Bradyrhizobium altum sp. nov., *Bradyrhizobium oropedii* sp. nov. and *Bradyrhizobium acaciae* sp. nov. from South Africa show locally restricted and pantropical *nodA* phylogeographic patterns

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Highlights

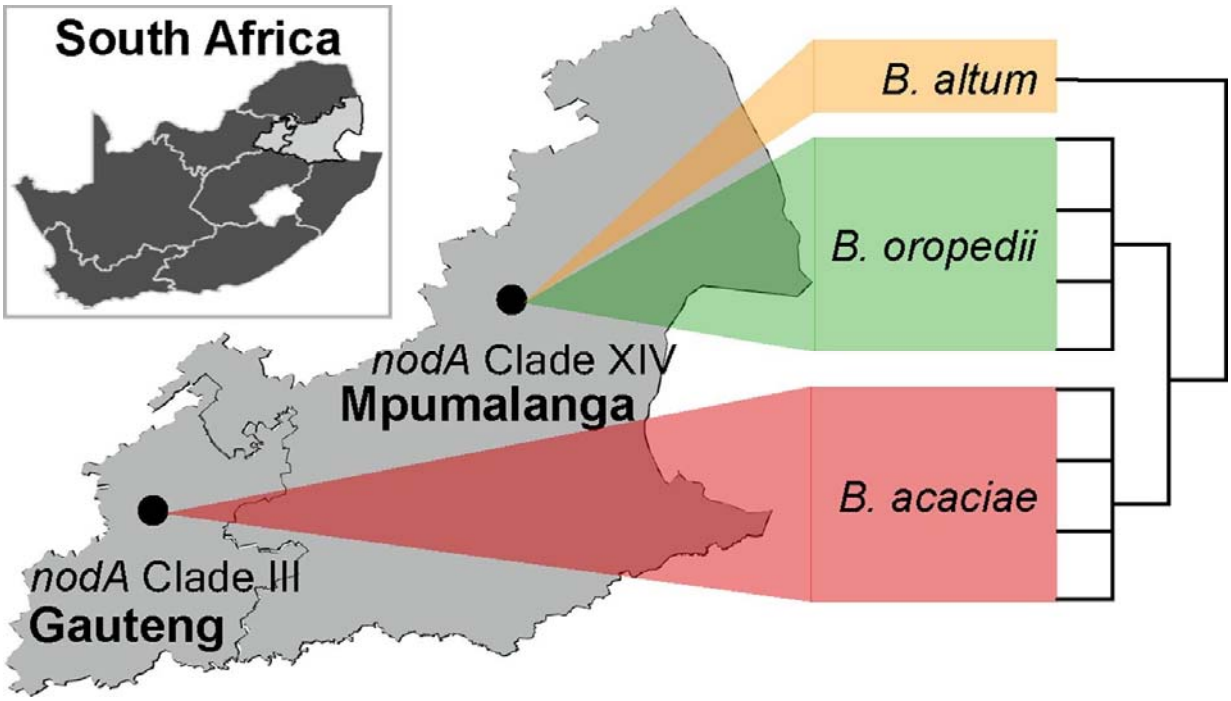
- *Bradyrhizobium* species isolated from diverse legumes in South Africa.
- Delineation of these species following the genealogical concordance approach.
- Novel *Bradyrhizobium* species represent *nodA* alleles specific to South African Clades.
- *Bradyrhizobium* species may have agricultural importance in sub-Saharan Africa.

Abstract

Africa is known for its rich legume diversity with a significant number of endemic species originating in South Africa. Many of these legumes associate with rhizobial symbionts of the genus *Bradyrhizobium*, of which most represent new species. Yet, none of the *Bradyrhizobium* species from South Africa have been described. In this study, phylogenetic analysis of 16S rRNA gene sequences of fourteen strains isolated in southern Africa from root nodules of diverse legumes (i.e., from the tribes Crotalarieae, Acacieae, Genisteae, Phaseoleae and Cassieae) revealed that

they belong to the *Bradyrhizobium elkanii* supergroup. The taxonomic position and possible novelty of these strains were further interrogated using genealogical concordance of five housekeeping genes (*atpD, dnaK, glnII, gyrB* and *rpoB*). These phylogenies consistently recovered four monophyletic groups and one singleton within *Bradyrhizobium*. Of these groups, two were conspecific with *Bradyrhizobium brasilense* UFLA 03-321^{T} and *Bradyrhizobium ivorense* CI-1B^{T}, while the remaining three represented novel taxa. Their existence was further supported with genome data, as well as metabolic and physiological traits. Analysis of *nodA* gene sequences further showed that the evolution of these bacteria likely involved adapting to local legume hosts and environmental conditions through the acquisition, via horizontal gene transfer, of optimal symbiotic loci. We accordingly propose the following names *Bradyrhizobium acaciae* sp. nov. 10BB^{T} (SARCC 730^{T}=LMG 31409^{T}), *Bradyrhizobium oropedii* sp. nov. Pear76^{T} (SARCC 731^{T}=LMG 31408^{T}), and *Bradyrhizobium altum* sp. nov. Pear77^{T} (SARCC 754^{T}=LMG 31407^{T}) to accommodate three novel species, all of which are symbionts of legumes in South Africa.

Graphical abstract



Keywords: *Bradyrhizobium elkanii* supergroup, species hypotheses, phylogeography, phylogenomics, nodulation

1. Introduction

Biological nitrogen fixation (BNF) plays a key role in environmental sustainability, especially in nitrogen-limited soil (Hungria et al., 2015, Lindström et al., 2010). In agriculture, for example, BNF associated with the legume-rhizobium symbiosis is essential for soil health, plant growth and food security in general (Gogoi et al., 2018, Jensen and Hauggaard-Nielsen, 2003). Among the diversity of known rhizobial bacteria (Howieson and Dilworth, 2016), those in the genus *Bradyrhizobium* are relatively well-studied. The genus represents a large and diverse assemblage of cosmopolitan bacteria capable of nodulating a variety of legumes from across the world (Parker, 2015, Sprent et al., 2017). It also includes non-nodulating endophytes of other plants (Chaintreuil et al., 2000), and non-diazotrophic strains that appear common in soils where legumes are absent (Jones et al., 2016, VanInsberghe et al., 2015).

Nodulating *Bradyrhizobium* species typically carry the loci conferring their symbiotic properties (e.g., nodulation or *nod* genes and nitrogen fixation or *nif* and *fix* genes) on so-called "symbiotic islands" within their genomes (Okazaki et al., 2015, Okubo et al., 2016). Because such genetic elements are prone to horizontal gene transfer (HGT), this process has greatly impacted the evolution and host range of *Bradyrhizobium* species (Beukes et al., 2016, Menna and Hungria, 2011, Moulin et al., 2004, Stępkowski et al., 2005,). By focussing on the *nodA* gene, for example, *Bradyrhizobium* strains may be separated into a range of distinct groups or clades that broadly reflect the geographic distribution of their legume hosts (e.g., Beukes et al., 2016, Menna and Hungria, 2011, Steenkamp et al., 2008, Stępkowski et al., 2012). Accordingly, agricultural practices involving the cultivation of non-native legumes, in locations outside their centres of origin and endemicity, are usually critically dependent on the application of suitable rhizobial inoculants (e.g., see Brockwell et al., 1995, Drew et al., 2012, Menna et al., 2006).

Bradyrhizobium was initially introduced to accommodate slow-growing rhizobia (Jordan, 1982) and, until about a decade ago, the genus consisted of relatively few species. Currently, there are more than 60 validly described *Bradyrhizobium* species (Bromfield et al., 2019a, 2019b, Bromfield and Cloutier, 2021, de Oliveira Urquiaga et al., 2019, Fossou et al., 2020, Helene et al., 2020a, Jang et al., 2018, Klepa et al., 2019, Klepa et al., 2021, Li et al., 2019, Rejili et al., 2020, Stępkowski et al., 2018, Wasai-Hara et al., 2020), which separate into at least seven phylogenetic clades or lineages that are typically referred to as "supergroups" (Avontuur et al., 2019, Hungria

et al., 2015, Menna et al., 2009, Stępkowski et al., 2012). Unlike the phylogenetic information contained within symbiotic gene sequences, these lineages or clades seem to reflect only broad geographic patterns and do not track the geographic distribution of the host plant associated with the bacteria (Beukes et al., 2016, Moulin et al., 2004, Steenkamp et al., 2008). Among the clades containing nodulating species, the *B. japonicum* supergroup has a broad distribution, occurring across various climatic zones and continents (Stępkowski et al., 2018, Vinuesa et al., 2008), the *B. elkanii* supergroup is more prevalent in subtropical and tropical regions (Delamuta et al., 2012, Delamuta et al., 2015, Vinuesa et al., 2008), while the *B. jicamae* and Kakadu supergroups generally occur in regions with Mediterranean and tropical climates (Guerrouj et al., 2013, Stępkowski et al., 2012). The only exception is the Photosynthetic supergroup that associates only with *Aeschynomene* species, although these distribution patterns might change as our knowledge of rhizobial diversity expands.

