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Bradyrhizobium xenonodulans sp. nov. isolated from nodules of Australian *Acacia* species invasive to South Africa

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ABSTRACT

A genealogical concordance approach was used to delineate strains isolated from *Acacia dealbata* and *Acacia mearnsii* root nodules in South Africa. These isolates form part of *Bradyrhizobium* based on 16S rRNA sequence similarity. Phylogenetic analysis of six housekeeping genes (*atpD, dnaK, glnII, gyrB, recA* and *rpoB*) confirmed that these isolates represent a novel species, while pairwise average nucleotide identity (ANIb) calculations with the closest type strains (*B. cosmicum* 58S1^T, *B. betae* PL7HG1^T, *B. ganzhouense* CCBAU 51670 ^{T}, *B. cytisi* CTAW11^T and *B. rifense* CTAW71^T) resulted in values well below 95–96%. We further performed phenotypic tests which revealed that there are high levels of intraspecies variation, while an additional analysis of the *nodA* and *nifD* loci indicated that the symbiotic loci of the strains are closely related to those of *Bradyrhizobium* isolates with an Australian origin. Strain 14AB^T (=LMG 31415 ^{T} = SARCC-753 ^{T}) is designated as the type strain of the novel species for which we propose the name *Bradyrhizobium xenonodulans* sp. nov.

Introduction

Acacia is a large genus in the *Fabaceae*, containing more than 1000 species, almost all of which are endemic to Australia (Beukes et al., 2019; Brockwell et al., 2005; Murphy, 2008; Thiele et al., 2011). Because of their commercial value, some *Acacia* species have been planted outside of Australia. In South Africa, for example, *A. mearnsii* (black wattle) was introduced in 1864 for the production of firewood and shade, but with the discovery of the high tannin levels in its bark, this species was planted widely in commercial plantations primarily for its use in leather tanning (De Beer, 1986). The closely related *A. dealbata* (silver wattle) was likely introduced into South Africa by accident, as it might have been confused with *A. mearnsii* (Campbell, 1988). Since *A. dealbata* has inferior properties compared to *A. mearnsii*, it was never planted commercially, but is rather used for windbreaks and firewood (Campbell, 1988; Keet et al., 2017). Both *A. mearnsii* and *A. dealbata* have invaded large areas of South Africa (De Beer, 1986; Ndlovu et al., 2013), where they encroach on native grasslands and threaten Fynbos shrubland (Kamutando et al., 2017). In its native South-East Australia, *A. dealbata* is even considered an invasive weed (De Beer, 1986), which demonstrates how effective *Acacia* species can be at spreading to and colonizing new geographic regions.

Acacia's symbiotic partnership with nitrogen-fixing bacteria has aided its rapid establishment and spread in new environments, especially when introduced concurrently with symbionts from its original environment (Le Roux et al., 2017). These symbionts are collectively termed rhizobia and are hosted in root nodules, which are specialised plant organs within which the bacteria catalyse the conversion of atmospheric dinitrogen to ammonia (Hungria et al., 2015). When introduced to a new environment, the *Acacia*-rhizobia symbiosis can disrupt microbial communities in resident soils by changing their functional structure and taxonomic composition (Kamutando et al., 2019, 2017; Lazzaro et al., 2014). This is due to changes in soil properties (e.g., pH, total carbon availability and type of carbon sources, levels of bioavailable nitrogen) caused by the accumulation of *Acacia* leaf litter and detritus (Lazzaro et al., 2014), as well as the chemicals and allelopathic compounds released in the root exudates of *Acacia* species (Lorenzo et al., 2013). Therefore, soil communities associated with *Acacia*

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invasions are often typified by an overrepresentation of certain bacterial species, and in extreme cases the complete homogenization of rhizobial communities, even across vast geographical ranges (Kamutando et al., 2019; Le Roux et al., 2018). *Acacia* and its rhizobial symbionts can further directly affect the distribution of native diazotrophs and rhizobia and their symbiotic legumes in these environments (Kamutando et al., 2017; Le Roux et al., 2016).

The preferred rhizobial symbiont of *Acacia* in both native and invasive settings are species from the genus *Bradyrhizobium* (Warrington et al., 2019). Like other rhizobia, members of this genus can share the loci responsible for the symbiotic interaction and nitrogen fixation via horizontal gene transfer (HGT) (Beukes et al., 2016). This is because these loci form part of symbiosis islands or are encoded on plasmids in some *Bradyrhizobium* strains (Kaneko et al., 2002; Okazaki et al., 2015). Therefore, DNA sequences of nodulation (*nod*) and nitrogen-fixation (*nif*) loci commonly provide a strong geographical signal, making them ideal markers for exploring the biogeography of *Bradyrhizobium* (Beukes et al., 2016; Parker and Rousteau, 2014; Steenkamp et al., 2008). Numerous studies have accordingly sequenced the *nodA* and *nifD* genes of these bacteria (encoding for an acyltransferase and dinitrogenase reductase, respectively) and their inferred genealogies provide a well-established biogeography framework (Beukes et al., 2016; Crisóstomo et al., 2013; Muñoz et al., 2011; Parker and Rousteau, 2014; Steenkamp et al., 2008; Stępkowski et al., 2018; Stepkowski et al., 2012, 2011). Indeed, various studies have used this framework to investigate the evolutionary history of *Bradyrhizobium*, while at the same time also investigating the processes shaping its geographic distribution.

Bradyrhizobium is regarded by some as the ancestral genus of nodulating bacterial species (Avontuur et al., 2019; Hungria et al., 2015; Parker, 2015). This notion is consistent with its broad genetic diversity, exemplified by the rapid rate of novel species descriptions in the last decade (Delamuta et al., 2012; Grönemeyer and Reinhold-Hurek, 2018). Recent genomic studies showed that the genus is characterized by a number of deep divergences that has given rise to seven well-supported clades that are commonly referred to as supergroups (Avontuur et al., 2022, 2019). Most of the described *Bradyrhizobium* species form part of the so-called *B. japonicum* supergroup, which has been recovered from a wide range of continents and climatic zones (Avontuur et al., 2019). Species from the *B. elkanii* supergroup are mostly isolated from legumes occurring in tropical and subtropical areas (Avontuur et al., 2019), and members of the *B. jicamae* and Kakadu supergroups mostly occur in Mediterranean and tropical climates (Avontuur et al., 2022; Stepkowski et al., 2012). Members of the Photosynthetic supergroup have thus far only been isolated from *Aeschynomene* species in tropical regions (Avontuur et al., 2019). Of the 83 described *Bradyrhizobium* species (Ahnia et al., 2018; Avontuur et al., 2022; Bromfield et al., 2019; Bünger et al., 2018; Cabral Michel et al., 2020; Helene et al., 2020; Jang et al., 2018; Klepa et al., 2019; Li et al., 2019; Stępkowski et al., 2018; de Urquiaga et al., 2019) (https://lpsn.dsmz.de/genus/bradyrhizobium; Accessed February 2023), the type strains of most originate in the Americas (34 species), Asia (20 species) and Africa (16 species), while some are from Europe (four species) and Australia (nine species). The fact that there are only nine species described from Australia is striking, since a remarkable proportion of *Bradyrhizobium* diversity has been recognised on the Australian continent (Stepkowski et al., 2012).

Here we focused on ten isolates obtained from root nodules of *A. mearnsii* and *A. dealbata* growing in invasive settings across three provinces in South Africa. Initial 16S rRNA sequence analysis indicated that they could represent a distinct and novel species of *Bradyrhizobium* that might have been co-introduced with their plant hosts (Boshoff, 2015). The first aim was therefore to determine whether this assemblage of isolates represent a unique new taxon and if so, to provide it with a detailed description. For this purpose, a polyphasic approach was used, which involved genealogical concordance analysis based on multiple core gene regions, genome comparisons and phenotypic characterization (Venter et al., 2017). Secondly, the possible origins of the isolates and their symbiotic loci were explored. This was done by incorporating *nodA* and *nifD* data for the ten *Acacia* strains into datasets containing all the available sequences for these genes in order to determine the strains' placement in the overall phylogeographic framework for *Bradyrhizobium*.

