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Estimated timeline for the evolution of symbiotic nitrogen fixing *Paraburkholderia*

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ABSTRACT

The nitrogen-fixing and nodule-forming symbionts of legumes, which belong to the class *Betaproteobacteria,* are informally known as beta-rhizobia. Thus far, members of this group have only been found in the genera *Paraburkholderia, Trinickia* and *Cupriavidus*. In this study, we investigate the poorly characterized evolutionary history of this trait in the predominant beta-rhizobial genus, *Paraburkholderia*. This was determined in the context of the current evolutionary theories and date estimates of rhizobia, the genus *Paraburkholderia* and the earth. Evolutionary divergence dates of rhizobial *Paraburkholderia* as well as their ancestral nodulation states were estimated using over 800 diverse proteobacterial genomes. Molecular dating was carried out using the software BEAST (Bayesian Evolutionary Analysis Sampling Trees) and APE (using the 'chronopl' function). Our results showed that the most recent common ancestor (MRCA) of the extant beta-rhizobial species emerged between 2744 and 1752 million years ago (Ma) and later (2135–514 Ma) diverged into the lineages *Cupriavidus*, *Trinickia* and *Paraburkholderia*. However, major diversifications of rhizobial *Paraburkholderia* occurred in three phases: (i) during the Permian and Triassic periods (400–200 Ma) when Pangaea was fully assembled and its landmass filling up with flora and fauna, (ii) during the Jurassic period (200–150 Ma) when fauna and flora were flourishing in Pangaea, and (iii) during the Cretaceous and Paleogene periods (150–23 Ma) when Gondwana was breaking up. Furthermore, *Paraburkholderia* were estimated to have acquired their precursor nodulation loci that evolved into their current nodulation loci from different sources between 103 and 48 Ma. Accordingly, our study describes the evolutionary history of rhizobial *Paraburkholderia*, thus enabling us to understand the past environmental factors that shaped the current geographical distribution of these agriculturally important bacteria, and to identify locations potentially rich in beta-rhizobia.

1. Introduction

As a member of the class *Betaproteobacteria*, *Paraburkholderia* forms part of the most well-represented and highly diverse bacterial phylum, the *Pseudomonadota* (formerly known as *Proteobacteria*) (List of Prokaryotic names with Standing in Nomenclature (LPSN); https://www.bacterio.net; Holt, 1984; Balows et al., 1992; Holt et al., 1994; Collier et al., 1998; Gupta, 2000; Parte, 2020; Oren et al., 2021). Like its phylum and class, *Paraburkholderia* is diverse and rich in species of biological and socioeconomic significance, ranging from plant beneficial taxa to phytopathogens (Coenye et al., 2001; Suárez-Moreno et al., 2012; Beukes et al., 2017; Ferro et al., 2019). Currently, the genus includes more than 90 species (LPSN, as of 7 August 2025) (Parte et al., 2020) that are globally distributed across a range of environments including niches associated with soils, water, plants, fungi and animals (Coenye et al., 2001; Depoorter et al., 2016; Beukes et al., 2017). Of the known *Paraburkholderia* species, 24 (up to June 2024) are rhizobial in nature as they can induce the formation of nitrogen-fixing nodules on the roots of certain legumes (Mavima et al., 2021, 2022; Belles-Sancho et al. 2023). Among *Betaproteobacteria*, this rhizobial trait also occurs in species of

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Cupriavidus and *Trinickia*, which together with nodulating *Paraburkholderia* are often referred to as beta-rhizobia (Barrett and Parker, 2005, 2006; Gyaneshwar et al., 2011; Estrada-de los Santos et al., 2018). Those residing in the *Alphaproteobacteria* (e.g., in the genera *Rhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Microvirga*, and *Sinorhizobium*) are referred to as alpha-rhizobia.

Based on their known geographic distribution, many beta-rhizobia are confined to specific regions (Barrett and Parker, 2005, 2006; Gyaneshwar et al., 2011; Platero et al., 2016; Estrada-de los Santos et al., 2018). For example, rhizobial *Paraburkholderia* are predominantly associated with legume-rich areas with acidic soils like the Fynbos biome in South Africa, and the Caatinga and Cerrado biomes in South America (Bontemps et al., 2010; Lemaire et al., 2015, Lemaire et al., 2016). However, information about the extant distribution of beta-rhizobia is severely limited since many parts of the world remain uncharted for these bacteria. Also, previous work on beta-rhizobia in their native environments (Bontemps et al., 2010, 2016; Mishra et al., 2012; Beukes et al., 2013; Bournaud et al., 2013; Lemaire et al., 2016; Pires et al., 2018; Silva et al., 2018; Zilli et al., 2021; Rouws et al. 2024) could not explain how and why these bacteria became endemic in particular regions, despite showing clear biogeographical signal. In other words, beta-rhizobial distribution patterns can generally not be explained through clear evolutionary hypotheses or scenarios (Mishra et al., 2012; Lammel et al., 2013; Lemaire et al., 2015, 2016; Mavima et al., 2022; Rouws et al. 2024).

Pinpointing the exact mechanisms driving the divergence and distribution of bacteria is typically scale-dependent. For instance, at local scales bacterial evolution is mostly determined by growth-limiting environmental factors, intrinsic dispersal ranges of the bacteria and, if relevant, the availability of compatible host species (Bahlaoui et al., 1998; Fierer et al., 2009; Custer et al., 2022). At these scales, adaptation to particular niches and/or the emergence of new species are ultimately mediated by mutations that accumulate over time and/or horizontal gene transfer (HGT) of adaptive elements (Fenchel and Finlay, 2004; Nemergut et al., 2011; Cohan, 2017). However, the forces driving bacterial distribution at broader scales (e.g., migration between niches or habitats) vary and are often uncertain (Nemergut et al., 2011; Custer et al., 2022). This is because phenomena such as wind, dust storms, oceanic drifts and movement of their hosts may result in long-distance spread of the bacteria in question (Griffin et al., 2006; Gorbushina et al., 2007; Gillespie et al., 2012). The latter (e.g., host plants) may also be mediated by vicariance due to geographical separation associated with tectonic plate or continental movements (Ali et al., 2013). Irrespective of the dispersal mechanisms involved, such long-distance distribution of bacterial species typically leads to the disjunction of distribution patterns and evolutionary relationships, with closely related taxa inhabiting distinct geographical areas (Nemergut et al., 2011; Hanson et al., 2012). In the case of rhizobial *Paraburkholderia*, any one or combinations of these long-distance dispersal mechanisms might explain the geographic distribution of extant species.

Various previous studies explored the evolutionary origins of rhizobia or the lineages harboring them. Based on sequences for conserved genes or regions, divergence times for lineages containing *Betaproteobacteria* and *Alphaproteobacteria* have been dated, respectively, to 2500 and 1500 million years ago [Ma] during the middle and early Proterozoic Aeon (Battistuzzi et al., 2004; Hedges, 2009). However, vastly different divergence times have been reported among independent studies for beta- and alpha-rhizobia. Sequence analyses using part of the ribosomal RNA (rRNA) operon placed divergence of the clade containing beta-rhizobia at around 747 Ma (https://www.timetree.org; Kumar et al 2017; Marin et al. 2016) or 114 Ma (https://www.timetree.org; Kumar et al 2017; Chriki-Adeeb and Chriki 2016) during the Proterozoic eon or Cretaceous period, respectively, and for the clade containing alpha-rhizobia at around 1040 Ma (Hedges, 2009) or 396 Ma (Chriki-Adeeb and Chriki 2016), during the Paleozoic era. More recently, Rahimlou et al. (2021) analyzed 92 core gene sequences and placed the divergence of the alpha-rhizobia-containing clade at around 1100 Ma and the beta-rhizobia-containing clade at around 250 Ma during the Permian. Nevertheless, differences between the various studies are less pronounced at the genus level; Rahimlou et al. (2021) estimated divergence within *Paraburkholderia*, *Rhizobium*, *Bradyrhizobium*, *Sinorhizobium* and *Mesorhizobium* at 51–4 Ma during the Miocene and late Eocene; Marin et al. (2016) suggested that they diverged 88–3 Ma; while Chriki-Adeeb and Chriki (2016) placed their divergence most likely during the Cretaceous period at around 150–70 Ma.

The overall goal of this study was to provide a robust explanation for the ancestry and extant geographic distribution of rhizobial *Paraburkholderia*. For this purpose, we used a combination of phylogenetic analyses, molecular dating and geographic distribution analyses to investigate the evolutionary history of these bacteria. Firstly, we estimated divergence dates of the genera *Paraburkholderia*, *Cupriavidus* (Chen et al., 2005; Liu et al., 2012; Melkonian et al., 2014; Platero et al., 2016) and *Trinickia* (Sheu et al., 2012; Estrada-de los Santos et al., 2018), as well as *Mesorhizobium*, *Rhizobium*, *Sinorhizobium* and *Bradyrhizobium* (Peix et al. 2015; Howieson and Dilworth, 2016). Secondly, to explore the evolutionary history of rhizobial *Paraburkholderia*, we estimated the divergence dates of species in the so-called *Paraburkholderia tuberum sensu lato* clade (De Meyer et al., 2013; Mavima et al. 2021; Mavima et al. 2022). This is an assemblage of closely related species with members capable of nodulation but that are geographically separated between continents (i.e., *P. tuberum sensu stricto*, "*P. podalyriae*" and *P. sprentiae* are native to Southern Africa, and *P. youngii* and "*P. atlantica*" are native to South/Central America). Lastly, we explored the origins of nodulation as a trait in *Paraburkholderia* using ancestral state reconstruction (ASR) analyses. Overall, our findings provided a snapshot into the likely factors that could have shaped the current geographical distribution of these agriculturally important bacteria.

