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RESEARCH ARTICLE

Recombination and horizontal transfer of nodulation and ACC deaminase (*acdS*) genes within *Alpha*- and *Betaproteobacteria* nodulating legumes of the Cape Fynbos biome

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One sentence summary: Rhizobia of the South African Fynbos biome are subjected to gene shuffling via transmission of symbiosis genes and homologous recombination of chromosomal genes.

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ABSTRACT

The goal of this work is to study the evolution and the degree of horizontal gene transfer (HGT) within rhizobial genera of both *Alphaproteobacteria* (*Mesorhizobium*, *Rhizobium*) and *Betaproteobacteria* (*Burkholderia*), originating from South African Fynbos legumes. By using a phylogenetic approach and comparing multiple chromosomal and symbiosis genes, we revealed conclusive evidence of high degrees of horizontal transfer of nodulation genes among closely related species of both groups of rhizobia, but also among species with distant genetic backgrounds (*Rhizobium* and *Mesorhizobium*), underscoring the importance of lateral transfer of symbiosis traits as an important evolutionary force among rhizobia of the Cape Fynbos biome. The extensive exchange of symbiosis genes in the Fynbos is in contrast with a lack of significant events of HGT among *Burkholderia* symbionts from the South American Cerrado and Caatinga biome. Furthermore, homologous recombination among selected housekeeping genes had a substantial impact on sequence evolution within *Burkholderia* and *Mesorhizobium*. Finally, phylogenetic analyses of the non-symbiosis *acdS* gene in *Mesorhizobium*, a gene often located on symbiosis islands, revealed distinct relationships compared to the chromosomal and symbiosis genes, suggesting a different evolutionary history and independent events of gene transfer. The observed events of HGT and incongruence between different genes necessitate caution in interpreting topologies from individual data types.

Keywords: *acdS*, *Burkholderia*; Fynbos legumes; horizontal gene transfer; *Mesorhizobium*; nodulation genes

INTRODUCTION

The large-scale availability and analyses of sequence information from individual genes and complete genomes has demonstrated significant amounts of gene movements or horizontal gene transfer (HGT) in bacterial evolution (Ochman, Lawrence and Groisman 2000). The acquisition of new genes and metabolic capabilities between a broad spectrum of bacteria in a non-parent-to-offspring manner impacts bacterial diversification (Jain et al. 2003; Vetsigian and Goldenfeld 2005; Boto 2010) and ecological adaptation of the recipient cells (Preston, Haubold and Rainey 1998; Goldenfeld and Woese 2007) and has been attributed to several mechanisms such as insertion sequences, transposons, integrons, bacteriophages, genomic islands and plasmids.

In rhizobia, the capacity to establish an effective symbiosis with the host plant and to fix atmospheric nitrogen involves the expression of nodulation (*nod*) and nitrogen fixation (*nif*) genes. These genes are part of the 'accessory' gene pool and are usually located as mobile genetic elements on either transferable plasmids (*Rhizobium* spp. and *Ensifer* spp.) or scattered across the chromosome(s) as genomic islands (*Mesorhizobium* spp. and *Bradyrhizobium* spp.) (Finan 2002; Masson-Boivin et al. 2009). In *Mesorhizobium* species (i.e. *Mesorhizobium amorphae* and *M. huakuii*), symbiosis genes have also been detected on plasmids (Xu and Murooka 1995; Wang et al. 1999; Zhang et al. 2000). Symbiosis-related genes of rhizobia of the beta-subclass of *Proteobacteria*, which so far consist of the genera *Burkholderia* and *Cupriavidus*, are found on plasmids (Chen et al. 2003; Amadou et al. 2008; Gyaneshwar et al. 2011).

Extensive evidence for horizontal transmission of symbiosis genes has been revealed by conflicting or incongruent sequence data of plasmid- and chromosomal-located genes among a wide range of rhizobial lineages including both alpha-rhizobia (Van Berkum et al. 2003; Ormeño-Orrillo et al. 2006, 2013; Barcellos et al. 2007) and beta-rhizobia (Andam, Mondo and Parker 2007; Liu et al. 2012).

In the Fynbos biome, rhizobial studies have recorded *Burkholderia* (Betaproteobacteria) symbionts as common root nodulating species associated with the papilionoid legume flora (Kock 2004; Elliott et al. 2007b; Garau et al. 2009; Gyaneshwar et al. 2011; Beukes et al. 2013; Howieson et al. 2013; Sprent, Ardley and James 2013; Lemaire et al. 2015), but the characterization of the symbiosis genes as an important basis for the understanding of gene transfer during rhizobial evolution remains elusive. A recent study of Beukes et al. (2013) revealed conflicting relationships between chromosomal (16S rRNA and *recA*) and nodulation (*nodA*) genes among rhizobia mainly from legumes of the tribe Podalyriaceae, suggesting HGT as an importance force in the evolution of South African *Burkholderia*. Interestingly, *Burkholderia* species isolated from native legume species from the Brazilian Cerrado/Caatinga biomes, another major biodiversity hotspot for *Burkholderia* dominated by a distinct legume flora (South American Mimosoideae versus South African Papilionoideae), are genetically different in terms of nodulation genes and appear not be subjected to the same levels of HGT (Bontemps et al. 2010; Bournaud et al. 2013).

In contrast to *Burkholderia*, only a handful of studies have focussed on the diversity of South African Alphaproteobacteria, such as *Mesorhizobium* (Gerding et al. 2012; Hassen et al. 2012; Kanu and Dakora 2012), which are largely underestimated in terms of diversity (Lemaire et al. 2015), but coexist as a dominant group with *Burkholderia* in the acidic and oligotrophic

Fynbos soils. The study of Lemaire et al. (2015) also demonstrated that most isolated Cape mesorhizobia were distantly related to known reference species. Although HGT has been widely described in *Mesorhizobium* (Kaneko et al. 2000; Sullivan et al. 2002; Nandasena et al. 2006, 2007), the occurrence and degree of HGT within the evolution of these putatively new *Mesorhizobium* lineages remains to be tested in the Fynbos.