Materials and methods

Isolation and culturing

The bacteria used in this study were obtained from previous studies (Boshoff, 2015; Joubert, 2002). They were isolated from the root nodules of *A. mearnsii* and *A. dealbata* that were collected in South Africa from various locations covering different edaphic conditions (Table 1). Isolates were routinely grown on Yeast Mannitol Agar (Somasegaran and Hoben, 2012) (YMA) and incubated at 28 °C for seven days. They were then stored in 15% (v/v) glycerol at −80 °C. Strain 14AB was designated as the type strain for the newly proposed species (see below) and was submitted to the Belgian Coordinated Collections of Microorganisms (BCCM; Universiteit Gent, Belgium; with number LMG 31415^T) and South African Rhizobium Culture Collection (SARCC, Pretoria, South Africa; with number SARCC-753^T).

PCR, sequencing and phylogenetic analysis

In this study, portions of six protein-coding housekeeping genes were amplified and sequenced. These were *atpD* encoding subunit beta of ATP synthase, *dnaK* encoding the Hsp70 chaperone, *glnII* encoding isoform II of glutamine synthetase, *gyrB* encoding subunit B of DNA gyrase, *recA* encoding recombinase A, and *rpoB* encoding subunit beta of RNA polymerase. To this end, we used the following primer sets: TSatpDf and TSatpDr (Stepkowski et al., 2005), TSdnaK4 and TSdnaK2 (Stępkowski et al., 2007), TSglnIIf and TSglnIIr (Stępkowski et al., 2005), AMgyrBf and AMgyrBr (Stepkowski et al., 2012), TSrecAf5 and TSrecAr3 (Beukes et al., 2016), and rpoB-456F and rpoB-1364R (Vinuesa et al., 2008, 2005). Portions of the two symbiotic genes, *nifD* and *nodA*, were also amplified using the primer sets TSnifDf1 and nifp12 (Beukes et al., 2016; Parker, 2000) and TSnodD1-1c, TSnodB2N and TSnodB7 (Stępkowski et al., 2005), respectively.

All PCRs utilized template DNA that were extracted with the Quick g-

Table 1
Isolates sampled from root nodules of *Acacia* trees growing in an invasive setting in South Africa.

Isolate number	Sampling location	Host	Soil pH[1]	Nodule formation[2]
2AA	Pretoria, Gauteng Province	*A. dealbata*	5.73	*A. mearnsii*
2AC	Pretoria, Gauteng Province	*A. dealbata*	5.73	*A. dealbata, A. mearnsii*
2BB	Pretoria, Gauteng Province	*A. dealbata*	5.73	None
7AB	Laingsnek, KwaZulu-Natal Province	*A. mearnsii*	5.71	None
7AC	Laingsnek, KwaZulu-Natal Province	*A. mearnsii*	5.71	*A. mearnsii*
14AB^T	Vandyksdrif, Mpumalanga Province	*A. dealbata*	6.03	*A. dealbata, A. mearnsii*
20BH	Kliprivier, Gauteng Province	*A. dealbata*	7.18	*A. mearnsii*
20BK	Kliprivier, Gauteng Province	*A. dealbata*	7.18	*A. mearnsii*
20CC	Kliprivier, Gauteng Province	*A. dealbata*	7.18	*A. mearnsii*
20DD	Kliprivier, Gauteng Province	*A. dealbata*	7.18	*A. dealbata, A. mearnsii*

[1] Data obtained and reported by Boshoff (2015) and Joubert (2002).
[2] Nodule formation as determined in this study (see Supplementary Fig. S3).

DNA MiniPrep kit (Zymo Research, USA) and quantified with a NanoDrop™ spectrophotometer (ThermoFisher Scientific, USA). Each PCR mixture contained 50–100 ng of genomic DNA, 0.1 U/µl SuperTherm *Taq* DNA Polymerase and reaction buffer (Southern Cross Biotechnology, SA), 10 mM each of the respective forward and reverse primers, 25 mM $MgCl_2$, 2.5 mM of each dNTP and sterile water added to a final volume of 25 µl. PCR cycle conditions for all housekeeping genes were as follows: denaturation at 95 °C for 2 min, then 35 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 2 min and elongation at 72 °C for 2 min, with a final elongation step at 72 °C for 5 min. The symbiotic gene regions were amplified using the following conditions: initial denaturation at 95 °C for 2 min, thereafter 35 cycles of denaturation at 95 °C for 2 min, annealing at 53 °C for 30 sec, an elongation step at 72 °C for 1 min, followed by the final elongation step at 72 °C for 10 min.

PCR products were cleaned with 0.1 U/µl FastAP Thermosensitive Alkaline Phosphatase (ThermoFisher Scientific, USA) and 0.5 U/µl Exonuclease I (ThermoFisher Scientific, USA). Cleaned products were then sequenced with the BigDye® Terminator v3.1 cycle sequencing kit, using the original PCR primers and an ABI3100 Automated Capillary DNA sequencer (Applied Biosystems, USA). The trace files were visualised and edited (where necessary) using ChromasLite v2.6.6 (Technelysium, Australia). To confirm gene identities, the sequences for all PCR products were compared to those in the nucleotide database of the National the National Centre for Biotechnology Information (NCBI; https://www.ncbi.nlm.nih.gov).

DNA sequences were aligned using the inferred amino acid sequences in BioEdit (Hall, 1999) for the housekeeping genes, and MAFFT (Multiple Alignment using Fast Fourier Transformation; https://mafft.cbrc.jp/alignment/server/) (Katoh et al., 2002) for the symbiotic genes. These datasets included the relevant sequences for the *Bradyrhizobium* type strains, according to the List of Prokaryotic names with Standing in Nomenclature (LPSN; https://lpsn.dsmz.de/; Accessed February 2023; (Euzéby, 1997; Parte, 2018, 2014; Parte et al., 2020)) and current literature. We also generated a 16S rRNA alignment using previously determined sequences (Boshoff, 2015) and the Q-INS-I strategy of MAFFT (Katoh et al., 2002). A multilocus sequence analysis (MLSA) dataset was constructed by concatenating the six protein-coding housekeeping gene sequence sets using SequenceMatrix (Vaidya et al., 2011).

To investigate the phylogeography of *Bradyrhizobium* we constructed three additional datasets that included all available *Bradyrhizobium* sequences for *nifD* and *nodA* in the NCBI nucleotide database (accessed 1 June 2020). A comparable dataset, containing aligned and concatenated *recA* and *glnII* sequences were also constructed. Where needed, the DNA sequences of the *nodA* and *nifD* datasets were translated to amino acid sequences using BioEdit. Both the latter datasets were also analysed for substitution saturation in DAMBE v7.0.68 (Xia, 2017; Xia et al., 2003), as the fast evolution associated with HGT-prone genes significantly impact the accuracy of phylogenetic analyses (Hao and Brian Golding, 2006; Philippe et al., 2011). Furthermore, to reduce limitations in computing resources, the *recA* + *glnII*, *nodA* and *nifD* datasets were converted into haplotype files using DNA Sequence Polymorphism v6.12.03 (Rozas et al., 2017).

Maximum likelihood (ML) phylogenetic analyses were performed with RAxML v8.2.12 (Stamatakis, 2014) on the respective nucleotide datasets and on the amino acid datasets for *nodA* and *nifD*. For the amino acid sequences, the best-fit JTT (Jones-Taylor-Thornton; (Jones et al., 1992)) substitution model was used, as determined by ProtTest v3.4 (Darriba et al., 2011). For the nucleotide sequences, the GTR model (Generalised Time Reversible, (Tavare, 1986)) was used. In all cases, independent parameter estimation was enabled, including for each partition of the concatenated dataset. RAxML was also used to estimate branch support by using the same model parameters as before, as well as rapid hill-climbing and 1000 repetitions of non-parametric bootstrap analysis. All ML trees were visualised in Mega X (Kumar et al., 2018), using *Rhodopseudomonas palustris* as outgroup in the housekeeping gene trees, *Bosea thiooxidans* DSM 9653^T as outgroup in the 16S rRNA phylogeny, and *Methylobacterium nodulans* ORS2060^T as the outgroup in the *nodA* and *nifD* trees. For the final visualisation of the trees, an in-house script was used to expand the haplotypes. All sequences used in this study as well as their GenBank accession numbers are listed in Supplementary Tables S1 and S2. Also, the phylogenetic trees produced were uploaded onto the Interactive Tree Of Life (iTOL) v6.3 (Letunic and Bork, 2021) and is available at https://itol.embl.de/shared/RicuClaassens.