2. Materials and methods

2.1. Taxon selection and datasets

For the various analyses conducted in this study, five different datasets were used. Four datasets consisted of the aligned nucleotide or amino acid sequences for 92 conserved loci (Na et al., 2018), to investigate the divergence of genera or species that currently contain members able to nodulate, and one consisted of the aligned amino acid sequences for the common nodulation loci, *nodACD*, specifically focusing on the divergence of the loci that evolved into the current loci encoding nodulation. The respective datasets were designated as the '*Pseudomonadota*', 'All *Paraburkholderia*', '*Paraburkholderia* species', 'Rhizobial *Paraburkholderia*' and '*nodACD*' (see sequence data at https://figshare.com/s/bda511ccb0470957d3ed). Among these, the '*Pseudomonadota*' dataset, containing 809 taxa distributed across >120 genera, was obtained from the work published by Rahimlou et al (2021). For the remaining datasets, the 92 conserved gene sequences were obtained from whole genome data publicly available at the National Centre for Biotechnology Information (NCBI; https://www.ncbi.nlm.nih.gov/genbank (Benson et al., 2017)). From each genome, nucleotide sequences for the 92 loci were extracted using the Up-to-date Bacterial Core Gene (UBCG) pipeline (Na et al., 2018). Following alignment with MAFFT (Multiple Alignment using Fast Fourier Transformation; https://mafft.cbrc.jp/alignment/server) (Katoh et al., 2002), aligned sequences were translated *in silico*, concatenated and partitioned using FASconCAT-G (Kück, 2010).

The 'All *Paraburkholderia*' dataset comprised 263 taxa presumably identified as *Paraburkholderia* and whose whole genome sequences were publicly available in the databases of the NCBI and the United States Department of Energy (DOE) Joint Genome Institute (https://jgi.doe.gov), as of June 2024. The dataset also included the type strains of

representatives from genera in *Burkholderia sensu lato* (Beukes et al. 2017) for outgroup purposes. These were *Caballeronia glathei* DSM 50014^T, *Burkholderia cepacia* ATCC 25416^T, *Pararobbsia alpina* LMG 28138^T and *Robbsia andropogonis* LMG 2129^T. This dataset was used to construct the phylogeny needed for the ASR analysis (see below).

The '*Paraburkholderia* species' dataset comprised 96 taxa and included the type strains of *Paraburkholderia* species with validly published names and species with names that are effectively published (being indicated with quotation marks), that have publicly available genome sequences (as of June 2024). For outgroup purposes, it included the type strains of representatives of the genera in *Burkholderia sensu lato* (Beukes et al. 2017). These were *Caballeronia glathei* DSM 50014^T, *Burkholderia cepacia* ATCC 25416^T, *Trinickia symbiotica* JPY-345^T, *Mycetohabitans rhizoxinica* HKI 454^T, *Pararobbsia alpina* LMG 28138^T and *Robbsia andropogonis* LMG 2129^T.

The 'Rhizobial *Paraburkholderia*' dataset consisted of 50 taxa, including all rhizobial *Paraburkholderia* species, various members of *Burkholderia sensu lato*, representatives of the alpha-rhizobia, and *Paraburkholderia* reference strains. For rhizobia, the type strains for the species were used, except in cases where they lack one or more of the common nodulation loci (instead we then used *P. phenoliruptrix* BR3459a, *P. caribensis* TJ182, *S. meliloti* 1021, and *R. leguminosarum* sv. trifolii WSM1325) or where a species is a known legume-nodulator but not yet described (i.e., *Paraburkholderia* sp. JPY105AMAC11-3, *Paraburkholderia* sp. JPY530, and *Cupriavidus* sp. JPY540). The dataset included strains of *Escherichia coli*, *Salmonella enterica* and *Cyanobacteria* for the purpose of calibrating the nodes of phylogenies for molecular clock dating. Also, a second version of this dataset with 55 taxa was created, where five *Mycobacterium* strains were added for comparitive purposes. Additionally, the 92 locus sequence alignments for these 'Rhizobial *Paraburkholderia*' datasets were refined to 63 locus sequence alignments, manually selected based on the completeness of the sequences.

The *'nodACD'* dataset consisted of amino acid sequences for the common nodulation loci (see sequence data at https://figshare.com/s/bda511ccb0470957d3ed). It was used to date the emergence of nodulation in rhizobial *Paraburkholderia*. Relevant sequences were either extracted from the whole genome sequences using Geneious Prime v.2023.2.4 and the local BLAST (Altschul et al., 1990) function in BioEdit (Hall, 1999) or were downloaded directly from the protein database of NCBI. The sequences were aligned and concatenated as before, and the final dataset consisted of 46 taxa spanning beta-rhizobia (i.e., genera *Paraburkholderia*, *Cupriavidus* and *Trinickia*) and alpha-rhizobia (i.e., genera *Mesorhizobium*, *Rhizobium*, *Sinorhizobium* and *Bradyrhizobium*).

In all datasets, we attempted to use as wide as possible a selection of taxa, taking into account phylogenetic diversity, nodulation capacity and geographical distribution, especially for *Paraburkholderia* and *P. tuberum sensu lato*. Additionally, the geographic distribution of rhizobial *Paraburkholderia* in this study comprised of isolates from South America, Central America and Africa. Isolates from South and Central America originated from a wide area spanning the entire South America and a small part of North America, while those from the continent of Africa were isolated from South Africa's Western Cape Province which is a much smaller area by comparison (Bontemps et al., 2010, 2016; Beukes 2013, 2019; Ormeño-Orrillo et al., 2012; Howieson et al., 2013; Lemaire et al., 2016; Rouws et al. 2024).

2.2. Phylogenetic analysis and molecular dating

Maximum-likelihood (ML) phylogenetic trees were constructed using IQ-TREE v.2.0.6 (Nguyen et al., 2015) and RAxML v.8.2 (Stamatakis, 2014). ModelFinder (Kalyaanamoorthy et al., 2017) and ProtTest v.3 (Guindon et al., 2003; Darriba et al., 2011) were employed to independently estimate substitution rates for each gene partition in IQ-TREE (−m MF) and RAxML (−m GTRGAMMA), respectively. For phylogenies generated with IQ-TREE, branch support was estimated using 1000 pseudoreplicates and both ultrafast bootstrap (UFBoot) (Hoang et al., 2018) and the Shimodaira-Hasegawa-like approximate likelihood-ratio test (SH-aLRT) (Guindon et al., 2010). For the RAxML phylogenies, rapid bootstrapping using 1000 pseudoreplicates was used to estimate branch support (Stamatakis et al., 2008; Stamatakis, 2014). Phylogenetic trees were visualized with either iTOL v.6.7 (https://itol.embl.de (Letunic and Bork, 2021)) or FigTree v.1.4.4 (https://tree.bio.ed.ac.uk), and manually edited in Inkscape v.0.92 (https://www.inkscape.org).

Divergence times for the '*Pseudomonadota*', '*Paraburkholderia* species' and 'Rhizobial *Paraburkholderia*' phylogenies were obtained using a penalized likelihood approach with the 'chronopl' function (Sanderson, 2002) in the Analyses of Phylogenetics and Evolution (APE) package in R v.4 (Paradis and Schliep, 2019). The 'lambda' parameter was set at 'zero' ($\lambda = 0$), which allowed substitution rates among tree branches to vary to the fullest (Sanderson, 2002). The '*Pseudomonadota*' chronogram was calibrated using two divergence times including the divergence of Hydrobacteria (represented by *Pseudomonadota)* and Terrabacteria (represented by the lineage containing *Acidobacteria*) at 3540–2830 Ma (Battistuzzi and Hedges, 2009), and the split between *Alphaproteobacteria* and *Betaproteobacteria* at 2928–2154 Ma (Battistuzzi et al., 2004) (Supplementary Tables S1 and S2). Divergence estimates obtained from the *Pseudomonadota* chronogram for the most recent common ancestor (MRCA) of *Burkholderia sensu lato* and the genus *Paraburkholderia* were subsequently used, as secondary calibration points, to calibrate the '*Paraburkholderia* species' chronogram. Two secondary calibration points were used, i.e., 860 Ma for the divergence of the genus *Robbsia* and the rest of *Betaproteobacteria*, and 550 Ma for MRCA of *Paraburkholderia* species (Table 1; Supplementary Tables S1 and S2). The 'Rhizobial *Paraburkholderia*' chronogram was calibrated using two calibration points, i.e., 3203–2490 Ma for the divergence of *Actinobacteria* and *Cyanobacteria* (Battistuzzi et al., 2004) and 3000–2300 Ma for the emergence of *Cyanobacteria* based on geologic and fossil records (Battistuzzi et al., 2004; Blank, 2010; Boden et al., 2021). In all cases, the ranges of all calibration points used in this study were based on the minimum and maximum date estimates from the respective references (Supplementary Tables S1 and S2).

For comparative purposes, divergence times were also estimated for the 'Rhizobial *Paraburkholderia*' dataset using the Bayesian methods in BEAST v.2.6.2 (Dos Reis et al., 2016; Bouckaert et al., 2019). Three calibration points were used sequentially in different combinations, i.e., 3540–2830 Ma for the MRCA of Hydrobacteria and Terrabacteria *sensu* (Battistuzzi et al., 2004), 3000–2300 Ma for the emergence of *Cyanobacteria* based on fossil records (Battistuzzi et al., 2004; Blank, 2010; Boden et al., 2021), and 176–57 Ma for the *Escherichia coli* and *Salmonella* split (Battistuzzi et al, 2004; Sŏrfová et al., 2008). The analysis utilized the following parameter settings: Site model = GTR (Gamma category = 4, Shape = 1.0 and Proportion invariant = 0.0), Clock model = relaxed clock log normal, Tree model = calibrated Yule process, MCMC (Markov Chain Monte Carlo) chains = 5000000 (logged every 1000 chains). Additionally, exponential distribution was applied on calibration points where the minimum date estimates were set as the 'offset' and the 'mean' adjusted to accommodate the date ranges obtained from the literature (Supplementary Tables S1 and S2). Also, the analysis constrained the topology of the tree generated in BEAST by utilizing a starting tree externally generated with IQ-TREE (Supplementary Fig. S1). The optimized relaxed molecular clock model allowed rates among branches to vary (Drummond et al., 2006), while the calibrated Yule process tree model was appropriate for our different species considering some had fossil records (Heled and Drummond, 2012). Convergence of the posterior distributions generated by BEAST was evaluated using Tracer v.1.7 (Rambaut et al., 2018). Individual trace files, which were obtained using different MCMC chains, priors and data were compared to determine if they converge to the same posterior distribution. Lastly, the BEAST-dated tree was curated using TreeAnnotator v.2.6.2 (Rambaut et al., 2018) (using 10 % burnin,

Table 1
Comparison of divergence times estimated in this study with those captured from literature in TIMETREE5.

