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RESEARCH ARTICLE

Symbiotic diversity, specificity and distribution of rhizobia in native legumes of the Core Cape Subregion (South Africa)

Benny Lemaire[1,2,*], Oscar Dlodlo[1], Samson Chimphango[1], Charles Stirton[1], Brian Schrire[3], James S. Boatwright[4], Olivier Honnay[2], Erik Smets[2,5], Janet Sprent[6], Euan K. James[7] and Abraham M. Muasya[1]

[1]Department of Biological Sciences, University of Cape Town, Private Bag X3, Rondebosch 7701, Cape Town, South Africa, [2]Plant Conservation and Population Biology, KU Leuven, Kasteelpark Arenberg 31, PO Box 2435, 3001 Heverlee, Belgium, [3]Royal Botanic Gardens, Kew, Richmond, Surrey TW9 3AB, UK, [4]Department of Biodiversity and Conservation Biology, University of the Western Cape, Private Bag X17, Bellville 7535, Cape Town, South Africa, [5]Naturalis Biodiversity Center, Leiden University, 2300 RA Leiden, The Netherlands, [6]Division of Plant Sciences, University of Dundee at JHI, Dundee DD2 5DA, UK and [7]The James Hutton Institute, Invergowrie, Dundee DD2 5DA, UK

*Corresponding author: Plant Conservation and Population Biology, KU Leuven, Kasteelpark Arenberg 31, PO Box 02435, 3001 Heverlee, Belgium. Tel: +32-16-32-86-36; Fax: +32 16 32 19 68; E-mail: benny.lemaire@bio.kuleuven.be

One sentence summary: This study is the most comprehensive phylogenetic assessment of rhizobia within the Fynbos biome, showing that legumes are specifically associated with *Burkholderia* and *Mesorhizobium*, the latter underestimated within Fynbos soils.

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ABSTRACT

Rhizobial diversity and host preferences were assessed in 65 native Fynbos legumes of the papilionoid legume tribes Astragaleae, Crotalarieae, Genisteae, Indigofereae, Millettieae, Phaseoleae, Podalyrieae, Psoraleeae and Sesbanieae. Sequence analyses of chromosomal 16S rRNA, *recA, atpD* and symbiosis-related *nodA*, *nifH* genes in parallel with immunogold labelling assays identified the symbionts as alpha- (*Azorhizobium*, *Bradyrhizobium*, *Ensifer, Mesorhizobium* and *Rhizobium*) and beta-rhizobial (*Burkholderia*) lineages with the majority placed in the genera *Mesorhizobium* and *Burkholderia* showing a wide range of host interactions. Despite a degree of symbiotic promiscuity in the tribes Crotalarieae and Indigofereae nodulating with both alpha- and beta-rhizobia, *Mesorhizobium* symbionts appeared to exhibit a general host preference for the tribe Psoraleeae, whereas *Burkholderia* prevailed in the Podalyrieae. Although host genotype was the main factor determining rhizobial diversity, ecological factors such as soil acidity and site elevation were positively correlated with genetic variation within *Mesorhizobium* and *Burkholderia*, respectively, indicating an interplay of host and environmental factors on the distribution of Fynbos rhizobia.

Key words: *Burkholderia*; Fynbos legumes; host preference; *in situ* immunogold labelling; *Mesorhizobium*; root nodulation

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INTRODUCTION

The Core Cape Subregion (CCR) formerly known as the Cape Floristic Region (Manning and Goldblatt 2012) has been recognized as one of the richest areas of flowering plants in the world with outstanding diversity, density and number of endemic species (Myers *et al.*, 2000, 2003). This biodiversity hotspot, with a unique assemblage of vascular plants referred to as the Fynbos (fine bush) biome, is probably the outcome of a combination of stable climatic conditions promoting a persistent speciation of the Cape flora and relatively recent and rapid radiations since the Miocene (Richardson *et al.*, 2001; Linder 2005; Schnitzler *et al.*, 2011). Furthermore, physical heterogeneity in topography and diverse soil types create a mosaic of sharply distinct microhabitats which also contribute to the elevated species richness (Linder 2003, 2005; Cowling, Procheşb and Partridge 2009). The CCR is largely semi-arid with generally nutrient-poor and acidic soils (Witkowski and Mitchell 1987; Manning and Goldblatt 2012). Similar to other (semi-) arid regions in the world, the Cape flora has a large component of Leguminosae (Fabaceae), with this family ranked as the second most species rich in the region, following the Asteraceae (Manning and Goldblatt 2012).

The Leguminosae, with about 19 500 species and 751 genera (LPWG 2013), have largely colonized the land surface on Earth (excluding Antarctica), and its species are adapted to most terrestrial ecosystems ranging from deserts to tropical rainforest, and tropical to arctic habitats (Doyle and Luckow 2003; Lavin, Herendeen and Wojciechowski 2005; Lewis *et al.*, 2005). In the Fynbos biome, about 750 native legume species are known to date, with the majority of these placed within the papilionoid tribes Crotalarieae, Podalyrieae, Psoraleeae and Indigofereae among which 634 species and 8 genera are endemic (Goldblatt and Manning 2002; Linder 2003; Manning and Goldblatt 2012).

All Leguminosae studied in the CCR nodulate with nitrogen-fixing soil bacteria, indicating that rhizobial symbionts are playing an important ecological role in this ecosystem (Sprent 2009; Sprent and Gehlot 2010; Sprent, Odee and Dakora 2010, Sprent, Ardley and James 2013). Over the last few decades, many rhizobial studies have surveyed and characterized the rhizobia–plant interaction within diverse Cape legume genera (Crotalarieae—*Aspalathus* L., *Lebeckia* Thunb.; Hypocalypteae—*Hypocalyptus* Thunb.; Phaseoleae—*Rhynchosia* Lour.; Podalyrieae—*Cyclopia* Vent., *Podalyria* Willd., *Virgilia* Poir.; Psoraleeae—*Otholobium* C.H.Stirt., *Psoralea* L.), describing a wide diversity of rhizobial lineages with most members placed in the genus *Burkholderia* (Elliott *et al.*, 2007a; Garau *et al.*, 2009; Gyaneshwar *et al.*, 2011; Beukes *et al.*, 2013; Howieson *et al.*, 2013), but relatively few within classical alpha-rhizobial groups (Kock 2004; Gerding *et al.*, 2012; Hassen *et al.*, 2012; Kanu and Dakora 2012).

The observed frequency and recent descriptions of new *Burkholderia* species among many legume species investigated indicate how successfully the Fynbos biome has been colonized by these belowground mutualists (Elliott *et al.*, 2007a; Beukes *et al.*, 2013; De Meyer *et al.*, 2013a,b, 2014; Howieson *et al.*, 2013). The dominance of *Burkholderia* species in root nodules has been attributed to their general ecological adaptation to acidic and nutrient-poor soils (Bontemps *et al.*, 2010; dos Reis Junior *et al.*, 2010; Mishra *et al.*, 2012). Similarly, the first major centre of diversity of the *Burkholderia*-legume association was reported in the arid and low pH soils of the Cerrado/Caatinga biomes in Brazil and in other parts of South America, particularly with *Mimosa* spp. and related genera in the tribe Mimoseae (Chen *et al.*, 2005a, 2006, 2007, 2008; Mishra *et al.*, 2012; Sheu *et al.*, 2012, 2013; Bournaud *et al.*, 2013).

The establishment of an effective symbiosis between legumes and rhizobia usually involves a complex molecular bacteria-plant dialogue based on rhizobial Nod factors and signal flavonoid-based molecules from the host plant (Masson-Boivin *et al.*, 2009). The secreted Nod factors (classified as lipochitooligosaccharides or LCOs) are mainly synthesized by common nodulation genes that are specifically recognized by the host. Consequently, symbiotic nodulation genes play a crucial role in establishing symbiosis in rhizobial species, and have been used as genetic markers to determine symbiotic diversity and host specificity (Perret, Staehelin and Broughton 2000).

Previous rhizobial studies in the CCR have focused on only a few legume genera, probably housing only a fraction of the total rhizobial diversity. Considering the high diversity of native legumes and the diverse low-nutrient habitats in the CCR which are positively linked with the belowground microbial communities (Slabbert *et al.*, 2010), it is likely that the rhizobial diversity is still largely unexplored. This study investigates the rhizobia–legume interaction in the Fynbos biome, by selecting a broad phylogenetic sample of legumes from different soil types and biogeographical regions. We surveyed and characterized rhizobia from 14 genera in 9 tribes (Crotalarieae—*Aspalathus*, *Crotalaria* L., *Rafnia* Thunb.; Astragaleae—*Lessertia* DC.; Genisteae—*Argyrolobium* Eckl. and Zeyh.; Indigofereae—*Indigofera* L.; Millettieae—*Tephrosia* Pers.; Phaseoleae—*Bolusafra* Kuntze; Podalyrieae—*Amphithalea* Eckl. and Zeyh., *Podalyria* Willd., *Virgilia* Poir.; Psoraleeae—*Otholobium* C.H. Stirt., *Psoralea* L.; Sesbanieae—*Sesbania* Scop.). Three housekeeping genes (16S rRNA, *recA* and *atpD*) were used to identify the symbiotic nitrogen-fixing bacteria and to elucidate their phylogenetic relationships. Symbiotic *nodA* and *nifH* genes were used to reveal the evolutionary history of the symbiotic traits. The specific aims of this study were 2-fold: (1) to identify the rhizobial symbionts within taxonomically diverse hosts and determine nodulation preferences for particular rhizobial lineages within the alpha- and beta-rhizobia; and (2) to examine the effect of ecological and geographic variables on the genetic structure of rhizobial populations.

MATERIALS AND METHODS

Legume and soil sampling

Legume species were collected from 2008 to 2012 in the field from different localities, ecoregions and soil types in the CCR (Fig. 1). In total, 65 host lineages were sampled and herbarium specimens of all collected plants were deposited at the Bolus Herbarium, University of Cape Town for reference and detailed taxonomic identification. For each legume tribe, one representative species is documented with photographs showing their native habitat (Fig. 2) and collected field nodules are illustrated in Fig. 3. At every collection site, the soil acidity (pH) was determined for three replicates of soil samples by suspending 10 g of soil in 25 ml 1 M KCl, shaking at 180 rpm for 60 min, centrifuging at 10 000 g for 10 min and measuring pH in the supernatant. Voucher information of rhizobial taxa with reference to geographical origin, soil type and acidity is listed in Table S1 (Supporting Information).