Whole genome sequencing and ANI analysis

High-quality DNA was extracted using the Qiagen genomic tip 20/G kit (Qiagen, Germany). For MinION Nanopore sequencing (Oxford Nanopore Technologies, UK) of the type strain, 14AB^T, libraries were prepared using the SQK-LSK108 ligation sequencing kit. This strain was also sequenced using the Illumina MiSeq platform at the Agricultural Research Council (ARC, South Africa) for which paired-end libraries of 300 bp were prepared. Quality control of the raw sequence data was done using FastQC version 0.11.9 (Andrews, et al., 2017). Low-quality regions and adapter sequences were trimmed using Trimmomatic version 0.36 (Bolger et al., 2014) on the Illumina Raw sequence data. For Oxford Nanopore raw sequence data, poor-quality regions and adapter sequences were trimmed using Porechop version 0.2.4 (https://github.com/rrwick/Porechop), using the default parameters. Both long and short sets of sequence data were used to construct a hybrid *de novo* assembly using Masurca version 3.3.4 (Zimin et al., 2013).

Genome sequences were subjected to Average Nucleotide Identity (ANI) analyses using the BLAST-based approach implemented in JSpecies v1.2.1 (Richter and Rosselló-Móra, 2009). The genome sequenced in the current study was also used to determine its position in the genome-based taxonomic framework introduced by Parks et al. (Parks et al., 2018). This was achieved using the Genome Taxonomy Database (GTDB) and associated taxonomic classification toolkit GTDB-Tk version 2 (Chaumeil et al., 2022), as implemented in KBase (https://www.kbase.us; (Arkin et al., 2018)).

Phenotypic and growth characteristics

Growth was evaluated by incubating the isolates on YMA for seven days at temperatures ranging from 10 °C to 35 °C in increments of 5 °C. Isolates were also grown at 28 °C as it is the optimal growth temperature for many *Bradyrhizobium* species (Howieson and Dilworth, 2016) and the incubation temperature at which other growth variables were determined. Salt tolerance was tested by growing the bacteria on YMA containing added NaCl concentrations ranging from 0% to 2.5% (w/v) in increments of 0.5%. The isolates were grown in yeast mannitol broth to determine the pH range in which they would grow. For this purpose, pH was adjusted using 1 M HCl to prepare broth of pH 4–7 and 1 M NaOH to prepare broth of pH 8–10. It is important to consider that the growth of rhizobia can lead to significant pH changes of the media due to metabolic activity. Hence, the growth observed at different pH values using unbuffered media should be regarded as an indication rather than a verified outcome. Motility of the isolates was evaluated using 15 ml test tubes containing 0.4% YMA (Gerhardt et al., 1994), which were then stab inoculated using an inoculation loop, and incubated for 7 days at 28 °C. Gram staining was performed according to the method specified for rhizobia (Somasegaran and Hoben, 2012).

The catalase test was done by adding a few colonies of the respective isolates to 10% hydrogen peroxide and recording bubble formation after 5 min (Gerhardt et al., 1994). The oxidase test was done by exposing the isolates (on sterile filter paper) to 1% tetramethyl-ρ-phenylenediamine in dimethyl sulfoxide (DMSO) and recording colour development after 20 s (Gerhardt et al., 1994). Susceptibility of isolates to eight different antibiotics was tested by spreading a suspension of the cells onto YMA and placing various bio-discs containing the antibiotics on the media

surface where after the plates were incubated at 28 °C for 7 days. The antibiotics included were ampicillin (10 μg), chloramphenicol (30 μg), erythromycin (30 μg), gentamycin (10 μg), kanamycin (10 μg), penicillin (10 μg), streptomycin (10 μg), and tetracycline (30 μg). To assess the strains' metabolic capabilities, API 20NE (BioMerieux, France) and BIOLOG GN2 microplates (Biolog, USA) were used according to the manufacturer's protocols, with the incubation period extended to seven days to compensate for the slow growth rate of *Bradyrhizobium* species.

Nodulation test

Seeds for *A. mearnsii* and *A. dealbata* were obtained from various locations in Pretoria, South Africa. Following surface-sterilization by immersion in 3.5% (m/v) NaOCl for 15 min and immersion in 90% (v/v) ethanol for another 15 min, seeds were submerged in boiling water and then left to cool overnight. They were then planted in a sterile sand substrate with the addition of 1 ml of nitrogen free Hoagland's solution (Supplied by the ARC of South Africa). Immediately following germination, suspensions of the respective bacteria were applied directly to the plant roots, after which the plants were left to grow for eight weeks in the laboratory and at room temperature. Nodules were removed and surface sterilized as described above. The clean nodules were then crushed onto YMA and the plates incubated at 28 °C. After re-streaking for pure colonies, the identities of the cultures were confirmed using sequence analysis of the *glnII* gene region as described above.

Results

Delineation of putative species

Phylogenetic analysis of the 1595-base pair (bp) 16S rRNA alignment confirmed that all the bacteria examined in this study are members of *Bradyrhizobium* (Supplementary Fig. S1). The 16S rRNA phylogeny also showed they form part of the *B. japonicum* supergroup, where they represent a distinct and exclusive group, albeit with no statistical support. Within the tree, this group is situated next to the type strains of *B. ottawaense, B. symbiodeficiens, B. amphicarpaeae, B. lupini, B. canariense, B. symbiodeficiens, B. betae* and *B. cosmicum*. To evaluate the exclusivity of the unique group of isolates identified, DNA sequences for six protein-coding housekeeping genes were used. The total alignment lengths were 586 bp for *atpD*, 687 bp for *dnaK*, 519 bp for *glnII*, 647 bp for *gyrB*, 435 bp for *recA*, and 819 bp for *rpoB*. See Supplementary Table S1 for European Nucleotide Archive (https://www.ebi.ac.uk/ena/data/view/LR899448-LR899517) accession numbers of the sequences determined in this study.

ML phylogenetic analysis of each single gene dataset generated genealogies in which the *Acacia* strains examined here formed an exclusive cluster (Supplementary Fig. S2). In all trees, the node received bootstrap support with values ranging from 70% (*atpD*) to 100% (*recA*). Also, no smaller groupings of isolates within this cluster were consistently supported and/or recovered in the six genealogies. In other words, relationships among the *Acacia* strains in the various trees only became concordant at the node uniting all of them into a cluster (Venter et al., 2017). This node is thus representative of the species-population interface (Baum and Shaw, 1995; Venter et al., 2017), suggesting that the group of bacteria examined form a distinct species.

The MLSA dataset (i.e., six-gene concatenated dataset) allowed resolution of relationships between the putative new species and other *Bradyrhizobium* species (Fig. 1). Based on this 3693-bp alignment, ML phylogenetic analysis recovered the cluster of *Acacia* strains as monophyletic (100% bootstrap support), forming a clade that is nested within the *B. japonicum* supergroup. This putative new species had a sister-group relationship (albeit lacking bootstrap support) containing 10 known species (*B. canariense, B. lupini, B. hipponense, B. japonicum, B. barranii, B. betae, B. cosmicum, B. ganzhouense, B. cytisi,* and *B. rifense*).

