Biogeographical Patterns of Legume-Nodulating *Burkholderia* spp.: from African Fynbos to Continental Scales

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ABSTRACT

Rhizobia of the genus *Burkholderia* have large-scale distribution ranges and are usually associated with South African papilionoid and South American mimosoid legumes, yet little is known about their genetic structuring at either local or global geographic scales. To understand variation at different spatial scales, from individual legumes in the fynbos (South Africa) to a global context, we analyzed chromosomal (16S rRNA, *recA*) and symbiosis (*nifH, nodA, nodC*) gene sequences. We showed that the global diversity of nodulation genes is generally grouped according to the South African papilionoid or South American mimosoid subfamilies, whereas chromosomal sequence data were unrelated to biogeography. While nodulation genes are structured on a continental scale, a geographic or host-specific distribution pattern was not detected in the fynbos region. In host range experiments, symbiotic promiscuity of *Burkholderia tumefaciens* STM678T and *B. phytofirmans* STM815T was discovered in selected fynbos species. Finally, a greenhouse experiment was undertaken to assess the ability of mimosoid (*Mimosa pudica*) and papilionoid (*Dipogon lignosus, Indigofera filifolia, Macroptilium atropurpureum*, and *Podalyria calytrata*) species to nodulate in South African (fynbos) and Malawian (savanna) soils. While the *Burkholderia*-philous fynbos legumes (*D. lignosus, I. filifolia*, and *P. calytrata*) nodulated only in their native soils, the invasive neotropical species *M. pudica* did not develop nodules in the African soils. The fynbos soil, notably rich in *Burkholderia*, seems to retain nodulation genes compatible with the local papilionoid legume flora but is incapable of nodulating mimosoid legumes that have their center of diversity in South America.

IMPORTANCE

This study is the most comprehensive phylogenetic assessment of root-nodulating *Burkholderia* and investigated biogeographic and host-related patterns of the legume-rhizobial symbiosis in the South African fynbos biome, as well as at global scales, including native species from the South American Caatinga and Cerrado biomes. While a global investigation of the rhizobial diversity revealed distinct nodulation and nitrogen fixation genes among South African and South American legumes, regionally distributed species in the Cape region were unrelated to geographic and host factors.

Microorganisms have been observed to vary in distribution, diversity, and species composition across spatial scales (1), challenging the long-held perception of a microbial cosmopolitanism driven by their high dispersal capacities (2). Although microorganisms can disperse over great distances, dispersal limitations have revealed spatially isolated microbial populations over multiple spatial scales (1, 3–5). For rhizobia (both the Alphaproteobacteria and Betaproteobacteria classes), similar geographic distribution patterns have been detected in different bacterial groups and over various spatial scales, showing a geographic structure preserved in phylogenies of both chromosomal and nodulation genes (6–11).

Root-nodulating species of the genus *Burkholderia* (Betaproteobacteria) have been described in different regions of the world, including parts of the Americas, Africa, Asia, and Australasia. The highest level of diversity has been reported in the South American Cerrado/Caatinga and South African fynbos biomes (12), together with Asian and Australian/New Zealand representatives so far described exclusively in nonnative invasive species such as the weeds *Mimosa dipotricha, M. pigra, M. pudica* (13–18), and *Dipogon lignosus* (19, 20). *Burkholderia* species isolated from native legumes from neotropical and African regions, which are dominated by distinct legume florals (South American Mimosoideae versus South African Papilionoideae), differ genetically in their nodulation genes (12, 21), suggesting that the legume host is shaping symbiotic diversity and that the biogeography of rhizobia is linked to the distribution of compatible legume hosts (22 and references therein). Despite many local surveys of *Burkholderia* interactions with papilionoids and mimosoids across the globe, our knowledge of the global distribution pattern is still frag-
mented and a spatial survey of the genus *Burkholderia* has never been conducted in a global context and across biomes. In South Africa, *Burkholderia* symbionts are widespread and associated with diverse lineages of the tribes Crotalarieae (23–26), Hypocalypeteae (27, 28), Indigofereae (26), Phaseoleae (26, 29, 30), and Podalyrieae (26, 27, 31, 32), indicating that the South African soils are an important reservoir for nodulating *Burkholderia*, and thus this needs to be explored further for new candidate species. With the exception of *Burkholderia phymatum* strains nodulating the nonnative crop species *Phaseolus vulgaris* (common bean) in Moroccan soils (33), the legume-*Burkholderia* symbiosis in Africa has only been reported in a range of sites within the fynbos region, supporting the idea of the Cape region as an exclusive biodiversity hot spot for the symbiosis (12).