Similar to nodulation and nitrogen fixation genes, the ACC deaminase (*acdS*) gene is prevalent in rhizobia, playing an ecological role for plant growth and nodulation via the reduction of deleterious ACC levels (referred to as 1-aminocyclopropane-1-carboxylate and considered as an ethylene precursor) in plants (Ma, Guinel and Glick 2003; Ma, Charles and Glick 2004; Glick et al. 2007; Conforte et al. 2010; Nascimento et al. 2012a,c). The *acdS* gene is also located on transmittable genetic elements (symbiosis islands) and has been previously shown to evolve via HGT in diverse bacterial groups, including *Mesorhizobium* (Hontzeas et al. 2005; Blaha et al. 2006; Nascimento et al. 2012b). In Fynbos mesorhizobia, however, the occurrence of the *acdS* gene, the degree of HGT and its potential use for phylogenetic reconstruction has never been investigated.

Homologous recombination is another common driving force for prokaryotic evolution (Didelot and Maiden 2010), diffusing genetic material or creating new allele combination throughout bacterial populations (Fraser, Hanage and Spratt 2007). In rhizobia, various degrees of homologous recombination have been demonstrated within species of *Bradyrhizobium* (Vinuesa et al. 2005b, 2008; Tang et al. 2012), *Ensifer* (Silva, Kan and Martínez-Romero 2007), *Mesorhizobium* (Li et al. 2009) and *Rhizobium* (Tian et al. 2010; Van Cauwenberghe et al. 2014).

In an attempt to shed some new light on the evolution of Fynbos rhizobia, phylogenies from both housekeeping and symbiosis genes were reconstructed in order to assess incongruent signals as a result of levels of HGT (transmission of symbiosis genes and homologous recombination of chromosomal genes). By using this retrospective approach (sensu Sørensen et al. 2005), the degree of HGT will be investigated in both alpha-rhizobia (*Mesorhizobium*) and beta-rhizobia (*Burkholderia*). The objectives of the study were (i) to examine whether HGT of symbiotic plasmids has occurred among rhizobial lineages of *Mesorhizobium* and *Burkholderia* (incongruences between chromosomal and *nod* sequence data), (ii) to investigate recombination rates between homologues of rhizobial strains (incongruences between housekeeping genes), (iii) to evaluate the prevalence of *acdS* and test whether this accessory gene is prone to HGT among *Mesorhizobium* lineages.

MATERIALS AND METHODS

Bacterial strains

A selection of 22 *Burkholderia* and 24 *Mesorhizobium* isolates from phylogenetically diverse lineages was obtained from previous rhizobial screenings and new collections in the Fynbos region (Lemaire et al. 2015) (Table S1, Supporting Information). The *Mesorhizobium* isolates originate from diverse host legumes of the tribes Crotonaceae (*Aspalathus*), Genisteae (*Argyrobolium*), Indigoferaceae (*Indigofera*) and Psoraleaceae (*Psoralea*, *Otholobium*), whereas all *Burkholderia* accessions are from *Podalyria calypttrata* populations (tribe Podalyriaceae) collected from 14 sites. One *Rhizobium* isolate (accession Dlodlo 49) from the study of Lemaire et al. (2015) with a nodulation gene related to *Mesorhizobium* was also included in this study. The *Rhizobium* and all *Mesorhizobium* strains were successfully authenticated on either the original

host, siratro or *Otholobium hirtum*, except for the strain isolated from the host species *Aspalathus spicata* Muasya 5440 (Lemaire et al. 2015). For *Burkholderia*, the ability to nodulate was verified on the original host *P. calyptrata* (data not presented).

Amplification and phylogenetic analyses

PCR reactions were performed in a standard 25 μ l reaction mixture (Kapa Biosystems), according to the manufacturer's instructions. All PCR amplifications were generated with primers listed in Table S2 (Supporting Information), following the PCR conditions as described by the authors. Amplified products were purified using the Exo/Sap enzyme cleaning protocol (Werle et al. 1994) and sent to the MacroGen sequence facility (MacroGen, the Netherlands), using the same PCR primers for sequencing. All GenBank accession numbers are listed in Table S1 (Supporting Information).

Sequence reads were edited, assembled and aligned in Geneious Pro v.5.1.7 (<http://www.geneious.com>). Alignments were subjected to phylogenetic analyses, 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 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. In the combined BI analyses, the multiple-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).

Testing phylogenetic incongruence between chromosomal and nodulation genes

Parallel evolution between chromosomal and plasmid gene (vertical transmission of accessory genes) trees was evaluated with a topology or cophylogeny mapping method in Jane v.4 (Conow et al. 2010). The degree of congruence between the topologies was assessed by maximizing the number of cospeciation (vertical gene transfer) and minimizing the possible number of non-codiversification events (HGT) under the default setting of event costs. A permutation procedure tested the null hypothesis that two phylogenies are randomly related or that the observed number of cospeciation events of the initial search was not larger than expected by chance alone. The best scoring ML trees were imported as input trees for the reconciliation analyses, comparing the scores of the optimal and initial reconstruction with those of randomly obtained topologies. Randomization tests were ran with 1000 randomly permuted trees and a population size of 100. The cost distribution of random sample solutions and statistical significance was calculated in a cost histogram in Jane.

Analysis of recombination

Sequence alignments of the housekeeping genes *recA*, *atpD*, *gyrB* and *glnA* were subjected to ClonalFrame analyses (Didelot and Falush 2007) to assess the effect of recombination estimated by the r/m (the ratio of probability that a nucleotide will be altered through recombination and point mutations) (Guttman and Dykhuizen 1994) and the ρ/θ (the frequency of occurrence of recombination relative to point mutations) (Milkman and Bridges 1990) statistics. Five independent ClonalFrame runs were performed each consisting of 1000 000 MCMC iterations (25% burn-in), and trees were sampled every 100 iterations, resulting in a sample size of 7500 trees. Convergence of the MCMC was confirmed by the Gelman and Rubin test (Gelman and Rubin 1992).