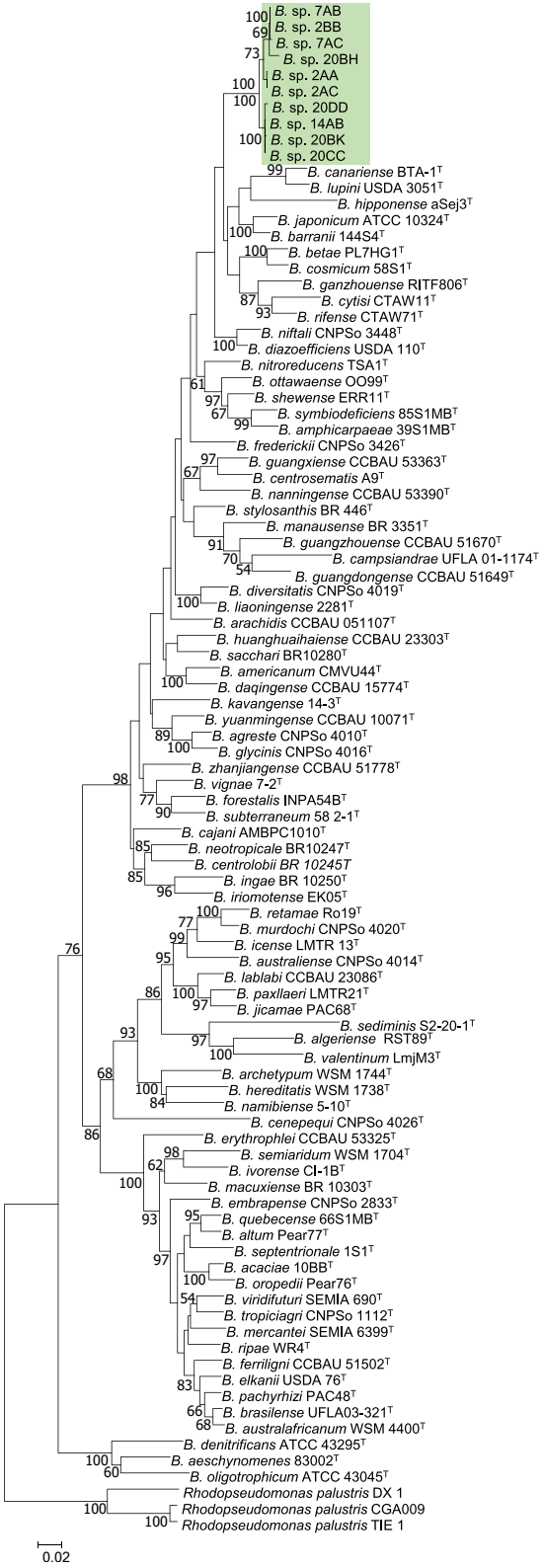

Fig. 1. A maximum likelihood phylogeny for *Bradyrhizobium* inferred from the concatenated *atpD* + *dnaK* + *glnII* + *gyrB* + *recA* + *rpoB* sequences. The ten isolates from the current study are indicated in green and bootstrap support ≥ 50% is indicated at the branches (see Supplementary Table S1 for sequence accession numbers), while the scale bar shows the number of nucleotide substitutions per site. Maximum likelihood phylogenetic trees for each of the individual housekeeping genes can be found in the Supplementary material. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Genomic, biochemical, and phenotypic support for the new species

To determine whether the putative new species is supported by genomic data, whole genome sequence information for the proposed type strain (14AB^T) was used in comparison with closely related *Bradyrhizobium* species. A total of 88,422 MinION reads (mean length: 7614 bp; minimum length: 1000 bp; maximum length: 204408 bp) and 7,084,106 paired-end Illumina MiSeq reads (mean length: 150 bp) were used to assemble the genome of 14AB^T. Its final genome assembly consisted of one, ungapped sequence with a size of 8276659 bp, G + C content of 63.95%, and an average coverage of 85.29X. Annotation with RAST predicted that the genome contains 10,049 coding sequences and 52 RNAs. The quality of the genome assembly for 14AB^T thus satisfied the suggested minimum standards for genome data to be used for taxonomic purposes (Chun et al., 2018). The genome assembly is available on the NCBI database with accession number CP089391.

Pairwise ANI comparisons were performed between the 14AB^T genome sequence and those of the type strains (where available) of its close relatives. Based on the MLSA tree, these were *B. betae* PL7HG1^T, *B. cosmicum* 58S1^T, *B. cytisi* CTAW11^T, *B. diazoefficiens* USDA 110^T, *B. hipponense* aSej3^T, *B. japonicum* ATCC 10324^T, *B. niftali* CNPSo 3448^T, *B. nitroreducens* TSA1^T, *B. ottawaense* OO99^T, *B. rifense* CTAW71^T and *B. shewense* ERR11^T. In all cases (Table 2), ANI values were well below the 95–96% value typically suggested for delineation of species (Richter and Rosselló-Móra, 2009). In fact, the highest value obtained (87.66%) was for the comparison between 14AB^T and *B. japonicum* ATCC 10324^T. The GTDB taxonomic classification based on topology and ANI values showed that there are no genomes in the database that could be categorised as a representative of the same species as 14AB^T. Taken together, the results of these genome-based comparisons indicated that the putative species recognized here represents a unique taxon in the genus *Bradyrhizobium*.

Various phenotypic and biochemical properties for each of the 10 *Acacia* strains were also used in comparisons with closely related *Bradyrhizobium* species. Many of these characters supported clustering of the strains. All ten isolates were Gram negative, motile rods that were catalase and oxidase positive, and that grew optimally at 28 °C and pH 7. Also, after 7 days of incubation on YMA, colonies appeared translucent and produced large amounts of slime, except for those of isolate 20BH that produced less slime and were pearl white in appearance. All of the isolates could grow at temperatures between 15 and 35 °C and at pH 5–9, with some also able to grow at 10 °C and at pH 4 and 10, although it should be noted that the growth at the extremes was not verified due to the use of unbuffered media (Supplementary Table S3). In terms of growth in the presence of salt, large variation was observed, but they could all grow in media containing 0% or 0.1 % (w/v) NaCl, with only a few capable of growing in 2.5% NaCl. As expected, antibiotic sensitivity among the isolates were highly variable, although all isolates appeared to be resistant to kanamycin.

Although the traits tested using the API 20NE and BIOLOG GN2 systems revealed a substantial level of among-isolate variation, a range of characters were common among the 10 *Acacia* strains (Table 3; Supplementary Table S3). These included the ability to assimilate esculin ferric citrate and the inability to assimilate L-tryptophan, as well as to utilise a range of compounds as carbon sources (i.e., D-fructose, L-fucose, methyl pyruvate, D-gluconic acid, β-hydroxy butyric acid, D,L-lactic acid, D-saccharic acid and L-pyroglutamic acid) while being incapable of using others (i.e., α-cyclodextrin, N-acetyl-D-galactosamine, D-melibiose, β-methyl-D-glucoside, sucrose, D-trehalose, α-hydroxy butyric acid, p-hydroxy phenylacetic acid, α-keto valeric acid, malonic acid, glycyl-L-aspartic acid, glycyl-L-glutamic acid, L-ornithine, L-threonine, thymidine, phenylethylamine, 2-aminoethanol, D,L-α-glycerol phosphate and glucose-1-phosphate). Additionally, some of these traits allowed discrimination between the putative new species and its close relatives (e.g., the metabolism of D-Trehalose, D-Fructose, and Sucrose).

We were unable to complete the BIOLOG tests for isolates 2BB and 7AC, as they repeatedly failed to grow in any of the wells. This result highlights the various difficulties of employing commercial test kits within rhizobial systematics (Avontuur et al., 2022; Sutcliffe, 2015; Sutcliffe et al., 2012). Many problems can arise when using such systems and it can result in poor reproducibility and large margins of error (Sutcliffe, 2015) which can be exacerbated when performing tests which are not taxon-specific (Sutcliffe et al., 2012).

Table 2
Pairwise ANIb values for strain 14AB^T and the type strains of closely related *Bradyrhizobium* species. The proportion of genomes (%) analysed are indicated in parentheses.