Apart from a few studies (e.g., Amrani et al., 2010, Puozaa et al., 2017, Steenkamp et al., 2008), rhizobial diversity are vastly underexplored in many regions in Africa (e.g., Grönemeyer and Reinhold-Hurek, 2018), including South Africa where three of the world's biodiversity hotspots are found (Hoveka et al., 2020). Also, economically important legumes such as those in the tribes Crotalarieae and Genisteae have their centres of origin or endemicity in southern Africa (Lewis, 2005). Both these tribes are predominantly nodulated by *Bradyrhizobium* symbionts (Ahnia et al., 2018, Beukes et al., 2016, Stępkowski et al., 2018, Stępkowski et al., 2007, Stępkowski et al., 2005, Stępkowski et al., 2012). However, knowledge regarding *Bradyrhizobium* nodulators of South African legumes is typically restricted to those associating with cultivated legumes (Helene et al., 2020b, Naamala et al., 2016, Steenkamp et al., 2008). Of the six species (three in each of the *B. japonicum* and *B. elkanii* supergroups) described from the region, most of them were isolated from agriculturally important legumes (e.g., *Arachis hypogaea*, *Vigna unguiculata* and *Cajanus cajan*) (Ahnia et al., 2018, Aserse et al., 2017, Bünger et al., 2018, Fossou et al., 2020, Grönemeyer et al., 2017, Grönemeyer et al., 2015a, 2015b, Grönemeyer et al., 2016). Ironically, southern African rhizobia are thought to have physiological properties that would make them ideal candidates for use in inoculants and biofertilizers (Grönemeyer and Reinhold-Hurek, 2018, Jaiswal and Dakora, 2019). Because of their ecological and agricultural importance (Grönemeyer and Reinhold-Hurek, 2018, Jaiswal and Dakora, 2019), knowledge about the identity and distribution of southern African rhizobia is thus imperative.

In this study, we sought to characterize putative new species in the *B. elkanii* supergroup that were previously collected from the nodules of various legumes in South Africa (Beukes et al., 2016, Boshoff, 2015, Steenkamp et al., 2008). We aimed to confirm the novelty of these *Bradyrhizobium* strains and to provide descriptions for them. For this purpose, genealogical concordance among five housekeeping genes (*atpD, dnaK, glnII, gyrB* and *rpoB*) was used to delineate putative species, after which evidence for these species hypotheses was sought using genome data and conventional phenotypic characters. For the respective strains included in the study we also confirmed their nodulation capabilities and determined the possible origins of their symbiotic loci. The results of this study therefore expand the number of described species from Africa and add to the growing body of information regarding the phylogeography of *Bradyrhizobium* and its symbiotic loci.

2. Materials and Methods

2.1. Bacterial isolations and growth conditions

A set of 14 strains potentially belonging to novel *Bradyrhizobium* species were used in this study (Beukes et al., 2016, Boshoff, 2015, Steenkamp et al., 2008). They originate from the root nodules of various legumes indigenous to South Africa, as well as cultivated or economically important legumes such as *V. unguiculata* and *Acacia* species (Table 1). The bacteria were grown on Yeast Mannitol (YM; 10 g/l D-mannitol, 0.5 g/l Yeast extract, 0.5 g/l K_2HPO_4, 0.2 g/l $MgSO_4$, 0.1 g/l NaCl) agar (15 g/l bacteriological agar) medium (Howieson and Dilworth, 2016). Growth was typically observed on this medium following incubation for 7 days at 28 °C. The selected type strains for each novel species were deposited at the Belgian Coordinated Collections of Microorganisms (BCCM/LMG) and the South African Rhizobium Culture Collection (SARCC).

2.2. DNA extractions, PCR and sequencing

This study employed DNA sequence information for five protein-coding housekeeping genes (*atpD, dnaK, glnII, gyrB* and *rpoB*), the 16S ribosomal RNA (16S rRNA) gene and nodulation gene *nodA*. For nine of the strains, sequences for the five protein-coding housekeeping genes and for *nodA* were available from a previous study (Beukes et al., 2016). For the remaining five strains, genomic DNA was extracted from YM agar cultures using Prepman™ Ultra (Applied Biosystems, USA), after which the missing *atpD, dnaK, glnII, gyrB*, *rpoB* and *nodA* sequences, as well as 16S

Table 1. Strain numbers, legume host, geographic origin and nodulation results for the proposed new *Bradyrhizobium* species described in this study.

Strain number	*Bradyrhizobium* species	Legume host	Geographic location[a]	Nodulation	Reference
19AJ	*B. acaciae* sp. nov.	*Acacia mearnsii*	Evaton, GP	cowpea, siratro	Boshoff, 2015
19AH		*Acacia mearnsii*	Evaton, GP	cowpea, siratro	Boshoff, 2015
16BA no1		*Acacia dealbata*	Vandyksdrift, MP	cowpea, siratro	Boshoff, 2015
10BB^T		*Acacia dealbata*	Wakkerstroom, MP	cowpea, siratro	Boshoff, 2015
Leo176	*B. oropedii* sp. nov.	*Leobordea lanceolata*	Dullstroom area, MP	cowpea, siratro	Beukes et al., 2016
Pear128		*Pearsonia obovata*	Dullstroom area, MP	cowpea, siratro	Beukes et al., 2016
Pear129		*Pearsonia obovata*	Dullstroom area, MP	cowpea, siratro	Beukes et al., 2016
Pear76^T		*Pearsonia obovata*	Dullstroom area, MP	cowpea, siratro	Beukes et al., 2016
Pear77^T	*B. altum* sp. nov.	*Pearsonia* obovata	Dullstroom area, MP	cowpea, siratro	Beukes et al., 2016
Arg68	*B. ivorense*	*Argyrolobium sericeum*	Badplaas area, MP	cowpea	Beukes et al., 2016
Cham227	*B. brasilense*	*Chamaecrista* sp.	Pretoria area, GP	cowpea, siratro	Beukes et al., 2016
Cham231		*Chamaecrista* sp.	Pretoria area, GP	cowpea, siratro	Beukes et al., 2016
Arg62		*Argyrolobium sericeum*	Badplaas area, MP	cowpea, siratro	Beukes et al., 2016
R5		*Vigna unguiculata*	Rasesa, Botswana[c]	cowpea, siratro	Steenkamp et al., 2008

[a] South African provinces are indicated as GP = Gauteng Province and MP = Mpumalanga Province.

rRNA sequences for all 13 strains, were determined by employing the PCR and sequencing protocols described by Beukes et al. (2016).

Electropherograms generated by the ABI3100 analyzer (Applied Biosystems, USA) were visualized and trimmed using ChromasLite v. 2.01 (Technelysium, Queensland, Australia) and BioEdit v. 7.05 (Hall, 1999). The sequences were then subjected to blastn searches to compare them to the available nucleotide sequences in GenBank (National Centre for Biotechnology Information (NCBI); http://www.ncbi.nlm.nih.gov/) (Altschul et al., 1990, Benson et al., 2004). This was done to confirm the strains' placement in *Bradyrhizobium* and the gene identity of the sequences. The sequences resulting from this study are available from the European Nucleotide Archive (https://www.ebi.ac.uk/ena) under the accession numbers listed in Supplementary Table S1.

2.3. Phylogenetic analysis

Single gene phylogenies were constructed for the *atpD, dnaK, glnII, gyrB*, *rpoB* and 16S rRNA gene sequence. The 16S rRNA dataset included the strains examined in this study, as well as the type strains of all described *Bradyrhizobium* species (until August 2020) with *Bosea thiooxidans* DSM 9653^T as the outgroup taxon. Individual datasets for *atpD*, *dnaK, gyrB, glnII* and *rpoB* were constructed to evaluate their genealogical concordance (Venter et al., 2017), while a concatenated dataset of the five genes were used for multi-locus sequence analysis (MLSA). These single protein-coding gene and concatenated datasets only included *Bradyrhizobium* type strains from the *B. elkanii* supergroup (Avontuur et al., 2019) with *B. japonicum* USDA6^T as the outgroup taxon. The nucleotide sequences of the protein-coding housekeeping genes that were not available in GenBank were extracted from relevant genome sequences, where available (Supplementary Table S1). This was done with local blastn searches in CLC Workbench v. 8.1 (CLC Bio; Aarhus, Denmark), using *B. elkanii* USDA76^T as reference. For those type strains that did not have genome sequences available, partial sequences of the respective housekeeping genes were obtained from GenBank (Supplementary Table S1). All the datasets were aligned with the MAFFT (Multiple Alignment using Fast Fourier Transform) online server (Katoh et al., 2017), using the default settings, except for the 16S rRNA dataset, for which the secondary structure of the RNA was considered, and the function Q-INS-I was used (Katoh and Toh, 2008). For generating the concatenated dataset, the nucleotide alignments for *atpD*, *dnaK, gyrB, glnII* and *rpoB* were

combined and partitioned using FASconCAT-G v. 1.02 (Kück and Longo, 2014). Maximum-likelihood (ML) analysis and non-parametric bootstrap analyses of 1000 repetitions were done in RaxML v. 8.2.1 (Stamatakis, 2014). The General Time Reversible (GTR) substitution model (Tavaré, 1986) with parameter optimization (Stamatakis, 2014) was employed for all ML analyses of the single gene datasets. ML analysis of the concatenated dataset also used this model, but with independent parameter optimization for each gene partition.