The general aims of the present study were to provide novel insights into the biogeography of *Burkholderia* and to elucidate the extent to which it exhibits a geographic pattern in relation to the distribution of its hosts. Because lineages vary in distribution and diversity over various spatial scales and spatial factors play a significant role in shaping microbial communities, it is clear that geographic patterning should be analyzed across multiple spatial scales (from local to broad geographic regions). We took advantage of the large record of root-nodulating *Burkholderia* established since the first reports of its nodulation ability (12 and references therein) supplemented with new sequence data of fynbos *Burkholderia*. Available sequence data for chromosomal 16S rRNA and symbiosis-related *nodA*, *nodC*, and *nifH* genes were analyzed in a worldwide perspective to assess geographic patterns at a continental scale, as well as the host-specific interactions with the legume subfamilies Mimosoideae and Papilionoideae.

The diversity, geographic distribution, and host associations were further investigated at a regional scale in the South African (Cape) fynbos biome. The *Burkholderia* symbionts from five Cape legume tribes and 11 genera of the subfamily Papilionoideae were investigated by phylogenetic analyses of two chromosomal genes (16S rRNA and *recA*) and one nodulation gene (*nodA*) in relation to their geography and host phylogeny. We hypothesize that the *Burkholderia* symbionts of native and invasive legume species reported in Africa, America, Asia, and Australasia exhibit a geographic distribution pattern with continents having their own subsets of symbionts. We also expect a geographic effect on the genetic variation of rhizobia at a regional scale within the fynbos. Our specific objectives were (i) to determine and compare the *Burkholderia* types for housekeeping and symbiosis loci recorded in mimosoids and papilionoids reported on four different continents, (ii) to investigate the distribution pattern of *Burkholderia* and its host associations within the fynbos biome by using field-collected nodules of indigenous papilionoids, (iii) to investigate the ability of South African papilionoid legume species (Indigofera filifolia, *D. lignosus*, *Podalyria calyptrata*, *Psoralea pinnata*) and the South American species *M. pudica* (subfamily Mimosoideae) to form nodules in South African (fynbos) and Malawian (savanna) soil, and (iv) to test and evaluate the host range of the *Burkholderia tuberum* STM678^T^ and *B. phymatum* STM815^T^ type strains on selected fynbos species that are known to exhibit different host affinities, as dictated by their genetically distinct nodulation genes. We expect that the tested papilionoid legumes from the fynbos are nodulated exclusively by the common and native symbiont *B. tuberum* STM678^T^.

**MATERIALS AND METHODS**

*Burkholderia* data sets and OTU-based analyses. Analyses of operational taxonomic units (OTUs) were used to cluster the 16S rRNA sequence data (see Table S1 in the supplemental material). A large 16S rRNA data set was constructed that comprised 1,121 sequences and 75 validly named *Burkholderia* species with multiple accession numbers per species. Sequences were aligned with available bacterial reference sequences via the Ribosomal Database Project (RDP pyrosequencing pipeline; http://pyro.cme .msu.edu). An uncorrected pairwise distance matrix was calculated, and the numbers of OTUs and rarefaction curves at various cutoff values (0.030 to 0.010) were calculated in mothur v.1.31.2 (34).