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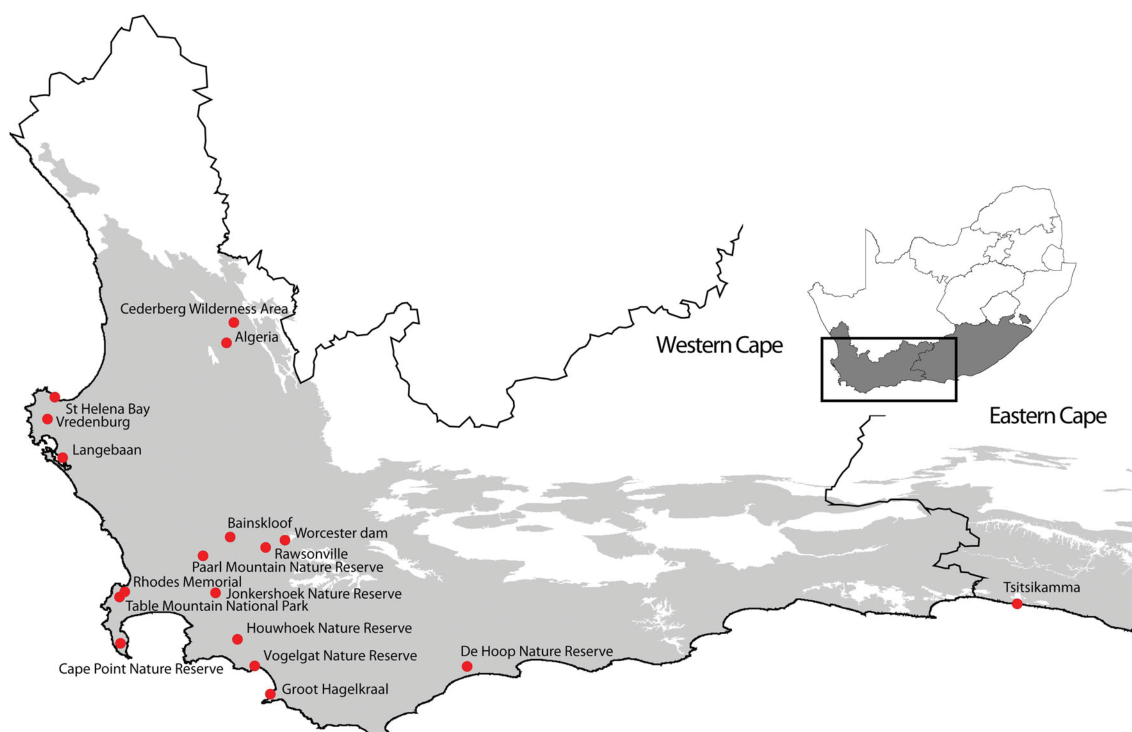

Figure 1. Map of South Africa showing the geographic distribution of sampling sites (red dots) within the Western and Eastern Cape Provinces. Fynbos vegetation is shown in light grey.

Isolation of rhizobia

One nodule was removed from the roots of each sampled legume plant to isolate the endosymbiont following standard protocols (Vincent 1970). Nodules were surface sterilized in 70% ethanol and 0.1% acidified mercuric chloride for 1 and 3 min, respectively. After washing six times in sterile distilled water, they were crushed in sterile sodium chloride buffer and the bacterial suspension was streaked on yeast extract mannitol agar (YEMA) plates. Bacterial strains were grown at 28°C for up to 10 days. Individual colonies were selected and re-streaked on new YEMA plates to obtain pure bacterial cultures. Two replicates of each strain were maintained for long-term storage in YEM broth containing 20% glycerol at −80°C.

Authentication experiments

Nodulation capabilities of rhizobia were verified through authentication experiments on the original host for a set of rhizobia and on three secondary host species i.e. siratro (*Macroptilium atropurpureum* (DC.) Urb.), *Podalyria calyptrata* (Retz.) Willd. and *Otholobium hirtum* (L.) C.H. Stirt. covering all rhizobial isolates. For the symbiont of *Sesbania punicea*, nodulation capabilities were also tested on *S. rostrata* Bremek. and Oberm. and *S. virgata* (Cav.) Pers. Available seeds for 16 legume species were obtained from the original host in the field or purchased from a commercial seed store (Silverhill Seeds, Kenilworth, Cape Town, South Africa). Nodulation was assessed by growing seedlings from surface-sterilized seeds in sterile Leonard jars (Vincent 1970) or in controlled aseptic open pot systems covered with sterilized nylon beads (Lomold group HQ, South Africa). Seedlings were inoculated with a 1 ml rhizobial culture of $OD_{600} = 0.8$, or left uninoculated as negative controls. The seedlings (three per pot) were watered with a nitrogen-free nutrient solution and the roots were harvested after six weeks, to assess nodulation. Authentication was confirmed if isolates nodulated the roots of inoculated plants and uninoculated plants remained nodule-free. Nodules in every treatment were checked visually for the presence of leghaemoglobin, which is an indication of nitrogen fixation. For rhizobia able to induce effective nodulation, hosts showed enhanced plant growth compared with ineffective or incompatible symbiotic associations and uninoculated controls.

DNA extraction, PCR amplification and sequencing

Total DNA was isolated by using a standard thermal lysis protocol, in which a loopful of bacterial culture was suspended in 20 μl lysis buffer (10% SDS, 1M NaOH) followed by incubation for 15 min at 95°C. The lysate was centrifuged briefly at 13 000 g, and 180 μl of sterile water was added. The DNA extract was centrifuged for another 5 min at 13 000 g and preserved at −20°C.

Each amplification reaction was done in a 25 μl reaction mix (Kapa Biosystems), according to the manufacturer's instructions. All amplification reactions of the housekeeping (16S rRNA, *recA*, *atpD*) and symbiosis (*nifH* and *nodA*) genes were performed using a GeneAmp PCR System 2700 (Applied Biosystems, Foster City, CA, USA) with PCR parameters as described by the authors (Table S2, Supporting Information): 16S rRNA (Weisburg *et al.*, 1991), *atpD*, *recA* (Gaunt *et al.*, 2001), *nodA* (Haukka, Lindström and Young 1998) and *nifH* (Boulygina *et al.*, 2002; De Meyer *et al.*, 2011). Amplified products were purified using a modification of the Exo/Sap enzyme cleaning protocol (Werle *et al.*, 1994). Purified PCR amplicons were sent to Macrogen for sequencing (Macrogen, the Netherlands) using the same sequencing primers as for the initial PCR. All lineages sampled with GenBank accession numbers are listed in (Table S3, Supporting Information).

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Figure 2. Representatives for the main legume groups occurring in the their native habitat of the Fynbos biome: **(A)** *Lessertia lanata* (Astragaleae) **(B)** *Aspalathus astroites* (Crotalarieae) **(C)** *Indigofera brachystachya* (Indigofereae) **(D)** *Tephrosia capensis* (Milletieae) **(E)** *Bolusafra bituminosa* (Phaseoleae) **(F)** *Podalyria calyptrata* (Podalyrieae) **(G)** *Psoralea azuroides* (Psoraleeae) **(H)** *Sesbania punicea* (Sesbanieae). Photographs: a-g Charles Stirton; h, Ispot—Tony Rebelo).

Phylogenetic analyses

Sequences were edited and assembled in Geneious Pro v.5.1.7 (http://www.geneious.com). A preliminary alignment of DNA sequences was created with Muscle v.4.0 (Edgar 2004), followed by manual adjustments in MacClade v.4.04 (Maddison and Maddison 2001) to adjust ambiguously aligned regions. Phylogenetic analyses were conducted using maximum likelihood (ML) and Bayesian inference (BI) criteria, both carried out on the CIPRES web portal (http://www.phylog.org). ML analyses were done with RAxML-VI-HPC v.2.2.3 using GTR-GAMMA as the most complex substitution model available (Stamatakis 2006). A multiparametric bootstrap resampling of 1000 pseudo-replicates was plotted onto the previously selected best-scored ML tree.

Model selection for the Bayesian analyses was conducted with MrModeltest v.3.06 (Posada and Crandall 1998) under the

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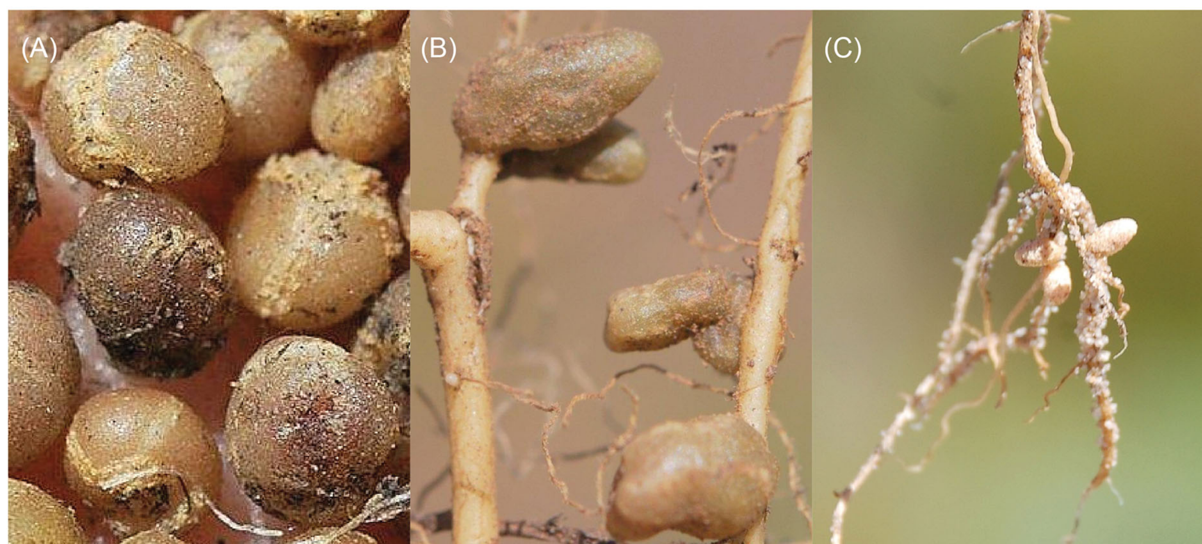

Figure 3. Detailed photographic illustrations of different nodule structures from field collected legume species (**A**) Determinate nodules of *Psoralea suaveolens* (Psoraleeae) (**B**) Indigoferoid nodules of *Indigofera incana* (Indigofereae) (**C**) Indeterminate unbranched nodules of *Podalyria sericea* (Podalyrieae).