RESULTS

Phylogenetic analyses of individual chromosomal and symbiosis genes

Four chromosomal (16S rRNA—1360 bp, *atpD*—650 bp, *recA*—620 bp, *gyrB* 650 bp) and four symbiosis (*nodA*—590 bp, *nodB*—250 bp, *nodC*—570 bp, *nifH*—310 bp) genes were sequenced and analysed for the *Burkholderia* isolates, including reference sequences of chromosomal and plasmid genes of available genomes of *Burkholderia graminis*, *B. phytofirmans*, *B. rhynchosiae*, *B. tuberum* and *B. xenovorans*. Phylogenetic reconstruction of the separate datasets produced similar groupings among genes of the core genome (Fig. S1A–D, Supporting Information), and among symbiosis-related genes (Fig. S1E–H, Supporting Information). Some discrepancies were detected, but most conflicts were not statistically supported, except for the placement of two taxa in the 16S rRNA (isolates 25I3R1 and 23I2R2) and one *nodC* lineage (29I6R2) relative to the housekeeping and nodulation gene trees, respectively. The *nifH* sequences within *Burkholderia* (Fig. S1H, Supporting Information) generated mostly an unresolved topology as a result of similar or identical (12 out of 21 isolates) amplicons (pairwise genetic similarity > 99%), comprising only 16 variable nucleotides in the dataset.

Similarly for *Mesorhizobium*, analyses of sequence data of five chromosomal (16S rRNA—1329 bp, *atpD*—516 bp, *recA*—458 bp, *gyrB*—637 bp, *glnA*—953 bp) and four symbiosis (*nodA*—621 bp, *nodB*—560 bp, *nodC*—592 bp, *nifH*—349 bp) genes generated consistent relationships (Fig. S2, Supporting Information) with only a few conflicts observed among different chromosomal loci (Fig. S2 A–E, Supporting Information), comprising the isolates of *Psoralea rigidula* 5343 (16S rRNA), *A. aurantiaca* 5397, *Ps. asarina* 15 (*recA*) and *Argyrolobium lunare* 5369 (*gyrB*). Incongruences among nodulation and nitrogen fixation gene trees (Fig. S2G–J, Supporting Information) were detected for the *Mesorhizobium* isolates of *Ps. asarina* 15 (*nodC*, *nifH*) and *O. hirtum* 5334 (*nodA*).

One *Rhizobium* isolate (accession 49) was included in the analyses, previously demonstrated to harbour *nodA* and *nifH* symbiosis genes from a *Mesorhizobium* strain (Lemaire et al. 2015). Sequencing of the *nodB* and *nodC* genes supports the identification of symbiosis genes related to *Mesorhizobium*, indicating horizontal transfer of symbiosis genes across different rhizobial genera (*Rhizobium/Mesorhizobium*).

Two strongly supported clades were recovered in the *nodA* and *nodC* *Mesorhizobium* gene trees, largely separating the isolates of *Otholobium* and *Psoralea* (tribe Psoraleeae) from nodule symbionts of the genera *Aspalathus* (tribe Crotalariaeae), *Argyrolobium* (tribe Genisteae) and *Indigofera* (tribe Indigoferaeae). Only