Four other *Burkholderia* data sets were obtained from the available 16S rRNA (365 sequences), *nifH* (246 sequences), *nodA* (152 sequences), and *nodC* (199 sequences) data, and all rhizobia were assigned to four geographic regions (Africa, America, Asia, and Australasia) and two legume subfamilies (Papilionoideae and Mimosoideae). The alignments were created with MUSCLE (35) by using Geneious v.5.1.7 (Biomatters Ltd., Auckland, New Zealand). The diversity of 16S rRNA sequences was clustered into OTUs by using the previous estimated cutoff value to delineate taxonomic identities at the species level. For the *nifH*, *nodA*, and *nodC* data sets, we used a similar conservative cutoff value in order to classify genetic groups of the more variable symbiosis genes. Types unique to and shared among different continents and subfamilies were identified in mothur.

Alignments for the NeighborNet analyses were compiled on the basis of the previous 16S rRNA, *nifH*, *nodA*, and *nodC* rhizobial data sets. One sequence representative per sequence cluster was manually selected from the original alignments and imported into SplitsTree v.4.12.8 (36) to display the phylogenetic relatedness among the clusters as a NeighborNet network (37) by using the most complex model of nucleotide substitution available (the general time reversible [GTR] model). Bootstrap confidence values were generated by using 1,000 permutations.

Nodule sampling, DNA extraction, amplification, cloning, and sequencing to identify fynbos rhizobia. In this study, we investigated 20 root nodulated fynbos species representing various localities (Fig. 1) and diverse host legumes (five legume tribes and 11 genera). Voucher information and GenBank accession numbers are listed in Table S2 in the supplemental material, and the geographic localities are shown in Fig. 1. Nodules were collected in the field from a broad geographic range at different localities covering diverse soil types ranging from limestone substrate (De Hoop Nature Reserve, Still Bay), granite substrate (Paarl Mountain Nature Reserve), and sandstone mountain slopes (Bainskloof Nature Reserve) to coastal deep sand (Cape Point Nature Reserve). Three to five nodules were removed from each host plant for isolation of rhizobia.

Rhizobia were identified by both standard culturing techniques (38) and direct genomic DNA extraction from nodules. The latter method enabled the unequivocal assessment of intranodular endophyte diversity, including unculturative endophytes that can be masked by culturing-based techniques because of the selective effects of growth media and incomplete sampling of colony morphotypes. For the standard culturing technique, rhizobia were isolated on yeast extract mannitol agar (YEMA) from a single bacterial colony type in accordance with standard procedures (38). Pure rhizobial cultures from single colonies were stored at −80°C in YEM broth containing 20% glycerol. Total DNA of the rhizobial cultures was obtained by the following thermal cell lysis procedure. A loopful of bacterial culture was suspended in 20 μl of lysis buffer (10% SDS, 1 M NaOH) and then incubated for 15 min at 95°C. The lysate was centrifuged at 10,000 × g for 45 s, and 180 μl of sterile water was added. The DNA extract was centrifuged for another 5 min at 10,000 × g at 4°C and preserved at −20°C. For direct DNA extraction from root nodules, genomic DNA of surface-sterilized nodules was obtained with the E.Z.N.A. HP Plant DNA minikit (Omega Bio-Tek) in accordance with the manufacturer’s instruction.

PCR amplification of 16S rRNA was done with universal bacterial
primers 27f and 1492r as previously described (39). Amplicons of nearly complete 16S rRNA were sequenced and subjected to BLAST analyses on GenBank as a first identification tool. Amplification of the recA housekeeping gene and the nodA nodulation gene was carried out with primers recA-63F, recA-504R, nodA-1F, and nodA-2R and the PCR parameters described by Gaunt et al. (40) and Haukka et al. (41). Amplification of the nodC nodulation gene was carried out for selected fynbos isolates with primers nodC-540 and nodC-1160. The sequences of all of the primers used are listed in Table S3 in the supplemental material.

Amplified 16S rRNA products from total genomic DNA extractions of the nodules were cloned into a pGEM-T vector (Promega) in accordance with the manufacturer’s instructions and transformed into *Escherichia coli* JM109 by heat shock (42). Purified plasmids and all PCR products were sent to Macrogen for sequencing (Macrogen Inc., Seoul, South Korea). The sequencing primers used for the 16S rRNA, recA, and nodA genes were the same as for the initial PCR.