To infer the *nodA* phylogeny, the dataset included sequences for all *Bradyrhizobium* isolates investigated by Beukes et al., (2016) (accession numbers of isolates included can be found in the Supplementary Table S2 of the Beukes et al., 2016 study). The alignment consisted of haplotypes (i.e., identical sequences were represented by a single sequence) determined with DnaSP v. 5 (Librado and Rozas, 2009), and was supplemented with available *nodA* sequences of newly described *Bradyrhizobium* species and sequences examined in this study (Supplementary Table 1). FASconCAT-G v1.02 was utilized to exclude the third codon position, as this position is known to be highly saturated in *Bradyrhizobium* (Moulin et al., 2004, Stępkowski et al., 2012). An ML phylogeny was constructed with RaxML v. 8.2.1 using optimized parameters, and branch support was inferred with non-parametric bootstrap analyses of 1000 pseudoreplicates, as described above.

2.4. Genome-based analyses

To allow for genome-based analyses, we determined the genome sequences for six strains included in this study. Of these, three (Pear76^T, 10BB^T, Pear77^T) were subsequently chosen as type strains for the new species recognized here (see below). High-quality DNA was extracted using the hexadecyltrimethylammonium bromide (CTAB) method (Cleenwerck et al., 2002) and subjected to Ion Torrent™ PGM (Thermo Fisher Scientific) whole-genome sequencing at the Central Analytical Facilities (CAF) at Stellenbosch University (Stellenbosch, South Africa). For this purpose, the Ion Torrent 200-base pair (bp) chemistry and P1-Proton-chip platform were used. The single-ended reads produced were then filtered using the FASTX Toolkit (http://hannonlab.cshl.edu/fastx_toolkit). Following removal of reads with an 85% coverage cut-off and a quality score below 20, the remaining sequences were assembled with Newbler v 2.9 assembler (Margulies et al., 2005), using infrastructure provided by the Bioinformatics and Computational Biology Unit of the University of Pretoria (Pretoria, South Africa).

All genome sequences examined in this study were subjected to Average Nucleotide Identity (ANI) analyses. Two ANI datasets were constructed, the first included a total of 18 genome sequences consisting of the six strains from this study and all available genomes of type strains in the *B. elkanii* supergroup. This dataset was then subjected to BLAST-based pairwise comparisons across shared genomic regions using JSpecies v1.2.1 (Richter and Rosselló-Móra, 2009). The second dataset included genome sequences generated in this study and all the representatives (i.e., described, and undescribed species) of the *B. elkanii* supergroup, corresponding to the phylogenomic tree reported by Avontuur et al. (2019). A total of 41 genome sequences was therefore subjected to FastANI (Jain et al., 2017) for pairwise comparisons, using Mashmap as a search algorithm (Jain et al., 2017, Jain et al., 2018). A custom script was used to compute multiple pairwise ANI-values against a single reference genome at a time in a sequential manner (Palmer et al., 2020).

We also examined the phylogenetic relationships among the new species recognized here and those known for the *B. elkanii* supergroup using genome data. For this purpose, for the Up-to-date Bacterial Core Gene (UBCG) pipeline (Na et al., 2018) was used, which allowed identification and extraction of 92 universal gene sequences from all the *Bradyrhizobium* strains in the *B. elkanii* supergroup with available genome information, as described before (Avontuur et al., 2019). The 92 aligned sequence sets were concatenated using FASconCAT-G v. 1.02 (Kück and Longo, 2014). The General Time Reversible (GTR) substitution model (Tavaré, 1986) was employed for each gene with independent parameter optimization according to the partitioning file produced. The dataset was then subjected to ML analysis with non-parametric bootstrapping based on 1000 replicates using RaxML v. 8.2.1 (Stamatakis, 2014).

2.5. Phenotypic characterization

A range of phenotypic characteristics was evaluated for the 14 strains investigated in this study. These tests included morphological characterization using Gram staining (Somasegaran & Hoben, 1994) and a Zeiss Stereo phase-contrast microscope with associated Auxiovision software. Different growth conditions were also tested using incubation at 28°C for 7 days. These included growth across a pH range of 4 to 10 in YM broth, and growth across a temperature range of 4 °C to 40 °C and at 0.3% to 2.5% (w/v) sodium chloride (NaCl) using YM agar medium. Motility, oxidase, and catalase tests were performed according to Gerhardt et al. (1994).

We subjected the strains to carbon source utilization tests using Biolog GN2 (bioMereux, France) and substrate assimilation tests using API 20NE (bioMereux, France), in both cases according to the manufacturer's protocol. Lastly, antibiotic resistance was evaluated after 28 °C for 4 to 7 days on YM agar medium using bio-discs (Mast Diagnostics and Oxoid) containing ampicillin (10 µg), kanamycin (30 µg), tetracycline (30 µg), gentamycin (10 µg), penicillin (10 µg), chloramphenicol (30 µg), streptomycin (10 µg and 25 µg), neomycin (10 µg) and erythromycin (30 µg), respectively.

2.6. Nodulation confirmation

The nodulation capability of the 14 strains was tested on the promiscuous legumes, cowpea (*V. unguiculata*) and siratro (*Macroptilium atropurpureum*), for which seeds were obtained from the Agricultural Research Council Plant Protection Research Institute (ARC-PPRI). Nodulation tests were performed as described previously (Beukes et al., 2016) but instead of Leonard jars, 50 ml conical polypropylene centrifuge tubes were used. Each strain was tested in triplicate on the two hosts, and the two sets of experiments each included a negative control where the plant was inoculated with sterile water. After 4 and 6 weeks, respectively, the cowpea and siratro plants were harvested, to observe nodules. The nodule interiors were examined for the presence of leghaemoglobin (i.e., nodule interior pink in colour), which is essential for nitrogen-fixation in legumes (Howieson and Dilworth, 2016). The identities of the resulting nodule isolates were confirmed by comparing their *glnII* sequences to those of the original strains.

3. Results

3.1. Delineation of putative species

Analyses of the 16S rRNA gene sequences showed that all of the strains examined in this study represented *Bradyrhizobium* (Supplementary Fig. 1). Although the 16S rRNA phylogeny generally lacked robust bootstrap support (BS), our isolates grouped close to species known to form part of the *B. elkanii* supergroup. Within this tree, eight of our strains (10BB^T, 19AH, 19AJ and 16BA no1, as well as Pear76^T, Leo176, Pear128 and Pear129) clustered with *B. uaiense* (UFLA03-164^T) and *B. algeriense* (RST89^T), while Pear77^T grouped with the type strains of *B. embrapense*, *B. mercantei*, *B. erythrophlei*, *B. viridifuturi* and *B. jicamae*. Strain Arg68 was closely related to *B. ivorense* CI-1B^T (88% BS), while the remaining four strains (R5, Cham227, Cham231, and Arg62)

clustered together with *B. brasilense* UFLA03-321^T, *B. elkanii* USDA76^T, *B. pachyrhizi* PAC48^T, *B. ripae* WR4^T, *B. macuxiense* BR10303^T, and *B. tropiciagri* SEMIA 6148^T (76% BS).

To further interrogate the potential novelty of these strains, we focused on the taxa of the *B. elkanii* supergroup and used DNA sequences for the genes *atpD, dnaK, glnII, gyrB* and *rpoB*. Evaluation of genealogical concordance among the five datasets allowed delineation of the 14 strains into five putative species (Fig. 1). Of these, five strains appeared to be conspecific with known species, i.e., strains Cham231, Cham227, R5 and Arg62 that grouped with *B. brasilense* UFLA03-321^T, and Arg68, which likely represents a member of *B. ivorense*. The remaining three species appeared to be novel as they were represented by strains from the current study only, for which we propose the names *B. acaciae* sp. nov. (containing 10BB^T, 19AJ, 19AH, and 16BA no1), *B. oropedii* sp. nov. (consisting of Pear76^T, Pear128, Pear129, and Leo176) and *B. altum* sp. nov. (Pear77^T). The single-strain taxon *B. altum* sp. nov. (Pear77^T) occupied varying positions within the five single-gene genealogies. However, in all five of the single-gene trees, strains forming part of each of *B. brasilense*, *B. ivorense*, *B. acaciae* sp. nov. and *B. oropedii* sp. nov. consistently formed clusters, mostly with BS-values exceeding 75%. The only exceptions were for the cluster containing *B. oropedii* sp. nov. strains in the *atpD* and *dnaK* trees, where these strains were situated on a polytomy at the basal node of a well-supported *B. acaciae* sp. nov. clade, to the exclusion of any *B. oropedii* sp. nov. strains.