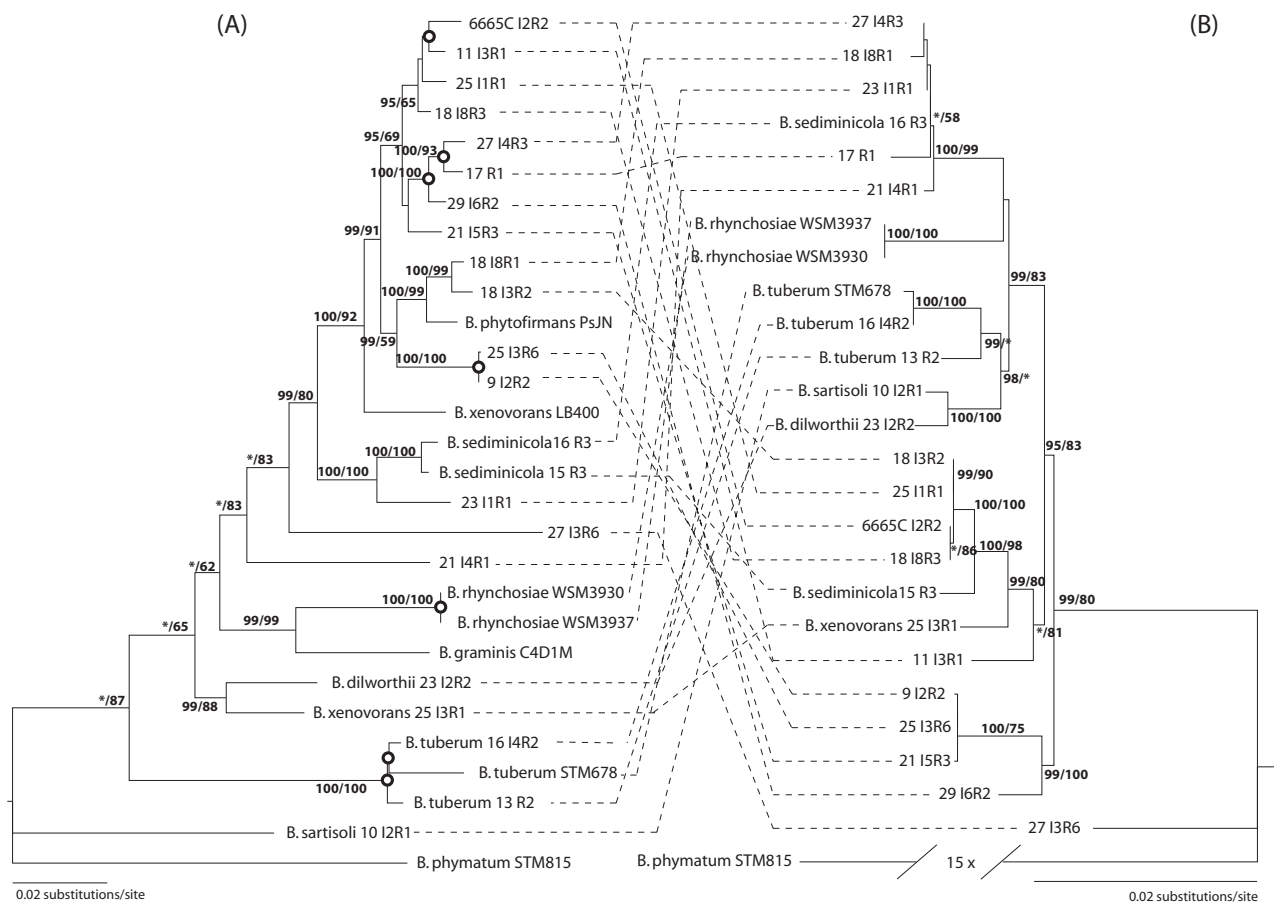


Figure 1. Phylogenetic incongruences between chromosomal and *nod* sequence data of *Burkholderia* isolates. Comparison of the best ML trees based on (A) chromosomal (16S rRNA, *recA*, *atpD*, *gyrB*) and (B) nodulation genes (*nodA*, *nodB*, *nodC*) (right tree). Support values for the BL and ML analyses are shown at the nodes. Dashed lines indicate the species association between the chromosomal and nodulation gene trees. Nodes highlighted by a circle represent events of cospeciation as revealed by the reconciliation analysis.

one *Aspalathus* symbiont (*Mesorhizobium* sp. 31) was found in the *Otholobium*-*Psoralea* clade. The grouping of nodulation genes according to the host was reflected by high-sequence divergence in the *nodA* (72.3% mean sequence similarity) and *nodC* (80.2% mean sequence similarity) datasets. Moreover, for *nodB*, none of the *Psoralea* or *Otholobium* isolates could be amplified (except for *Mesorhizobium* sp. 5462) (Table S1, Supporting Information), suggesting that the used primers are not suitable for rhizobia of the tribe *Psoraleae*, which have probably too diverged *nodB* genes.

In contrast to the symbiosis genes (Fig. S2G–J, Supporting Information), which are correlated with the host range, the housekeeping genes (Fig. S2 A–E, Supporting Information) of *Mesorhizobium* species differ from the host phylogeny, showing an intermingled pattern of isolates from *Argyrolobium*, *Aspalathus*, *Otholobium* and *Psoralea* legumes. This result indicates that legume species form symbiosis with *Mesorhizobium* lineages with diverse genetic backgrounds.

Concatenated sequence analyses

Analyses of the concatenated sequences were generally congruent with those of the individual gene trees. Because short gene fragments appear to lack sufficient phylogenetic information to provide well-resolved trees, combination of single genes with unequal evolutionary rates has been recommended to give a more robust evolutionary tree and to level out conflicting signals

of homoplastic character states (Gaunt *et al.* 2001; Gadagkar *et al.* 2005; Vinuesa *et al.* 2005a; Rivas *et al.* 2009; Laranjo, Young and Oliveira 2012). In this study, the concatenated sequences of single gene markers of chromosomal (16S rRNA, *recA*, *atpD*, *gyrB*) and nodulation (*nodA*, *nodB*, *nodC*) genes produced robust phylogenies, resolving relationships among most isolates with high support values for both genera of *Burkholderia* and *Mesorhizobium* (Figs 1 and 2). Because the 16S rRNA matrix of the *Mesorhizobium* isolates lacks phylogenetic information (pairwise genetic similarity > 99%), comprising only 5 variable nucleotides within 18 out of 24 sequences, we excluded 16S rRNA from the concatenated chromosomal gene analysis (Fig. 2).

A considerable number of *Mesorhizobium* (Fig. S3, Supporting Information) and *Burkholderia* (Fig. S4, Supporting Information) isolates were not related to 16S rRNA sequences of reference type strains, suggesting novel rhizobial species. For *Burkholderia* (Fig. S4, Supporting Information), only seven isolates were highly related (>99% 16S rRNA sequence similarity) to the common South African rhizobial species *B. tuberum* and *B. dilworthii* (Gyaneshwar *et al.* 2011; De Meyer *et al.* 2014), and the diazotrophic species *B. xenovorans* and *B. sediminicola*, which were previously isolated from Fynbos legumes (Beukes *et al.* 2013). One *Burkholderia* isolate was related to *B. sartisoli*, which has never been isolated from root nodules. Similarly for *Mesorhizobium* (Fig. S3, Supporting Information) we showed that only one strain was conspecific to *M. chacoense* (symbionts of

