubated for up to ten days, and after this time, individual colonies were collected to obtain a pure culture of each rhizobial isolate. All the pure cultures were grown on HPC agar plates supplemented with bromothymol blue as described in Somasegaran and Hoben [67] for separating *Bradyrhizobium* strains from other bacteria.

Molecular methods

DNA isolation from soil samples was performed with MO BIO PowerSoil® DNA Isolation Kit (Qiagen, Germany). The concentration of DNA samples was measured using an Eppendorf BioSpectrometer® basic (Eppendorf, Germany) spectrophotometer. Total genomic DNA of *Bradyrhizobium* isolates obtained from root nodules of legume traps was extracted from their fresh cultures (grown on agar HPC medium), using the PrepMan® Ultra Sample Preparation Reagent (Applied Biosystems, USA) [6]. Before *nifD* amplification, the purity of DNA samples was assessed with PCR tests targeting the amplification of partial sequences of 16S rRNA as described elsewhere [81]. The FastStart™ High Fidelity PCR System (Merck, Germany) was used in all amplification assays, and the primers used are listed in Supplementary Table S1. Briefly, universal primers TSnifDf1 and TSnifDr1 were used for the soil samples tested and for all nodule isolates. In addition, some modified *nifD* primers were used to detect members of Clade VII and subclade III.4 [69]. Amplicon sizes were estimated using 1% agarose gel

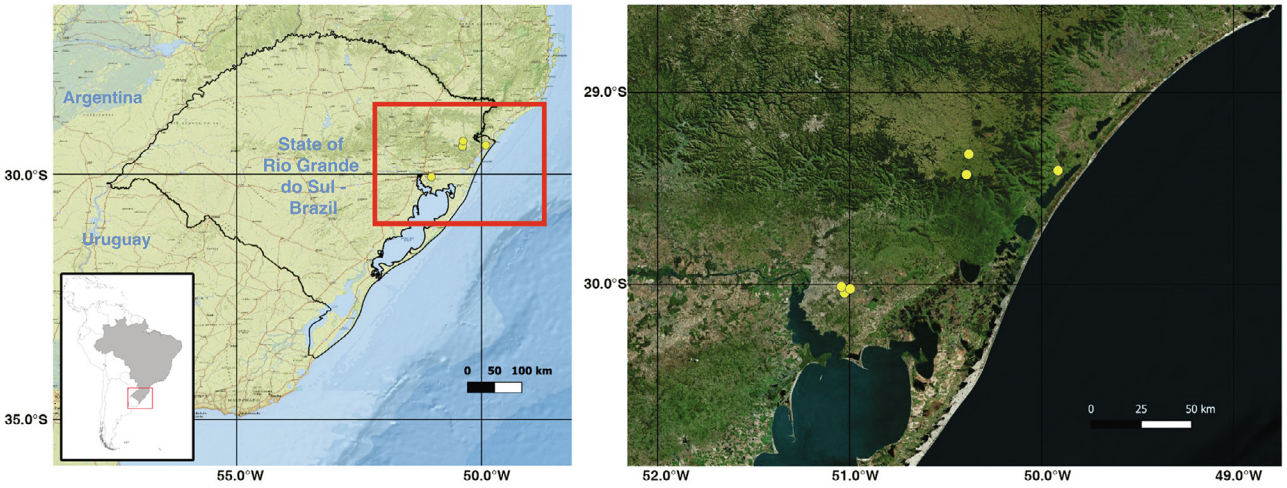

Fig. 1. Locations where the samples were collected (yellow points on the map).

Table 1
Characteristics of the sampling sites and the soil samples with their geographic coordinates, type of soils and the nearest host plants.

Site	Coordinates	Elevation (m a.s.l.)	USDA soil taxonomy (ST)	Geology	Sampling year	Soil sample	Legume plant rhizosphere
A	S30° 2′37.49″/W51° 1′19.42″	55	Ultisol	Granite	2017	17-1	*Desmodium* sp.
						17-2	*Aeschynomene falcata*
						17-3	*Desmodium* sp.
						17-4	*Crotalaria tweediana*
						17-5	*Desmodium* sp.
						17-6	*Rhynchosia* sp.
						17-7	*Macroptilium prostratum*
					2016	16-53	*Macroptilium* sp.
B	S30° 2′43.10″/W51° 1′21.90″	55	Ultisol	Granite	2017	17-8	*Rynchosia corylifolia*
					2016	16-57	out crop soil
						16-58	*Stylosanthes* sp.
C	S30° 2′43.94″ /W51° 1′27.70″	48	Ultisol	Granite	2017	17-9	*Acacia mearnsii*
						17-10	*Acacia mearnsii*
						17-11	*Desmodium barbatum*
D	S29°24′24.6″/ W49° 54′43.3″	15	Mollisol	Basalt	2016	16-1	*Chamecrista* sp.
E	S29° 19′20.17″/W50° 22′41.52″	930	Inceptisol	Rhyolite	2016	16-36	*Lupinus reitzii*
						16-37	*Lupinus* sp.
F	S29° 25′26.89″/W50° 23′23.16″	910	Inceptisol	Rhyolite	2016	16-44	*Machaerium* sp.
						16-45	*Desmodium* sp.
						16-46	*Araucaria* deadwood soil