Taxon divergence	Date estimates in million years[φ]			
	BEAST[∞]	APE 'chronopl'[†]	Midpoint [Lower–Upper] [*]	TIMETREE5[#]
Proteobacteria and *Cyanobacteria*	2466–3444	3050	2955 [2466–3444]	3134 [1007–3186]
MRCA of *Cyanobacteria* spp.	2200–2501	2500	2351 [2200–2501]	2619 [1180–2790]
Alphaproteobacteria and *Betaproteobacteria*	1752–2465	2273, 2744	2248 [1752–2744]	2504 [606–2819]
Bradyrhizobium and *Mesorhizobium*	996–1939	1627, 2196	1596 [996–2196]	1104 [396–1241]
Cupriavidus and *Paraburkholderia*	514–867	916, 2135	1325 [514–2135]	562 [114–747]
Genus *Robbsia* and the rest of *Betaproteobacteria*	577–1830	844, 860, 1830	1204 [577–1830]	<562[θ]
Mesorhizobium and *Rhizobium*	508–1280	1221, 1647	1078 [508–1647]	509 [356–509]
MRCA of *Bradyrhizobium*	348–1200	500, 1098	723 [348–1098]	1167 [1147–1187]
Rhizobium and *Sinorhizobium*	308–766	939, 1098	703 [308–1098]	201
MRCA of *Paraburkholderia*	285–397	542, 550, 555	422 [285–555]	<20
Escherichia and *Salmonella*	55–62	550, 1220	638 [55–1220]	106 [49–636]

[φ] Divergence time estimates obtained from analyses in this study, with the exception of those from TIMETREE5.

[∞] Dates estimated with BEAST v2.6.2 [Bouckaert et al., 2019] using the following parameters: Clock model = relaxed clock log normal, Tree model = calibrated Yule process, MCMC chains = 5000000. The minimum and maximum dates were generated with TreeAnnotator v.2.6.2 [Rambaut et al., 2018] from 10,000 trees using 10 % burnin, Maximum clade credibility tree and Common Ancestor heights.

[†] Dates estimated with the chronopl function in APE in R v.4 [Paradis and Schliep, 2019] using the following parameter: λ = 0. The individual date values were obtained from the three analyses run in this study (Fig. 1; Supplementary Figs. S2 and S5).

[*] Date ranges were derived from the lowest and highest date estimates obtained from all of the applicable analyses carried out in this study.

[#] Dates were retrieved from the online resource TIMETREE 5 (https://www.timetree.org), for comparative purposes. The date estimates were obtained from multiple published articles [Battistuzzi et al., 2004; Hedges and Kumar, 2009; Hedges et al., 2015; Kumar et al., 2017, Kumar et al., 2022]. Dates represent adjusted medians with the upper and lower bounds in brackets.

[θ] The divergence of the genus *Robbsia* and the rest of *Betaproteobacteria* was unavailable (as of July 2025) on TIMETREE 5 (https://www.timetree.org), therefore, the divergence of the sister genera *Cupriavidus* and *Burkholderia* was used to get a rough maximum date estimate for the divergence in question.

Maximum clade credibility tree and Common Ancestor heights) and visualized in FigTree v.1.4.4 (https://tree.bio.ed.ac.uk).

2.3. Ancestral state reconstruction (ASR) and molecular dating of the nodulation trait

ASR of the ability to nodulate legumes was performed using Mr Bayes Ancestral States in the R (MBASR) toolkit (Heritage, 2021). We define the ability to nodulate in the context of ASR as the genetic predisposition of the bacterium to harbor nodulation genes. We traced the phenotype through the presence of the *nodC* gene or known nodulation ability. The phylogenetic trees generated from the '*Pseudomonadota*' (Supplementary Fig. S2) and 'All *Paraburkholderia*' (Supplementary Fig. S3) datasets were used as phylogenetic frameworks. Nodulation ability was binary encoded (non-nodulating = 0, nodulating = 1, unknown / uncertain =?) and treated as unordered. Here, we inferred nodulation ability from known records or from the presence of *nodC* in a taxon's genome. The product of this gene is the N-acetylglucosaminyltransferase responsible for synthesis of the lipochitooligosaccharide core of the rhizobial Nod Factor needed for root nodulation and establishment of the nitrogen-fixing symbiosis (Broughton et al., 2000). Markov chain Monte Carlo (MCMC) sampling was set to 1000 (= 100 000 generations). A continuous-time Markov model was utilised across the phylogenetic trees to obtain posterior probability estimates for the character states at the nodes. The results were then used to determine the ancestral nodulation state of extant rhizobial lineages.

Finally, we dated the emergence of the precursors of nodulation loci in *Paraburkholderia.* For this purpose, the '*nodACD*' phylogeny generated by IQ-TREE was analyzed in APE with the 'chronopl' function as above. Two calibration points were used. The first was determined in the current study, corresponded with the time of geographical separation between certain groups of rhizobial *Paraburkholderia* species and was fixed at 140–100 Ma (Supplementary Tables S1 and S2). The second calibration point corresponded with the divergence of *Bradyrhizobium japonicum* and *B. elkanii* according to date estimates provided by https://www.timetree.org and was fixed at 71–7 Ma (Supplementary Tables S1 and S2).

3. Results

3.1. Divergence times for the Betaproteobacteria and Paraburkholderia

The ML phylogenetic tree inferred from the '*Pseudomonadota*' dataset grouped the 809 representative strains into two main supported groups representing the *Alphaproteobacteria* (red circle, Supplementary Fig. S2) and a lineage containing the *Betaproteobacteria* and *Gammaproteobacteria* (blue circle, Supplementary Fig. S2). The lineage containing the classes *Betaproteobacteria* (purple circle, Supplementary Fig. S2) and *Gammaproteobacteria* (green circle, Supplementary Fig. S2) was treated as one class (i.e., *Gammaproteobacteria*), which is consistent with the notion that these two taxa may represent a single class (Parks et al., 2018). The '*Pseudomonadota*' phylogeny (Supplementary Fig. S2) was also broadly congruent with those of Rahimlou et al. (2021) and several previous studies (Battistuzzi and Hedges, 2009; Hedges et al., 2015; Parks et al., 2018, Parks et al., 2019). However, the topology of the *Burkholderia sensu lato* group differs slightly among our phylogenies, depending on whether the analyses were based upon nucleotide or amino acid sequence alignments, as clearly shown in Supplementary Figs. S6 and S7. Although the nucleotide-based phylogenies were better supported, we used amino acid sequence-based phylogenies for the purpose of estimating more accurate divergence dates. In all phylogenies (whether based on amino acid or nucleotide sequences), the species *Robbsia andropogonis* remained the basal taxon (Fig. 2; Supplementary Figs. S1, S2 and S5–S7) and was, therefore, used as an outgroup in phylogenies including only *Burkholderia sensu lato* (Fig. 1; Supplementary Figs. S3 and S4). Furthermore, ML phylogenies inferred from '*Paraburkholderia* species' and '*All Paraburkholderia*' datasets showed that the lineage of *Paraburkholderia* formed a well-supported monophyletic group, which further split into two clearly distinct clusters separated by *P. phosphatilytica* (Fig. 1; Supplementary Figs. S3 and S4).

Our divergence estimates also suggested that the genus *Paraburkholderia* emerged around 555–285 Ma (Figs. 1 & 2, Supplementary Fig. S5), whereas most of its rhizobial species appeared around 140–100 Ma (around the time South African and South American rhizobial *Paraburkholderia* split) (Fig. 2). However, bacteria with the predisposition to harbor nodulation genes most likely appeared in the alphaproteobacterial genus *Bradyrhizobium* around 1098–348 Ma (Fig. 2,