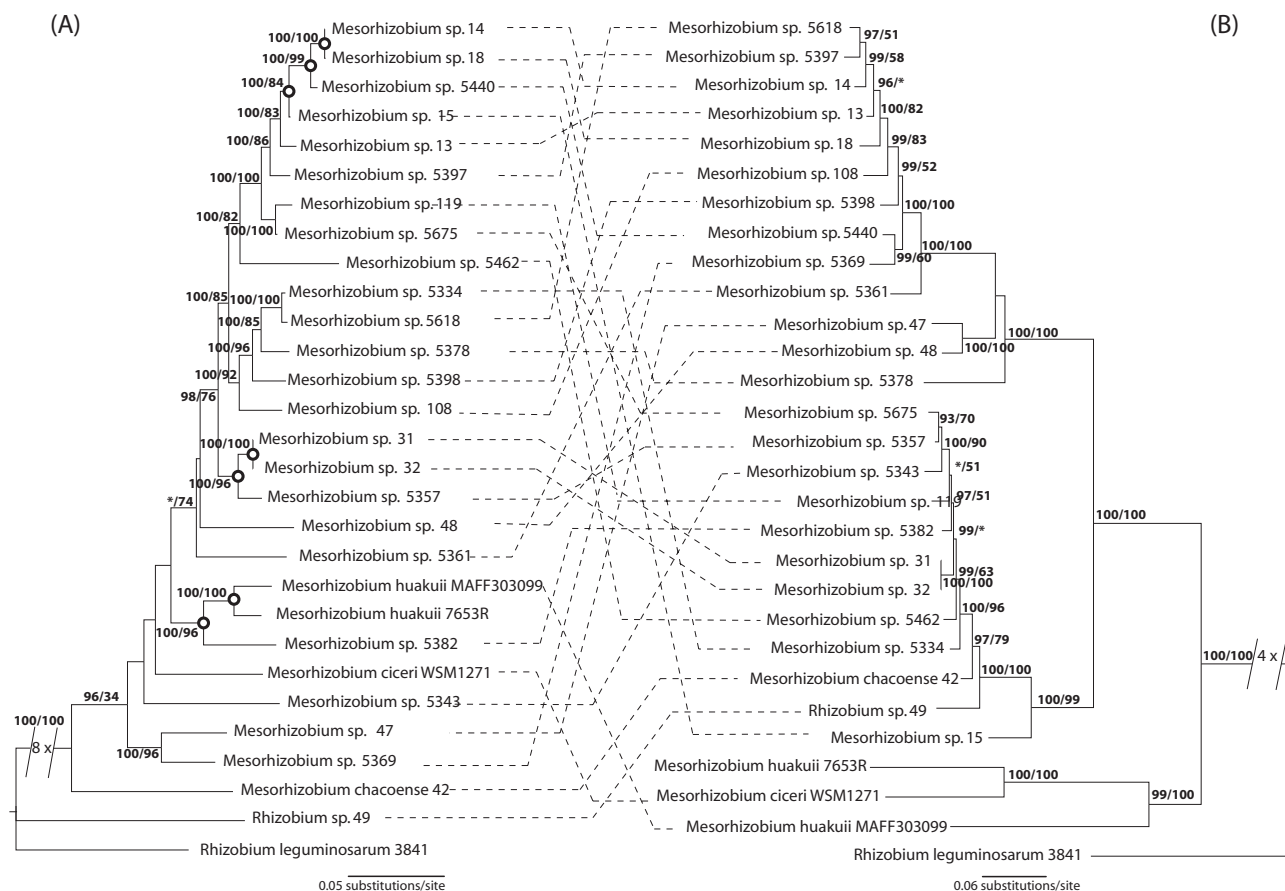


Figure 2. Phylogenetic incongruences between chromosomal and *nod* sequence data of *Mesorhizobium* isolates. Comparison of best ML trees based on (A) chromosomal (*recA*, *atpD*, *gyrB*, *glnA*) and (B) nodulation genes (*nodA*, *nodB*, *nodC*) (right tree). Support values for the BL and ML analyses are shown at the nodes. Dashed lines indicate the species association between the chromosomal and nodulation gene trees. Nodes highlighted by a circle represent events of cospeciation as revealed by the reconciliation analysis.

O. bracteolatum 42), placing the remaining isolates in distinct phylogenetic clades unrelated to any reference *Mesorhizobium* species. These mesorhizobia isolates are most likely new species as previously suggested by Lemaire et al. (2015).

Incongruence between chromosomal and symbiosis genes

Phylogenetic relationships of the chromosomal genes were largely inconsistent with the nodulation genes for both *Burkholderia* (Fig. 1) and *Mesorhizobium* (Fig. 2). Visual inspection of the combined trees of chromosomal versus nodulation genes revealed congruent relationships for only three *Burkholderia* clades (clade 1: 16I4R2/13R2/*B. tuberum*, clade 2: 25I3R6/9I2R2, clade 3: 25I1R1/18I8R3/6665CI2R2) and one *Mesorhizobium* grouping (*A. ericifolia* 31/*O. hirtum* 32). To estimate the degree of parallel evolution between chromosomal and nodulation genes, a reconciliation analysis was performed for the *Burkholderia* and *Mesorhizobium* datasets, mapping the nodulation gene tree on the chromosomal gene phylogeny (Fig. S5, Supporting Information). The codivergence approach estimates the maximum number of cospeciation events and visualizes all solutions by introducing a minimum number of non-cospeciation events (duplication, host-switch and sorting events) on the nodulation gene tree. For *Burkholderia*, 8336 equal cost solutions were recovered with cospeciation events ranging

between seven and nine, and a total cost of 36 for 18 events of host switches/duplications. A similar degree of non-parallel evolution was also observed for the *Mesorhizobium* analyses, revealing 6432 equal cost solutions with six cospeciation events and 22 lateral transfers/duplications (cost = 44) (Fig. S5, Supporting Information). Topological congruence (vertical inheritance/parallel evolution) was further statistically examined with randomization tests (Fig. S6, Supporting Information), providing evidence to reject the null hypothesis of random relationships for both gene phylogenies ($P < 0.01$). Despite large-scale symbiosis-gene transfers, the overall chromosomal and symbiosis topology shares a significant number of codivergence, indicating that events of parallel evolution occur more frequently than we would expect purely by chance.