electrophoresis and purified with the ExtractMe DNA-gel-out kit (BLIRT, Poland).

The 35 *nifD* amplicon libraries were generated using DNA isolated from soil samples as a template. This was done by cloning the soil-derived PCR products using the CloneJET PCR Cloning Kit (Thermo Scientific, USA). For this purpose, competent *E. coli* TOP10 (Str^r) cells were prepared using the calcium-chloride method described by Chang et al. [15]. Transformants were cultured on LB-agar medium with ampicillin (100 μg/ml) and streptomycin (20 μg/ml) at 37 °C for 24 h. Each bacterial colony was transferred and grown on the same substrate for the next 24 h, after which DNA was extracted with the PrepMan® Ultra Sample Preparation Reagent. The extracted DNA was used for PCR reactions with pJET1.2 Forward Sequencing Primer and pJET1.2 Reverse Sequencing Primer (Table S1), applying DreamTaq™ DNA Polymerase (Thermo Fisher Scientific, USA). Amplicons were then purified with the ExtractMe DNA-gel-out kit and sequenced on an ABI3100 Automated Capillary DNA sequencer (Applied Biosystems, USA) using the two pJET1.2-specific primers. Chromatograms of *nifD* sequences were manually edited using BioEdit 7.2.3 [29], MultAlin software [17] and subjected to BLASTn searches [1] in order to compare them to the nucleotide sequences deposited in GenBank (National Centre for Biotechnology Information, http://www.ncbi.nlm.nih.gov/) [7]. Nucleotide sequence data reported are available in the NCBI GenBank database under the accession numbers MW436758 – MW437534 and MW455191 – MW455318 (Tables S2 and S3).

Phylogenetic analyses

The *nifD* sequences were aligned using ClustalX software [76], while ML phylogenies were inferred with Mega 6 [75] using the best-fit nucleotide substitution model (i.e., HKY + I + G) as indicated by jModelTest 2.1.4 [53]. Before the tree construction, the substitution rate was estimated at each codon position using DAMBE 5 [44,82]. Given that the substitution saturation associated with the third codon position in the *nifD* dataset was revealed, this position was excluded from further analysis. Branch support was estimated using nonparametric bootstrap analyses based on 500 replicates [25]. The datasets used in phylogenetic reconstructions included type strains of classified *Bradyrhizobium* species as well as a number of unclassified *Bradyrhizobium* strains obtained from GenBank using Blastn and Blast-microbes searching tools [1].

Statistical analyses

According to Clade and primer set, the association of sequence frequency was performed with the mosaic function from VCD package v1.4-4 [26]. Both Permutational Multivariate Analysis Of Variance (PERMANOVA) performed with the adonis function, and Non-Metric Multidimensional Scaling (NMDS) performed with the metaMDS function were based on Vegan package v2.4-2 [24]. Principal Component Analysis (PCA) was carried out with the prcomp function from the Stats package 3.5.2 [55]. All analyses were performed on R and are available on https://github.com/PedroBeschoren/Banasiewicz-et-al-2019/. See also Statistical analyses in Supplementary Material.

Results

nifD amplification strategy

A great number of microbial species present in the soil makes it one of the most challenging environments in any metagenomic research. To limit the probability of obtaining non-specific products, we used the FastStartTM High Fidelity PCR System (Merck, Germany) that is well suited for amplification of "difficult" targets, such as nodulation genes [72]. Initial amplification trials were carried out using pairs of various "external" and "internal" primers (not shown). However, we soon found out that the primers TSnifDf1 and TSnifDr1 (Table S1) that were applied in our previous works [8,74] enabled the generation of specific PCR products in a single amplification step on DNA templates isolated from most soil samples. The second round of amplification with the same primers was required only for DNA templates having low DNA concentrations. We also designed two pairs of forward primers that were specific for subclade III.4 (nifDf5fo and nifDf5fis) and Clade VII (nifDf7fo and nifDf7fi), i.e., for bradyrhizobia that were expected to occur in studied soils [70]. Although the designed primers cannot be regarded as clade-specific, we could generate *nifD* sequences specific for subclade III.4 and Clade VII, respectively, in soil samples originating from the Campos de Cima da Serra. The application of all these primers led to the generation of 35 distinct *nifD* libraries following the cloning of *nifD* amplicons in pJET1.2 vector. Finally, we generated 876 specific *nifD* sequences from almost all soil samples (except sample B-16-57, Table S2). In addition, 29 *nifD* sequences originated from the root nodule-isolates were obtained in the trapping experiments (Table S3).