	1	2	3	4	5	6	7	8	9	10	11	12
1	*	87.35 (69.57)	87.13 (61.54)	85.48 (63.10)	87.26 (68.26)	87.18 (69.07)	86.90 (65.57)	86.05 (67.30)	87.31 (69.51)	85.94 (65.45)	86.90 (64.33)	88.04 (69.46)
2	86.62 (59.97)	*	86.45 (51.62)	85.51 (57.76)	86.52 (60.27)	87.02 (62.61)	86.31 (55.89)	85.73 (59.58)	91.42 (68.05)	85.55 (56.76)	86.37 (53.76)	87.23 (61.54)
3	87.64 (69.66)	87.62 (68.07)	*	85.70 (62.63)	86.92 (69.12)	86.94 (67.82)	86.70 (69.90)	86.52 (67.34)	87.76 (70.20)	86.39 (63.98)	93.20 (77.60)	88.17 (70.35)
4	85.25 (59.86)	85.80 (64.23)	85.11 (52.49)	*	84.95 (60.24)	84.96 (61.75)	84.85 (56.97)	85.30 (60.75)	85.91 (61.59)	85.69 (61.53)	85.21 (54.57)	85.41 (59.27)
5	87.05 (65.81)	87.06 (67.66)	86.45 (58.59)	85.10 (61.71)	*	93.43 (77.89)	88.69 (65.83)	85.25 (61.16)	88.87 (74.39)	85.28 (59.85)	86.31 (61.15)	88.52 (70.59)
6	86.71 (63.11)	87.22 (66.65)	86.06 (54.50)	84.73 (59.21)	93.16 (73.05)	*	88.15 (61.58)	84.69 (59.39)	86.69 (65.16)	84.73 (57.31)	85.90 (57.76)	86.60 (64.04)
7	86.90 (65.48)	87.02 (65.23)	86.15 (62.10)	85.02 (60.53)	88.74 (68.76)	88.58 (67.96)	*	85.28 (62.42)	87.05 (68.22)	85.24 (60.20)	86.33 (65.25)	86.64 (64.45)
8	85.16 (57.53)	85.65 (58.65)	85.06 (51.09)	84.68 (54.30)	84.41 (53.91)	84.36 (55.24)	84.50 (53.02)	*	85.38 (57.85)	89.49 (63.24)	84.80 (54.48)	85.42 (59.47)
9	86.87 (63.65)	91.76 (72.25)	86.80 (56.66)	85.86 (59.40)	88.41 (70.55)	86.62 (65.37)	86.63 (62.04)	85.82 (62.37)	*	85.73 (59.70)	86.67 (59.48)	89.04 (71.74)
10	85.55 (62.15)	86.00 (62.76)	85.64 (53.37)	85.72 (61.26)	85.16 (58.08)	84.93 (59.25)	85.10 (56.23)	90.31 (69.91)	85.92 (61.30)	*	85.50 (55.67)	85.76 (61.33)
11	87.27 (71.34)	87.35 (69.39)	92.90 (76.07)	85.54 (64.44)	86.70 (70.58)	86.62 (70.26)	86.62 (72.52)	86.06 (70.64)	87.42 (72.53)	86.03 (65.44)	*	87.76 (72.36)
12	87.66 (63.49)	87.54 (65.54)	87.24 (56.82)	85.28 (57.20)	88.14 (66.81)	86.71 (64.29)	86.22 (59.05)	85.97 (63.47)	89.06 (71.59)	85.79 (59.12)	87.04 (59.72)	*

1 – *B.* sp. 14AB^T, 2 – *B. niftali* CNPSo3448^T, 3 – *B. betae* PL7HG1^T, 4 – *B. hipponense* aSej3^T, 5 – *B. ottawaense* OO99^T, 6 – *B. shewense* ERR11^T, 7 – *B. nitroreducens* TSA1^T, 8 – *B. rifense* CTAW71^T, 9 – *B. diazoefficiens* USDA110^T, 10 – *B. cytisi* CTAW11^T, 11 – *B. cosmicum* 58S1^T, 12 – *B. japonicum* USDA6^T.

Table 3
Distinguishing characteristics with regards to closely related species.

Characteristics[1]	*Bradyrhizobium* species[2]							
	***B. xenonodulans* sp. nov.**	***B. canariense***	***B. betae***	***B. cytisi***	***B. rifense***	***B. japonicum***	***B. cosmicum***	***B. ganzhouense***
Growth at pH 4	v	+	nd	–	–	+	nd	–
Growth in the presence of 1% NaCl	v	–	+	–	–	+	–	+
Carbon Utilization:								
D-Fructose	+	+	v	+	+	–	–	+
Sucrose	–	–	–	–	–	+	–	–
D-Trehalose	–	–	–	–	–	+	–	nd
Number of strains used for characterization	10	4	4	6	2	Not known	2	3

[1] Characteristics scores are as follows: + indicates a positive result; - indicates a negative result; w indicates weak growth; nd indicates the result was not determined; v indicates that the results were variable.

[2] Relevant data were extracted from previous publications as follows: *B. canariense* (Vinuesa et al., 2005); *B. betae* (Rivas et al., 2004); *B. cytisi* (Chahboune et al., 2011); *B. rifense* (Chahboune et al., 2012); *B. japonicum* (Jordan, 1982); *B. cosmicum* (Wasai-Hara et al., 2020); *B. ganzhouense* (Lu et al., 2014).

Confirmation of nodulation capability

Nodulation tests were performed to confirm the nodulation ability of the novel species (Table 1). Except for isolates 2BB and 7AB, all of the bacteria examined induced formation of effective nodules on the roots of *A. mearnsii*. Three isolates (2AC, 14AB^T, 20DD) also nodulated *A. dealbata*. On both plant hosts, the nodules were indeterminate (Supplementary Fig. S3), suggesting that they have a persistent apical meristem (Sprent and James, 2007). In all nodulation experiments, *glnII* sequence analysis confirmed that the bacteria obtained from the induced nodules, corresponded with those of the isolates used as inoculum.

Inferred geographic origins

In order to shed light on the possible geographic origins of the 10 *Acacia* strains or their symbiotic loci, broadly sampled phylogenies were computed for *nifD* and *nodA*, as well as a concatenated *recA* + *glnII* dataset. The latter consisted of 3464 taxa (1684 haplotypes) and 948 aligned nucleotides. The *nifD* dataset consisted of 2021 taxa (represented by 1028 unique sequences or haplotypes) and 909 aligned nucleotides, while the *nodA* dataset consisted of 1433 taxa (847 haplotypes) and 693 aligned nucleotides. Because DAMBE showed that substitutions at third codon positions were highly saturated in both the *nodA* and *nifD* datasets (Supplementary Fig. S4), subsequent phylogenetic analyses employed the inferred amino acid sequences for both datasets.

Inspection of the *recA* + *glnII* ML phylogeny provided limited information regarding the geographic origin of the novel species relative to the origins of the taxa included (Fig. 2). In general, the observed clustering patterns did not reflect the geographic origin of strains nor the origin of their host plant. The majority of the *recA* + *glnII* tree consisted of groups that contain isolates from multiple geographic origins, with a few notable groups that consisted of isolates from the same geographical region. Overall, these sequences, which are representative of the conserved core genome of *Bradyrhizobium*, mostly lacked phylogeographic signal.

By contrast, the *nodA* and *nifD* data both contained a strong phylogeographic signal (Fig. 2). Although the *nifD* phylogeny was skewed by the large number of isolates from the Americas, it still contained clusters that are homogenous or near homogenous with regards to the geographic origin of the species. Overall, however, the *nodA* phylogeny showed a better balance between isolates from different continents and thus formed many large clusters containing isolates only from specific regions. Both of these ML phylogenies also corresponded broadly regarding the geographic origin of the *Bradyrhizobium* isolates and the geographic origin of the host plant species (Fig. 2).

The *nodA* ML phylogeny contained all 16 clades previously identified for *Bradyrhizobium* (i.e., Clades I to XVI) (Beukes et al., 2016; Bromfield et al., 2019; Muñoz et al., 2011; Steenkamp et al., 2008; Stępkowski et al., 2018), with the 10 isolates from the current study grouping exclusively in Clade I (Supplementary Fig. S5). Within Clade I (Fig. 3), isolates 2AA, 2AC, 20DD, 20CC and 20BK were represented by a single haplotype, which is most closely related to BDV5029 (originating from *Bossiaea ensata*, Australia), USDA 3002 (from *Acacia decurrens*, Brazil),