Akaike information criterion. For all datasets, MrModeltest selected the general time reversible (GTR) model of DNA substitutions with gamma-distributed rate variation across invariant sites. This best-fitting model of DNA substitution was applied for each separate dataset (16S rRNA, *recA*, *atpD*, *nodA*, *nifH*). In the combined BI analyses, the three-gene dataset was partitioned and the same models were assigned to separate unlinked partitions. BI analyses were carried out using MrBayes v.3.1 (Ronquist and Huelsenbeck 2003) running four Markov chains (one cold and three heated) simultaneously for five million generations. Conservatively, 25% of the first trees sampled were regarded as 'burnin' and discarded. Convergence of the chains was checked using Tracer v.1.4 (Rambaut and Drummond 2007).

Statistical analyses

Genotypes of rhizobial isolates were identified for 16S rRNA, *nodA* and *nifH* sequence data in GenAlEx (Peakall and Smouse 2006), and the extend of genetic variation among isolates representing soil types, geographical provenance and host genotype was statistically analysed by analysis of molecular variance (AMOVA) (Excoffier, Smouse and Quattro 1992). The statistical significance of the pairwise φ_{ST} components of variance was tested using 9999 permutations, and adjusted by the Bonferroni method for multiple comparisons. For soil type and geographic provenance, AMOVA was applied separately for the *Burkholderia* and *Mesorhizobium* datasets.

Mantel tests were performed to examine associations between pairwise genetic distances on the one side and pairwise geographical distances, pairwise differences in soil pH and pairwise differences in site elevation on the other side. The statistical significances were based on 9999 permutations in GenAlEx.

Nodule structure and immunogold labelling

In parallel to the molecular identification of *Burkholderia* rhizobia, samples of nodules that had been collected in parallel to those that had been used for isolation of bacteria were prepared for microscopy to determine the symbiotic effectiveness of the nodules and for *in situ* immunogold labelling assays to confirm (or not) their symbiotic association with *Burkholderia*. Nodules from 45 different lineages comprising legumes from 10 tribes and 16 genera (Table S4, Supporting Information) were treated according to the methods of Elliott *et al*., (2007b) and Dos Reis Junior *et al*., (2010). Nodules were fixed and embedded in resin, sectioned longitudinally, stained with toluidine blue for general structural observations and then probed with *Burkholderia*- and *Cupriavidus taiwanensis*-specific antibodies. Sections incubated in non-immune serum served as negative controls.

RESULTS

Identification of rhizobia in Cape legumes using 16S rRNA, *recA* and *atpD* data

An initial classification of the 65 isolates into rhizobial genera was assessed by 16S rRNA sequence comparison using BLASTN search facilities on the public GenBank database (Altschul *et al*., 1990). The 16S rRNA BLAST results placed the 65 bacterial strains in different genera of both *Alpha*- (*Azorhizobium*, *Bradyrhizobium*, *Ensifer*, *Mesorhizobium*, *Rhizobium*) and *Beta*- (*Burkholderia*) classes of the *Proteobacteria* and revealed that the isolated strains shared highly similar (99–100%) 16S rRNA sequences with known bacterial species (Table S5, Supporting Information). For all *Burkholderia* isolates (except for *Rafnia* sp. Dlodlo 28, *Indigofera* sp. Muasya 5748, *Bolusafra bituminosa* Dlodlo 29 and *Virgilia oroboides* Muasya 5366), known South African strains from *Lebeckia*, *Cyclopia* and *Rhynchosia* hosts were recovered as closest relatives, while all classical alpha-rhizobial strains showed highly scored BLAST similarities with non-South African strains.

In addition to 16S rRNA data, *recA* and *atpD* housekeeping genes were amplified and sequenced (16S rRNA, 1347 base pairs (bp); *recA*, 527 bp; *atpD*, 457 bp) to reveal the phylogenetic position of the 65 novel isolates. An initial phylogenetic analysis including all available reference strains for the three genes was run to identify closely related sister taxa of our isolates (data not shown). These closest relatives are included in the current multilocus sequence analysis. The Bayesian majority rule consensus tree with branch lengths optimized from partial 16S rRNA, *recA* and *atpD* data was congruent with the ML analysis and provided clearly resolved clusters (Figs 4 and S1, Supporting Information), placing the rhizobial lineages in distinct phylogenetic groups

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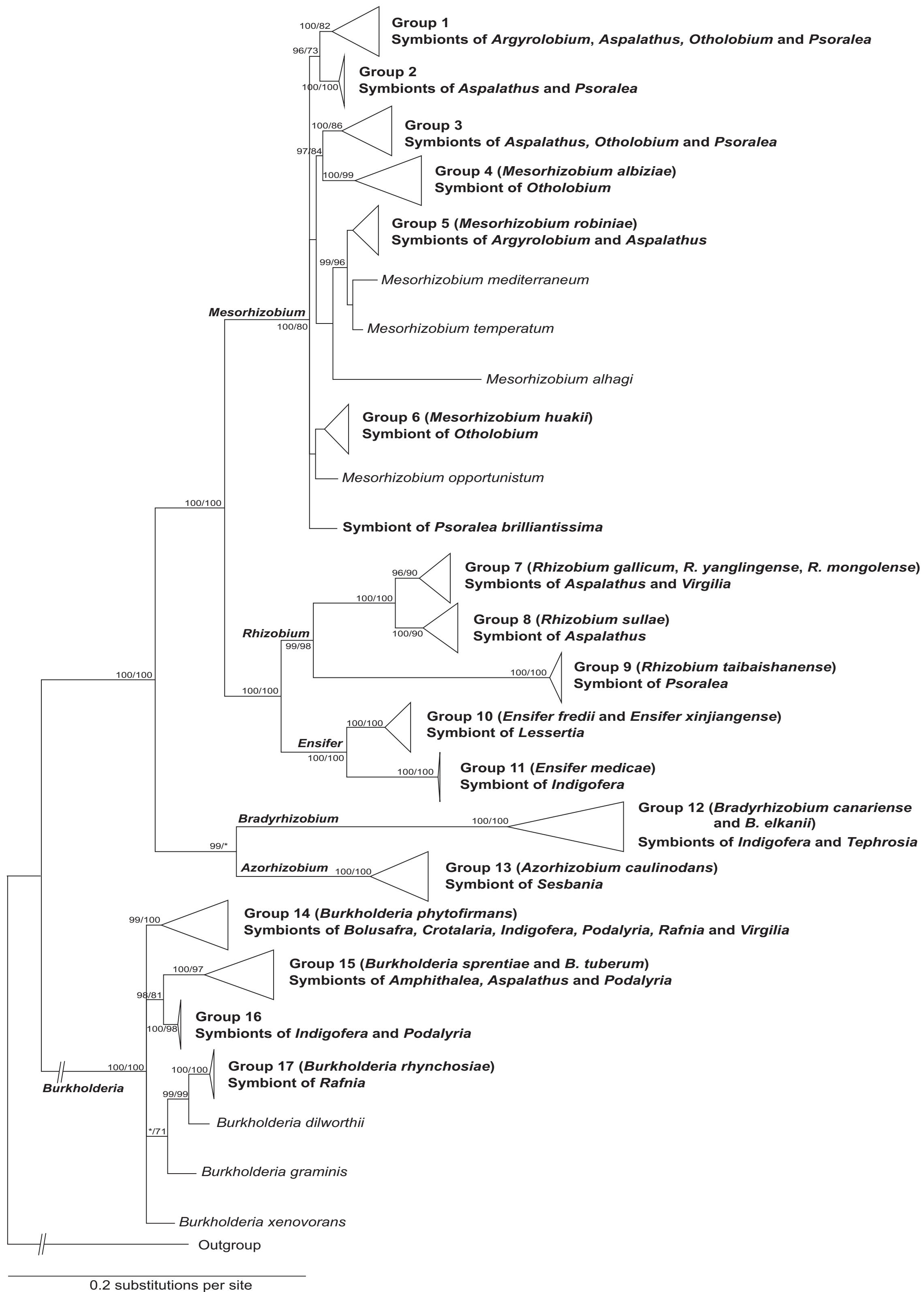

Figure 4. Phylogenetic tree of rhizobial endosymbionts based on 16S rRNA, *recA* and *atpD* data. Support values for the Bayesian and ML analyses are given at the nodes (Bayesian posterior probabilities—bootstrap support values for the ML analysis). Isolated strains are shown in bold or placed in phylogenetic groups (Group 1–17).

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of *Alpha*- and *Beta-Proteobacteria*. Isolates from *Amphithalea*, *Bolusafra*, *Crotalaria*, *Podalyria*, *Rafnia* and from four species of the genera *Virgilia*, *Indigofera* and *Aspalathus* were associated with *Burkholderia* with the majority of rhizobia related to *B. tuberum/B. sprentiae* (Group 15–100% Bayesian posterior probability-BPP, 97% bootstrap support-BS), *B. phytofirmans* (Group 14–99% BPP, 100% BS), and *B. rhynchosiae/B. dilworthii* (Group 17–99% BPP, 99% BS). *Burkholderia tuberum* is recognized as a widespread symbiont in South African soils, mostly associated with *Cyclopia* species (Elliott *et al*., 2007a; Gyaneshwar *et al*., 2011), but also found to nodulate South American *Mimosa* species (Mishra *et al*., 2012), showing that this *Burkholderia* species can nodulate legumes in both subfamilies Papilionoideae and Mimosoideae (the host ranges of *B. tuberum* referred to as biovar papilionoideae and biovar mimosae, respectively). *Burkholderia sprentiae*, *B. rhynchosiae* and *B. dilworthii* are newly described species from the South African legumes *Lebeckia ambigua* (Crotalarieae) (*B. sprentiae/B. dilworthii*) (De Meyer *et al*., 2013b, 2014) and *Rhynchosia ferulifolia* (Phaseoleae) (*B. rhynchosiae*) (De Meyer *et al*., 2013a), respectively. *Burkholderia phytofirmans,* initially described as a plant-growth promoting symbiont without either nitrogen fixation or nodulation capacity (Compant *et al*., 2005), has been recently isolated as a beneficial (non-nodulating) root nodule endophyte from South African and Australian *Acacia* species (Ndlovu *et al*., 2013). Most *Burkholderia* rhizobia were closely related to known reference strains (Group 14–15–17, Fig. 4), although two isolates (symbionts from *Indigofera angustifolia* Muasya 5878 and *Po. burchellii* Muasya 5875) were placed within a separate clade (Group 16) without closely related reference strains and may represent novel *Burkholderia* species.