MLSA analysis based on the concatenated sequences for *atpD, dnaK, glnII, gyrB* and *rpoB* also allowed robust delineation of the three putative species (Fig. 2). Clades representing *B. brasilense*, *B. ivorense*, *B. acaciae* sp. nov. and *B. oropedii* sp. nov. received 99-100% BS. MLSA also confirmed that *B. acaciae* sp. nov. and *B. oropedii* sp. nov. are closely related to each other and to *B. uaiense* UFLA03-164^T (Fig. 1, 2 and Supplementary Fig S2). MLSA further showed that *B. embrapense* SEMIA6208^T is the closest known relative of *B. altum* sp. nov.

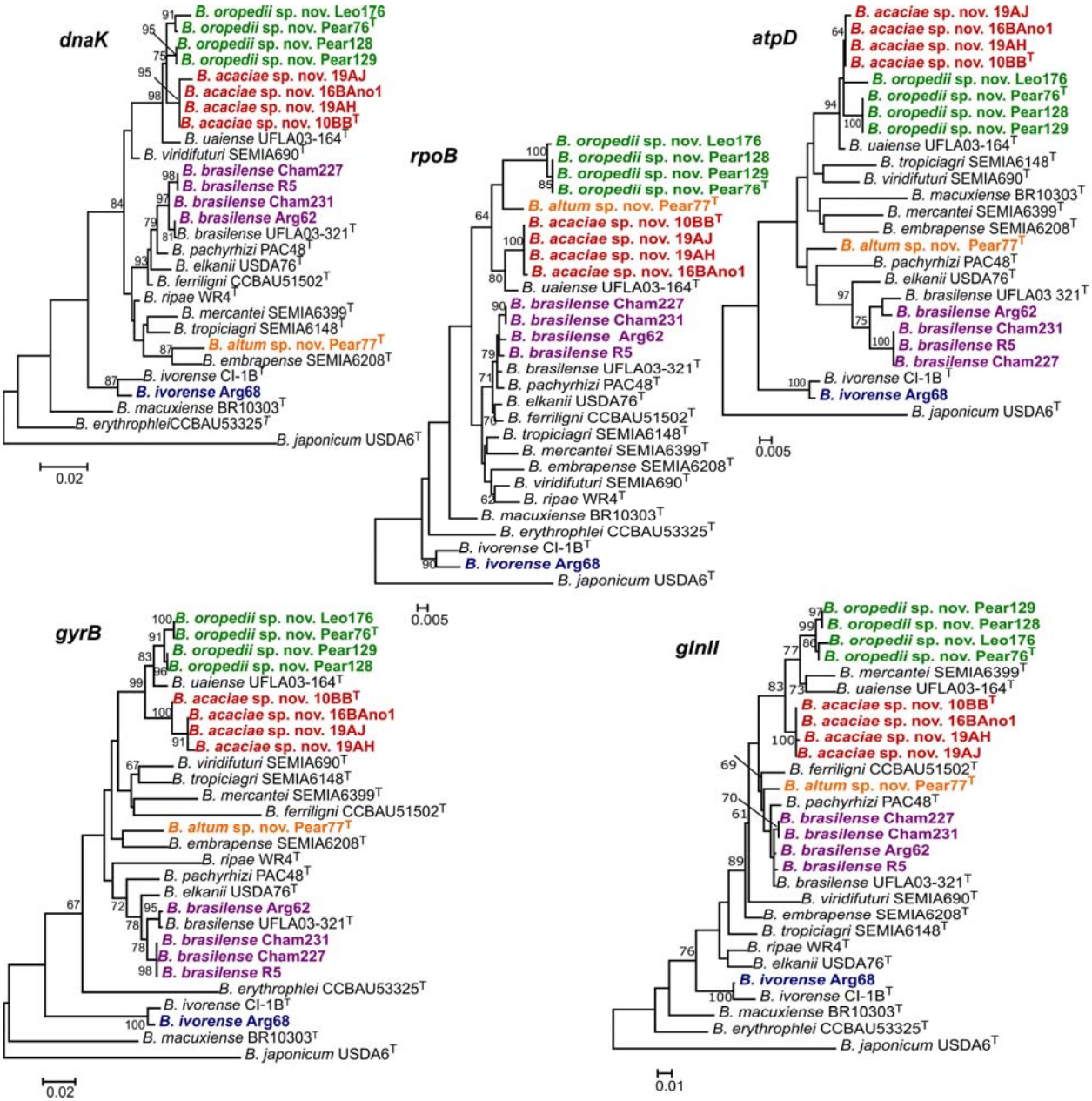


Fig 1: Individual maximum-likelihood phylogenies of *atpD, dnaK, glnII, gyrB* and *rpoB* housekeeping genes. These phylogenies only include *Bradyrhizobium* type strains from the *B. elkanii* supergroup, although the *atpD* phylogeny did not include *B. ripae* WT4^T, *B. erythrophlei* CCBAU 53325^T, and *B. ferriligni* CCBAU 51502^T due to the lack of sequence data. In each phylogeny *B. japonicum* USDA6^T was used as the outgroup. Strains of the species investigated in this study are indicated in colour. Bootstrap values were inferred from 1000 replicates and only those greater than 60% are shown on the nodes. The scalebars indicates nucleotide substitutions per site.

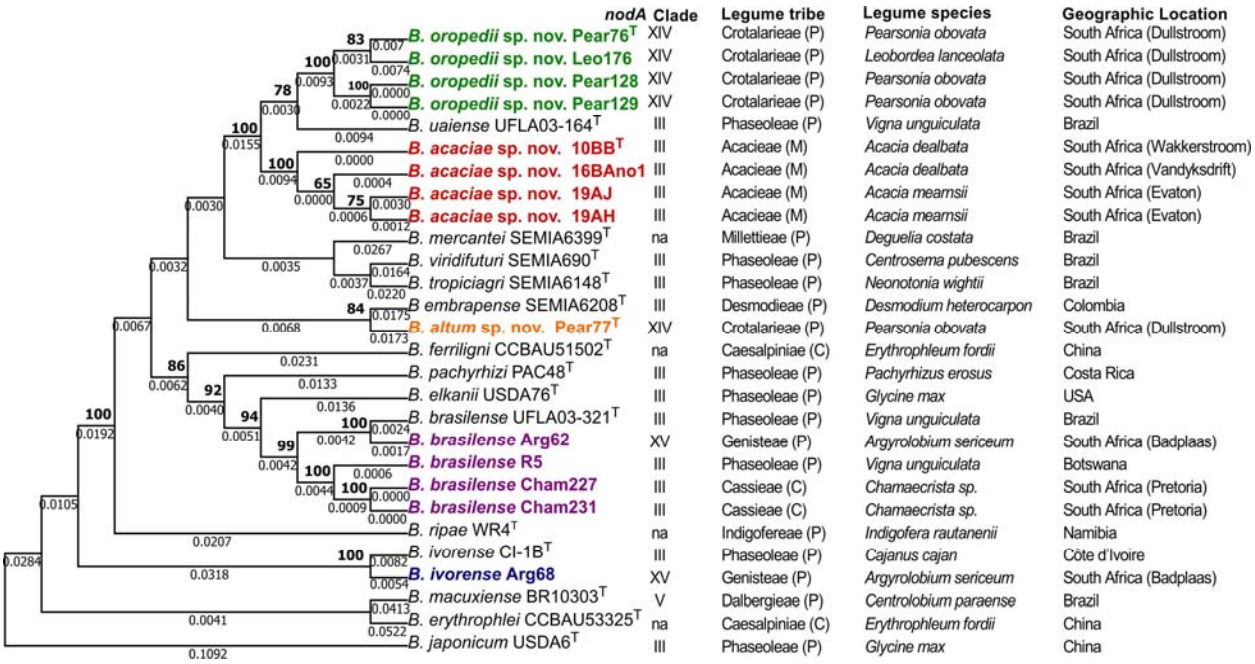


Fig 2: Cladogram inferred from the concatenated (*atpD, dnaK, glnII, gyrB* and *rpoB*) maximum-likelihood phylogeny. Only type strains from the *B. elkanii* supergroup were included in the analysis. *Bradyrhizobium japonicum* USDA6^T was used as an outgroup. Bootstrap support values were inferred from 1000 replicates and only values greater than 60% are indicated in bold at the nodes, while branch lengths (i.e. indicative of the nucleotide substitutions per site) are shown below the branches. Strains of the species investigated here are indicated in their respective colours. For each taxon, the legume tribe, host and geographic location from where it was isolated is also listed, including their *nodA* clade affiliation. *Bradyrhizobium* species with no available *nodA* sequence is indicated as "na" under *nodA* Clade. The P and C in brackets behind the Tribe indicate subfamily Papilionoideae or Caesalpinioideae, while M indicates the Mimosoid Clade within the Caesalpinioideae. The closest town names from which the South African strains were isolated were also included under geographic location.