Phylogenetic analysis of nifD partial sequences

The *nifD* dataset included both complete (genomic) and partial sequences deposited in the GenBank database. Our *nifD* dataset comprised all major symbiotic bradyrhizobial groups that have been identified so far [69]. Apart from the symbiotic reference genera (i.e., *Rhizobium*, *Ensifer*, *Paraburkholderia*, *Mesorhizobium*, and *Microvirga*), we included *nifD* sequences from the related genus *Rhodopseudomonas*. Given that the Blast searches revealed the similarity of bradyrhizobial sequences to *nifD* sequences of other proteobacteria, we included representatives of the genera *Methylococcus*, *Nitrospirillum*, *Methylocella*, *Methylosinus*, *Methylocystis*, *Sphingomonas*, *Xanthobacter*, *Methylovirgula*, and *Azorhizobium*. The final dataset consisted of 1 649 partial *nifD* sequences (751 bp in length) used for phylogenetic analysis, including the 876 metagenomic sequences and the 29 sequences obtained from legume traps (Fig. 2).

Within the *nifD* phylogeny (rooted with sequences from *Rhizobium*, *Sinorhizobium*, *Mesorhizobium*, and *Microvirga*) (Fig. 2), we observed clusters similar to groups described previously in phylogenies of the *nodA* and *nifD* genes [6,8,69]. The 905 *Bradyrhizobium* sequences generated in this work clustered in 22 major groups containing two or more sequences and 21 singletons. Of these 22 groups, five corresponded to Clade I, Clade III, Clade VII, Clade XVII, and Clade XX that have already been reported [6,44,69,70,74]. Eighteen clades representing novel *nifD* groups were referred to as Clade XXI-Clade XXXVIII, although Clade XXIX does not contain sequences obtained in this work (see Fig. 2). The number of sequences in these 22 groups ranged from 479 (including 29 sequences of the nodule isolates) for Clade III and 159 for Clade XXXVIII, to two sequences for Clade VII, Clade XXI, Clade XXIV, Clade XXVIII, Clade XXXIII, and Clade XXXIV (Table S2 and S3).

For convenience, the *nifD* phylogeny was divided into three parts, 1–3 (Fig. 2). The assemblage comprising part 1 corresponds to Clade III, the most divergent of the major symbiotic groups within the genus *Bradyrhizobium* [8,19,72]. Part 2 spans twelve clades, including Clade I, Clade V, Clade X, and Clade XVIII, all of which probably have an Australian origin [69,72], the pantropical Clade IV, as well as Clade VII, Clade XVI, and Clade XIX containing strains mainly from tropical parts of the Americas. Part 2 also includes Clade II and Clade XI containing Genisteae and Loteae isolates. Part 3 comprises 25 clades, including Clade VI and Clade XVII [69,74], encompassing both symbiotic and non-symbiotic diazotrophs [27,74].

Affinities within parts 1–3 of the nifD phylogeny

Clade III (portion 1 of the phylogeny) has been divided into four subclades, out of which subclade III.1 contains only two isolates from Senegal, subgroups III.2 and III.4 comprise strains of mainly an American origin, while the most diverse subgroup III.3 includes strains from all populated by Fabaceae continents, probably, except Europe [8,19,72]. All of the *nifD* sequences generated in this study were in subclades III.3, III.4. The published phylogenies split subgroup III.3 further into eight branches (III.3A-III.3H), forming a pattern reflecting partially the strains' geographical origin [69]. In the current work, we detected seven new branches (III.3I-III.3O), and five single-sequence lineages, out of which branches III.3I-III.3L were composed of the sequences obtained in this study (see Fig. 2).