Within the genus *Mesorhizobium,* at least six major monophyletic groups were recovered with symbionts from *Aspalathus*, *Psoralea*, *Argyrolobium*, *Otholobium* and *Indigofera* (Fig. 4). The well-supported monophyletic genus *Mesorhizobium* (100% BPP, 83% BS) contains two major groups (Group 1–100% BPP, 82% BS and Group 2–100% BPP, 100% BS) with highly related lineages, which might represent putative novel *Mesorhizobium* species based on the DNA similarity results and the absence of closely related reference strains. All remaining *Mesorhizobium* species, isolated from nodules of *Aspalathus*, *Otholobium*, *Psoralea* and *Argyrolobium* species, were placed in clusters 3–6 (Fig. 4), and were intermingled with known reference strains (*M. albiziae, M. huakuii*, *M. mediterraneum, M. opportunistum*, *M. robiniae*, *M. temperatum*) all of which have been shown to nodulate various hosts (Jarvis, Downer and Young 1992; Gao *et al*., 2004; Wang *et al*., 2007; Nandasena *et al*., 2009; Zhou, Chen and Wei 2010).

Within the genus *Ensifer* (syn. *Sinorhizobium*), the strain isolated from *Indigofera* sp. (Dlodlo 45) was closely related to the named *Medicago*-nodulating species *Ensifer medicae* (100% BPP, 100% BS) (Rome *et al*., 1996), while the symbiont of *Lessertia* sp. (Dlodlo 46) formed a supported clade with the soybean nodulators *E. fredii* and *E. xinjiangense* (100% BPP, 100% BS) (Jarvis *et al*., 1992; Peng *et al*., 2002).

In the genus *Rhizobium*, the endosymbionts of *Aspalathus* sp. (Dlodlo 49) and *V. divaricata* (Dlodlo 123) were placed within a monophyletic group together with the known rhizobial species *Rhizobium gallicum*, *R. yanglingense* and *R. mongolense* (96% BBP; 90% BS) (Amarger, Macheret and Laguerre 1997; van Berkum *et al*., 1998; Tan *et al*., 2001). The symbiont of *Aspalathus* sp. (Dlodlo 53) was closely related to the nodulating *R. sullae* (100% BPP; 90% BS) (Squartini *et al*., 2002), and the *Rhizobium* strain of *Psoralea gigantea* (Dlodlo 24) was closely related to *R. taibaishanense* (100% BPP; 100% BS) which nodulates *Kummerowia striata* (Yao *et al*., 2012).

In the genus *Bradyrhizobium*, the highly acid-tolerant *Br. canariense* (Vinuesa *et al*., 2005a) was a sister group to the endosymbiont of *I. gracilis* (Muasya 5621) (100% BPP; 100% BS). The rhizobia of *I. frutescens* (Muasya 5392) and *Tephrosia capensis* (Muasya 5405) were placed as a sister group to *Bradyrhizobium elkanii* (Kuykendall *et al*., 1992). Finally, the symbiont of *S. punicea* (Muasya 5717) was related to the stem-nodulating bacterium *Azorhizobium caulinodans* (100% BBP; 100% BS) (Dreyfus, Garcia and Gillis 1988).

Nodulation capabilities assessed by authentication experiments and *nodA* sequencing

Authentication experiments were performed to fulfil Koch's postulates, thereby verifying the ability of the strain to nodulate. Nodulation tests were carried out on the original host for selected species where seeds were available and on alternative legume hosts for all species (Table 1). The ability of all rhizobial isolates to nodulate was verified on siratro, a host plant known to be symbiotically promiscuous, whereas beta- and alpha-rhizobia were tested on *Po. calyptrata* (tribe Podalyrieae) and *O. hirtum* (tribe Psoraleeae), respectively. These legume hosts are naturally associated with *Burkholderia* and *Mesorhizobium*, respectively (Fig. 4) and may represent suitable alternative hosts for either beta- or alpha-rhizobia of the CCR. Siratro nodulated with most of the rhizobial isolates (55 out of 65) confirming its utility as a widely used promiscuous trapping species (Trinick, Miller and Hadobas 1991; Mishra *et al*., 2012). Similarly *Po. calyptrata* was shown to be very promiscuous with regards to *Burkholderia* symbionts and nodulated with all burkholderias except for the isolate from *Rafnia acuminata*, whereas *O. hirtum* was a more specific host plant and only nodulated with *Mesorhizobium* strains from *Otholobium* (seven strains), *Psoralea* (three strains) and *Aspalathus* (three strains) (Table 1). The *Azorhizobium* symbiont of *S. punicea* nodulated its parent host and could induce nodules on the roots or stems of *S. virgata* and *S. rostrata*, respectively. However, the *Azorhizobium* (Muasya 5717)–*S. virgata* interaction did not result in effective nitrogen fixation, confirming the high stringent specificity of *S. virgata* towards its symbiont, *A. doebereinerae* (Moreira *et al*., 2006). Overall, by using a combination of original and alternative legume hosts, all rhizobial isolates were successfully authenticated, with the exception of six isolates: *Aspalathus spicata* Muasya 5440, *O. hirtum* Muasya 5376, *P. asarina* Muasya 5360, *P. gigantea* Dlodlo 24, *P. usitata* Muasya 5364 and *Ra. acuminata* Dlodlo 22.

In addition to nodulation experiments, isolates were screened for their nodulation genes as a general indication of their ability to nodulate. *nodA* sequences were determined for most species, although some lineages failed to generate nodulation amplicons (Table S3, Supporting Information). The inability to amplify the *nodA* gene in selected rhizobia does not necessarily imply that these isolates are ineffective endophytes, but may be attributed to different causes, such as primer mismatches by degenerated primers, a Nod factor-independent symbiotic signalling pathway, and the release of plasmid carrying symbiotic determinants upon cultivation (Howieson *et al*., 2013). In seven cases, where *nodA* amplification failed, the rhizobial strains successfully nodulated their host within the authentication experiments (i.e. *A. ciliaris* Stirton 13166, *A. laricifolia* Muasya 5372, *Otholobium* sp. Muasya 5370, *O. virgatum* Muasya 5333, *P. pinnata* Muasya 5336, *P. pullata* Muasya 5413 and *S. punicea* Muasya 5717). This observation clearly indicates that these strains harbour nodulation-related genes or possess an alternative nodulation signalling pathway (Giraud *et al*.,

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Table 1. Authentication experiments of isolated bacterial strains on their original host and alternative host species: siratro (*M. atropurpureum*), *O. hirtum* and *Po. calyptrata* (+ = nodulated; - = not nodulated; / = strains were not authenticated on this host; n/a = seeds were not available to authenticate on the original host). *A.* = *Aspalathus; Ar.* = *Argyrolobium; I.* = *Indigofera; O.* = *Otholobium; P.* = *Psoralea; Po.* = *Podalyria; Ra.* = *Rafnia; S.* = *Sesbania; T.* = *Tephrosia; V.* = *Virgilia*

Tribe and species	Strain	siratro	*O. hirtum*	*Po. calyptrata*	Original host
Astragaleae					
Lessertia sp.	Dlodlo 46	+	-	/	+
Crotalarieae					
A. astroites	Dlodlo 18	+	-	/	n/a
A. aurantiaca	Muasya 5397	+	-	/	n/a
A. bracteata	Muasya 5618	+	-	/	n/a
A. callosa	Muasya 5477	+	/	+	+
A. carnosa	Muasya 5496	+	/	+	n/a
A. ciliaris	Dlodlo 108	+	-	/	n/a
A. ciliaris	Muasya 5361	+	+	/	n/a
A. ciliaris	Stirton 13166	+	-	/	+
A. cordata	Dlodlo 13	+	-	/	n/a
A. ericifolia	Dlodlo 31	+	+	/	n/a
A. ericifolia	Muasya 5352	+	-	/	n/a
A. laricifolia	Muasya 5372	+	-	/	n/a
A. perfoliata	Muasya 5757	+	-	/	n/a
Aspalathus sp.	Dlodlo 49	+	/	+	n/a
Aspalathus sp.	Dlodlo 53	+	/	+	n/a
A. spicata	Muasya 5398	+	+	/	n/a
A. spicata	Muasya 5440	-	-	/	n/a
A. uniflora	Dlodlo 26	+	-	/	+
A. uniflora	Muasya 5734	+	-	/	n/a
Crotalaria sp.	Dlodlo 120	+	/	+	+
Ra. acuminata	Dlodlo 22	-	/	-	n/a
Rafnia sp.	Dlodlo 28	+	/	+	+
Ra. triflora	Dlodlo 55	+	/	+	n/a
Genisteae					
Argyrolobium sp.	Dlodlo 14	+	-	/	+
Ar. lunare	Dlodlo 48	+	-	/	n/a
Ar. lunare	Muasya 5369	+	-	/	n/a
Ar. velutinum	Dlodlo 47	+	-	/	n/a
Indigofereae					
I. angustifolia	Muasya 5878	+	/	+	+
I. frutescens	Muasya 5392	+	-	/	n/a
I. gracilis	Muasya 5621	+	-	/	n/a
Indigofera sp.	Dlodlo 45	+	-	/	n/a
Indigofera sp.	Muasya 5748	+	/	+	n/a
I. venusta	Muasya 5377	+	-	/	n/a
Millettieae					
T. capensis	Muasya 5405	+	-	/	+
Phaseoleae					
Bo. bituminosa	Dlodlo 29	+	/	+	+
Podalyrieae					
Amphithalea ericifolia	Muasya 5482	+	/	+	n/a
Po. burchellii	Muasya 5875	+	/	+	n/a
Po. calyptrata	Dlodlo 25	+	/	+	n/a
Po. calyptrata	Muasya 5337	-	/	+	+
Po. sericea	Muasya 5384	+	/	+	n/a
V. divaricata	Dlodlo 123	+	/	/	+
V. oroboides	Muasya 5366	+	/	+	+
Psoraleeae					
O. bracteolatum	Dlodlo 42	-	+	/	n/a
O. hirtum	Dlodlo 32	+	+	/	+
O. hirtum	Muasya 5334	+	+	/	n/a
O. hirtum	Muasya 5376	-	-	/	n/a
O. hirtum	Muasya 5382	+	+	/	n/a
O. sp.	Muasya 5370	+	-	/	n/a