3.2. Putative species supported by genome data

In order to interrogate the species hypotheses generated using housekeeping gene sequences, we employed genome data. For this purpose, the draft genomes for the strains 10BB^T, Pear76^T, Pear77^T, Cham227, Arg62 and Arg68 were assembled, while the genome sequence for strain R5 was previously generated by the Joint Genome Institute (JGI) as part of Phase III (KMG-III) of the Genomic Encyclopedia of Bacteria and Archaea (GEBA) project (Avontuur et al., 2019, Whitman et al., 2015). Overall, the sequencing depth for these 8.5-9.8 Mb genomes ranged between 63x and 119x, consisted of 371-1240 contigs, with GC content ranging between 63.3%

and 64.1%. Also, given the high N-50 values for these assemblies (Table 2), the quality and contiguity of the genome data generated here were thus in accordance with the recommendations for species delineation in taxonomic studies (Chun et al., 2018, de Lajudie et al., 2019). All six of the genomes were submitted to NCBI (see Supplementary Table S1 for accession numbers).

Table 2. General information for the seven draft genomes assembled in this study.

***Bradyrhizobium* strain**	**Number of contigs**	**N50**	**Coverage**	**Genome size (bp)**	**%G+C**	**NCBI Accession number**
B. acaciae sp. nov., 10BB^T	371	47328	89x	8,480,983	63.6	JACMYK000000000
B. oropedii sp. nov., Pear76^T	1053	36050	103x	9,199,930	63.5	JACMYO000000000
B. altum sp. nov., Pear77^T	685	28950	83x	9,781,279	63.3	JACMYP000000000
B. brasilense Cham227^T	571	33778	119x	9,121,295	63.7	JACMYN000000000
B. brasilense Arg62	703	28398	85x	9,261,942	63.7	JACMYL000000000
B. ivorense Arg68	1240	19201	92x	9,509,457	64.1	JACMYM000000000

ANIb analysis using the genome sequences for strains 10BB^T, Pear76^T, Pear77^T, Cham227, Arg62, Arg68 and R5, as well as those of the described *Bradyrhizobium* type strains within the *B. elkanii* supergroup supported our species hypotheses (Fig. 3). Values for ANIb were mostly below 92% between the proposed new species and the type strains for known species. The only exceptions were for comparisons involving *B. acaciae* 10BB^T, *B. oropedii* Pear76^T and *B. uaiense* UFLA03-164^T, which produced ANIb-values ranging from 94.18 % to 95.37%, reflecting their close phylogenetic relationship. The ANIb values among strains Cham227, Arg62, R5 and *B. brasilense* UFLA03-321^T yielded values of 95.9-98%. Strain Arg68 and *B. ivorense* CI-1B^T had an ANIb value of 96%. A similar pattern was observed with FastANI, although the identity values produced were generally slightly higher than those recorded for ANIb (Fig. 3, Supplementary Fig. S3 and Supplementary Table S3).

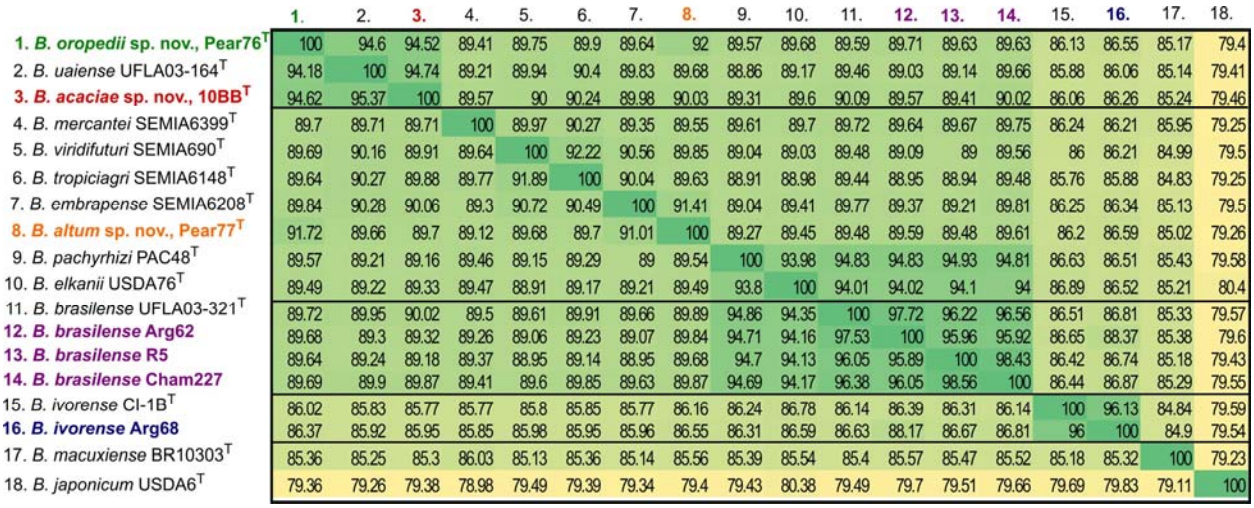


Fig 3: A heatmap illustrating the Average Nucleotide Identity (ANIb) similarity between *Bradyrhizobium* type strains within the *B. elkanii* supergroup. ANIb pairwise values were calculated using BLAST in JSpecies. Strains of the species investigated here are indicated in their respective colours. The black boxes around the strains highlight the strains investigated in this study and their closest relatives.

Genome-based phylogenetic relationships among our new species and those in the larger *B. elkanii* supergroup were examined using the UBCG pipeline to generate a 92-gene dataset (Supplementary Table S2). This nucleotide dataset included 41 taxa and was 92 769 bp in length and produced an ML phylogeny confirming the close relationship among *B. acaciae* sp. nov., *B. oropedii* sp. nov. and *B. uaiense*, as well as the grouping of strain Arg68 with the type strain (CI-1B^T) of *B. ivorense*. (Supplementary Fig. S2). This phylogeny further supported a monophyletic clade containing strains Cham227, Arg62 and R5 and other strains of *B. brasilense* (one of which, strain BR3262, requires taxonomic revision) with a bootstrap support of 100%. In the case of *B. altum* Pear77 ^{T}, its closest known relative was *B. embrapense* SEMIA 6208^T. Although other strains were more closely related to it, their taxonomic status likely requires revision. For example, strain MT12 was isolated from Forest soil in the USA (JGI Project ID: 1111457) and assigned to *B. erythrophlei*, but it is not conspecific with the type strain of the species (results not shown). The same is also true for strain BR3262 (results not shown) that has previously been assigned to *B. pachyrhizi*, but clearly belongs to *B. brasilense* based on the results from this study.

3.3. Putative species supported by phenotypic data

All 14 strains displayed growth on YM agar medium only after 7 days of incubation at 28 °C, which is consistent with generation times of 8-18 hours typical of *Bradyrhizobium* (Howieson and Dilworth, 2016). Colonies were cream in colour and ranged from 0.08 to 1 mm in size (Supplementary Table S4). Microscopically, these strains represented Gram-negative, rod-shaped, motile bacteria that ranged in size from 1.29 x 0.45 to 2.68 x 0.75 µm (Supplementary Table S4). In general, the new species showed high resistance to most of the antibiotics tested, except for Pear77^T that was susceptible to most of the antibiotics (Supplementary Table S4). Certain *B. acaciae* sp. nov. strains showed growth between 37-40 °C, while strains of *B. oropedii* sp. nov. and *B. altum* sp. nov. could only grow up to 35 and 37 °C, respectively. Additionally, Pear77^T showed growth in the presence of 2% NaCl, while most of the other strains could only withstand 0.5% NaCl concentration or below (Table 3). All strains were tolerant to pH levels ranging between 5 to 10 (Table 3).

Among the strains representing *B. brasilense*, Cham227, Cham231, R5 and Arg62 had similar growth abilities to those reported for its type strain UFLA03-321^T (e.g., growth at 15-37 °C and NaCl tolerance < 1.0%). However, strains Cham231 and Cham227 had a narrower pH range (6 - 10), compared to the other strains and what is known for *B. brasilense* (i.e., pH 4-10). The characteristics of strain Arg68 also corresponded to those reported for *B. ivorense*, although Arg68 had a broader temperature range (10-35 °C) and could grow at <0.5% NaCl.