Branch III.3D of Clade III contained 364 *nifD* sequences (representing 237 haplotypes) that were generated on DNA templates isolated from soil samples collected at Viamão tropical pasture (Fig. 2 and S1). Notably, this branch encompasses type strains of *B. embrapense* (SEMIA 6208 ^{T}), *B. tropiciagri* (SEMIA 6148 ^{T}), and *B. viridifuturi* (SEMIA 690 ^{T}), as well as type strains of *B. brasilense* and *B. uaiense* (the latter two are not included in the *nifD* phylogeny) [6] – all five species described in Brazil [21,31]. Branch III.3D also comprised 78 nodule isolates, including 27 strains recovered in this study from the nodules which formed on *Macroptilium atropurpureum* when used as a trap plant grown in soil collected at Viamão (Fig. S1, Table S3). Apart from Brazil, the strains clustering within branch III.3D originated from the United States, Mexico, and the Island of Guadalupe (France) [37,48,51]. Two strains, Cham227 and Cham231, were isolated in South Africa [8], while strain Wall12 originated from the Philippines [2] (see the list of subclade III.3D strains in Supplementary Table S2 and S3). The subclade III.3D sequences obtained in this work showed considerable diversity, usually occupying separate positions from the sequences obtained by elsewhere (Fig. S1).

Branch III.3A contained 26 of our metagenomic sequences, representing 26 haplotypes (Figs. 2 and 3). This branch comprises sequences obtained from *Bradyrhizobium* strains originating mainly from tropical and subtropical areas, many of which cluster with *B. elkanii* and *B. pachyrhizi*. However, the *nifD* sequences obtained from Brazilian soil samples grouped separately from these two species, albeit close to *Bradyrhizobium* isolates originat-