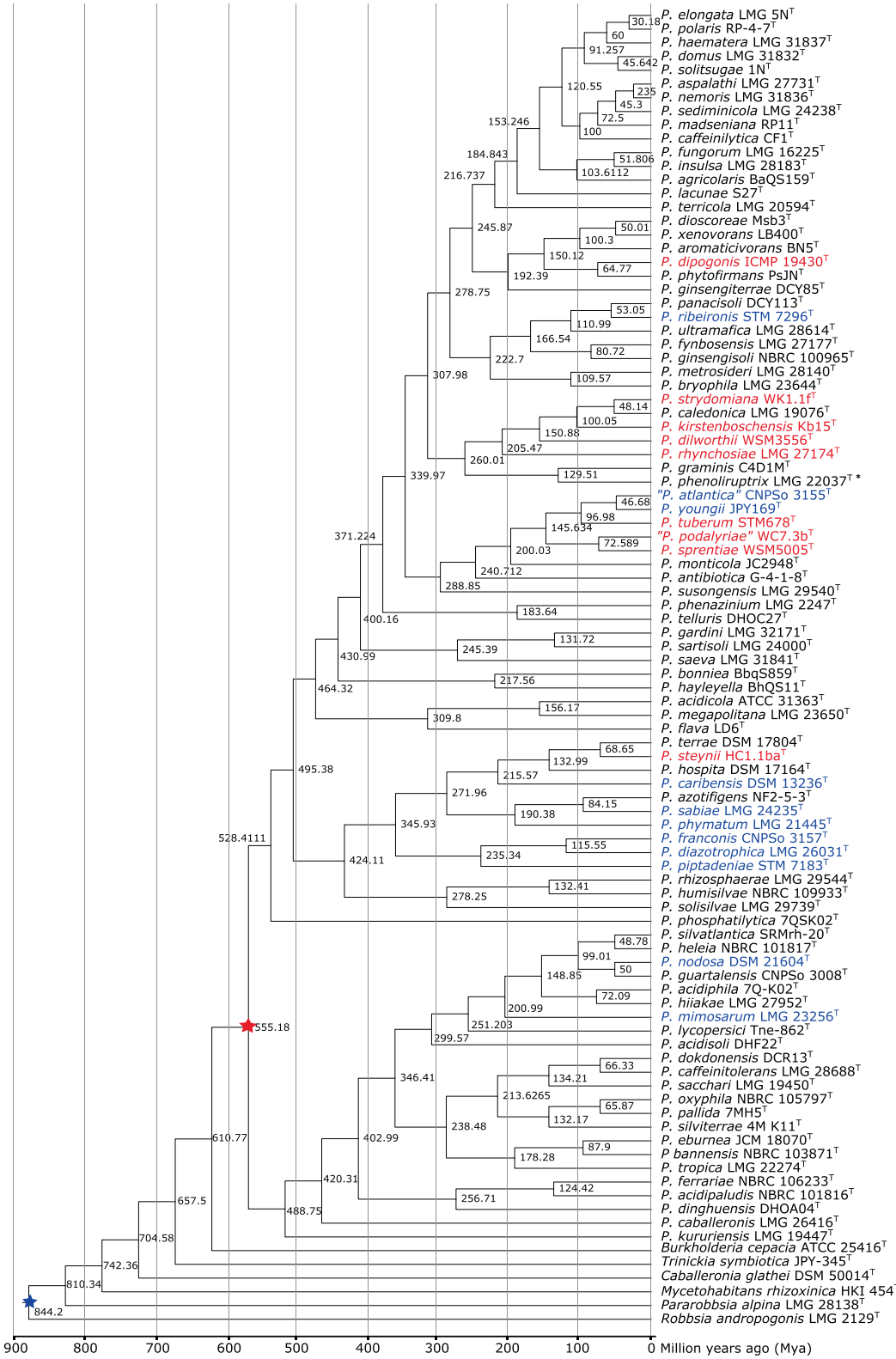

Fig. 1. A timeline showing the divergence dates of *Paraburkholderia* species. Divergence times were estimated using the 'chronopl' function (with $\lambda = 0$) from the APE package using R (4). The dated phylogeny included 96 taxa of the *Paraburkholderia* species and was inferred using amino acid sequences. The type strains of type species of genera belonging to the *Burkholderia sensu lato* group (Beukes et al 2017; Estrada-de los Santos et al 2018) were used for outgroup purposes, of which *Robbsia andropogonis* LMG 2129^T (Lopes-Santos et al., 2017) was used to root the tree. The blue and red stars indicate the secondary calibration points derived from Supplementary Fig. S2. Isolates in red and blue font are native to South Africa and South/ Central America, respectively. The strain *P. phenoliruptrix* LMG 22037^T (marked with *) represent the species *P. phenoliruptrix* as a type strain; however, unlike *P. phenoliruptrix* BR3459a used in other phylogenies to represent the species *P. phenoliruptrix* as a legume nodulator, *P. phenoliruptrix* LMG 22037^T neither nodulates legumes nor originate from South America. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

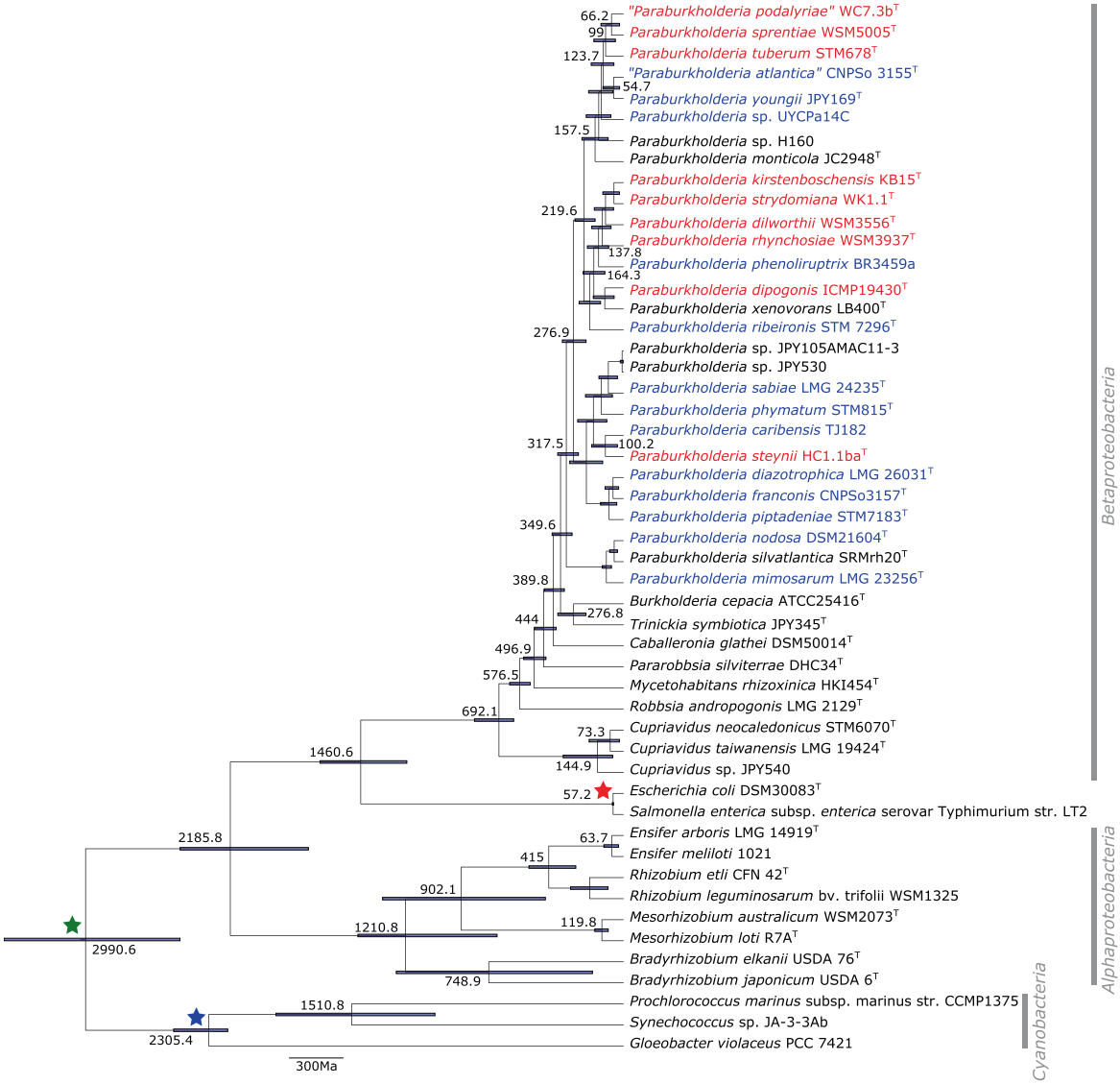

Fig. 2. An evolutionary timeline for rhizobial *Paraburkholderia* estimated with BEAST (2.6.2) using a relaxed clock lognormal model, WAG site model, calibrated yule tree model and 5 million MCMC chains (Bouckaert et al., 2019). The phylogeny included 50 taxa from the Rhizobial *Paraburkholderia* dataset, which also included enterobacteria, alpha-rhizobia and cyanobacteria for outgroup and tree calibration purposes. The BEAST analysis used a starting tree inferred with IQ-TREE (Nguyen et al., 2015) using amino acid sequences of 63 core loci extracted from whole genome sequences using the UBCG pipeline (Na et al., 2018). The vertical blue lines on the nodes represent the range of the estimated dates, where the lengths of the blue lines are directly proportional to both the date ranges and scale bar at the bottom of the phylogenetic tree; while, the numbers on the nodes represent the median of the estimated dates. The stars indicate the nodes whose dates were fixed for calibration purposes (Battistuzzi et al., 2004, Sorfova et al., 2008, Blank 2010, Boden et al., 2021). The green star indicates the divergence of Hydrobacteria and Terrabacteria (Supplementary Table S1). The blue star indicates the emergence of *Cyanobacteria* (Supplementary Table S1), while the red star indicates the divergence of *Escherichia coli* and *Salmonella typhimurium* (Supplementary Table S1). The species in red and blue font are rhizobial *Paraburkholderia* species native to South Africa and South/Central America, respectively. Whereas species in black font include rhizobial non-*Paraburkholderia* and non-rhizobia reference species. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Supplementary Figs. S2 and S5), and around 1423–75 Ma in the first betaproteobacterial genus *Cupriavidus* (Fig. 2, Supplementary Fig. S5). Additionally, we estimated the divergence of the alpha-rhizobial genera *Bradyrhizobium* and *Mesorhizobium* to have occurred 2196–996 Ma, while that of *Mesorhizobium* and *Rhizobium* split 1647–508 Ma (Fig. 2, Supplementary Figs. S2 and S5, Table 1, Supplementary Table S3). These dates were mostly also consistent with the divergence estimates captured on TIMETREE5 (Kumar et al., 2017, Kumar et al., 2022) (Table 1, Supplementary Table S3), which for instance, dated the divergence of *Alphaproteobacteria* and *Betaproteobacteria* at 2819–606 Ma compared to the range 2744–1752 Ma estimated during this study.