Using Biolog GN2 and API 20NE, a range of biochemical characters were determined for the 14 strains. These analyses included the closest relatives for each of the new species proposed (Supplementary Table S5). We observed considerable intraspecific variation for *B. acaciae* sp. nov., *B. oropedii* sp. nov., *B. ivorense* and *B. brasilense* (Table 3 and Supplementary Table S5). Accordingly, few differential characters were found for *B. acaciae* sp. nov., *B. oropedii* sp. nov. and *B. uaiense* UFLA03-321^T. Nevertheless, the strains generally differed with regards to utilization of D-fructose, D-arabinose and D-mannitol. In the case of *B. ivorense*, strains Arg68 and CI-1B^T had over 15 traits in common (Supplementary Table S5).

Table 3. Distinctive phenotypes for the proposed new *Bradyrhizobium* species compared to their closest known relatives.

	B. *acaciae* (4 strains)	***B. oropedii* (4 strains)**	***B. uaiense* (2 strains)**	***B. viridifuturi* (3 strains)**	***B. tropiciagri* SEMIA 6148^T**	***B. mercantei* (2 strains)**	***B. altum* Pear77^T**	***B. embrapense* SEMIA 6208^T**	***B. pachyrhizi* (3 strains)**	***B. elkanii* USDA76^T**
Growth at:										
Temperature (°C)	15-40	15-35	15-37	<37	<37	<37	15-30	≤37	5-37	<37
pH	5-10	5-10	4-10	4.5-8	4.5-8	4.5-8	5-10	4.5-8	4.5-8	>4.5
NaCl (w/v %)	<0.5	<0.5	<0.75	<1	<1	<1	<2	<1	<1	≤ 1
Carbon utilization:										
L-Arabinose	+	v	+	+	+	+	+	+	+	+
D-mannose	v	v		+	+	+	-	+	-	-
D-Mannitol	-	v	+	w	w	+	-	w	+	+
N-acetyl-glucosamine	-	-		-	-	-	-	-	-	-
Potassium gluconate	+	v		-	-	-	-	+		+
Glycogen	-	-		-	-	v	-	+		w
D-Arabitol	-	v		w	w	+	+	w		+
D-Cellobiose	-	-		-	-	-	+	-	-	-
i-erythritol	-	-		-	-	v	-	-		-
D-Fructose	v	+	w	w	w	+	+	w	+	+
Gentiobiose	-	v		-	-	-	+	+		-
Maltose	-	-	-	-	-	v	+	-	v	-
D-Melibiose	-	-		-	-	-	+	+		-
D-Raffinose	-	-		-	-	-	+	-		-
α-hydroxy butyric acid	+	+					-			+
Sucrose	-	-	-	-	-	-	+	-		-
D-Trehalose	-	-		-	-	-	+	-	w	-
Turanose	v	-		-	-	w	+	-		-
Xylitol	-	-		w	-	+	+	-		-
Glycerol	+	v	w	w	w	+	+	w		+

Results are indicated as '+' positive for carbon utilization, 'W' weak for carbon utilization, '-' negative for carbon utilization, 'v' for variable results between strains within a species and grey blocks where no data are available.

Results of carbon utilization test for each known *Bradyrhizobium* type strain was obtained from respective published species descriptions. *B. viridifuturi* (Helene et al., 2015), *B. embrapense* SEMIA 6208^T and *B. tropiciagri* SEMIA 6148^T (Delamuta et al., 2015), *B. pachyrhizi* (Ramírez-Bahena et al., 2009), *B. elkanii* USDA76^T (This study), *B. mercantei* (Helene et al., 2017) and *B. uaiense* (Michel et al., 2020).

Results based on multiple strains for a species are indicated in brackets.

3.4. nodA-*based phylogeographic patterns*

The possible origins of the symbiotic locus of the 14 strains were evaluated using the *nodA*-based phylogeographic framework that has been developed for *Bradyrhizobium* (Beukes et al., 2016, Steenkamp et al., 2008, Stępkowski et al., 2012, Stępkowski et al., 2011). After supplementing the *nodA* dataset employed by Beukes et al. (2016) with relevant sequences that have been published subsequently, as well as *B. acaciae* sp. nov., strains (10BB^T, 19AH, 19AJ and 16BA no1) examined in this study, the final dataset analyzed consisted of 523 taxa (330 haplotypes) and 642 aligned nucleotides.

ML analysis generated a phylogeny containing the 16 known clades (I-XVI) of *nodA* (Moulin et al., 2004; Stępkowski et al., 2005; Steenkamp et al., 2008 and Beukes et al., 2016) (Supplementary Fig. S4). All the strains of *B. acaciae* sp. nov. isolated from the root nodules of *Acacia dealbata* and *A. mearnsii* in South Africa grouped together in the cosmopolitan Clade III (Fig. 2 and Supplementary Fig. S4). All of the strains originating from legumes in the tribe Crotalarieae (i.e., *B. oropedii* sp. nov. and *B. altum* sp. nov.) were represented by the same haplotype and formed part of the South African Clade XIV. However, the origins of *nodA* in *B. brasilense* and *B. ivorense* varied. In the case of *B. brasilense*, strains Cham231 and Cham227 (isolated from *Chamaecrista* species collected in the Pretoria area of South Africa), and strains R5 and UFLA03-321^T (isolated from *V. unguiculata* in Botswana and Brazil, respectively) all formed part of Clade III, while strain Arg62 (isolated from the root nodules of a genistoid legume in South Africa) formed part of the South African Clade XV. A similar pattern was observed for *B. ivorense*, where strain CI-1B^T originating from *Cajanus cajan* in Northern Africa formed part of Clade III, while strain Arg68 isolated from a genistoid legume in South Africa formed part of Clade XV.

3.5. Nodulation confirmation

All 14 strains considered in this study were capable of nodulating *V. unguiculata* and *M. atropurpureum* by six weeks after inoculation. The only exception was *B. ivorense* strain Arg68, which could not nodulate *M. atropurpureum*. The nodules induced on *V. unguiculata* and *M. atropurpureum* were spherical and desmodioid-type determinate nodules with lenticels, which is typical for these legumes belonging to the Phaseoleae tribe (Sprent, 2007, Sprent et al., 2017). In all cases, the harvested nodules had pink interiors, indicative of effective nitrogen fixation

(Howieson and Dilworth, 2016). The *glnII* sequences from the pure cultures re-isolated from these nodules, were identical to those of the original strains, confirming their nodulation ability.

4. Discussion

In this study, we identified new species in the *B. elkanii* supergroup of *Bradyrhizobium*. This was achieved with a multi-step polyphasic workflow that involved genealogical concordance analysis for generating species hypotheses, followed by an assay of various additional biological properties as supporting evidence (Venter et al., 2017). Although the sequence information for the 16S rRNA gene is often used for generating species hypotheses (Gevers et al., 2006), its conserved nature precludes resolution of *Bradyrhizobium* strains to species level (Menna et al., 2009, Rivas et al., 2009, Vinuesa et al., 2008). In contrast, our analysis of *atpD, dnaK, glnII, gyrB* and *rpoB* gene sequences allowed for the recognition of five putative species among the 14 strains examined. This was evident in the congruent genealogical patterns observed in single-gene trees, where particular sets of strains formed consistent groups across all phylogenies, with one strain representing a singleton. Based on these data, three of the putative species were likely novel, while the remaining two probably represented *B. brasilense*, originally isolated from *V. unguiculata* nodules in Brazil (Da Costa et al., 2017), and *B. ivorense*, originally isolated from *Cajanus cajan* nodules in Côte d'Ivoire (Fossou et al., 2020). Having identified the putative boundary between divergent and reticulate evolution through genealogical concordance, we accordingly set out to interrogate other lines of evidence for the delineated taxa.

Among the suite of biological properties available for studying bacterial systematics (de Lajudie et al., 2019, Gevers et al., 2006, Goris et al., 2007, Konstantinidis and Tiedje, 2005, Richter and Rosselló-Móra, 2009, Venter et al., 2017), we included those related to genome data. This was done using the widely used ANI sequence similarity metric (de Lajudie et al., 2019, Delamuta et al., 2013, Estrada-de los Santos et al., 2018, Goris et al., 2007, Konstantinidis and Tiedje, 2005, Steenkamp et al., 2015) and a phylogenomic approach (Avontuur et al., 2019, Estrada-de los Santos et al., 2018, Na et al., 2018, Palmer et al., 2017). Consistent with the recommended ANI threshold for conspecific strains (Goris et al., 2007, Konstantinidis and Tiedje, 2005), our strains which likely represent *B. brasilense* and *B. ivorense* displayed values of ≥95.9% in pairwise comparisons with the type strains of these species. These relationships were also supported by phylogenomic data that monophyletically clustered the relevant strains with the respective species.

Strains representing the three novel putative species all showed ANI values below the 95% guideline, when compared to the type strains of known *Bradyrhizobium* species. The only exception was comparisons between *B. uaiense* and 10BB^T that yielded ANI values of 94.74% and 95.37%. Such borderline estimates for intra-species comparisons are not uncommon (Ciufo et al., 2018, Kim et al., 2014, Parks et al., 2020) and have been documented in *Bradyrhizobium* (Palmer et al., 2020). Our phylogenomic data also reflected the close relationship between the two taxa (*B. uaiense* and 10BB^T) and highlighted the uniqueness of our three novel species. We accordingly proposed *B. oropedii* sp. nov., *B. acaciae* sp. nov. and *B. altum* sp. nov. for the three new species delineated in this study.