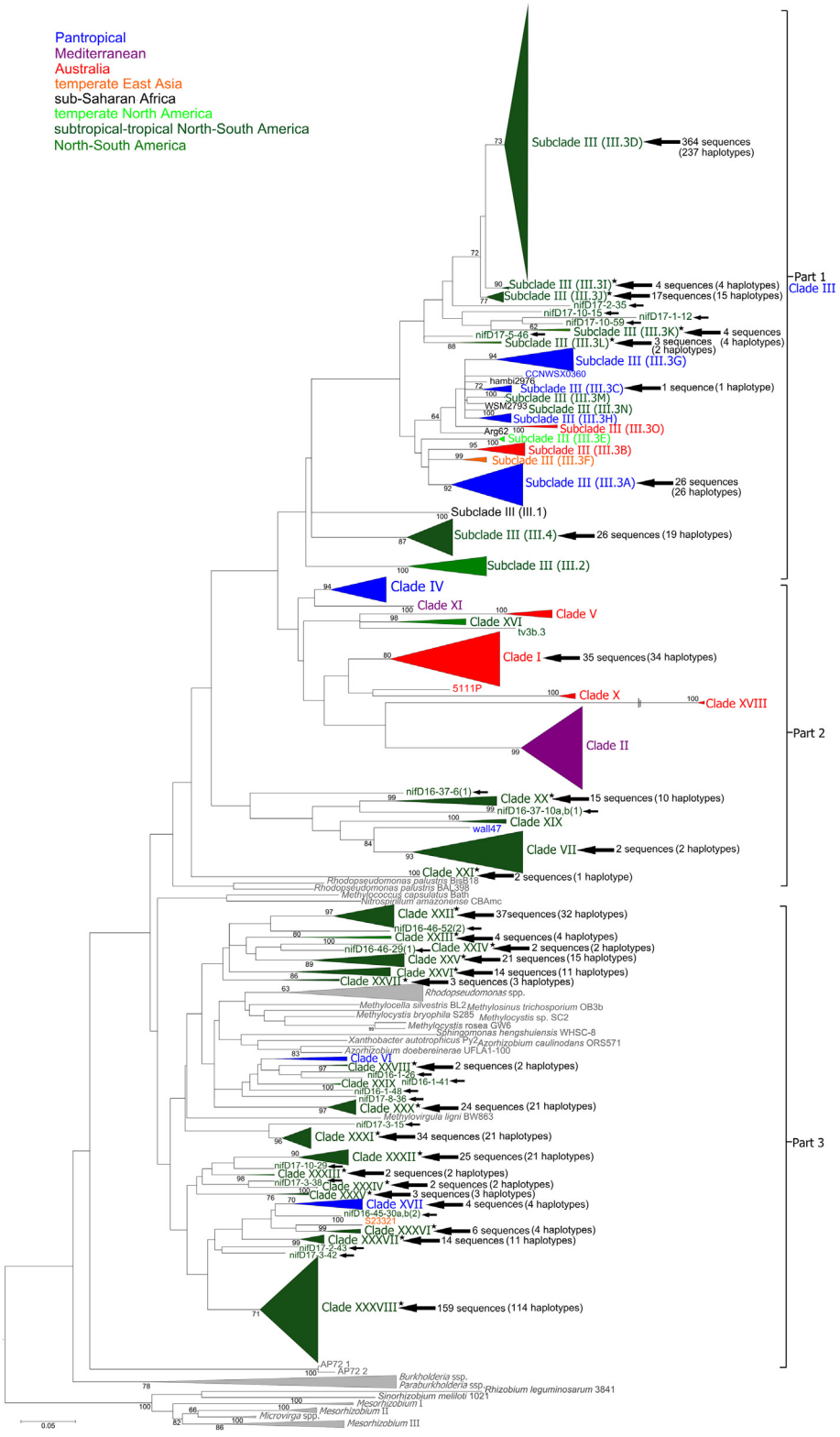

Fig. 2. Maximum likelihood (ML) tree based on partial *nifD* gene sequences (751 bp). The significance of each branch is indicated by the bootstrap percentage calculated for 500 bootstraps. Bootstrap values $\geq$ 60% are given at the branching nodes. The total number of sequences used in the construction of this phylogenetic tree is 1 649, including our 876 metagenomic sequences indicated with arrows. The phylogenies were also created with the use of *nifD* sequences obtained from 29 root nodule isolates; 27 (20 haplotypes) of them were affiliated to clade III.3D, 2 (2 haplotypes) to clade III.4 (not shown on the tree; see Table S3). Asterisks denote the new *nifD* groups that were obtained in this work.

ing from tropical northern Australia [72]. Seventeen *nifD* sequences (representing 15 haplotypes) detected at Viamão soils clustered within a new branch III.3J (Fig. 2).

Within part 2 of the *nifD* phylogeny, sequences generated in the current study clustered in Clades I and VII, as well as in new Clade XX and Clade XXI (Fig. 2). Clade I is the dominant *Bradyrhizobium* symbiotic group in Australia. Our previous work revealed two main subgroups, of which subclade I.1 strains originated from both temperate and tropical environments, whereas I.2 strains were so far detected only in tropical Australia [72]. The Clade I sequences (35 sequences, representing 34 haplotypes) obtained here showed considerable diversity, clustering within both the I.1 (31 sequences) and I.2 (four sequences). Apparently, Clade I *Bradyrhizobium* community at Viamão is divergent, and has not descended from a single clone (Fig. 4).

Part 3 of the *nifD* phylogeny contained most of our new clades (Clade XXII – XXXVIII) and also included previously identified Clade VI and Clade XVII haplotypes [69]. The metagenomic sequences generated in this work were restricted solely to Clade XVII (Fig. 2). Remarkably, part 3 of the *nifD* genealogy included sequences from *Azorhizobium caulinodans* ORS571, *A. doebereinerae* UFLA1-100 and non-symbiotic *Xanthobacter* sp. Furthermore, in this group the sequences originating from methylotrophic diazotrophs also clustered, including the genera *Methylocystis*, *Methylocella*, *Methylosinus*, *Methylovirgula* (all in the class α-Proteobacteria), and *Methylococcus* (γ-Proteobacteria). These sequences occupied internal positions within part 3 of the phylogeny, indicating that they may have a bradyrhizobial origin and were acquired via horizontal gene transfer events [4].