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Table 1. *continued*

Tribe and species	Strain	siratro	*O. hirtum*	*Po. calyptrata*	Original host
O. virgatum	Muasya 5333	+	+	/	n/a
O. virgatum	Muasya 5357	+	+	/	n/a
O. zeyheri	Muasya 5675	-	+	/	n/a
P. asarina	Dlodlo 15	+	+	/	n/a
P. asarina	Muasya 5360	-	-	/	n/a
P. brilliantissima sp. nov. ined.	Dlodlo 52	+	-	/	n/a
P. congesta sp. nov. ined.	Muasya 5462	+	+	/	n/a
P. gigantea sp. nov. ined.	Dlodlo 24	-	-	/	n/a
P. laxa	Dlodlo 119	+	+	/	n/a
P. oligophylla	Dlodlo 118	+	-	/	n/a
P. pinnata	Muasya 5336	+	-	/	+
P. pullata sp. nov. ined.	Muasya 5413	+	-	/	n/a
P. rigidula sp. nov. ined.	Muasya 5343	+	-	/	n/a
P. usitata sp. nov. ined.	Muasya 5364	-	-	/	n/a
Sesbanieae					
S. punicea	Muasya 5717	-	-	/	+

2007). For five rhizobial strains isolated from *Ra. acuminata* (Dlodlo 22), *O. hirtum* (Muasya 5376), *P. asarina* (Muasya 5360), *P. gigantea* (Dlodlo 24) and *P. usitata* (Muasya 5364), however, both *nodA* amplification and authentication were not successful, and hence these strains should not be credited as nodulating symbionts.

Nodule structure and immunogold labelling

Nodule structure as observed under the light and electron microscopes has been used as an indicator of the effectiveness of nodules collected in the field, including those containing burkholderias, and correlates well with the expression of nitrogenase (Dos Reis Junior *et al.*, 2010). That being the case, it is apparent that the nodules that were collected from the CCR were all effective (Fig. 5, Table S4, Supporting Information). In addition, immunogold labelling experiments with antibodies raised against *B. phymatum* STM815 and *C. taiwanensis* LMG19424 (Elliott *et al.*, 2007b) have recently been used to demonstrate *in situ* the presence of beta-rhizobial symbionts in field-collected *Mimosa* nodules in Brazil (dos Reis Junior *et al.*, 2010) and India (Gehlot *et al.*, 2013), as well as in nodules of *Dipogon lignosus* in New Zealand (Liu *et al.*, 2014). The same approach and antibodies were used in the present study to investigate if field-collected nodules from legumes of the tribes Crotalarieae, Hypocalypteae, Indigofereae, Loteae, Millettieae, Phaseoleae, Podalyrieae, Psoraleeae and Sesbanieae might also contain beta-rhizobial symbionts (Fig. 5, Table S4, Supporting Information). This culture-independent *in situ* microscopy technique not only corroborated previous *Burkholderia* identifications by DNA sequencing but also extended them into legume species from which rhizobia were not obtained. These data indicated the prevalence of *Burkholderia* (none contained *C. taiwanensis*; data not shown) as endosymbionts in diverse legume groups, including species of *Aspalathus*, *Bolusafra*, *Dipogon*, *Hypocalyptus*, *Indigofera*, *Podalyria*, *Rhynchosia*, *Stirtonanthus* and *Virgilia*. Specific examples are shown in Fig. 5, along with positive control sections of nodules of *Hypocalyptus sophoroides* that were formed after inoculation by *B. tuberum* STM678 (Fig. 5A and B), and include *Burkholderia*-positive sections of *Po. myrtillifolia* (Fig. 5C and D) and *I. angustifolia* nodules (Fig. 5E and F), as well as *Burkholderia*-negative sections of nodules *of A. teres* (Fig. 5G and H). Multiple nodule accessions per host species e.g. *A. cordata*, *A. hispida*, *A. hystrix*, *Po. calyptrata*, *P. myrtillifolia, V. oroboides* and *O. swartbergense* showed consistent results proving the robustness of the immunogold labelling technique to confirm *Burkholderia* (or not) in nodules. The comprehensive species sampling within a genus allowed assessment of the predominance or lack of *Burkholderia* symbionts in certain legume groups; the tribes Podalyrieae (*Podalyria*, *Stirtonanthus* and *Virgilia*) and Psoraleeae (*Otholobium* and *Psoralea*) clearly exhibit a degree of host preference, as all Podalyrieae nodules associate exclusively with *Burkholderia,* whereas Psoraleeae do not form *Burkholderia* interactions. For other tribes (i.e. Crotalarieae, Indigofereae and Phaseoleae) and genera (i.e. *Aspalathus*, *Indigofera*, *Rhynchosia*) legume species were associated with either *Burkholderia* or alpha-rhizobia, thus indicating a more diffuse bacteria–legume interaction.

NodA and *nifH* sequence data and phylogenetic reconstruction

The nodulation gene *nodA* and nitrogen-fixation gene *nifH* were determined for 52 and 54 isolates, respectively. Numerous isolates (32 out of 52) harboured *nodA* genes which were highly related (97–100% sequence similarities) to strains recorded in GenBank, including mostly representatives previously identified from South African legumes (e.g. *A. linearis*, *Cyclopia galioides*, *C. genistoides*, *C. pubescens*, *C. subternata*, *Lessertia annularis*, *L. excisa*, *L. herbacea*, *L. microphylla*, *Rh. ferulifolia*). However, for the alpha-rhizobia of *Lessertia* sp. (Dlodlo 46), *Indigofera* sp. (Dlodlo 45) and *P. brilliantissima* (Dlodlo 52) *nodA* types were recovered that were identical to *Ensifer* strains collected in Asia and North Africa (Table S1, Supporting Information). The remaining 17 isolates revealed distinct *nodA* amplicons without known references in GenBank with low sequence similarity values ranging from 79 to 94%.

Similar to *nodA*, the *nifH* BLASTN results (data not shown) indicate for most isolates high sequence similarities (96–100%)

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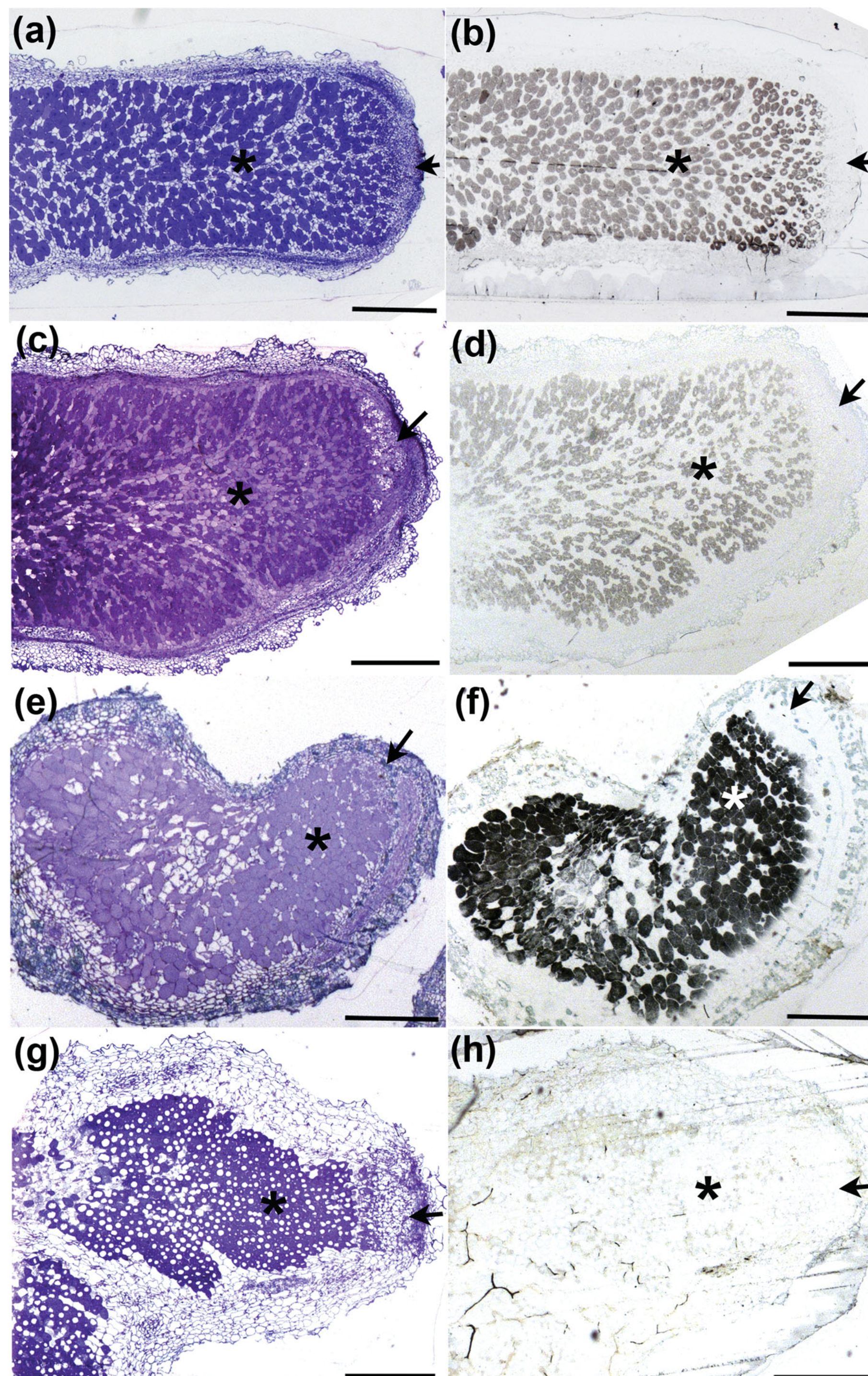