**Authentication of cultured rhizobia from field nodules.** Nodulation capabilities of isolates from field nodules were tested on siratro (*Macrop-tium atropurpureum*) (38). Table S2 in the supplemental material lists the authenticated isolates in this study together with previously tested strains (26). Rhizobial isolates from nodules of legume species (*D. lignosus*, *Indigofera filifolia*, *P. caLPYRata*, and *P. pinnata*) grown in a greenhouse were authenticated on their respective hosts. Nodulation (three replicates) was assessed, relying on the GTR-gamma model of evolution. Support values were estimated by a multiparametric bootstrap resampling with 1,000 pseudoreplicates.

**Geographic distances among fynbos representatives.** The genetic variation of rhizobia across spatial scales in the fynbos was calculated on both chromosomal (*recA*) and nodulation (*nodA*) data. Genetic distance matrices for both sets of genes was constructed by including our fynbos isolates and supplemented with previously described rhizobial strains (26). The *recA* and *nodA* data sets comprised 134 and 128 sequences, respectively, covering genera of the tribes Podalyrieae (*Aspalathus*, *Crotalaria*), Crotalarieae (*Crotalaria*, *Rafinia*), Hypocalyeae (*Hypocalypsis*), Phaseoleae (*Bolus*), and Indigoferae (*Indigofera*). Genetic variation of all pairs of isolates was linked with a geographic distance matrix calculated from their geographic coordinates with the Geographic Distance Matrix Generator, v.1.2.3 (Peter Ersts, Center for Biodiversity and Conservation, American Museum of Natural History). Values of genetic variations were grouped within geo-
graphic distance classes (0 to 200, 201 to 400, 401 to 600, and 601 to 800 km) and plotted as box plots in R v.2.15.3 (46). The correlation between genetic similarities and geographic distances was investigated with a Mantel test in GenALEX 6.501 (47), and its significance was tested on 9,999 permutations.

**Trapping experiment.** The legume species *D. linowus* (L.) Verd. (Phaseoleae), *Indigofera filifolia* Thunb. (trigue Indigofore), *Macrop- tulum atropurreatum* (DC.) Urb. (siratro; phaseoleae), *M. pudica* L. (trigue Mimoseae), *P. caileytrata* C.A. Sm. (Podalyriae), and *P. pinnata* L. (Psoraleae) were grown in soil samples from Malawi (Chinyongwa, Blantyre, S15.819431, E35.041753) and South Africa (Table Mountain National Park, S33.952532, E18.456871). Both sites are part of natural ecosystems with no history of cultivation or rhizobial inoculation. At each locality, soils were sampled from the top 0 to 20 cm of at least three field sites and bulked to generate a composite sample for rhizobial isolation. The pH of 4-g samples of sieved (1-mm mesh) soil mixed in 40 ml of 1 M KCl was determined.

The Cape legume species *I. filifolia* and *P. caileytrata* are endemic to the Western Cape Province of South Africa. *P. pinnata*, also endemic to the fynbos, became naturalized and invasive in South Australia and New Zea- land (48). *D. linowus* and *M. pudica*, which are native to South Africa and South America, respectively, are also considered invasive (19, 49). All of the legume species in the trapping experiment, except for *P. pinnata*, which is strictly associated with *Mesorhizobium* (class Alphaproteobacteria) (26), have been shown to form associations with *Burkholderia*. (19, 26, 50). Siratro is a widely used species known to be very promiscuous with regard to symbionts (51) and was proven previously to be effectively nodulated by *B. tuberum* (31, 52).

Nodulation was assessed by growing germinated seedlings (three replicates) in 20-cm-diameter plastic pots filled with acid-washed sterile sand and a layer of 200 g of soil (the layer of soil was omitted from negative-control pots). Seeds were surface sterilized in 4% (wt/vol) sodium hypochlorite for 10 min, rinsed in six changes of sterile water, soaked in boiled water, and pregerminated at room temperature on 1.5% (wt/vol) agar plates until root emergence. Pots were covered with a layer of nylon PA6 beads (Lomold group HQ, South Africa) and provided with a sterile wa- tering tube to prevent cross-contamination. All plants were watered with sterile deionized water every 2 days. Nodules were harvested from seed- lings after 2 months, and rhizobia were isolated on YEMA as previously described.