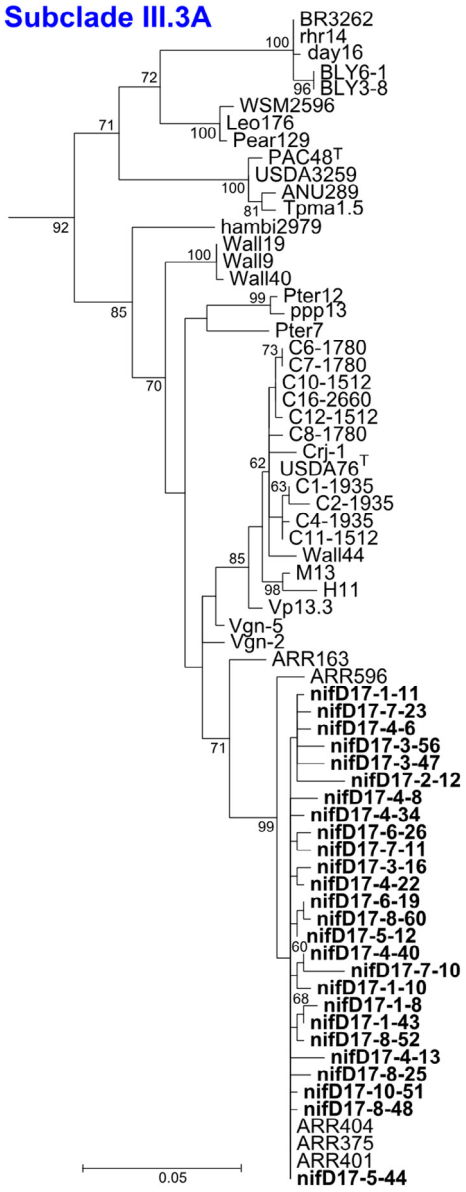

Fig. 3. Subclade III.3A of the *nifD* maximum likelihood (ML) phylogenetic tree. Bootstrap values ≥ 60% are given at the branching nodes. Our bradyrhizobial sequences are marked in bold.

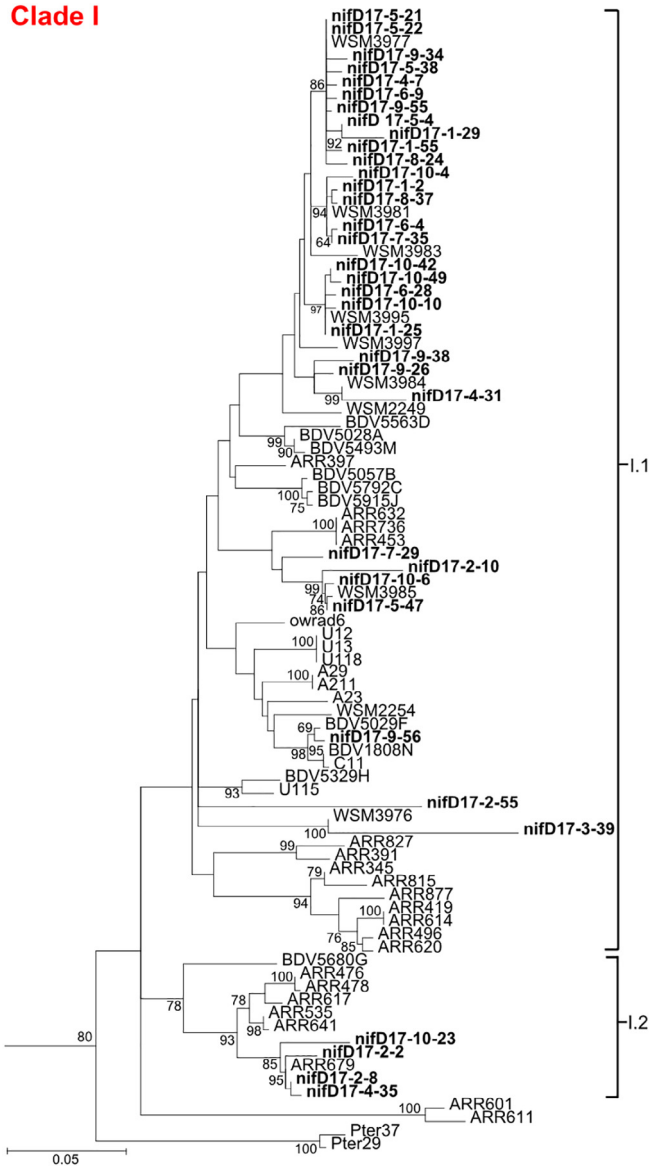

Fig. 4. Clade I of the *nifD* maximum likelihood (ML) phylogenetic tree. Bootstrap values ≥ 60% are given at the branching nodes. Our bradyrhizobial sequences are marked in bold.

Amplification of American-symbiotic gene groups

Blast searches revealed the lack of sequences showing similarity to some of the symbiotic clades we anticipated to detect. For instance, we expected to obtain a significant portion of *nifD* sequences belonging to Clade VII, a highly divergent group that seems to have a Neotropical origin [69]. So far, Clade VII isolates have been identified in south-eastern Brazil among lupin-nodulating bradyrhizobia and other, mainly tropical parts of the Americas [28,70]. In the GenBank, Clade VII is represented by over 70 sequences, which renders this group one of the largest among tropical *Bradyrhizobium* isolates. Unfortunately, the aligned *nifD* sequences lacked motifs that could be helpful in designing Clade VII-specific primers, therefore the primers used (see Table S1) were not regarded as specific for this group. Before the amplification tests were carried out on DNA templates isolated from soil samples, the primers' applicability was evaluated with positive results on genomic DNAs isolated from several Clade VII strains (data not presented). The application of the new primers using DNA isolated from six soil samples collected in the Campos de Cima da Serra resulted in the generation of 49 *nifD* sequences, of which only two (as revealed Blast searches) clustered in Clade VII (Fig. 2).