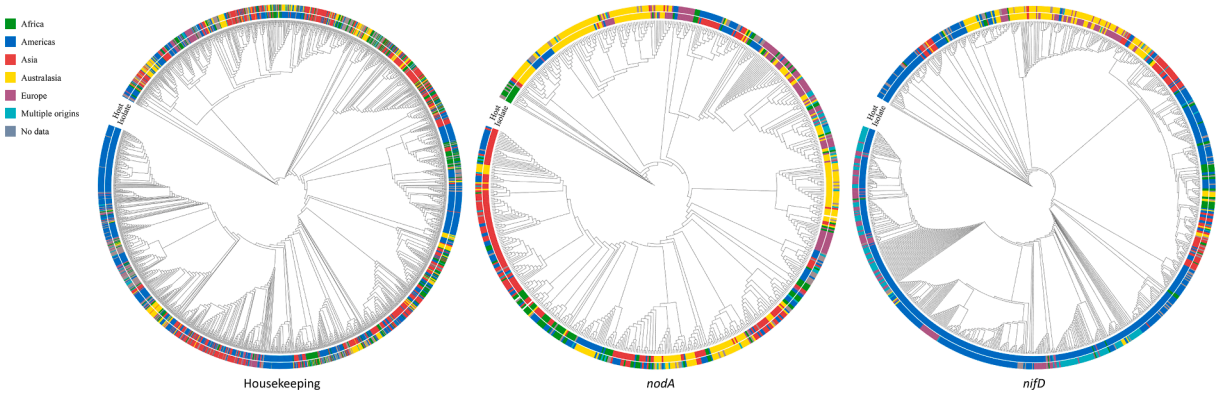

Fig. 2. Maximum likelihood phylogenetic trees for *recA* + *glnII*, *nodA* and *nifD*, which were inferred using all available sequences for *Bradyrhizobium* species and strains. Geographic origin of the isolates is indicated on the inside track, while the geographic origin of the specific plant host species is indicated on the outside track for phylogeny. More detailed information for the taxa included in these analyses are listed in Supplementary Table S2 such as the strain number, accession numbers for the individual loci, the country of origin for the bacteria, as well as the isolation source (e.g., nodules), the names of their legume hosts, the origin of that host as well as the reference associated with the isolation of the bacteria. Also, the two phylogenies can be inspected online, using various iTOL display options available at https://itol.embl.de/shared/RicuClaassens.

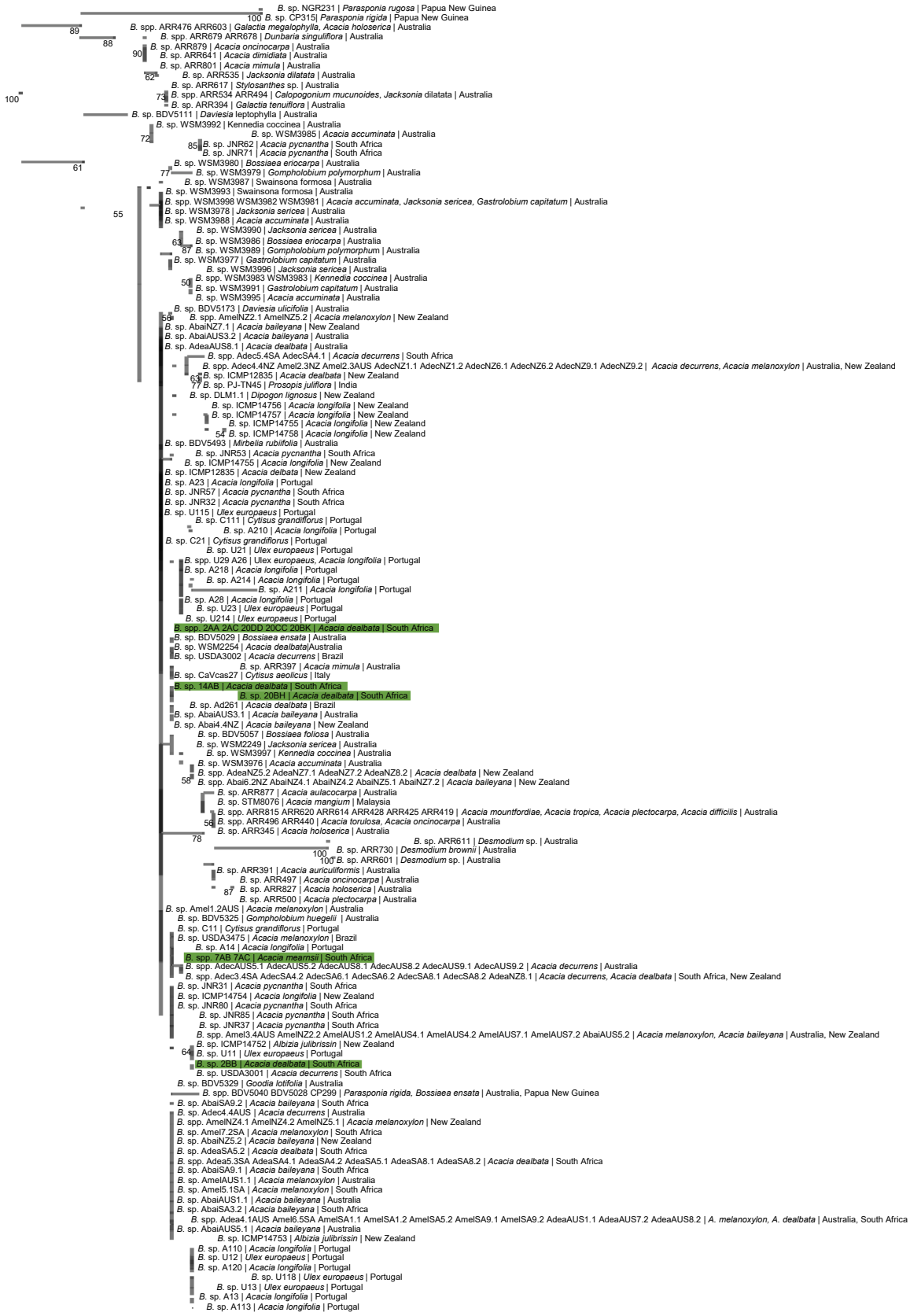

Fig. 3. A maximum likelihood phylogenetic tree showing Clade I of the *nodA* locus (inferred amino acid sequence) containing the ten isolates from this study (highlighted in green) as well as *Bradyrhizobium nodA* sequences available on NCBI (Accessed 1 June 2020). Only support $\geq$ 50% is indicated. Accession numbers for the relevant sequences can be found in Supplementary Table S2. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

and WSM2254 (from *Acacia dealbata*, Australia), which is also used as a commercial inoculant (Reeve et al., 2015). Isolates 20BH and 14AB^T grouped together and are closely related to ARR397 (from *Acacia mimuli*, Australia), Ad261 (from *Acacia dealbata,* Brazil), CaVcas27 (from *Cytisus aeolicus*, Italy), Abai4.4NZ (from *Acacia baileyana*, New Zealand), and AbaiAUS3.1 (from *Acacia baileyana*, Australia). Isolates 7AB and 7AC formed a group that also included 13 other obtained from *A. dealbata* and *A. decurrens* in Australia, South Africa, and New Zealand. Finally, isolate 2BB formed part of a group (with 64% bootstrap support) that contained eight strains (represented by a single haplotype) isolated from *Acacia melanoxylon* and *Acacia baileyana* in New Zealand and Australia, respectively, as well as USDA 3001 isolated from *A. decurrens* in South Africa, U11 from *Ulex europaeus* in Portugal and ICMP14752 isolated from *Albizia julibrissin* in New Zealand.

Although Clade I mostly contained isolates originating in Australia (Fig. 3), it was somewhat more diverse in terms of the isolates' geographic origins. Of the 207 *Bradyrhizobium* strains included in Clade I, 89 came from Australia, 46 from South Africa, 38 from New Zealand, 25 from Portugal, three each from Papua New Guinea and Brazil, and one each from Italy, India, and Malaysia. As far as host species are concerned, *Acacia* species are the most prevalent (146 strains), with most being from *A. dealbata* (28 isolates), *A. melanoxylon* (25 isolates) and *A. decurrens* (24 isolates). Additionally, a further 29 host species from 20 genera were also represented in this clade.

Apart from Clade I, South African strains are typically also included in Clades III, XIII, XIV and XV. Clade III is large and cosmopolitan and from its 753 isolates, 23 originate from South Africa and 91 from Australia. By contrast Clades XIII, XIV and XV are relatively small and were only identified recently (Beukes et al., 2016). They appear to be unique to South Africa where they are associated with certain legumes indigenous to the region.

In terms of host species, Clades III, V, VI and X also included *Bradyrhizobium* strains from *Acacia*. Of the 753 Clade III isolates, 67 were isolated from the nodules of *Acacia* species, while the remainder came from diverse legumes species spanning 58 different genera. Clade V consisted of 69 isolates, mostly from *Acacia* spp. (52 isolates), and primarily from Brazil (49 isolates) and Australia (15 isolates). Clade VI included nine strains, all being photosynthetic strains isolated in Senegal from *Aeschynomene afraspera*, *Aeschynomene nilotica*, *Aeschynomene sensitiva* and *Aeschynomene indica*. Clade X contained 33 isolates from Australia, of which 15 came from *Acacia* species, while the others came from 14 different legume species across nine different genera.