Figure 5. Light micrographs of nodules of *Hypocalyptus sophoroides* (**A** and**B**), *Podalyria myrtillifolia* (**C** and **D**), *Indigofera angustifolia* (**E** and **F**), *Aspalathus teres* (**G** and **H**) stained with toluidine blue (A, C, E, G) and immunogold labelled with a *Burkholderia*-specific antibody (B, D, F, H). All the nodules were collected from plants growing in the field in the CCR, except for those on *H. sophoroides* which were formed on plants grown from seedlings under sterile conditions in glass tubes and which were inoculated with *B. tuberum* STM678^{T}. Longitudinal sections through mature indeterminate nodules show an apical meristem (arrow) at the nodule tip and a large and heavily stained nitrogen-fixing zone (*) containing rhizobial-infected (stained) and uninfected (unstained) cells. The infected cells with *Burkholderia* bacteroids are clearly visible within the immunogold labelled sections of nodules of *H. sophoroides* + *B. tuberum* STM678, which are positive controls for the *Burkholderia*-specific antibody (B), and in nodules on *Po. myrtillifolia* (D) and *I. angustifolia* (F), whereas the nodules on *A. teres* (H) gave no signal with the antibody, and were similar to negative control sections of *H. sophoroides* + *B. tuberum* STM678 nodules probed with an antibody against *C. taiwanensis* or with pre-immune serum (not shown, but see Fig. 3 in dos Reis Junior *et al.*, 2010). Bars, 500 μm.

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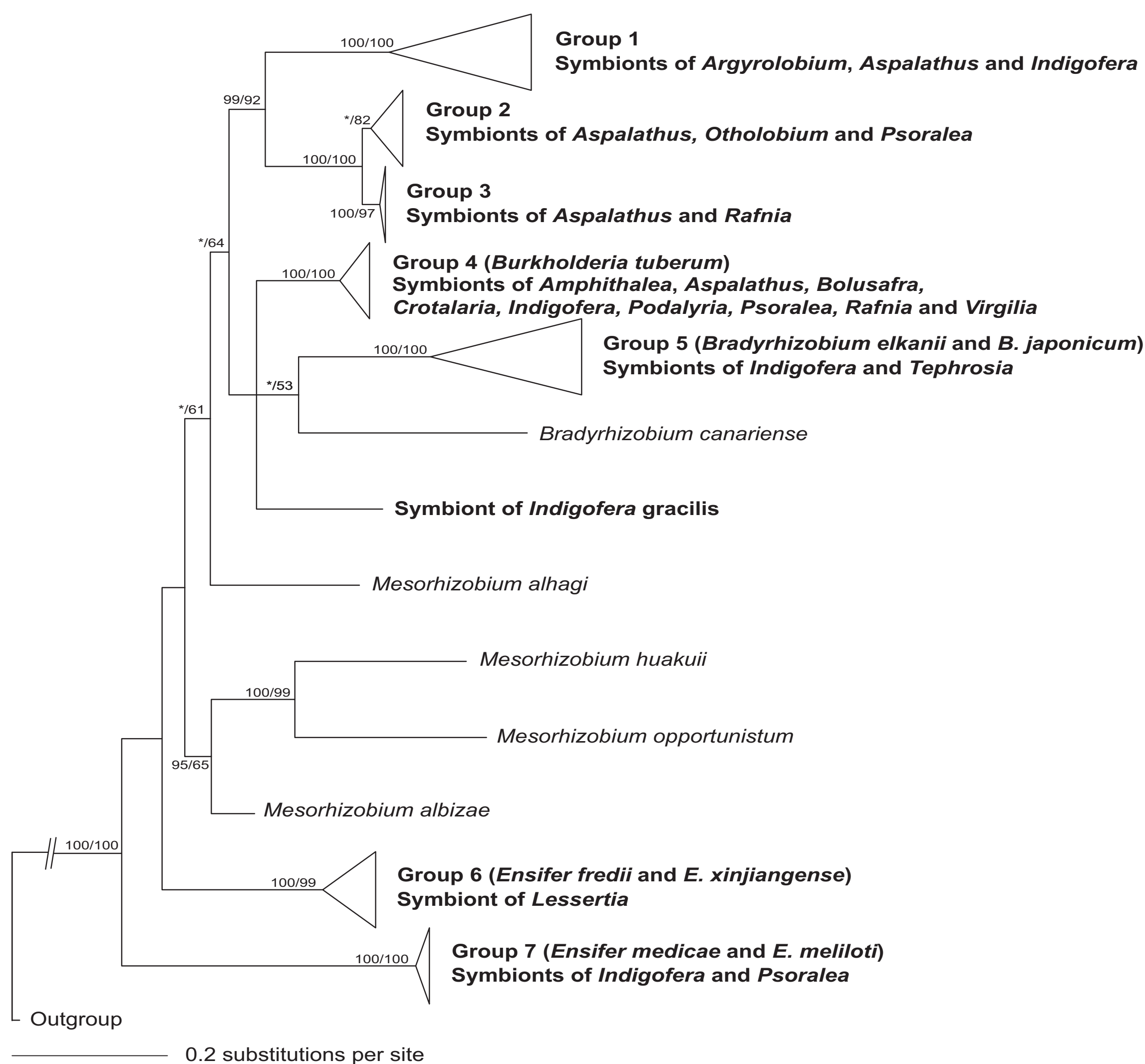

Figure 6. Phylogenetic tree based on partial *nodA* sequences of rhizobial endosymbionts. Support values for the Bayesian and ML analyses are given at the nodes (Bayesian posterior probabilities—bootstrap support values for the ML analysis). Isolated strains are shown in bold or placed in phylogenetic groups (Group 1–7).

with known South African *nifH* copies, predominantly originating from *Mesorhizobium* sp. Xhj23 (AF484646), *Burkholderia* sp. WC7.3c (HF544478), *Burkholderia* sp. UCT56 (HF544499), *Burkholderia* sp. RAU2d2 (HF544414) and *Burkholderia* sp. UCT71 (HF544495). The remaining *nifH* sequences included only alpha-rhizobia, and showed first hit results with 94–99% sequence similarities to non-South African isolates and represent novel *nifH* types for the CCR.

Phylogenetic analyses of the symbiosis-related gene phylogenies (*nodA*, Fig. 6; *nifH*, Figs 7, S2 and S3, Supporting Information) revealed complex relationships that were generally similar to their core gene data (16S rRNA, *recA* and *atpD*) such as the sistergroup relationships within the genera *Bradyrhizobium* (symbionts of *I. frutescens* Muasya 5392 and *T. capensis* Muasya 5405), *Burkholderia* (symbionts of *Bo. bituminosa* Dlodlo 29 and *Indigofera* sp. Muasya 5748) and *Mesorhizobium* (symbionts of *A. ciliaris* Muasya 5361 and *A. ericifolia* Muasya 5352; symbionts of *Argyrolobium velutinum* Dlodlo 47 and *Ar. lunare* Dlodlo 48; symbionts of *A. ericifolia* Dlodlo 31 and *O. hirtum* Dlodlo 32).

Although phylogenetic trees of the *nodA* and *nifH* genes are less resolved, especially for the *nifH* marker, the symbiosis genes were grouped into major clades with sufficient resolution to discriminate among closely related isolates. The *nodA* phylogeny grouped 28 *Mesorhizobium* lineages within two clades without any reference strain (Group 1 and 2). Two *Mesorhizobium* isolates of *P. oligophylla* (Group 4) and *P. brilliantissima* (Group 7), however, were placed within a separate position as sistergroup to *Burkholderia* and *Ensifer*, respectively, suggesting exchange of plasmids or other transferable genetic elements among these rhizobial groups (*Mesorhizobium*–*Ensifer*–*Burkholderia*). Similarly, although all *Burkholderia nodA* genes of the isolates were recovered as a monophyletic group (100% BBP, 98% BS) including the reference strain *B. tuberum* STM678, another *Burkholderia* isolate (Group 3, 100% BBP, 97% BS) appeared to be related to *Mesorhizobium* species (Group 2, 100% BBP, 100% BS), suggesting a separate acquisition of nodulation genes. For most isolates, the *nifH* phylogeny (Fig. 7) revealed similar relationships to those in the *nodA* gene tree. All *nifH* sequences obtained from *Mesorhizobium* isolates were grouped within a monophyletic group (100% BBP, 98% BS) with mostly congruent interspecific relationships as revealed within the *nodA* phylogeny. Consistent with the observation of separate *nodA* origins (see above), the *nifH* gene tree also provides evidence for exchange of symbiotic traits.