The application using primers specific for subclade III.4 were more successful (see Table S1 – the list of primers), yielding 26 III.4-specific sequences (19 haplotypes), which confirmed our assumption concerning the presence of subclade III.4 in the soils of the Campos de Cima da Serra (Fig. 5). As in the case of Clade VII, part of the generated sequences belonged to non-related to III.4 phylogenetic groups (Table S2). However, neither of the clade-specific primers made it possible to detect III.4 and Clade VII bradyrhizobia in soils originating from Viamão.

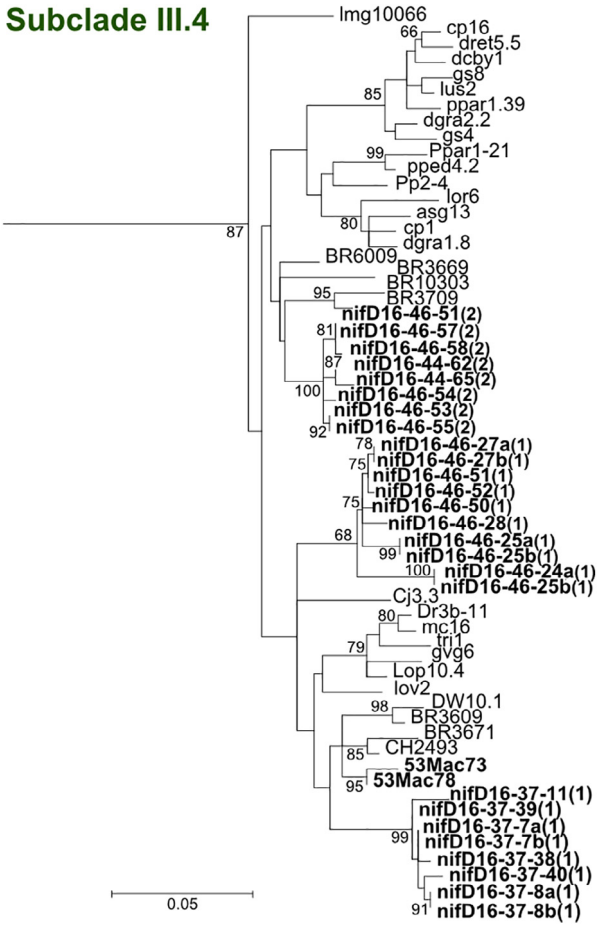

Fig. 5. Subclade III.4 of the *nifD* maximum likelihood (ML) phylogenetic tree. Bootstrap values ≥ 60% are given at the branching nodes. Our bradyrhizobial sequences are marked in bold.

Discussion

Application of the nifD gene in gene-directed metagenomics

In this work, the main objective was to develop an effective method for the characterization of Bradyrhizobium diazotrophic communities using genus-specific *nifD* primers. This method could complement the traditional approaches by making it easier to identify non-symbiotic diazotrophs or rare groups that often cannot be recovered from the root or stem nodules. We perceive it as a first step in the development of methods adapted to current massive DNA sequencing technologies. Our study is one of a few works where culture-independent approaches were employed in the characterization of rhizobial communities [42].

Notably, we uncovered 18 new phylogenetic groups out of the 38 clades within the diazotrophic bradyrhizobia, which is quite remarkable given the limited sequencing scale of this project. Some of the newly detected clades may belong to non-bradyrhizobial spp., as suggested by the placement of non-symbiotic methylotrophic diazotrophs and *Azorhizobium* spp. in the *Bradyrhizobium* branch. They have acquired their *nif* loci from bradyrhizobial lineages, a phenomenon that may have been facilitated by the co-occurrence of bradyrhizobia and methylotrophic Proteobacteria in plant tissues and the rhizosphere [83]. Likewise, the presence of the *Bradyrhizobium*-like *nifD* haplotypes in *Azorhizobium caulinodans* ORS571 and *A. doebereinerae* UFLA1-100 implies the acquisition of *nif* genes, a fact strengthened by the separate placement of the *nod* and *nif* genes in ORS571 chromosome [39].