Based on the physiological and metabolic characteristics investigated, there were differences between the strains of our proposed species and those reported for species in the *B. elkanii* supergroup (i.e., *B. uaiense* UFLA03-164^T, *B. embrapense* SEMIA 6208^T, *B. mercantei* SEMIA 6399^T, *B. tropiciagri* SEMIA 6148^T, *B. elkanii* USDA76^T and *B. pachyrhizi* PAC48^T). However, the types of phenotypic traits used, especially those evaluated using commercial test kits (e.g., API and Biolog) are difficult to rationalize and apply within the context of rhizobial systematics (Sutcliffe, 2015, Sutcliffe et al., 2012). Many of the traits tested for may be encoded by genes located on accessory genomes and could potentially vary among strains (Ormeno-Orrillo and Martínez-Romero, 2013). Also, a variety of such tests are being used for *Bradyrhizobium*, thus hampering phenotype comparisons between studies. The matter is further exacerbated by poor agreement of some tests among different studies (Sutcliffe, 2015, Sutcliffe et al., 2012). These challenges could potentially be surmounted with the use of genome-based *in silico* determined phenotypes, especially when they are incorporated into an evolutionary framework (Palmer et al., 2018, Steenkamp et al., 2015).

No clear patterns regarding legume host or geography were apparent in our MLSA and phylogenomic tree. For instance, *B. altum* sp. nov. was isolated from a root nodule of *Pearsonia obovata* (tribe Crotalarieae) in the Great Escarpment region in South Africa (Beukes et al., 2016), while its closest known relative, *B. embrapense*, was originally isolated from a root nodule of *Desmodium heterocarpon* in Colombia (Delamuta et al., 2015). Members of *B. oropedii* sp. nov. also originated in the Great Escarpment from nodules formed on Crotalarieae species, while its close relative, *B. acaciae* sp. nov., originates from root nodules of Australian *Acacia* species

occurring in South Africa (Boshoff, 2015), and *B. uaiense* is a rhizobial symbiont of *V. unguiculata* that was originally isolated in Brazil (Michel et al. 2017). Additionally, our strain of *B. ivorense* was isolated from *Argyrolobium sericeum* in the Great Escarpment in South Africa, while the type strain of the species was originally reported from *C. cajan* in West Africa (Fossou et al., 2020). Our strains of *B. brasilense* originate from root nodules of different legumes (i.e., *A. sericeum*, *V. unguiculata*, *Chamaecrista* sp.) collected in Botswana and various locations in South Africa, while the species was originally described from root nodules of *V. unguiculata* in Brazil (Da Costa et al., 2017). Furthermore, inoculation studies showed that our strains of *B. oropedii* sp. nov., *B. acaciae* sp. nov., *B. altum* sp. nov., and *B. brasilense* were capable of nodulating *V. unguiculata* and *M. atropurpureum*. For *B. brasilense*, this is similar to a previous report showing it being capable of nodulating *Stizolobium aterrimum* and *Glycine max* in addition to *M. atropurpureum* (Da Costa et al., 2017). However, our strain of *B. ivorense* could nodulate *V. unguiculata*, but not *M. atropurpureum,* despite previous reports of this species being capable of nodulating *M. atropurpureum* (Fossou et al., 2020).

For exploring the evolutionary origins of symbiotic traits in the *Bradyrhizobium* species characterized here, we employed phylogenetic analyses of the *nodA* gene. In contrast to the five clusters identified using the five housekeeping gene sequences, the *nodA* phylogeny separated our 14 isolates into three broad groups. These corresponded respectively to *nodA* Clades III, XIV and XV of *Bradyrhizobium*. As expected, all of our *B. brasilense* strains from *Chamaecrista* sp. and *V. unguiculata* had *nodA* alleles belonging to Clade III. This was also true for the type strains of *B. tropiciagri*, *B. uaiense*, *B. viridifuturi* and *B. embrapense*, which have all been reported to have significant agricultural importance (Delamuta e al., 2015, Helene et al. 2015, Michel et al., 2020). It is also not unusual for *Bradyrhizobium* strains from Clade III to be isolated from legumes in sub-Saharan Africa (Aserse et al., 2012, Degefu et al., 2017, Steenkamp et al., 2008). Members of this cosmopolitan clade associate with the largest diversity of legumes, both native and cultivated (Beukes et al., 2016, Stępkowski et al., 2007, 2012). Clade III might therefore represent one of the oldest *nodA* lineages in the genus (Steenkamp et al., 2008).

All of the *B. acaciae* sp. nov. strains harboured the Clade III allele of *nodA*. These strains were isolated in South Africa from root nodules of *A. mearnsii* and *A. dealbata*, legumes that are native to the temperate regions of Australia (Lewis, 2005). The *Bradyrhizobium* symbionts of these

legumes, and other legumes native to Australia, often encode *nodA* sequences belonging to Clades I, IV or X that are all thought to have Australian origins (Beukes et al., 2016, Stępkowski et al., 2012, Stępkowski et al., 2005). Previous studies showed that *nodA* of *Acacia* symbionts outside of their native range (e.g., strains isolated from *A. decurrens* in South Africa, and from *A. melanoxylon* and *A. mearnsii* in Brazil) typically belonged to Clade I (Banasiewicz et al., 2021, Menna and Hungria, 2011, Moulin et al., 2004). In these non-native situations, it is thought that the symbiotic loci enabling the *Acacia-Bradyrhizobium* association were co-introduced from Australia into the new geographic region (Banasiewicz et al., 2021, Rodríguez-Echeverría et al., 2011, Warrington et al., 2019). Such an introduction may involve the original bacterium carrying its specific symbiotic loci, or the latter could have been acquired by a local species via horizontal gene transfer (Warrington et al., 2019).

In southern Africa, *Bradyrhizobium* strains possessing *nodA* Clade III alleles are associated with native and non-native legume species (Aserse et al., 2012; Beukes et al., 2016; Steenkamp et al., 2008). However, strains with *nodA* alleles from Clade III, in addition to Clades I, IV and X, have been identified in *Bradyrhizobium* associated with legumes native to tropical and subtropical regions of Australia (Stępkowski et al., 2012). Therefore, the origins of *B. acaciae* symbionts of Australian *Acacia* species introduced into South Africa, and which have Clade III *nodA* alleles, remain unclear as they could have originated from either region. Based on our findings, the presence of *nodA* Clade III alleles in the genomes of *B. acaciae*, may be explained by the co-introduction into South Africa of *nodA* Clade III-bearing *B. acaciae* and its Australian host. Alternatively, strains belonging to Clade I, IV or X were introduced into South Africa and the symbiotic loci were subsequently replaced (via horizontal gene transfer) with those of locally adapted Clade III strains. One can also not exclude the possibility that *B. acaciae* strains carrying Clade III *nodA* alleles are indigenous to South Africa, encoding Nod Factor that is recognizable by these non-native plants. To fully address these hypotheses, it would be necessary to compare *Bradyrhizobium* species (and their *nodA* alleles) from *Acacia* species grown in both South Africa and Australia, something that is not currently possible due to the small number of *Bradyrhizobium* species described from both regions, where most members of this genus likely represent novel taxa (Beukes et al. 2016; Stepkowski et al., 2012).

Strains isolated from the Great Escarpment in South Africa all harboured *nodA* alleles belonging to Clades XIV and XV that have so far only been reported from this region (Beukes et al., 2016). These Clades have also not yet been recorded outside of South Africa. In fact, the Great Escarpment has been shown to represent a unique center of diversity for *Bradyrhizobium* (Beukes et al., 2016). As with the Clade III patterns mentioned above, distribution of the two *nodA* alleles also crossed species limits, occurring in a strain each of *B. brasilense*, *B. ivorense* and *B. altum* sp. nov., as well as all of our *B. oropedii* sp. nov. strains. In other words, *Bradyrhizobium* strains from the same species can have vastly different origins for their *nodA* and accompanying symbiotic loci (Guerrouj et al., 2013). These bacteria thus appear capable of adapting to local hosts and environmental conditions through the acquisition of optimal symbiotic loci. Such explanations are in-line with previous conclusions that local *Bradyrhizobium* populations are shaped by the combined effects of prevailing edaphic, geographic and climatic processes, as well as the intrinsic processes governing the evolution of their legume hosts (e.g., Beukes et al. 2016; Stępkowski et al., 2012).