**Host range study.** Seeds of legume species from the tribes Crotalar- ieae, Hypocarpaeae, Indigofore, and Podalyriae were used for this study. Seeds were surface sterilized with concentrated sulfuric acid for 10 min, followed by 4% sodium hypochlorite for 10 min. Seedlings were grown in glass tubes with a sterile mixture of vermiculite and perlite as a rooting medium and fed with Jensen’s N-free plant nutrient medium under acetic conditions (53). After 1 week of plant cultivation, seedlings were inoculated with wild-type strains *B. tuberum* STM678(2) and *B. phynatum* STM815(5) (54). Plants were harvested after 6 weeks and inspected for nodule formation, and the potential ability to perform symbiotic nitrogen fixation was assessed by the presence of leghemoglobin. In addition, nodules were fixed and embedded for light microscopy to assess their 3D structure and determine the potential ability to perform symbiotic nitrogen fixation. 

**Accession number(s).** The 16S rRNA gene sequences determined in this study were deposited in the GenBank database under accession numbers KF791602 to KF791673 and KF824727 to KF824733. The recA sequences were deposited under accession numbers KF791796 to KF791864, KF824747 to KF824733, KP013139 to KP013158, and KT700208 to KT700213. The nodA sequences were deposited under accession numbers KF791743 to KF791795, KF824740 to KF824746, KP013159 to KP013178, and KT700202 to KT700207. All OTUs were deposited under accession numbers KP013126 to KP013137.

**RESULTS.**

**16S rRNA gene sequence cutoff levels used for (putative new) species delineation.** A large 16S rRNA data set comprising 75 validly named *Burkholderia* species was constructed to evaluate the genetic diversity of *Burkholderia* at five different sequence similarity threshold values ranging from 97 to 99% (see Fig. S1 in the supplemental material). A sequence similarity level to delineate the true number of sequences at the species level was obtained between cutoff values of 98.5 and 99%, resulting in 59 and 96 OTUs. Although there is some controversy about the concept of a species in prokaryotes (56–59), the results of the empirical clustering analysis, using 16S rDNA data, support 98.5% as a conservative threshold value for species level definitions within *Burk- holderia*, and this corresponds to the general threshold value of 98.65% estimated to delineate global prokaryotic diversity (60). A 98.5% threshold value was used for further diversity calculations of 16S rRNA data sets.

**Phylogenetic clustering of *Burkholderia* richness according to geography and legume subfamily.** The diversity of root-nodul- ulating *Burkholderia* was classified according to geography and the hosts for different DNA regions (16S rRNA, nifH, nodA, and nodC). Table 1 shows the 16S rRNA OTUs and clusters of symbiosis genes calculated at a cutoff value of 98.5% that were identified on different continents and the host associations occurring across continents and legume subfamilies. From a total of 23 16S rRNA OTUs, eight groups occurred on more than one continent, including one OTU (number 5) that is globally distributed across all four of the continents assessed and three OTUs (numbers 1, 5, and 12) that are associated with both legume subfamilies (Table 1). *B. tuberum* (OTU number 1) was a highly recorded species (107 16S rRNA gene sequences) associated with eight South African genera (*Amphithalea*, *Aspalathus*, *Cyclopia*, *Hypocapsus*, *Lebeckia*, *Po- dalyria*, *Rhyynchosia*, and *Virgilia*) and with field nodules of the South American genus *Mimosa* (Table 1). Six OTUs (numbers 4 to 8 and 15) comprised symbionts of invasive *Mimosa* species re- corded in South America, as well as their invasive regions in Asia and Australia (see Table S1 in the supplemental material).