Recombination in *Mesorhizobium* and *Burkholderia*

The frequency and relative impact of recombination on the evolution of housekeeping genes was assessed using the Clonal-Frame approach (Didelot and Falush 2007). Recombination frequencies were estimated for the *Mesorhizobium* and *Burkholderia* datasets, comprising similar levels of genetic divergence with the lowest sequence similarity of 90% for both rhizobial groups. For the *Mesorhizobium* dataset ($n = 28$ strains), the r/m and ρ/θ values were 11.81 (7.52, 16.01; 95% CI) and 2.62 (1.73, 2.91; 95% CI), respectively, implying recombination rather than mutations

as predominant contribution to the evolution among the tested regions of the *Mesorhizobium* strains. Similarly, substantial levels of recombination were observed among the *Burkholderia* strains ($n = 28$), with $r/m = 7.80$ (2.47, 16.42; 95% CI) and $\rho/\theta = 2.18$ (0.68, 3.76; 95% CI).

Phylogenetic analysis of the *acdS* gene

The presence of *acdS* in one *Rhizobium* (49), one *Burkholderia* (25I3R1) and all *Mesorhizobium* isolates was confirmed by sequence analyses. Phylogenetic analyses of the *acdS* isolates and closely related reference sequences of *Mesorhizobium*, *Rhizobium* and *Burkholderia* placed all *Mesorhizobium* isolates within a monophyletic group (100% Bayesian support value—BS, 100% bootstrap support value—BSS) as a sister group to *M. chacoense* (98% BS, 90 BSS) (Fig. 3 and Fig. S7, Supporting Information). The sequence divergence between the *acdS* *Mesorhizobium* isolates (mean pairwise sequence similarity >95%) generated well-resolved relationships with high support values for most nodes. The *acdS* gene phylogeny revealed significant incongruent groupings in comparison to both chromosomal and symbiosis-related genes, indicating a different evolutionary history prone to HGT. Concordant relationships between the *acdS* tree (Fig. S2F, Supporting Information) and the chromosomal gene trees (Figs S2A–E, Supporting Information) were only detected among a few sister group relationships (e.g. *Mesorhizobium* spp. 31/32, *Mesorhizobium* spp. 5382/5343, *Mesorhizobium* spp. 5361/5357 and *Mesorhizobium* spp. 5378/5334). One similar relationship (*Mesorhizobium* sp. 31R1–31R2) was observed between the *acdS* and nodulation gene trees (Figs 2 and 3).

The *acdS* sequence of the *Rhizobium* isolate was placed as a sister group to the *Mesorhizobium* spp., although this relationship is not supported (Fig. 3 and Fig. S7, Supporting Information). Sequence analysis of the *Rhizobium* isolates detected low levels of similarity among available reference strains, showing the highest similarity value with *Rhizobium gallicum* (83.6% sequence similarity).

Amplification of the *acdS* region in *Burkholderia* resulted in only one amplicon, suggesting that the primers originally designed for *Mesorhizobium* (*Alphaproteobacteria*) (Nascimento et al. 2012b) are not suitable for *Burkholderia* genus (*Betaproteobacteria*) due to primer mismatches. The *acdS* sequence of *Burkholderia* was placed as a sister group to the reference strain *B. ginsengisoli* NBRC100965 (100% BS, 100 BSS). This species was originally recovered from the rhizosphere of plants from a ginseng field (Kim et al. 2006).

DISCUSSION

HGT of symbiosis genes among rhizobia of the Fynbos biome

Extensive incongruence between phylogenies of nodulation and chromosomal genes of members of the genera *Burkholderia* and *Mesorhizobium* provides evidence for frequent exchange of nodulation genes among rhizobial lineages of the South African Cape Fynbos biome. Within the *Burkholderia* species of the Cape, HGT of symbiosis genes has been previously suggested to explain discordant relationships between the nodulation (*nodA*) gene in comparison to chromosomal genes (Beukes et al. 2013). The observed exchange of nodulation genes located on plasmids and symbiosis islands in *Burkholderia* and *Mesorhizobium*, respectively, indicates that HGT is not restricted to one rhizobial group (i.e. *Burkholderia*), but also occurs among rhizobia of the *Alphapro-*

teobacteria, suggesting HGT as a common feature in the Fynbos biome. This observation is in contrast with rare events of HGT of symbiosis genes among South American *Burkholderia*, which are associated with *Mimosa* spp. mostly endemic to the Cerrado and Caatinga biomes of Brazil (Bontemps et al. 2010; Mishra et al. 2012). Recently, alpha- and beta-rhizobia of Mexican *Mimosa* spp. were also characterized without an indication of gene exchange of nodulation genes (Bontemps et al. 2015).

The symbiotic nodulation genes, which are involved in host recognition by the synthesis of signalling molecules (Nod factors), are expected to evolve under constraints imposed by the interaction with the host plant (Perret, Staehelin and Broughton 2000; Spaink 2000). Hence, the evolutionary history of nodulation genes is usually related to the host plant (Haukka, Lindström and Young 1998; Laguerre et al. 2001; Suominen et al. 2001; Lu et al. 2009). In Fynbos mesorhizobia, two distinct symbiosis clades (*nodA-nodB-nodC-nifH*) were recovered, largely grouped by the host tribes Psoraleeae and Crotalariaeae/Genisteae (Fig. 2); all symbionts of the legumes of *Otholobium* and *Psoralea* (tribe Psoraleeae) were clustered within a monophyletic group, while all *Aspalathus* (tribe Crotalariaeae) and *Argyrolobium* (tribe Genisteae) symbionts were placed in a clade with distinct nodulation and nitrogen fixation genes, except for one *Aspalathus* symbiont (accession 31). While the *nod* gene phylogenies of mesorhizobia are strongly aligned with the host, at least at tribal (but not generic) level, it does not explain the complex evolutionary history of nodulation genes for *Burkholderia*, which were all originally isolated from the same host *P. calyptata*. In previous studies, the association between *Burkholderia* isolates and the host was not strong, showing one *Burkholderia* species nodulating diverse host species from different legume tribes and genera (Beukes et al. 2013). Host range studies confirmed the aspecificity of the *Burkholderia*-legume interaction, showing one rhizobial strain able to form effective nodules in various legume species (Gyaneshwar et al. 2011; Liu et al. 2012; Angus et al. 2013; Sprent et al. 2013). This observation may indicate that South African legumes do not have stringent requirements for a particular *Burkholderia* genotype and allow relaxed coevolution between the symbiotic partners.