Time estimates obtained with BEAST for the 'Rhizobial *Paraburkholderia*' generally corresponded with those determined with APE (Table 1). Divergence time analyses performed with BEAST showed convergence between 4×10^6 and 5×10^6 MCMC chains, while the analyses performed with APE also showed consistent results from multiple runs. However, according to our divergence time estimates based on both the 'chronopl' function in APE, and BEAST, the divergence of *Cupriavidus* and *Paraburkholderia* occurred between 2135 and 514 Ma (Fig. 2, Supplementary Figs. S2 and S5, Table 1, Supplementary Table S3), which is much earlier than the 717–114 Ma suggested on TIMETREE5 using the Marin et al. (2016) dataset. Also, based on this published dataset, TIMETREE5 suggested that the *Paraburkholderia* MRCA diverged during the last 20 Ma, which is much later than our estimates, i.e., 397–285 Ma for BEAST (Fig. 2) and 555–542 Ma for APE (Fig. 1, Supplementary Fig. S5).

3.2. Divergence times for closely related but geographically separated Paraburkholderia rhizobia

All of our phylogenies showed that the *Paraburkholderia tuberum sensu lato* group, consisting of species *P. tuberum*, *P. sprentiae*, "*P. atlantica*", *P. youngii* and "*P. podalyriae*", is monophyletic (Figs. 1 & 2, Supplementary Figs. S1, S4–S6). In these trees, the lineage containing *P. tuberum*, which is native to South Africa, always diverges from the one containing "*P. atlantica*" and *P. youngii*, which are native to South and Central America (Fig. 2, Supplementary Figs. S1, S4–S6). Such a geographically disjunct distribution of closely related species was also observed in other clades (Figs. 1 & 2, Supplementary Figs. S4–S6). These included the lineage containing South American *P. phenoliruptrix* BR3459a and the South African *P. rhynchosiae* WSM3937^T, *P. dilworthii* WSM3556^T, *P. strydomiana* WK1.1f^T and *P. kirstenboschensis* Kb15^T, as well as the lineage containing South African *P. steynii* HC1.1ba^T and that

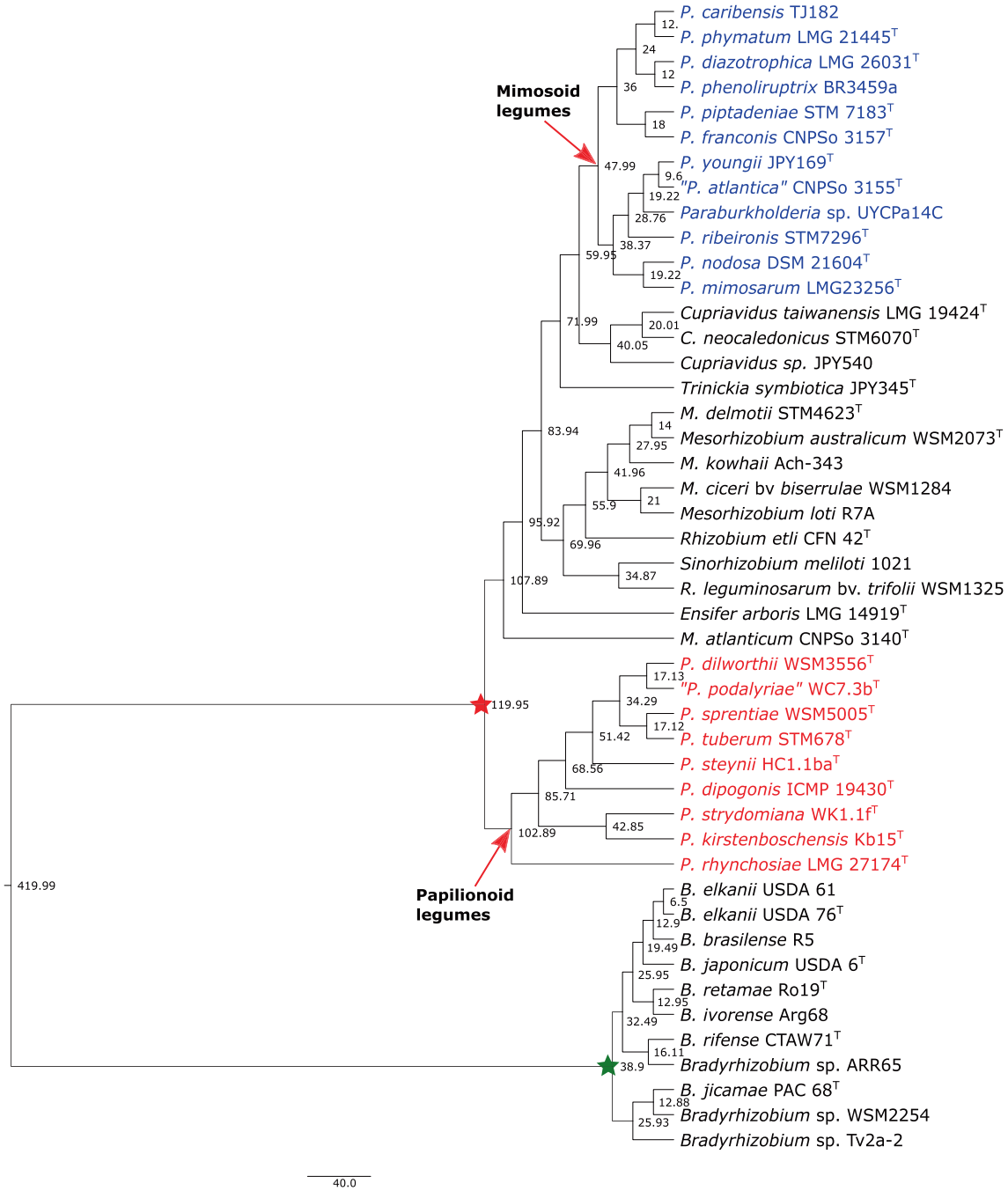

Fig. 3. A ML phylogeny of *nodACD* loci for 46 rhizobial taxa. The phylogeny shows the evolutionary timeline of the nodulation trait estimated using the chronopl function of the APE package in R. The dated *nodACD* phylogeny was calibrated using two points. The first was placed at the divergence of South African and South American *Paraburkholderia* species (red star) which corresponded with the split of the African and South American continents (140–100 Ma) (Supplementary Table S1). The second was placed at the divergence of *Bradyrhizobium japonicum* and *B. elkanii* around 71–7 Ma (green star) (Supplementary Table S1). The phylogeny was rooted with the *Bradyrhizobium* lineage. The species in blue and red font are rhizobial *Paraburkholderia* species native to South/Central America and South Africa, respectively. Whereas species in black font are non-*Paraburkholderia* rhizobial species. The red arrows indicate the points in time when rhizobial *Paraburkholderia* may have first associated with their legume hosts. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

of South American *P. caribensis* TJ182 (Figs. 1 & 2, Supplementary Figs. S4–S6).

Our results also revealed that the MRCA of the geographically separated lineages of *Paraburkholderia tuberum sensu lato* (which includes *P. tuberum sensu stricto*, "*P. podalyriae*", *P. sprentiae*, *P. youngii* and "*P. atlantica*") diverged between 150 and 80 Ma (Figs. 1 & 2, Supplementary Fig. S5). These dates coincide with the early split of the continents of Africa and South America, which occurred during a process lasting from 140 to 130 Ma (McLoughlin, 2001; Nie et al., 2012). This implies that the closely related lineages of *Paraburkholderia tuberum sensu lato* could have separated into different geographical regions by the continental drift that caused the split of the supercontinent Gondwana. Additionally, the divergence of other geographically separated lineages of *Paraburkholderia* like that of *P. phenoliruptrix* and *P. rhynchosiae*, as well as that of *P. steynii* and *P. caribensis* were estimated to have occurred 259–100 Ma and 213–80 Ma, respectively (Figs. 1 & 2, Supplementary Fig. S5), suggesting that continental drift could have also contributed to their divergence.

3.3. Origins of nodulation in Paraburkholderia

Phylogenetic analysis of the '*nodACD*' dataset separated the sequences of extant rhizobial *Paraburkholderia* species according to their geographic origin (Fig. 3, Supplementary Fig. S8). Strains indigenous to South America, such as *Paraburkholderia* sp. UYCPa14C and the type strains (or nodulating strains where the respective type strain is non-symbiotic) of 11 validly published *Paraburkholderia* species (i.e., *P. caribensis* TJ182 (Bournaud et al., 2013), *P. mimosarum* LMG 23256^T (Chen et al., 2006), *P. phymatum* LMG 21445^T (Elliott et al., 2007a), *P. nodosa* DSM 21604^T (Chen et al., 2007), *P. phenoliruptrix* BR3459 (de Oliveira Cunha et al., 2012), *P. piptadeniae* STM7183^T (Bournaud et al., 2017), *P. ribeironis* STM7296^T (Bournaud et al., 2017), *P. diazotrophica* LMG 26031^T (Sheu et al., 2013), *P. franconis* CNPSo3157^T (Paulitsch et al., 2020b), "*P. atlantica*" CNPSo3155^T (Paulitsch et al., 2020b) and *P. youngii* JPY169^T (Mavima et al., 2021)) formed a monophyletic cluster. Also, this monophyletic cluster is more closely related to sequences from *Cupriavidus*-nodulating strains than to its rhizobial *Paraburkholderia* counterparts indigenous to South Africa. The type strains of the nine rhizobial *Paraburkholderia* species indigenous to South Africa (i.e., *P. tuberum sensu stricto* STM678^T (Vandamme et al., 2002), *P. sprentiae* WSM5005^T (De Meyer et al., 2013), *P. kirstenboschensis* Kb15^T (Steenkamp et al., 2015), *P. dilworthii* WSM3556^T (De Meyer et al., 2014), *P. rhynchosiae* LMG 27174^T (De Meyer et al., 2013), *P. dipogonis* ICMP19430^T (Liu et al., 2014; Sheu et al., 2015), *P. strydomiana* WK1.1f^T (Beukes et al., 2019), *P. steynii* HC1.1ba^T (Beukes et al., 2019) and "*P. podalyriae*" WC7.3b^T (Mavima et al., 2022)) formed their own separate monophyletic cluster. These branching patterns confirm separate acquisitions of these nodulation loci (at different dates and from different sources) in the extant *Paraburkholderia* strains sampled in these two regions.