Branch III.3D as the predominant group in the subtropical pasture soils

The identification of branch III.3D as the predominant group in the *Bradyrhizobium* community at Viamão corroborates our recent study focused on the characterization of nodule isolates recovered from soil samples using trap legumes [6]. These two studies reveal that both the traditional and culture-independent approaches give similar results, exposing the prevalence of branch III.3D bradyrhizobia in the tropical pasture soils at Viamão, as we could confirm with an NMDS ordination (see Supplementary Fig. S2). Notably, the *nifD* sequences of *B. brasilense*, *B. embrapense*, *B. tropiciagri*, *B. uaiense*, and *B. viridifuturi* also cluster in branch III.3D. Given that type strains of these five species were described in various parts of Brazil, this group may be widespread not only in southern Brazil [11,21,31,41].

Nonetheless, this work's outcome implies that the application of next-generation sequencing technology would be valuable for elucidation of the structure of rhizobium communities and uncovering low-abundance groups that may constitute the bulk of diversity in local rhizobial communities [33]. It became apparent when we initially failed to detect several *nifD* groups, which were expected to occur in the characterized soil habitats. This required the application of more specific primers, resulting in the detection of Clade VII and subclade III.4 sequences in the soils of the Campos de Cima da Serra but not at Viamão (see also Supplementary Fig. S3). Given that Clade VII and subclade III.4 strains were earlier detected in southern Brazil, we considered their identification important for the evaluation of our culture-independent method. Moreover, bradyrhizobia belonging to these two symbiotic groups appear to be associated with legumes that diversified in the Americas [28,69,70].

The co-occurrence of Acacia mearnsii and Bradyrhizobium Clade I strains in the lowland pasture soils

It can be assumed that the presence of *nifD* Clade I haplotypes in Brazil's pasture soils is due to the co-introduction of Australian *Bradyrhizobium* strains with their *Acacia* hosts [80]. What is worth emphasizing, strains of this symbiotic gene group predominate in both temperate and tropical Australia. It seems that their predominance results from this group's symbiotic association with the endemic Mirbelieae and Bossiaeeae tribes and the genus *Acacia*, constituting the dominant elements of the Fabaceae flora [18,72]. Although some *Acacia* species establish effective nitrogen-fixing symbiosis with indigenous rhizobia when introduced to non-native ranges, most species in this genus show a preference for their natural microsymbionts [80]. Thus, the high specificity of this symbiosis contributes to the spreading of Clade I bradyrhizobia with their acacia hosts, which can be inferred from the occurrence of Australian *Acacia* species in their non-native ranges and the presence of Clade I strains. So far, Clade I isolates have been reported in Portugal, South Africa, and New Zealand, always in areas infested by Australian acacias. The detailed studies carried out in Portugal and South Africa revealed that "exotic" Clade I bradyrhizobia established stable populations in the non-native areas where they even infect native legumes [59,60,61,80].

There has been a lack of studies focused on rhizobia nodulating Australian acacias in Brazil, where they pose a severe threat to the native ecosystems and have substantial commercial significance [14]. Despite these two contrasting factors, very little is known about rhizobia nodulating wattles in Brazil [43]. Our work indicates that the co-introduction with Australian *Acacia* spp. is the most likely mechanism leading to the dissemination of Clade I bradyrhizobia in Brazil. Given that the Clade I community in the pasture soil is highly divergent, it appears that a divergent group of Clade I bradyrhizobia has been introduced in Brazil, presumably, with soil-contaminated acacia seeds or saplings.

Although invasive legumes can be infected by indigenous rhizobia, they are often nodulated by their specific rhizobia if such are only available in the soil [38]. This preference explains the presence of lupin and serradella-nodulating Clade II bradyrhizobia in Western Australia and South Africa. In these countries, the introduced lupins and yellow serradella (*Ornithopus campestris*) plants form an effective nitrogen-fixing symbiosis with the divergent group of strains, all of which show phylogenetic affinity to European bradyrhizobia [71]. A similar correlation has been detected in the case of gorse (*Ulex europaeus*) and Scotch broom (*Cytisus scoparius*) in the Americas, where these legumes are nodulated by "European" Clade II strains, even though related "American" bradyrhizobia (nodulating local legumes) occur in the infested areas [6,32,38]. Thus, one can conclude that soil-contaminated seeds remain a primary source of exotic rhizobia accidentally introduced into new habitats [52].

Importantly, this sequencing project enabled the detection of new *nifD* gene groups, significantly expanding our knowledge about the scale of diversity within this genus while confirming the prevalence of the III.3D branch bradyrhizobia in the lowland tropical pasture soils. Furthermore, the identification of Clade I *nifD* haplotypes indicates that invasive Australian acacia plants impact the composition of *Bradyrhizobium* communities in southern Brazil. Our study is in line with earlier reports showing that the introduction of new plant species causes significant changes in soil microbiota, including the composition of rhizobial communities [46].

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.syapm.2021.126228.

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