In contrast to 16S rRNA, fewer nodulation and nitrogen fixation types were shared among continents, including only four *nifH* (numbers 2, 4, 5, and 9), five *nodA* (numbers 3, 5, 6, 10, and 16), and three *nodC* (numbers 4, 9, and 10) types. One group of *nodC* sequences (type number 4) and one group of *nifH* sequences (type number 4) were globally distributed on all the four conti- nents. A total of five sequence clusters of *nifH* (numbers 1 and 4), *nodA* (numbers 3 and 14), and *nodC* (number 4) were shared by both subfamilies. All *nodA* and *nodC* sequence clusters associated with both legume subfamilies originate from mimosoids and from the papilionoid hosts *Macrophtium* and *Phaseolus*.

**Phylogenetic NeighborNet analyses for chromosomal (16S rRNA), nitrogen fixation (*nifH*), and nodulation (*nodA* and *nodC*) genes revealed the genetic divergence and clustering among sequence types and their affinities for a geographic locality and a legume subfamily (Fig. 2).** The genetic distances, proportional to evolutionary divergences, were more pronounced for the symbiosis genes (*nifH, nodA, and nodC*) than for the conservative 16S rRNA gene. For the 16S rRNA gene, phylogenetic relationships...
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**16S rRNA OTU**

### nifH sequence cluster

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### nodA sequence cluster

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### nodC sequence cluster

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**a** SAM, South America; AFR, Africa; AUS, Australasia; ASI, Asia.

**b** MIM, Mimosoideae; PAP, Papilionoideae.

**c** The host genera and reference strains of *Burkholderia* are listed per group (98.5% sequence similarity threshold).

**d** —, not present.

**e** *Burkholderia phymatum* STM815T was allegedly isolated from the papilionoid *Machaerium lunatum* in French Guiana but has never been proven to nodulate its original host (*M. brasilense* [17]).
among OTUs were not structured by geography or host (Fig. 2A). Large genetic clusters contained OTUs from different continents and subfamilies, confirming the previous observation of shared 16S rRNA types across localities and hosts (Table 1). In contrast to 16S rRNA, NeighborNet analyses of nitrogen fixation (nifH) (Fig. 2B) and nodulation (nodA and nodC) genes (Fig. 2C and D) identified a strong pattern according to geography and host. Genetic clusters were identified, separating the African papilionoids from the South American and Asian mimosoid representatives.

**Burkholderia diversity, specificity, and geographic distribution in legumes of the fynbos biome.** In order to investigate the biodiversity and geographic distribution of *Burkholderia* at a smaller spatial scale, rhizobia of diverse indigenous fynbos species were sampled and analyzed by using a combination of culture-based and non-culture-based identification techniques. Initially, a standard culture method was used to select legume lineages covering most of the legume groups (*Amphithalea, Aspalathus, Bolusafra, Crotalaria, Dipogon, Hypocalyptus, Indigofera, Podas...*)

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**FIG 2** NeighborNet networks of 16S rRNA gene (A), nifH (B), nodA (C), and nodC (D) sequence types. Sequence types exclusively recorded on one continent are shown by colored circles (Africa, green circles; South America, red circles; Asia, blue circles; Australasia, yellow circles). Numbers of sequence clusters sharing isolates from different continents and/or legume subfamilies are shown in gray squares as listed in Table 1. Bootstrap support values below and above 50% are shown with gray and black branches, respectively. The scale bar represents the number of substitutions per site.
lyria, Rafnia, Rhynchosia, and Virgilia; see Table S2 in the supplemental material), and all rhizobia were identified as *Burkholderia*, showing only a single colony morphotype in each root nodule. All cultured strains were authenticated by using siratro (see Table S2 in the supplemental material), showing effective nodules and enhanced plant growth compared with that of nodule-free controls. Only the strain from *Rafnia acuminata* (Dlodlo 22) failed to form effective nodules on siratro and so was not considered a rhizobial symbiont.

In addition, a culture-independent analysis was performed by direct PCR to assess nodule rhizobial diversity and to confirm single-strain occupation within a nodule. PCR amplifications of the total genomic DNA extracted from intranodular tissue produced high-quality and single-copy sequences for all of the genetic markers investigated, suggesting one dominant *Burkholderia* strain as nodule resident. Amplified 16S rRNA gene products were cloned for available nodules in selected species within genera of two legume tribes (*Podalyria* [A. M. Muasya, 6490 and 6463] and *Indigofera* [A. M. Muasya and C. Stirton, 6502B and 6502C]) to test the one-symbiont–one-nodule specificity. For all of the samples investigated, similar 16S rRNA clones (20 per sample) were obtained showing a single bacterial endosymbiont in each nodule.