ASR analyses showed that the genetic predisposition to harbor nodulation genes emerged multiple times in the *Pseudomonadota* (Supplementary Fig. S9). This predisposition appeared first in *Bradyrhizobium*, which diverged around 1098–348 Ma (Supplementary Fig. S2, Table 1). Therefore, to date the emergence of precursors of nodulation loci in *Paraburkholderia* species, the '*nodACD*' chronogram was calibrated using the divergence of geographically separated members of this genus, which we dated to 140–100 Ma with BEAST (Fig. 2), and corresponded with the early split of the African and South American continents 140–130 Ma (McLoughlin, 2001; Nie et al., 2012). The results showed that the genetic predisposition to harbor nodulation genes in rhizobial *Paraburkholderia* native to South/Central America started diverging at around 48 Ma, while that of rhizobial *Paraburkholderia* species native to South Africa diverged around 103 Ma (Fig. 3). Interestingly, our *nodACD* chronogram also suggested that the precursor nodulation loci first appeared around 420 Ma (Fig. 3).

Focusing on *Paraburkholderia* and based on results from our ASR analysis, the genetic predisposition to harbor nodulation loci was probably first acquired by the lineage containing the rhizobial species *P. steynii*, *P. caribensis*, *P. sabiae*, *P. phymatum*, *P. franconis*, *P. diazotrophica* and *P. piptadeniae* (Fig. 4), as well as *P. azotifigens* (Rouws et al., 2024). The same lineage also demonstrated the geographical separation of rhizobial *Paraburkholderia*, as shown by the divergence of *P. steynii* and *P. caribensis* species that are native to southern Africa and South America, respectively (Fig. 4). Similar geographical separation patterns were also observed between the South American *P. phenoliruptrix* BR3459a and the lineage containing South African *P. rhynchosiae* WSM3937^T, *P. dilworthii* WSM3556^T, *P. strydomiana* WK1.1f^T and *P. kirstenboschensis* Kb15^T, as well as between the lineage containing the South/ Central American *P. youngii* JPY169^T and "*P. atlantica*" CNPSo 3155^T and the one containing the South African *P. tuberum* STM678^T, "*P. podalyriae*" WC7.3b^T and *P. sprentiae* WSM5005^T (Fig. 4). Furthermore, the MRCAs of these geographically separated rhizobial *Paraburkholderia* were most likely nodulators (Fig. 4). This suggests that, if nodulation was transferred vertically, the geographically separated rhizobial *Paraburkholderia* may have acquired the nodulation loci before or during the time they separated (Fig. 4).

The geographically separated rhizobial *Paraburkholderia* species associate with different host legumes, as shown in Supplementary Fig. S10. The species native to South Africa associate almost exclusively with papilionoid species (i.e., *Aspalathus*, *Dipogon*, *Hypocalyptus*, *Lebeckia*, *Podalyria*, *Rhynchosia* and *Virgilia* (Beukes et al., 2013; Beukes et al., 2019; Howieson et al., 2013; Lemaire et al., 2016)), although some strains of *P. tuberum sensu stricto* have been isolated from a mimosoid species *Vachellia* karroo (Beukes et al., 2019; Mavima et al., 2022) (Supplementary Fig. S10). On the other hand, the species native to South/Central America generally associate with legumes from the Mimosae tribe of the Caesalpinioideae (i.e., *Calliandra*, *Mimosa* and *Piptadenia* (Bontemps et al., 2010; Bontemps et al., 2016; Rouws et al. 2024)) (Supplementary Fig. S10). This suggests that the geographically separated rhizobial *Paraburkholderia* species may have acquired nodulation horizontally from different sources following their geographical separation.

4. Discussion

The findings of this study provide a robust evolutionary timeline for rhizobial *Paraburkholderia* (see Fig. 5). Many published chronograms containing rhizobial taxa differ markedly from one another, both at higher taxonomic ranks such as classes and families and at lower ranks such as genera and species (Turner and Young 2000; Chriki-Adeeb and Chriki, 2016; Kumar et al., 2017, 2022; Wang et al., 2020a; Rahimlou et al., 2021). Although lack of suitable fossil records for calibrating recent evolutionary times might have contributed to such inconsistencies (Brasier et al., 2006; Schopf 2006; Guindon, 2020), the molecular sequences used for these estimations might also be important (Sauquet, 2013). We have accordingly estimated divergence times among species or lineages using information from fossil records, geologic events and geographical distributions of extant rhizobial *Paraburkholderia*, as well as the inferred amino acid sequences for 92 conserved loci common to all bacteria.

According to our divergence time estimates, *Pseudomonadota* diverged into the classes *Alphaproteobacteria* and *Betaproteobacteria* around 2744–1752 Ma. This was later followed by the divergence of *Betaproteobacteria* around 2175–1000 Ma, following the Great Oxidation Event (2300 Ma) (Holland, 2002). Our results also showed that divergence of the MRCA of beta-rhizobia occurred 2135–514 Ma during the Precambrian Period, creating the basal lineage of *Cupriavidus* and that of *Trinickia* and *Paraburkholderia*. Altogether, these dates span from the Proterozoic Aeon (2500–550 Ma) to the Paleozoic Era (550–250 Ma) of the Phanerozoic Aeon (Walker and Geissman, 2009). At that time in

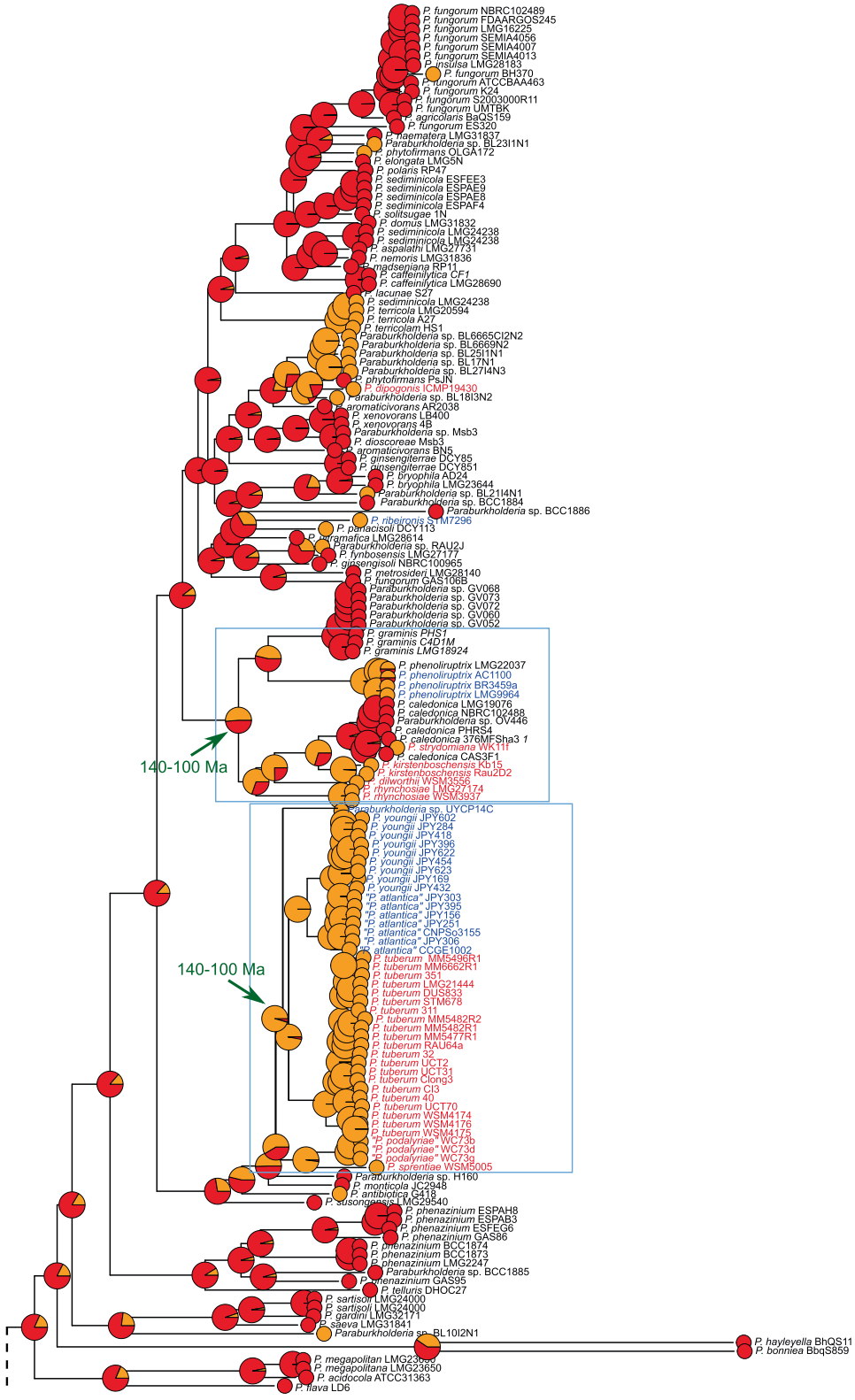

Fig. 4. A and B A ML phylogeny of the genus *Paraburkholderia* (corresponding to Supplementary Fig. S3) (spread across panels A and B, to be able to increase font size), showing the ancestral state reconstruction of the nodulation function performed using MrBayes Ancestral States in R. Assuming that nodulation was transferred vertically, the diagram illustrates that rhizobial *Paraburkholderia* from South/ Central America (blue font) and South Africa (red font) could have acquired nodulation during or after their geographical separation. This is highlighted in the blue rectangles where the MRCAs (marked with green arrows) that diverged into geographically separated rhizobial *Paraburkholderia* were likely nodulators, as indicated by the nodulation state probabilities in the form of pie charts at the nodes. According to the date estimates from BEAST (Fig. 2) these divergence points were dated to be about 140–100 Ma, which corresponds with the early split of the African and South American continents 140–130 Ma (McLoughlin, 2001; Nie et al., 2012). The species in black font are non-rhizobial species. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