From a practical point of view, the bacteria characterized in this study may have substantial agricultural benefits in sub-Saharan Africa. Many of our strains, together with those reported previously (Grönemeyer et al., 2016, Grönemeyer and Reinhold-Hurek, 2018), can tolerate relatively high temperatures (from 35 °C up to 40 °C). Also, some of our strains, like others reported before (da Costa et al., 2018, Da Costa et al., 2017, de Matos et al., 2017, Zilli et al., 2014), can grow in both acidic and alkaline media (i.e., from pH 4 to pH 10). One of our strains was additionally highly salt tolerant by being capable of growth in the presence of as much as 2% (w/v) NaCl. In most cases, *Bradyrhizobium* is salt sensitive, however, interrelated factors such as carbon source, pH and incubation temperature have been shown to have an effect on salt tolerance on rhizobia (Elsheikh, 1998). These bacteria thus have potential value in harsh sub-Saharan environments, especially under conditions of climate change. Here they could be used for the development of rhizobial inoculants for legume cultivation or as biofertilizer, particularly in small-scale farming systems and in low-fertility, nitrogen-deficient soils (Grönemeyer and Reinhold-Hurek, 2018, Jaiswal and Dakora, 2019).

4.1. Description of Bradyrhizobium acaciae sp. nov.

Bradyrhizobium acaciae (a.ca'ci.ae. N.L. fem. gen. n. *acaciae*, referring to the host plant genus *Acacia*, from which the species was isolated).

Cells are Gram-negative, motile, rod-shaped and approximately 2.35 x 0.65 μm in size. Colonies when grown on YMA at 28 °C after 7 days are convex and can either be translucent or opaque, cream in colour and less than 1 mm in diameter. Isolates within this species can grow in temperatures ranging from 10 to 40 °C and at a pH between 5 to 10 and cannot grow in the presence of more than 1.5% NaCl (w/v). They are positive for oxidase, urease, β-glucosidase and β-galactosidase activity. Positive reactions were recorded for the following carbon utilization: L-arabinose, D-mannose, potassium gluconate, Tween 40, L-asparagine, Tween 80, L-frucotose, D-galactose, D-galacturonic acid, α-D-glucose, L-arabinose, L-rhamnose, formic acid, D-saccharic acid, glucuronamide, D and L- alanine, and L-aspartic acid. Carbon utilization reactions was negative for L-tryptophane, glucose fermentation, hydrolysis of gelatin, D-mannitol, D-maltose, i-erythritol, gentibiose, m-inositol, D-melibiose, L-ornithine, capric acid, adipic acid, citrate utilization, phenylacetic acid, malic acid, α-cyclodextrin, glycogen, N-acetyl-D-galactosamine, glucosamine, D-raffinose, adonitol, D-arabitol, D-cellibiose, D-glucoside, D-psicose, sucrose, D-trehalose, xylitol, D-sorbitol, D-glucoronic acid, glucose-1-phosphate and glucose-6-phosphate. Resistance to ampicillin (10 μg/ml), penicillin (10 μg/ml), erythromycin (30 μg/ml), streptomycin (25 μg/ml), tetracycline (30 μg/ml), neomycin (10 μg/ml), chloramphenicol (30 μg/ml), kanamycin (30 μg/ml) and susceptible to gentamycin (10 μg/ml) and streptomycin (10 μg/ml).

The type strain, 10BB^{T} (=SARCC 730=LMG 31409), was isolated from nodules of *Acacia dealbata* growing in the Gauteng Province of South Africa and can form effective nodules on cowpea (*Vigna unguiculata*) and siratro (*Macroptilium atropurpureum*). The DNA G+C content of the type strain is 63.6 mol%. GenBank/EMBL/DDBJ accession numbers for the genome sequence and gene sequences of this type strain are: whole genome sequence (JACMYK000000000), 16S rRNA (LR877293), *atpD* (LR877274), *glnII* (LR877284), *dnaK* (LR877288), *gyrB* (LR877279), *rpoB* (LR877266) and *nodA* (LR880919).

4.2. Description of Bradyrhizobium oropedii sp. nov.

Bradyrhizobium oropedii (o.ro.pe'di.i. Gr. neut. n. *oropedion* mountain plain; N.L. gen. n. *oropedii*, of the central Southern African plateau, from where the species was isolated).

Cells are motile, Gram-negative, rod shaped and approximately 1.87 x 0.48 µm in size. Growth occurs on YMA at 28 °C after 7 days and colonies are translucent to opaque, circular, convex and cream in colour. Grows at pH values ranging from 5 to 10 and at temperatures ranging from 15 to 35 °C. Isolates are unable to grow in the presence of more than 0.5% NaCl (w/v). Strains tested positive for oxidase, catalase, urease and β-glucosidase activity as well as the production of Tween 40 and Tween 80. Tested positive for the following carbon sources: D-fructose, L-fructose, D-gluconic acid, D-mannitol, α and β-hydroxy butyric acid, p-hydroxy phenylacetic acid, L-alaninamide D, L-lactic acid, L-leucine, D-saccharic acid, and urocanic acid. Negative reactions were recorded for indole production and the assimilation of capric acid, adipic acid, malic acid, glycogen, α-D-lactose, N-acetyl-D-galactosamine, α-cyclodextrin, Adonitol, D-cellobiose, D-raffinose, i-erythritol, maltose, lactulose, D-melibiose, sucrose, D-trehalose, turanose, xylitol, L-threonine, uridine, inosine, thymine, glucose-1-phosphate and glucose-6-phosphate. m-inositol. Resistant to ampicillin (10 µg/ml), chloramphenicol (30 µg/ml), tetracycline (30 µg/ml), penicillin (10 µg/ml), and susceptible to streptomycin (25 µg/ml).

The type strain, Pear76^{T} (=SARCC 731=LMG 31408) was isolated from root nodules of *Pearsonia obovata* in the Dullstroom area of the Mpumalanga Province, South Africa and forms effective nodules on cowpea (*Vigna unguiculata*) and siratro (*Macroptilium atropurpureum*). The DNA G+C content of the type strain is 63.5 mol%. GenBank/EMBL/DDBJ accession numbers for the genome sequence and the gene sequences of this type strain are: whole genome (JACMYO000000000) and 16S rRNA (LR877297), *atpD* (LN650103), *glnII* (LN650145), *dnaK* (LN650124), *gyrB* (LN650208), *rpoB* (LN650187) and *nodA* (LN650250).

4.3. Description of Bradyrhizobium altum sp. nov.

Bradyrhizobium altum (al'tum. L. neut. adj. *altum*, high, referring to the high-altitude region where this species was isolated from).

Cells are motile, Gram-negative rods and is approximately 1.29 x 0.45 µm in size. Colonies are circular, convex, opaque, cream in colour and less than 1 mm in diameter on YMA after 7days incubation at 28 °C. The pH range for growth on YMA is 5 to 10 and growth can occur between 15 to 37 °C and these strains can grow in the presence of up to 2.0 % (w/v) NaCl. Reduction of nitrates, β-glucosidase urease, tested positive. It is also positive for the production of L-arabinose, Tween 40, Tween 80, adonitol, N-acetyl-D-glucosamine, D-cellobiose, D-arabitol, D-galactose, D

and L-fructose, m-inositol, D-mannitol, maltose, D-mannose, D-raffinose, D-melibiose, L-rhamnose, D-sorbitol, sucrose, xylitol, D-trehalose, turanose, methyl pyruvate, D-galacturonic acid, acetic acid, malonic acid, propionic acid, D-saccharic acid, quinic acid, sebacic acid, L-asparagine, D and L-alanine, L-leucine, L-proline, L-ornithine and glycerol. Negative reactions were recorded for glucose fermentation, indole production, β-galactosidase activity and L-arginine dihydrolase. It is also negative for the assimilation of N-acetyl-glucosamine, potassium gluconate, D-maltose, adipic acid, capric acid, malic acid, phenylacetic acid, glycogen, α-cyclodextrin, L-arabinose, i-erythritol, L-threonine, D and L-serine, glucose-1-phosphate and glucose-6-phosphate. Strains is resistant to penicillin (10 µg/ml), ampicillin (10 µg/ml), gentamycin (10 µg/ml), chloramphenicol (30 µg/ml) and neomycin (10 µg/ml) and is susceptible to kanamycin (30 µg/ml), erythromycin (30 µg/ml). streptomycin (10 and 25 µg/ml) and tetracycline (30 µg/ml).

The type strain Pear77^T (=SARCC 754=LMG 31407), was isolated from root nodules of *Pearsonia obovata* collected in the Dullstroom area of the Mpumalanga Province, South Africa. This species forms effective nodules on cowpea (*Vigna unguiculata*) and siratro (*Macroptilium atropurpureum*). The DNA G+C content of the type strain is 63.3 mol%. The GenBank/EMBL/DDBJ accession numbers for the genome sequence and gene sequences for this type strain are: whole genome (JACMYP000000000) and 16S rRNA (LR877298), *atpD* (LN650104), *glnII* (LN650146), *dnaK* (LN650125), *gyrB* (LN650209), *rpoB* (LN650188) and *nodA* (LN650251).

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Declaration of interest

The authors have no interest to declare.

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