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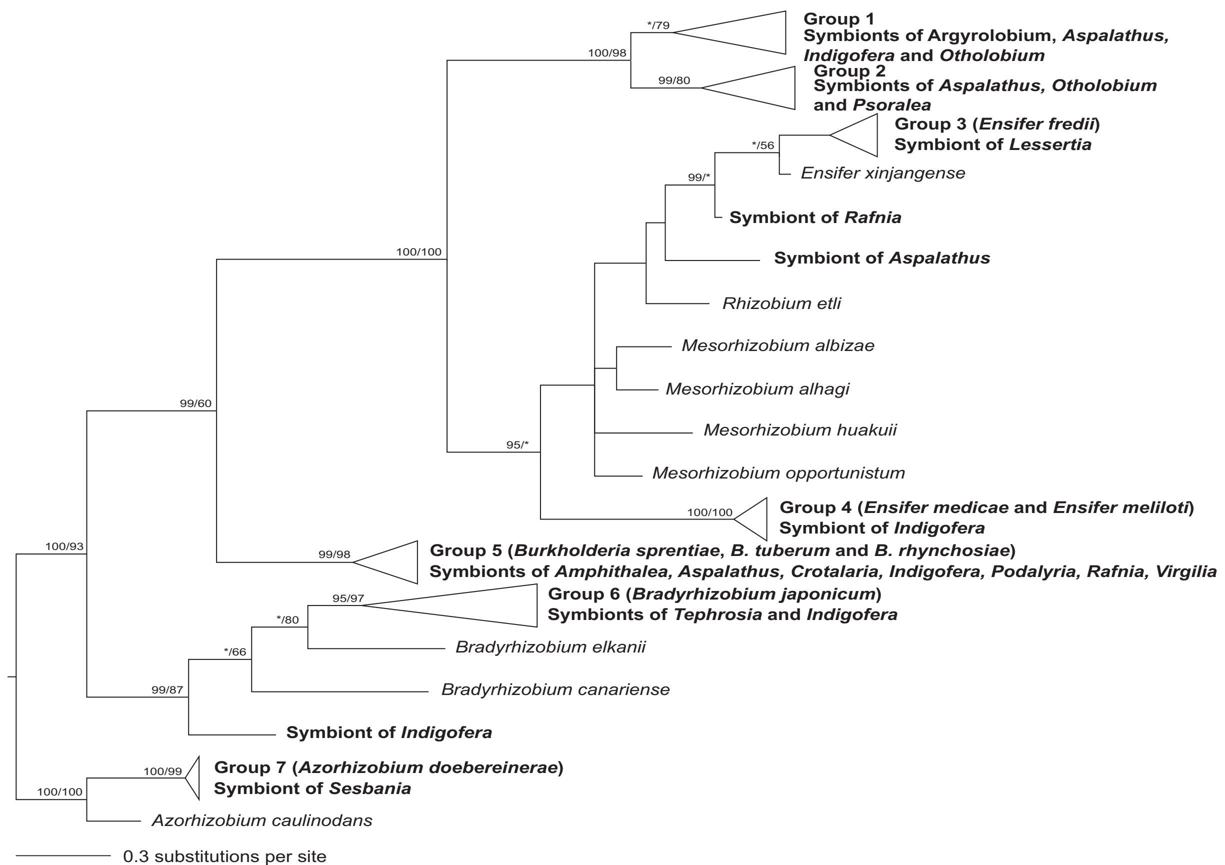

Figure 7. Phylogenetic tree based on partial *nifH* sequences of rhizobial endosymbionts. Support values for the Bayesian and ML analyses are given at the nodes (Bayesian posterior probabilities—bootstrap support values for the ML analysis). Isolated strains are shown in bold or placed in phylogenetic groups (Group 1–7).

More specifically, the *Burkholderia* symbiont of *Ra. triflora* carries a *nifH* fragment related to *E. fredii* and *E. xinjiangense* (99% BBP).

Genetic variation of rhizobia across host genotype and environmental variables

AMOVA was performed to test genetic variation of both chromosomal (16S rRNA) and symbiotic (*nodA* and *nifH*) genes among different legume groups, soil types and geographical origins (Table 2). There was a significant genetic differentiation among host types, for the three data sets. Rhizobial isolates were strongly structured with significant pairwise φ_{ST} values for a large number of legume tribes and genera.

Differences in geographical origin and soil type did not affect rhizobial genetic variation with low φ_{ST} values ranging from 0.006 to 0.087 (Table 2). However, when *Mesorhizobium* and *Burkholderia* rhizobia were analysed separately, they revealed that soil type and geographical origin may explain 16S rRNA genetic structuring within the *Burkholderia* dataset.

The effect of geographical distance, soil acidity and site elevation on the genetic variation of *Burkholderia* and *Mesorhizobium* was further explored using Mantel statistics. A strong positive correlation was found between phylogenetic relatedness of *Mesorhizobium* and soil acidity for all genetic markers (16S rRNA, *nodA* and *nifH*) (Table 3). Differences in soil pH were not related to genetic variation in *Burkholderia*, but site elevation differences were positively correlated with genetic differentiation for the 16S rRNA and *nifH* datasets (*nodA* was marginally significant). The geographic distance matrix was not linked with the *Burkholderia* and *Mesorhizobium* genetic datasets, except for *nodA* from the mesorhizobia.

DISCUSSION

Rhizobial diversity in the CCR

Most of the 750 Cape legumes described to date are placed within the tribes Psoraleeae (Stirton 2005), Crotalariae (Boatwright *et al.*, 2008a), Podalyrieae (van der Bank *et al.*, 2002; Boatwright *et al.*, 2008b) and Indigofereae (Schrire *et al.*, 2009), each containing a large proportion of endemic CCR lineages which are associated with certain groups of rhizobia. Until now, the diversity of rhizobia associated with Cape legumes has been determined in only a small number of host lineages. Many symbionts of other legume species and genera of the Fynbos still need to be discovered to fully understand the extent of rhizobial interactions. Furthermore, previous surveys on Fynbos legumes have demonstrated *Burkholderia* as a common root-nodulating genus (Kock 2004; Elliott *et al.*, 2007a; Garau *et al.*, 2009; Gyaneshwar *et al.*, 2011; Beukes *et al.*, 2013; Howieson *et al.*, 2013; Sprent *et al.*, 2013) and the abundance of this genus has been attributed to its adaptation to acidic conditions in Fynbos soils. The observation that *Burkholderia* is able to successfully colonize legume lineages within four distinct legume tribes (e.g. Crotalarieae, Hypocalypteae, Phaseoleae and Podalyrieae) underlines the hypothesis that beta-rhizobia are widely adapted to the CCR flora, suggesting that the known '*Burkholderia*-philous' legumes to date are only

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Table 2. AMOVA evaluating statistical significance of rhizobial differentiation among host tribe, host genus, soil type and geographic localities. φ_{ST} values and significance level are shown for 16S rRNA, *nodA* and *nifH* datasets: *P < 0.05, **P ≤ 0.01. N/A = group not present for calculation.

	16S rRNA	*nodA*	*nifH*
Host tribe	0.361**	0.365**	0.502**
Podalyrieae/Crotalarieae	0.407**	0.447**	0.566*
Podalyrieae/Genisteae	0.729**	0.801**	0.925
Podalyrieae/Psoraleeae	0.835**	0.646**	0.909**
Podalyrieae/Indigofereae	0.255	0.305	0.345
Psoraleeae/Crotalarieae	0.164	0.333**	0.411**
Psoraleeae/Indigofereae	0.480**	0.293*	0.588**
Psoraleeae/Genisteae	0.003	0.557**	0.659*
Indigofereae/Crotalarieae	0.025	0.187	0.198
Indigofereae/Genisteae	0.222	0.234**	0.341
Host genus	0.51*	0.372*	0.522**
Indigofera/Aspalathus	0.196	0.262*	0.303
Indigofera/Otholobium	0.37**	0.416*	0.473**
Indigofera/Psoralea	0.355	0.087	0.503*
Indigofera/Argyrolobium	0.222	0.234**	0.341
Podalyria/Argyrolobium	0.938	0.765	0.903
Podalyria/Aspalathus	0.742**	0.528**	0.653
Podalyria/Otholobium	0.943*	0.914	0.878
Podalyria/Psoralea	0.911**	0.461*	0.947
Rafnia/Argyrolobium	0.961	0.426	N/A
Rafnia/Aspalathus	0.740*	0.265	N/A
Rafnia/Otholobium	0.955**	0.440	N/A
Rafnia/Psoralea	0.918*	0.001	N/A
Virgilia/Aspalathus	0.283	0.468	0.636
Virgilia/Otholobium	0.653	0.901	0.871
Psoralea/Argyrolobium	0.001	0.422*	0.747*
Psoralea/Aspalathus	0.001	0.321*	0.453**
Psoralea/Virgilia	0.598	0.327	0.947*
Otholobium/Aspalathus	0.001	0.497**	0.367**
Otholobium/Argyrolobium	0.001	0.768	0.562
Soil type	0.033	0.059	0.087
Mesorhizobium isolates	0.007	0.177	0.193
Burkholderia isolates	0.368*	0.199	0.14
Locality	0.006	0.04	0.019
Mesorhizobium isolates	0.166	0.182	0.209
Burkholderia isolates	0.704*	0.076	0.057

Table 3. Mantel test (r_{Mantel}) showing the correlation between phylogenetic distance (16S rRNA, *nodA* and *nifH*), environmental variables (site elevation, and soil pH) and geographical distance. Correlations were investigated separately for *Mesorhizobium* and *Burkholderia* isolates. Significance: *P < 0.05, **P ≤ 0.01.

	16S rRNA	*nodA*	*nifH*
Mesorhizobium			
pH	0.208*	0.230**	0.115**
Geographic distance	0.1	0.178*	0.03
Altitude	−0.02	0.119	0.051
Burkholderia			
pH	−0.83	0.26	−0.172
Geographic distance	0.17	0.179	−0.144
Altitude	0.286*	0.103	0.306*

a small fraction of the true diversity. Following this hypothesis, we predicted that this survey, comprising a wide taxonomic sampling of Fynbos species, would reveal novel associations between *Burkholderia* and legumes.

Our study demonstrates the presence and prevalence of *Burkholderia* in different legume groups and extends the number of genera to include *Bolusafra*, *Rafnia*, and *Indigofera* that are associated with beta-rhizobia, thus confirming that soils of the CCR are a remarkable global biodiversity hotspot for *Burkholderia* (Gyaneshwar *et al.*, 2011). However, in terms of the number of hosts involved, isolates of *Mesorhizobium* were more frequently observed (39 strains) across a wider variety of legumes (*Aspalathus*, *Argyrolobium*, *Indigofera*, *Otholobium* and *Psoralea*); the previously known *Mesorhizobium* diversity of the Fynbos biome being limited to *Psoralea* (Kanu and Dakora 2012), *Aspalathus* (Hassen *et al.*, 2012) and *Lessertia* (Gerding *et al.*, 2012). The previous underreporting of *Mesorhizobium* in Fynbos legumes is in contrast with our observed diversity and clearly indicates that (i) this genus is largely underestimated as a symbiont and (ii) it is well adapted to form symbioses with a wide range of CCR legume species (see above). Our isolates of *Mesorhizobium* cover distinct geographical localities and co-exist with legumes associated with acid-adapted *Burkholderia* species; both genera occurring in similar soil types with similar acidities (pH ranging from 3.18 to 6.5 for *Burkholderia*, 3.18 to 6.7 for *Mesorhizobium*) (Table S1, Supporting Information).