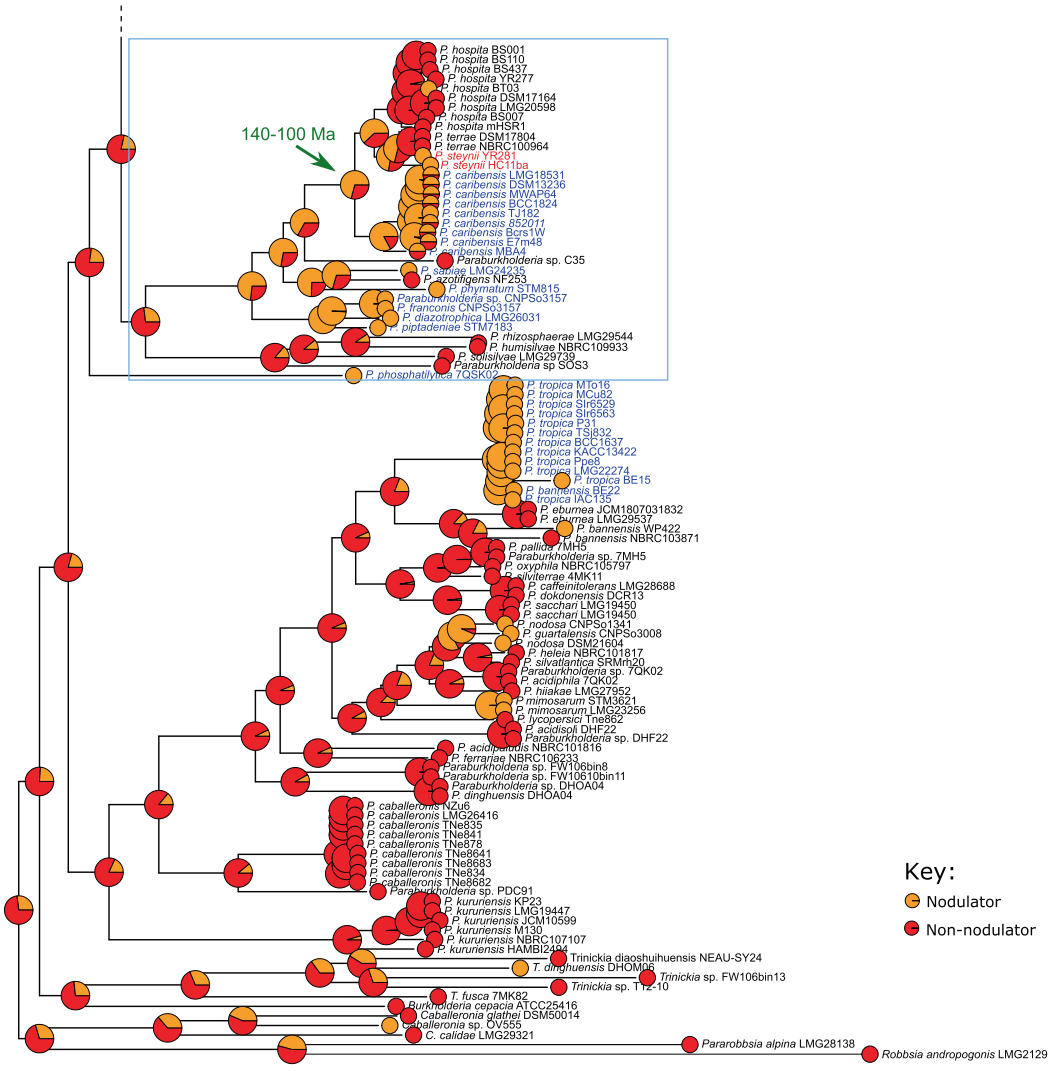

Fig. 4. (*continued*).

prehistory, bacteria had already colonized land (3540–2660 Ma) (Battistuzzi and Hedges, 2009), suggesting that extant beta-rhizobial lineages could have evolved on land spanning three major diversification phases: (i) during the Permian and Triassic periods (400–200 Ma) when Pangaea was fully assembled and its landmass filling up with flora and fauna, (ii) during the Jurassic period (200–150 Ma) when fauna and flora were flourishing in Pangaea, and (iii) during the Cretaceous and Paleogene periods (150–23 Ma) when Gondwana was breaking up (McLoughlin et al., 2001; Walker and Geissman, 2009). Furthermore, our data show that *Bradyrhizobium* and *Cupriavidus* likely represent the oldest lineages of alpha- and beta-rhizobia, respectively.

Based on our time estimates, we propose that *Paraburkholderia* emerged on the supercontinent Pangaea 600–400 Ma during the late Precambrian and early Paleozoic Era (https://www.britannica.com; Scotese, 2001, 2004; Walker and Geissman, 2009). Pangaea's assembly and breakup respectively began around 600 Ma and 200 Ma (Mitchell et al., 2021). This supercontinent existed at the time when atmospheric carbon dioxide levels had become conducive for plant growth, which subsequently increased the atmospheric oxygen to levels conducive for animal life on land (Berner, 2009; McLoughlin et al., 2001). However, considering that the genus *Paraburkholderia* consists largely of plant beneficial and environmental species (Beukes et al., 2017) that also represent Hydrobacteria (originally adapted to water) (Battistuzzi and Hedges, 2009), it is interesting to note that the early diversification of *Paraburkholderia* (555–285 Ma) coincides with the time flora was flourishing and also colonizing land (430–390 Ma) (McLoughlin et al., 2001). This could imply that *Paraburkholderia* first associated with plants along the ocean shores as plants began to colonize land. Subsequently, as these early land plants spread inland from the coastal areas, they carried their *Paraburkholderia* symbionts with them, causing the diversification of plants to directly influence that of *Paraburkholderia*. Furthermore, we observed that some major diversification of *Paraburkholderia* occurred during the Permian (300–250 Ma) and Triassic (250–200) Periods (https://www.britannica.com; Scotese, 2001, 2004; Walker and Geissman, 2009) when Pangaea was fully assembled (Mitchell et al., 2021). This could be attributed to the fact that both plants and animals flourished and diversified during the Permian and Triassic Periods due to the high levels of atmospheric oxygen and carbon dioxide, and due to the lack of dispersal limitation for living organisms as all landmasses were assembled into a single continent (Mitchell et al., 2021).

The breaking up and drifting apart of Pangaea (Wang et al., 2020b; Mitchell et al., 2021) likely influenced the evolution of rhizobial *Paraburkholderia* as their diversification increased during this period. For example, the separation of the supercontinent Gondwana may have caused the lineage comprising the South African species *P. tuberum*,