While *Burkholderia* seems to have a broad host range with local papilionoid species, they appear incapable of nodulating South American mimosoid hosts (Gyaneshwar et al. 2011). Interestingly, mimosoid-nodulating *Burkholderia* from the South Americas exhibit a broader host range, which are able to form interactions with papilionoid species (Martínez-Romero 2009; Talbi et al. 2010; Gyaneshwar et al. 2011; Liu et al. 2014; Moulin et al. 2014). The naturally broader host range of these *Burkholderia* species (e.g. *B. phymatum*) and consequently the low pressure of the bacterial symbiont to adapt to a legume host by the exchange of symbiosis-specific genes (Segovia et al. 1991) might explain the relative lack of HGT observed in South American *Mimosa* symbionts (Bontemps et al. 2010; 2015; Mishra et al. 2012).

In the Fynbos biome, the lateral transfer of nodulation genes in *Burkholderia* might also be the result of other factors, such as the flexibility and adaptability of the genome to highly diverse ecological environments (Miché et al. 2002). Rhizobial populations seem to interact reciprocally by exchanging symbiotic elements, comprising genes related to nodulation, nitrogen fixation, auxin synthesis, hydrogenase components and ACC deaminase activity (de Oliveira Cunha et al. 2012; Zuleta et al. 2014), in order to respond to highly diverse and changeable environments, and extend their capacity to colonize new habitats, which allow the host plants to associate with the most adapted

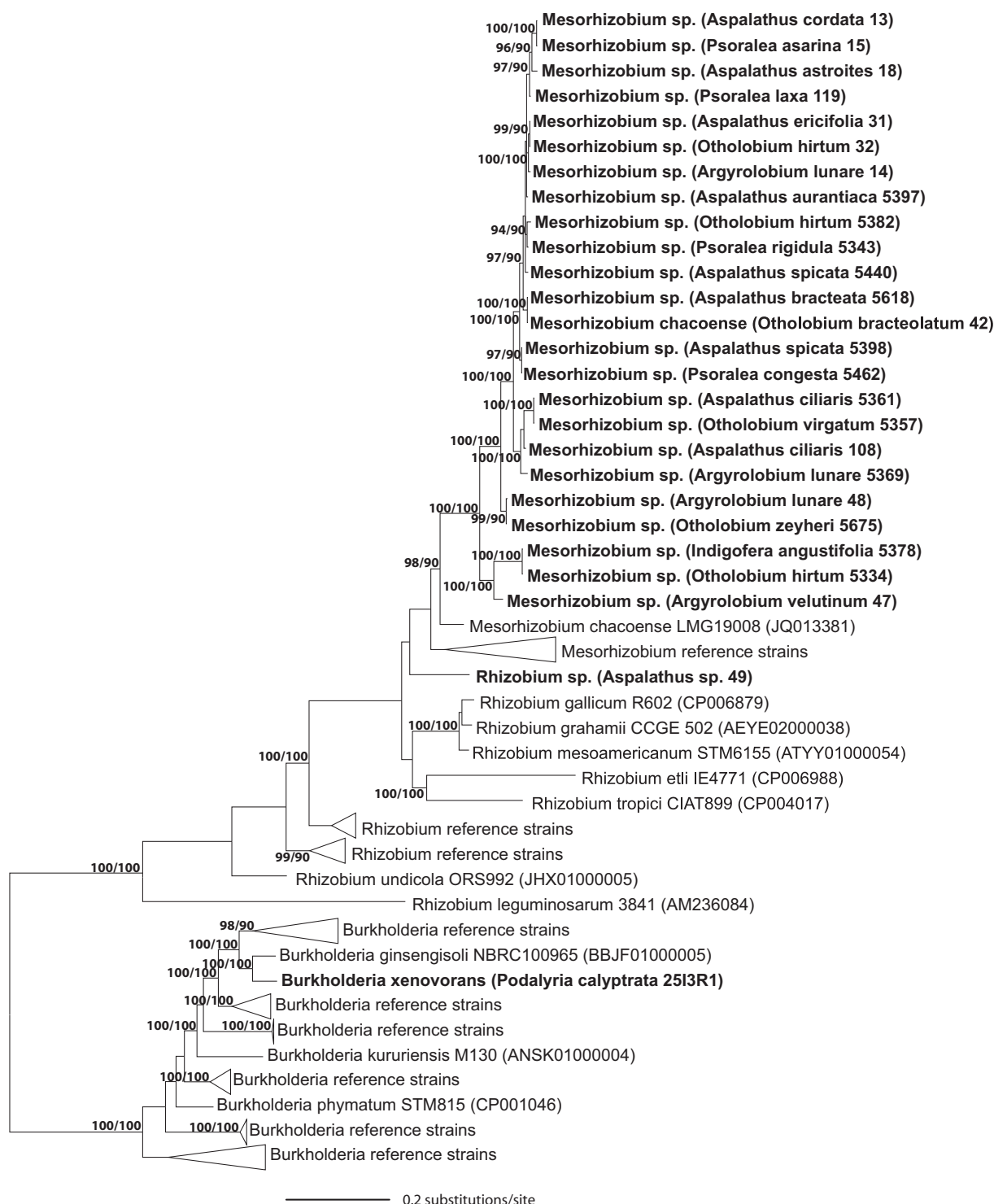


Figure 3. Phylogenetic relationships based on *acdS* sequences of *Mesorhizobium*, *Burkholderia* and *Rhizobium* isolates. Major lineages are schematically represented by triangles. Support values for the BL and ML analyses are shown at the nodes. A full phylogram is presented in Fig. S7 (Supporting Information).

rhizobia to the environment (Suominen et al. 2001; Moulin et al. 2004; Vinuesa et al. 2005b; Zhao et al. 2008).