In terms of the geographic origin of isolates and host legumes in the remaining clades, Clade II contained 220 isolates, mostly from countries in Europe, the Mediterranean, as well as Australia (25). Clade IV contained 74 isolates from 12 different countries, including Australia (17 isolates). Clade VII contained 31 isolates, mostly from tropical and subtropical regions, with one isolate from Australia. Clade VIII contained 55 isolates, also with tropical and subtropical origins. Clade IX contained a single strain from Zimbabwe, Clade XI contained 10 isolates from Spain, while Clade XII contained nine isolates from Spain and Nepal. Clade XVI contained four isolates from *Pachyrhizus erosus* in Honduras. Clades II and IV taxa were all isolated from diverse legumes species spanning numerous genera, while those in Clades VIII and IX all came from nodules of *Arachis hypogaea*, those in Clade XI came from *Lupinus mariae-josephae*, and those in Clade XII were isolated from *Cytisus scoparius* and *Chamaecrista fasciculata.*

The *nifD* phylogeny did not have previously defined clades like the *nodA* phylogeny. The smallest possible group containing all the isolates examined here consist of 95 isolates (Fig. 4). Of these, 71 were isolated from *Acacia* species in Australia and Portugal, as well as two isolated from *Acacia koa* in Hawaii. Within this group, isolates 7AB and 7AC clustered together, while 2BB is closely related to strain A14 (from *Acacia longifolia*, Portugal), C111 (from *Cytisus grandifloras*, Portugal), 5029F (from *Bossiaea ensata*, Australia), and 0401 m (from *Acacia implexa*, Australia). Isolates 2AA, 2AC, and 14AB^T formed a single haplotype as did 20BH, 20BK, and 20CC, which both grouped with isolate U115 from *Ulex europaeus* (Portugal).

Discussion

Here we describe the delineation of a new *Bradyrhizobium* species associated with *A. dealbata* and *A. mearnsii* growing in an invasive setting in South Africa using the taxonomic workflow proposed by Venter et al. (Venter et al., 2017). This involved genealogical concordance analysis for generating an initial species hypothesis, and then confirming its plausibility using additional lines of biological evidence. Despite differences among the members of this new taxon in one or more of the six housekeeping gene sequences analysed, all of the inferred single-gene genealogies showed that the ten strains constitute a unique and well-supported clade that does not include any of the previously described *Bradyrhizobium* species. It was recovered when we analysed these six genes in a concatenated dataset. Accordingly, these ten strains were recognized as a coherent group and possible new *Bradyrhizobium* species.

Validity of this species hypothesis was interrogated using a range of biological traits. As expected, its members were phenotypically diverse (de Lajudie et al., 2019), but they had many traits in common, especially in terms of the temperature and pH ranges supporting their growth. They also shared numerous biochemical features (i.e., carbon source utilization and ability to assimilate certain compounds), of which some allowed discrimination from other closely related *Bradyrhizobium* species. Most of the strains were further capable of inducing indeterminate nodules on the roots of either (or both) *A. dealbata* and *A. mearnsii*. The uniqueness of the proposed new taxon was also supported based on overall genome relatedness with known *Bradyrhizobium*. All JSpecies comparisons with the genome sequence of its chosen type strain produced ANI values well below the 95–96% range used to delineate species (Richter and Rosselló-Móra, 2009), and the GTDB results similarly showed that there are no available genomes that can be deemed to represent the same species.

The data presented here satisfied all of the so-called "minimal standards" for describing a new rhizobial species, as proposed by the Subcommittee on Taxonomy of Rhizobia and Agrobacteria of the International Committee on Systematics of Prokaryotes (de Lajudie et al., 2019). This included the essential requirement of showing the new taxon's unambiguous differentiation from other species based on genome sequence comparisons. Variation among multiple strains was clearly documented using sequence data and phenotypic traits, and their nodulation phenotypes were also described. Accordingly, the novel group of rhizobial strains obtained from the root nodules of *A. dealbata* and *A. mearnsii*, collected from diverse locations in South Africa, was named *Bradyrhizobium xenonodulans* sp. nov. with type strain 14AB^T (=LMG 31415 ^{T} = SARCC-753 ^{T}). Other known *Bradyrhizobium* species capable of nodulating *Acacia* species include *B. acaciae* (Avontuur et al., 2022), *B. ganzhouense* (Lu et al., 2014), and *B. viridifuturi* (Helene et al., 2015).

For exploring the biogeographic origin of *B. xenonodulans* sp. nov., two possible hypotheses were considered for explaining why an introduced legume can fix nitrogen in a non-native setting (Klock et al., 2015). The first is known as the "Accompanying Mutualist Hypothesis" and predicts that the original rhizobial symbiont was co-introduced with the legume host, and that the symbiotic relationship continues as in their native range (Crisóstomo et al., 2013; Klock et al., 2015; Ndlovu et al., 2013; Rodríguez-Echeverría et al., 2012). According to the "Generalist Host Hypothesis" the legume host can interact efficiently with a wide range of rhizobia, possibly even some that are native to the new area (Klock et al., 2015; Richardson et al., 2000). In this scenario it would be irrelevant whether the original rhizobial symbiont is co-introduced into the new area, as the legume's promiscuity would allow it to engage with new symbiotic partners. Although evaluation of these two hypotheses is relatively straightforward, the interpretation of experimental data