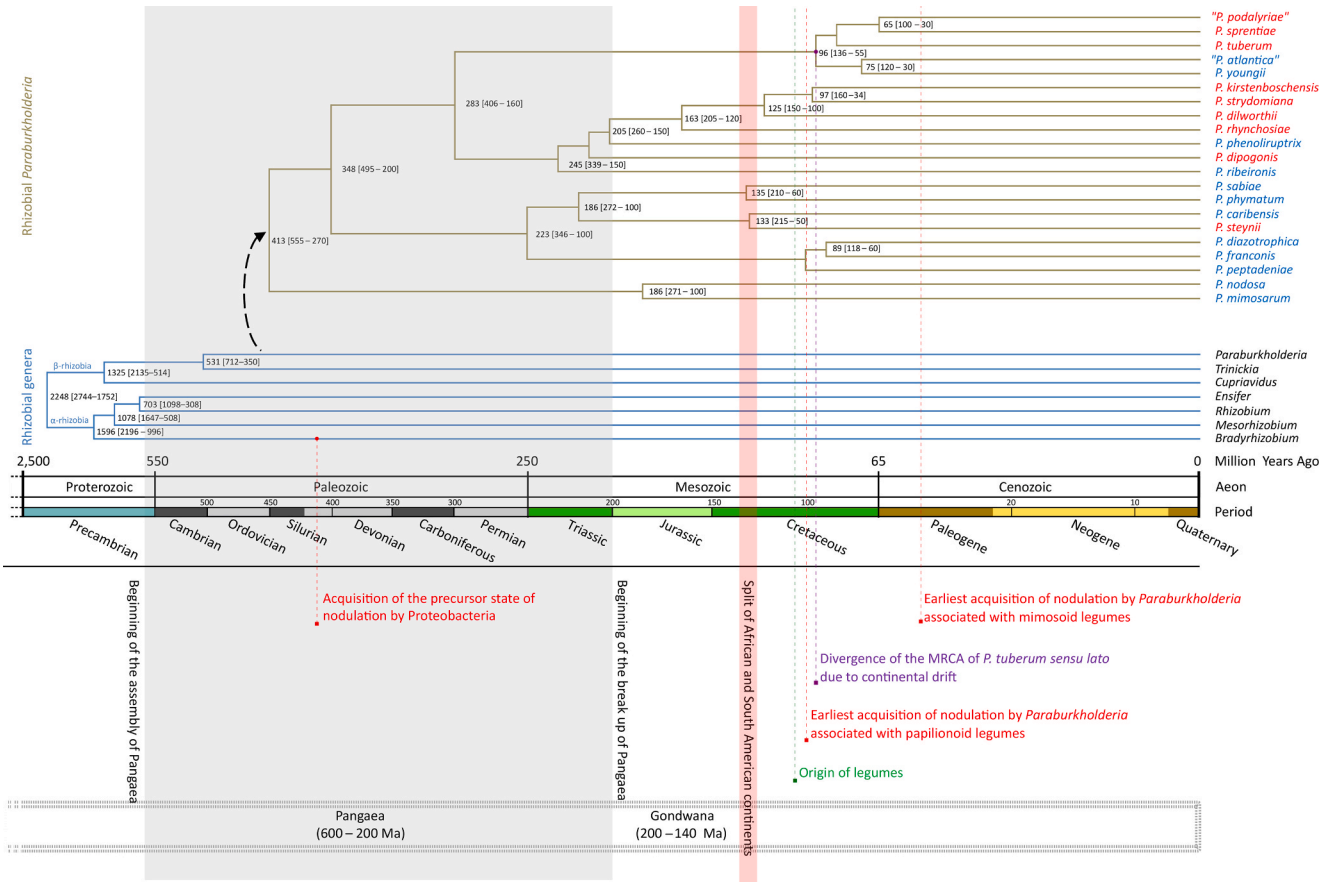

Fig. 5. A schematic diagram showing the average divergence dates on the lineages of rhizobial genera and beta-rhizobial *Paraburkholderia*. The phylogenies were manually created in Inkscape (https://www.inkscape.org) to correspond with the overall date estimates from our study. The diagram also shows the geological events from the time the supercontinent Pangaea formed to the time the mega-continent of Gondwana split into the South American and African continents. Date estimates at which alpha-rhizobia and beta-rhizobial *Paraburkholderia* acquired nodulation are indicated with red dotted lines. Additionally, the point at which continental drift drove geographical separation of *P. tuberum sensu lato* species is indicated with a purple dotted line. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

"*P. podalyriae*" and *P. sprentiae* to diverge from the lineage comprising the South American species "*P. atlantica*" and *P. youngii*. These divergence events support the notion that the rhizobial *Paraburkholderia* lineages could have diverged during or slightly after the early split of the continents of Africa and South America, which occurred around 140–130 Ma (McLoughlin, 2001; Nie et al., 2012). Therefore, the split of the African and South American continents may have geographically separated populations of several ancestral rhizobial *Paraburkholderia* species, causing allopatric speciation and subsequent emergence of various extant species. However, since our divergence time estimates are fairly broad, we cannot dismiss the possibility that closely related rhizobial *Paraburkholderia* species might have been geographically separated by long-distance dispersal, as opposed to vicariance or continental drift (Nemergut et al., 2011). Lastly, to the best of our knowledge, our study is the first to demonstrate the possible effect of continental drift on the divergence, speciation and geographical distribution of rhizobia, or any bacteria.

Indigenous beta-rhizobia, represented by the abundant genus *Paraburkholderia*, have predominantly been isolated from southern Africa and South and Central America (Bontemps et al., 2010; Beukes et al., 2013, 2021; Lemaire et al., 2016; Belles-Sancho et al. 2023). As indigenous bacteria, they have only been found in legume hotspots like the South African Fynbos biome and the South American Caatinga and Cerrado biomes (Bontemps et al., 2010; Lemaire et al., 2016; Pires et al. 2018; Rouws et al. 2024). This geographical distribution of rhizobial *Paraburkholderia* species more or less corresponds with the evolutionary history of the nodulation loci of beta-rhizobia (Paulitsch et al., 2020a; Mavima et al 2022). This suggests that nodulation as a trait coevolved with *Paraburkholderia* species and their legume-hosts for quite some time, which could also explain why rhizobial *Paraburkholderia* exhibit some degree of host specificity as reported by Elliott et al. (2007a, b, 2009), Mishra et al. (2012), Beukes et al. (2021) and Rahimlou et al. (2021). Also, rhizobial *Paraburkholderia*, which are tolerant to poor-nutrient conditions (Beukes et al., 2013; de Meyer et al., 2013; Lemaire et al., 2016; Dludlu et al., 2018), may have contributed immensely to the establishment and evolution of legumes in the Fynbos, Caatinga and Cerrado biomes whose soils are usually acidic and lack nitrogen and phosphorus (Witkowski and Mitchell, 1987; dos Reis Junior et al., 2010; Pires et al. 2018). In this regard, the western African coastal regions which were once connected to the continent of South America, have similar edaphic properties and legume diversity such as the Fynbos, Caatinga and Cerrado biomes, and thus are potentially rich in beta-rhizobial *Paraburkholderia* (Dludlu et al., 2018).

Although results from our ASR analysis suggest that the geographically separated rhizobial *Paraburkholderia* species (e.g., *P. steynii* and *P. caribensis*) acquired the predisposition to harbor nodulation genes before the split of the African and South American continents (140–130 Ma) (McLoughlin, 2001; Nie et al., 2012), our *nodACD* phylogeny does not support this idea. The nodulation predisposition arose in the MRCA of the South African *P. steynii* and South American *P. caribensis*, however their nodulation loci had separate evolutionary origins. Given that nodulation loci are prone to HGT, we assume that rhizobial *Paraburkholderia* acquired nodulation loci horizontally from various sources after individual species were geographically separated. The rhizobial

Paraburkholderia native to South Africa have since acquired their nodulation loci from the South African alpha-rhizobia, while those native to South America have since acquired theirs from South American alpha-rhizobia (Elliott et al. 2009; Bontemps et al., 2010; Beukes et al., 2013, 2019; Melkonian et al. 2014; De Meyer et al., 2016; Lemaire et al., 2016; Platero et al. 2016; Estrada-de los Santos et al., 2018). After their separation, both the South African and South American rhizobial *Paraburkholderia* species generally coevolved with the nodulation loci they acquired, as they form separate monophyletic clusters in the nodulation phylogenies (see Fig. 3 and Supplementary Fig. S8). These findings are also congruent with the notion that rhizobial *Paraburkholderia* initially associated with ancient legume lineages prior to the formation of the Fynbos, Caatinga and Cerrado biomes which occurred around 99.5–19.4 Ma (Quint and Classen-Bockhoff, 2004; Verboom et al., 2009; Bytebier et al., 2011), 20 Ma (Fernandes, 2022) and 10–1 Ma (Fernandes, 2022), respectively.

Our dated *NodACD* chronogram suggests the precursor nodulation locus emerged around 420 Ma, much earlier than generally assumed (van Velzen et al., 2019; Wang et al., 2020a). Our ancestral state reconstruction analysis also supports an earlier origin for the predisposition of bacteria to harbor nodulation genes by showing that it first emerged around 1098–348 Ma prior to being distributed to other rhizobial lineages. However, some authors argue that the precursor state of nodulation may have already existed in non-proteobacteria sources such as *Frankia* species, which are gram-positive actinobacterial symbionts nodulating actinorhizal plants that are related to legumes in the Nitrogen Fixing Clade (NFC) (van Velzen et al., 2019). One would expect acquisition of nodulation in rhizobia to correspond with the emergence of the MRCA of the NFC, 110–85 Ma (van Velzen et al., 2019), as the extant rhizobia form symbiotic interactions solely with the NFC (comprising orders *Fabales*, *Fagales*, *Cucurbitales* and *Rosales*) (van Velzen et al., 2019; Wang et al., 2020a; Koenen et al., 2021; Doyle et al. 2025). This hypothesis is widely accepted, as it is presumed to be based on empirical data from both fossil records of plant nodules and plant-based ASR studies (van Velzen et al., 2019). Indeed, our study indicates that precursor nodulation loci could be fairly old with an earlier origin predating the NFC of angiosperms, which is in agreement with previous suggestions (van Velzen et al., 2019; Wang et al., 2020a).

In conclusion, our study describes the evolutionary history of rhizobial *Paraburkholderia* in the context of the evolution of rhizobia, *Paraburkholderia* and the earth. In this regard, we have demonstrated how HGT together with vicariance shaped the current geographical distribution of rhizobial *Paraburkholderia*. We have also shown that the South African and South/Central American rhizobial *Paraburkholderia* acquired nodulation traits after the split of the African and South American continents, and that this happened before the origin of the Fynbos, Caatinga and Cerrado biomes. These findings suggest that the association of rhizobial *Paraburkholderia* with the early lineages of legumes started soon after the continents of Africa and South America split, creating centers of diversity for rhizobium-legume symbiosis which contributed to the emergence and evolution of the biomes mentioned above. Also, our study has estimated the origin of nodulation to be older than generally assumed. Since this trait predates the emergence of legumes at around 110–65 Ma (Barba-Montoya et al., 2018; Wang et al., 2020b), we posit that ancestral or precursor nodulation loci were likely involved in other functions at the time of their horizontal acquisition from *Alphaproteobacteria*. This information gives crucial insights into how the physical environment influenced the evolution and diversity of these agricultural important beta-rhizobia.

CRediT authorship contribution statement

Lazarus Mavima: Writing – review & editing, Writing – original draft, Visualization, Formal analysis, Data curation, Conceptualization. **Emma T. Steenkamp:** Writing – review & editing, Supervision, Investigation, Conceptualization. **Chrizelle W. Beukes:** Writing – review & editing, Supervision, Investigation. **Marike Palmer:** Writing – review & editing. **Sofie E. De Meyer:** Writing – review & editing, Investigation. **Euan K. James:** Writing – review & editing, Investigation. **Stephanus N. Venter:** Writing – review & editing, Supervision, Project administration, Investigation, Funding acquisition, Conceptualization. **Martin P.A. Coetzee:** Conceptualization, Investigation, Writing – review & editing, Supervision.

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.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ympev.2025.108447.

Data availability

The link to the data is provided in the manuscript

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