In contrast to the high diversity of *Burkholderia* and *Mesorhizobium*, only a few alpha-rhizobia of the genera *Ensifer*, *Rhizobium* and *Bradyrhizobium* were isolated in this study. Nevertheless, *Bradyrhizobium* and *Rhizobium* species have been previously recorded in Fynbos species (Kock 2004), suggesting that an increased sampling effort may uncover the true diversity of these Cape legume symbionts.

We also discovered a partnership between *Azorhizobium* and the invasive *S. punicea* (tribe Sesbanieae). As far as we know, this is the first African *Azorhizobium*–legume symbiosis discovered outside West Africa. The South American species *S. punicea* is known as a selective host towards *Mesorhizobium* and was previously considered unable to establish an effective symbiosis with *Azorhizobium* (Vinuesa *et al.*, 2005b; Cummings *et al.*, 2009). Our result indicates a potential wider host range of *S. punicea* and supports previous studies showing evidence for *Azorhizobium* interactions with different *Sesbania* species (Dreyfus *et al.*, 1988; Moreira *et al.*, 2006; Blanco *et al.*, 2008).

Distinct symbiotic preferences within the legume tribes Podalyrieae, Psoraleeae, Indigofereae, Crotalarieae and Phaseoleae

Phylogenetic analyses revealed *Burkholderia* as the dominant symbionts within *Amphithalea*, *Podalyria* and *Virgilia* (tribe Podalyrieae) and an exclusive association of *Mesorhizobium* with *Otholobium*, *Psoralea* and *Argyrolobium* species (tribes Psoraleeae and Genisteae) (Table S1, Supporting Information). In addition to molecular sequence data, *in situ* immunogold labelling assays allowed screening for the presence or absence of *Burkholderia* in a comprehensive sampling of root nodules (Table S4, Supporting Information), which confirmed the previous observations of a distinct rhizobial preference of the tribe Podalyrieae for *Burkholderia* (Elliott *et al.*, 2007a; Beukes *et al.*, 2013) with the predominantly tropical genus *Calpurnia* being the only exception so far reported with regard to this (Sprent *et al.*, 2013). Despite the observed narrow host range of *Mesorhizobium* within *Psoralea*, Kanu and Dakora (2012) demonstrated a degree of promiscuity

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as they isolated *Mesorhizobium*, *Rhizobium* and *Burkholderia* from eight *Psoralea* species, although most of the strains failed to renodulate *Psoralea* or the promiscuous host legume siratro. This underlines the importance of authentication of strains as nodules can house many bacteria that are not capable of nodulating that host (Sprent 2009; Gyaneshwar *et al*., 2011).

In contrast to the symbiotic specificity in the genera of *Psoraleeae and Podalyrieae*, species of *Aspalathus* (tribe Crotalarieae) were associated with a diverse range of rhizobia (*Burkholderia*, *Mesorhizobium*, *Rhizobium*). Originally, root nodules of the South African legume *A. carnosa* (Crotalarieae) were reported to house *Burkholderia* (*B. tuberum* STM678), although it was not shown to renodulate its host, but instead was confirmed as a symbiont of *Cyclopia* spp. (Podalyrieae) (Elliott *et al*., 2007a). Since then *Burkholderia* has been isolated from other Crotalarieae, such as *Lebeckia* (De Meyer *et al*., 2013b, 2014; Howieson *et al*., 2013;). In this study, we report *Burkholderia* to be isolated from (and capable of nodulating) one *Crotalaria* and three *Rafnia* species (tribe Crotalarieae), and we confirm previous observations of *Burkholderia* symbionts from *A. carnosa* and *A. callosa*, which are species known to be associated with *B. tuberum* STM678 and DUS833, respectively (Vandamme *et al*., 2002; Elliott *et al*., 2007a). Interestingly, however, and in spite of the above observations, this study has actually demonstrated that the occurrence of *Burkholderia* as symbionts within Fynbos Crotalarieae is relatively low, particularly within the genus *Aspalathus* in which 15 out of 19 species were nodulated by *Mesorhizobium* and 2 by *Rhizobium*. Although speculative, the more relaxed mutualistic associations observed in *Aspalathus*, but also in *Indigofera* (see below), may provide them with an ecological advantage to colonize different habitats without being restricted to geographically limited and/or incompatible below-ground mutualists. *Indigofera* and *Aspalathus* are the most speciose in the CCR, the latter ranked as the second largest genus in the CCR (Manning and Goldblatt 2012).

Within the tribe Phaseoleae, *Burkholderia* was previously isolated from various hosts such as *Rh. ferulifolia*, a native Fynbos species adapted to the acidic Cape soils (Garau *et al*., 2009). In the same study, however, bradyrhizobia were associated with related *Rhynchosia* species collected outside the Cape, *Rh. totta* within northern South Africa (Transvaal) and *Rh. minima* in Australia (where it has become naturalised). Similarly, burkholderias were found within nodules of *D. lignosus*, a South African species from tribe Phaseoleae now invasive in New Zealand and Australia, whereas other nodules from different field sites were associated (albeit less frequently than with *Burkholderia*) with *Bradyrhizobium* and *Rhizobium* (Liu *et al*., 2014). In our study, although only one individual plant was sampled, *Bo. bituminosa* (Phaseoleae) was also shown to nodulate with *Burkholderia*, and hence our field (immunogold) data showing nodulation of three genera (*Bolusafra*, *Dipogon* and *Rhynchosia*), taken together with laboratory studies on effective nodulation of siratro by *B. tuberum* (Elliott *et al*., 2007a; Angus *et al*., 2013), clearly supports an affinity of some Phaseoleae species for these beta-rhizobia.

Similar to the Crotalariae and Phaseoleae, the tribe Indigofereae, represented by six *Indigofera* species, was associated with a variety of symbionts, with four distinct groups of beta- (*Burkholderia*) and alpha-rhizobia (*Bradyrhizobium*, *Ensifer* and *Mesorhizobium*) and is the most promiscuous legume group in the Fynbos biome known to date.

Ecological drivers for genetic variation

The host preference of Podalyrieae and Psoraleeae for beta- and alpha-rhizobia, respectively, and the pattern of genetic diversity of rhizobia among numerous legume genera demonstrates that host genotype is one of the main drivers regulating the observed genetic variation. However, some legume groups are nodulated by a broad range of rhizobia suggesting that environmental factors, rather than host genotype alone, affect the rhizobia–legume interaction. A relaxed specificity within *Aspalathus* species, for example, is possibly linked to physicochemical factors. Indeed, burkholderias were isolated from *Aspalathus* spp. growing in sand substrates at the Cape of Good Hope Nature Reserve, mesorhizobia were mainly isolated from alluvium/sandstone at Jonkershoek Nature Reserve and the Cederberg Wilderness Area, and *Rhizobium* was isolated from plants growing in limestone soils at De Hoop Nature Reserve, suggesting that geographic site and/or soil characteristics may also be valuable predictors of rhizobial distributions. Environmental conditions such as pH, site elevation and geology have been previously reported to affect diversity and biogeography of alpha- and beta-rhizobia (Bala, Murphy and Giller 2003; Bontemps *et al*., 2010; Liu *et al*., 2012; Mishra *et al*., 2012; Rahi *et al*., 2012; Bournaud *et al*., 2013; Gehlot *et al*., 2013). We detected a similar effect of soil pH and site elevation, which correlated positively with the genetic variation in *Mesorhizobium* and *Burkholderia*, respectively, suggesting that different environmental factors act differently on various rhizobial groups (alpha- versus beta-rhizobia).

Burkholderia has been frequently isolated from low pH environments, suggesting that the genus is widely adapted and tolerant to acidic soils (Garau *et al*., 2009). However, despite low pH being a general feature and predictor for the presence of *Burkholderia*, the intrageneric community composition and distribution is not correlated with pH (Stopnisek *et al*., 2014) as also observed in this study. In contrast to *Burkholderia*, genetic differentiation of mesorhizobia correlated strongly with soil pH and supports a previous study with related Mediterranean chickpea (*Cicer arietinum*, tribe Cicereae) mesorhizobia (Alexandre *et al*., 2009), underpinning the idea of soil acidity as a general key parameter for the geographical distribution of microorganisms (Fierer and Jackson 2006).

Site elevation correlated well with the genetic clusters within *Burkholderia*. A similar pattern of altitudinal replacement of *Burkholderia* species has been previously reported by Bontemps *et al*., (2010). These authors showed a strong geographical pattern with an association between altitude and *Burkholderia* diversity in the Cerrado/Caatinga biome, suggesting an indirect effect of temperature or humidity as important ecological drivers.

The genetic variation of Cape *Burkholderia* and *Mesorhizobium* isolates was largely unrelated to geographical distance suggesting high rates of rhizobial dispersal in the Fynbos biome. The enormous dispersal and colonization capabilities of microorganisms into new localities allow high rates of gene flow and thus prevents spatial differentiation (Martiny *et al*., 2006). *Burkholderia,* for example, has been recorded on four continents indicating their unlimited dispersal and adaptation capabilities. It is worth noting that long-distance dispersal events of *Burkholderia* as a result of comigration with their host seed allows them to colonize new locations and avoid inhospitable habitats (Chen *et al*., 2005b; Liu *et al*., 2012, 2014).

Concluding remarks

In summary, the broad phylogenetic sampling of legume genera and tribes in the CCR has revealed distinct symbiotic preferences and clearly indicates a high degree of host preference within

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the tribes Psoraleeae and Podalyrieae for *Mesorhizobium* and *Burkholderia*, respectively. Despite a predominance of *Burkholderia* within Fynbos soils, known as one of the major biodiversity hotspots, *Mesorhizobium* was remarkably abundant in *Otholobium*, *Psoralea* (tribe Psoraleeae) and *Argyrolobium* (tribe Genisteae), and some represented genotypes unrelated to the known reference strains. These putative novel Fynbos mesorhizobia are underestimated and are clearly well-adapted nodulators able to compete with *Burkholderia* within the acidic and nutrient-poor soils of the Fynbos. Furthermore, our study demonstrated that environmental factors such as soil pH and site elevation are positively linked with rhizobial differentiation and are important ecological drivers which interact with patterns of host specificities.

SUPPLEMENTARY DATA

Supplementary data is available at FEMSEC online.

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