Role of recombination in Fynbos rhizobia

The rates of recombination relative to those of mutation showed similar results of recombination for *Mesorhizobium* and

Burkholderia strains, indicating a high impact of homologous recombination or low mutation rates. The ratio of the probabilities that a given nucleotide is changed by recombination or mutation (r/m) is roughly eleven and seven for the *Mesorhizobium* and *Burkholderia* isolates, respectively. Although similar high values of recombination relative to mutation ($r/m = 2-10$, *sensu* Vos and Didelot 2009) have been recorded in many rhizobial groups (e.g.

Tian et al. 2010, 2012; Van Cauwenberghe et al. 2014), the observation of recombination among distinct *Mesorhizobium* (*M. ciceri*, *M. loti*, *M. huakuii*) and *Burkholderia* species (including at least five species) is remarkable, because the success rate of exchange of homologous genetic material decreases exponentially with the genetic distance of interacting species (Majewski 2001). Consequently, high rates of recombination occur more frequently between close relatives than among divergent organisms (Didelot and Maiden 2010; Popa et al. 2011). Nevertheless, events of recombination across bacterial divisions and domains have been reported (Garcia-Vallve, Romeu and Palau 2000; Rest and Mindell 2003).

Why these *Mesorhizobium* and *Burkholderia* are shuffling around alleles by homologous recombination is still an open question. Although *Mesorhizobium* and *Burkholderia* are phylogenetically distinct (alpha- and beta-subclass of *Proteobacteria*), similar recombination rates in both rhizobial groups could imply that events of recombination are more related with comparable ecologies rather than to genetic background (Wiedenbeck and Cohan 2011). Although speculative, the considerable level of recombination (and gene movement of symbiosis genes; see above) in *Burkholderia*, and linked to its renowned genomic plasticity (Miché et al. 2002; Vial et al. 2007), is not evolutionary constrained to beta-rhizobia, but is a common feature in the Fynbos, occurring among mesorhizobia adapted to the same ecological environment.

Evolution, occurrence and ecological significance of the ACC deaminase (*acdS*) gene

The location of the *acdS* gene varies in different species but is often located on transferable elements such as plasmids in *Rhizobium* and *Ensifer* (Ma et al. 2003; Young et al. 2006; Kuhn et al. 2008) and symbiosis islands in *Mesorhizobium* (Sullivan et al. 2002; Nascimento et al. 2012b). In *Burkholderia*, analyses of genome data identified *acdS* on the chromosome, except for *B. phymatum* STM815^T and *B. phenoliruptrix* BR3459a, which have two copies of the *acdS* gene, one on the chromosome and the other on the plasmid (Nascimento et al. 2014). Despite the variation of the position on transmittable elements, the *acdS* gene is expected to evolve mainly through HGT, at least for species having *acdS* on plasmids and symbiosis islands, as previously demonstrated in phylogenetic studies of both *Alpha*- and *Betaproteobacteria* (Hontzeas et al. 2005; Blaha et al. 2006; Glick et al. 2007; Nandasena et al. 2007; Nascimento et al. 2012b).

In *Mesorhizobium*, *acdS* has been reported in many species, which are shown to be prone to HGT, most likely through symbiotic island exchange (Nascimento et al. 2012b; Laranjo, Alexandre and Oliveira 2014). In the study of Nascimento et al. (2012b), the *acdS* tree revealed similar relationships in comparison to the symbiosis gene trees and correlates well with the host range, rather than the 16S rRNA phylogeny. In the current study, few congruent relationships were observed between the *acdS*, housekeeping and nodulation gene trees, indicating that ACC deaminase genes of these South African *Proteobacteria* are extensively subjected to HGT with genes on transmittable elements (i.e. plasmids and symbiotic islands) being prone to such different evolutionary histories. Future genome studies are needed to investigate the genome characteristics and the exact location of the *acdS* gene within the multipartite genome; a genome arrangement prevalent among plant-associated symbionts (Harrison et al. 2010; Landeta et al. 2011). It is also important to note that the genes located on accessory replicons or smaller chromosomes may evolve at a higher substitution rate

compared to genes present within the larger primary chromosomes (Cooper et al. 2010; MacLean et al. 2014) and hence may consequently affect the inference of phylogenetic relationships between different sets of genes (i.e. housekeeping, nodulation, *acdS*).

The ecological significance of the microbial ACC deaminase activity to stimulate plant growth (Glick et al. 2007) and the observed prevalence of the ACC deaminase gene throughout all *Mesorhizobium* spp. indicate that this enzyme is playing an important role in the nodulation process of these strains by increasing their ecological competitiveness and symbiotic performance (Ma et al. 2003, 2004; Uchiumi et al. 2004; Nascimento et al. 2012b,c; Brígido et al. 2013). The presence of *acdS* genes in all *Mesorhizobium* and one *Rhizobium* and *Burkholderia* strain, originating from different geographical locations and diverse legume groups of the tribes *Crotalariaeae*, *Genisteeae*, *Podalyriaceae* and *Psoraleeae*, indicates that ACC deaminase is a common and important plant-beneficial property among Fynbos rhizobia, particularly for lineages of the genus *Mesorhizobium*.

CONCLUSION

In this multilocus sequence analysis, we provided phylogenetic evidence for horizontal transfer of plasmid located genes within species of *Burkholderia* and *Mesorhizobium*, and extensive exchange of housekeeping genes through homologous recombination. No evidence of HGT between alpha- and beta-rhizobia was observed. The dynamic nature of gene transfer and acquisition observed in selected 'core' and 'accessory' genes among *Burkholderia* and *Mesorhizobium* in the Fynbos biome is most likely only the tip of the iceberg, and future genomic work is necessary to reveal the true extent of the migratory lifestyle of (accessory) genes among rhizobia of the Fynbos biome.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSEC online.

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