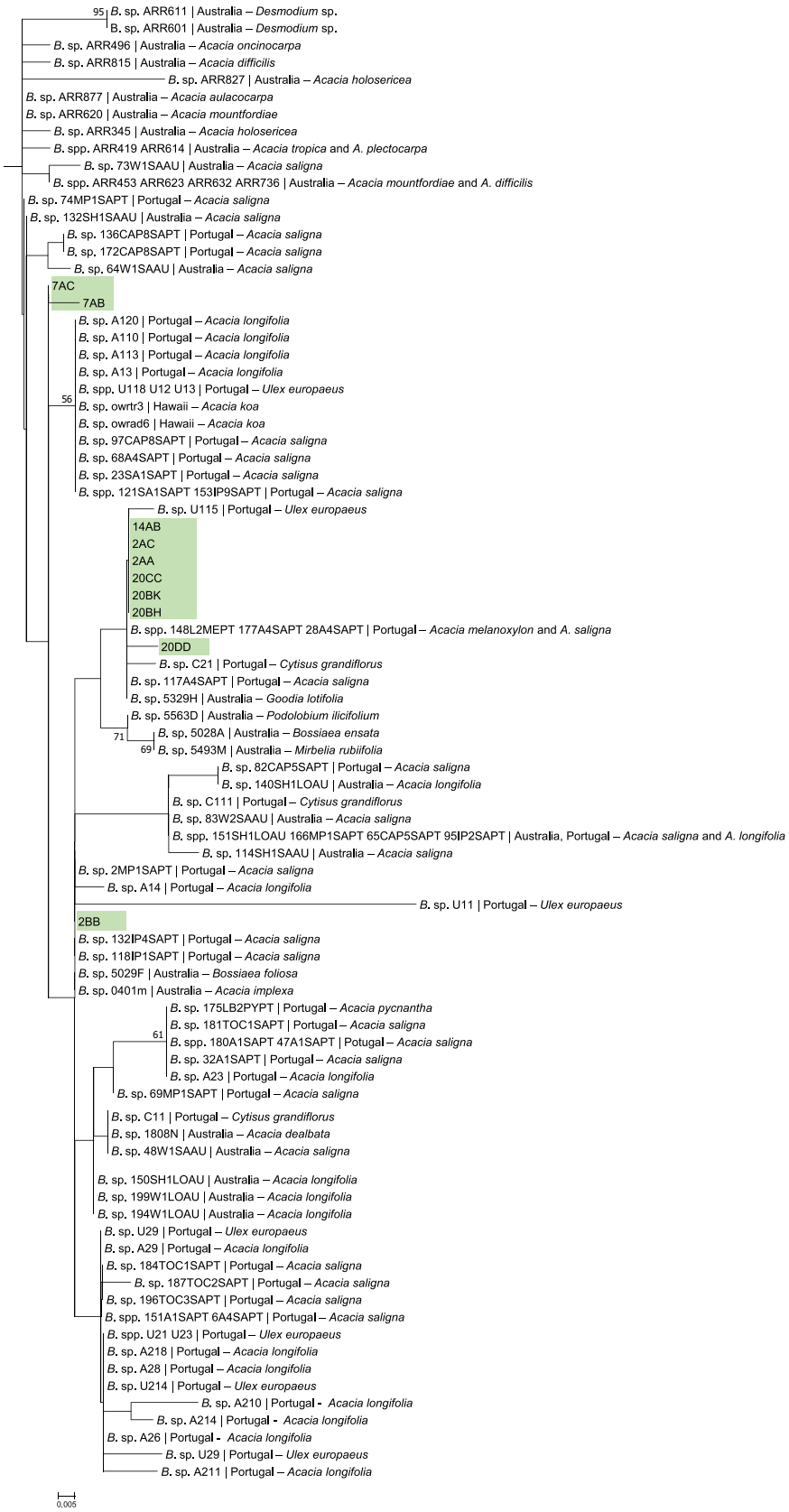

Fig. 4. A maximum likelihood (ML) tree showing a subgroup of the *nifD* locus (inferred amino acid sequence) containing the ten isolates from this study (highlighted in green) as well as *Bradyrhizobium nifD* sequences available on NCBI (Accessed 20 January 2020). Only support $\geq$ 50% is indicated, while the scale bar shows the number of nucleotide substitutions per site. Accession numbers for the relevant sequences can be found in Supplementary Table S2. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

would be complicated by the prevalence of HGT at symbiotic loci in rhizobia (Greenlon et al., 2019; Wardell et al., 2022). Therefore, strict interpretation of the "Accompanying Mutualist Hypothesis" would dictate that both bacterium and its original symbiotic loci were introduced into the new region (and for the "Generalist Host Hypothesis" both would be native to the new region). However, this is not always the case in practice, as the symbiotic loci of co-introduced rhizobia may spread through native rhizobial populations via HGT. In the new region, the legume host can thus initiate new symbiotic partnerships with rhizobia that are already well-adapted to local edaphic conditions, but that have acquired the requisite symbiotic loci (Greenlon et al., 2019; Wardell et al., 2022). To account for these complexities, the two hypotheses were evaluated using a global collection of *Bradyrhizobium nodA* and *nifD* sequences and of *Bradyrhizobium recA* and *glnII* sequences to trace the biogeographic origins of *B. xenonodulans* sp. nov. and its symbiotic loci.

Despite being obtained from the root nodules of *A. dealbata* and *A. mearnsii* growing in South Africa, the *nodA* of the *B. xenonodulans* sp. nov isolates did not originate in the country. Our global *nodA* phylogeny contained 1433 taxa and separated all 16 of the biogeographic clades known for *Bradyrhizobium* (Beukes et al., 2016), but was substantially more inclusive than the one reported by Beukes et al. (Beukes et al., 2016) who included only 499 taxa. Within this well-represented phylogeny, *B. xenonodulans* sp. nov isolates formed part of Clade I, which was mostly populated by Australian isolates. A number of Clade I isolates also came from South Africa, New Zealand, Portugal, Papua New Guinea, Brazil and Malaysia. However, most of the non-Australian isolates in Clade I had been isolated from Australian *Acacia* species or the *Ulex europaeus*-associated isolates in Portugal, which were shown to have been co-introduced with Australian *Acacia* (Crisóstomo et al., 2013; Rodríguez-Echeverría, 2010). Although our *nifD* phylogeny was taxonomically less well-represented, it revealed similar distribution patterns. These data thus showed that the *B. xenonodulans* sp. nov isolates examined in the current study have *nodA* and *nifD* alleles that are native to Australia. Their presence in root nodules of Australian *Acacia* species growing in South Africa is most likely due to co-introduction with the legume into the region.

Co-introduction of *Bradyrhizobium* isolates with Australian *Acacia* species into South Africa was initially thought to be unlikely (Parker, 2001). Many legumes native to South Africa are closely related to Australian *Acacia* and Parker (Parker, 2001) argued that their *Bradyrhizobium* symbionts would be compatible with the introduced *Acacia*, thereby hampering local establishment of co-introduced *Bradyrhizobium*. However, more recent studies showed that African relatives of the Australian *Acacia* species (i.e. *Vachellia* and *Senegalia* species) preferentially interact with symbionts from the genera *Mesorhizobium* and *Ensifer* (Bakhoum et al., 2015; Beukes et al., 2019; Ngwenya et al., 2016). In contrast to Parker's prediction (Parker, 2001), the current study and that of Ndlovu et al. (Ndlovu et al., 2013) and Le Roux et al. (Le Roux et al., 2016) clearly demonstrated co-introduction of an *Acacia* symbiont (or at least its symbiotic loci) into South Africa. Given the prevalence of *Bradyrhizobium* carrying Australian *nodA* alleles in certain South African locations, such co-introductions with *Acacia* might have been common, and these introduced symbionts are likely key to the establishment of *Acacia* in invasive settings (Warrington et al., 2019).

The findings presented here, together with those from previous studies (Le Roux et al., 2016; Ndlovu et al., 2013) allowed inferences regarding the origins of *nod* and *nif* loci carried by *Bradyrhizobium* symbionts of invasive *Acacia*, but the origin of the species itself remains unclear. Under the current taxonomic systems utilised for rhizobia and other bacteria (Hedlund et al., 2022; Parker et al., 2016), species affinities are inferred from the stable core component of the organism's genome. To address this issue, we constructed a *Bradyrhizobium* phylogeny based on all available sequences for *recA* and *glnII* in 3464 taxa. However, this housekeeping gene tree generally did not reveal biogeographic patterns, as it lacked isolate groupings linked to particular geographic locations (see Fig. 2). Therefore, we cannot employ a strict interpretation of the "Accompanying Mutualist Hypothesis" to explain the presence of *B. xenonodulans* sp. nov. in South Africa. The possibility cannot be excluded that the bacterium is native to southern Africa, and that the strains examined acquired their Australian symbiotic loci from an unrelated bacterium previously introduced to the region.

Description of Bradyrhizobium xenonodulans sp. nov

Bradyrhizobium xenonodulans (xe.no.no'du.lans. Gr. masc. adj. *xenos*, foreign; N.L. part. adj. *nodulans*, nodulating; N.L. part. adj. *xenonodulans*, foreigner that nodulates).

The cells are Gram-negative, motile rods that are 1 μm in length. On YMA the colonies appear mucoid, white, approximately 1 mm in diameter, circular and convex with an entire margin. Growth occurs at pH 4 – 10 and at temperatures ranging from 10 to 35 °C, while optimal growth conditions occur at a temperature of 28 °C and at pH 7.0. Grows on YMA at a salt concentration ranging from 0 to 2,5% NaCl. Catalase and oxidase positive. Strains use D-Fructose, L-Fucose, Methyl Pyruvate, D-Gluconic Acid, β-Hydroxy Butyric Acid, D,L-Lactic Acid, Quinic Acid, D-Saccharic Acid and L-Pyroglutamic Acid as a carbon source but not α-cyclodextrin, N-Acetyl-D-galactosamine, D-Melibiose, β-Methyl-D-Glucoside, Sucrose, D-Trehalose, Mono-Methyl-Succinate, α-Hydroxy Butyric Acid, p-Hydroxy Phenylacetic Acid, p-Hydroxy Phenylacetic Acid, α-Keto Valeric Acid, Malonic Acid, Glycyl-L-Aspartic Acid, L-Ornithine, L-Threonine, Thymidine, Phenyethylamine, 2-Aminoethanol, D,L-α-Glycerol Phosphate and Glucose-1-Phosphate. Assimilates Esculin ferric citrate. Can form nodules on *Acacia dealbata* and *Acacia mearnsii*.

The type strain 14AB^T (=LMG 31415 ^{T} = SARCC-753 ^{T}) was isolated in 2002 (Joubert, 2002) from a root nodule on *Acacia dealbata* in South Africa. The complete genome of strain 14AB^T is 8,27 mbp in length and has a G + C content of 63,95%.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.syapm.2023.126452.

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