16S rRNA, recA, and *nodA* sequence data from rhizobia of 26 *Podalyrieae* (15 individuals, 3 genera), 11 *Indigoferae* (8 individuals, 1 genus), 4 *Hypocynateae* (2 individuals, 1 genus), 15 *Crotalarieae* (6 individuals, 3 genera), and 16 *Phaseoleae* (9 individuals, 3 genera) family members were subjected to ML and Bayesian phylogenetic analyses (Fig. 3 and 4), clustering the isolates within diverse reference strains comprising root-nodulating (*B. dilworthii* WSM3556T, *B. dipogonis* LMG19430T, *B. kirschenbothemsis* Kbb15T, *B. rhynchosiae* WSM3937T, *B. sperata* WSM5005T, *B. tuberum* STM6785T) and plant-beneficial (*B. phytofirmans* PsNNT, *B. xenovorans* LB400T) lineages. Our isolates from various host legumes (e.g., *Amphithalea*, *Aspalathus*, *Indigofera*, *Rafnia*, *Rhynchosia*, *Podalyria*) were closely related to nodulated representatives (*B. kirschenbothemsis*, *B. rhynchosiae*, *B. tuberum*) of the current fynbos nodule, but the majority of the isolates appeared to be related to bacteria without generally nodulating traits (*B. phytofirmans*, *B. xenovorans*) or were grouped apart into clusters without known reference species (Fig. 3).

Analyses of rhizobial lineages in relation to their geographic provenance showed many widely distributed 16S rRNA OTU types, suggesting genetic similarity of *Burkholderia* in fynbos soils. To evaluate the diversity of fynbos rhizobia in relation to geography at a regional scale, we investigated spatial structuring by the common approach of isolation by distance (61), assuming that phy at a regional scale, we investigated spatial structuring by the one-symbiont–one-nodule approach of isolation by distance (61), assuming that phy at a regional scale, we investigated spatial structuring by the effective fynbos lineage and nodulation data (Fig. 3 and 4). Sequence analyses showed that a given *Burkholderia* lineage was associated with different legume lineages and, in turn, these host plants accommodated genetically diverse symbionts.

**Nodulation of Cape legumes in African soils and identity of rhizobial groups.** Nodulation of the legumes *I. filifolia*, *P. calytrata*, and *P. pinnata*, which are restricted in distribution to the Cape fynbos biome, and the widely distributed species *D. lignosus* and *M. pudica* was assessed in South African (fynbos region) and Malawian (savanna grassland) soils. The pH of the soil from the fynbos (pH 4.6 ± 0.2) was substantially lower than that at the savanna site (pH 7.1 ± 0.3). Distinct symbiotic associations were found among the legumes, with a strong influence of the soil source on the rhizobia sampled (Fig. 6 and 7). *P. calytrata* (*Podalyrieae*, *I. filifolia* (*Indigoferae*), and *D. lignosus* (*Phaseoleae*)) were nodulated exclusively by *Burkholderia* in fynbos soil, with the exception of one *Bradyrhizobium* isolate associated with *D. lignosus* that was from fynbos soil. None of these legume species nodulated in Malawian soil, except *P. pinnata* (*Psoraleae*) and siratro (*Phaseoleae*), which were able to form nodules in both soils (see Table S4 in the supplemental material) with isolates identified as *Mesorhizobium* (*P. pinnata*, fynbos), *Burkholderia* (*siratro*, fynbos), and *Bradyrhizobium* (*P. pinnata*, siratro, Malawi). *M. pudica* formed no nodules in either South African or Malawian soil.

The *Burkholderia* and *Mesorhizobium* symbionts isolated from legumes growing in fynbos soils were placed in different clades (Fig. 6 and 7) and were highly related (99 to 100% sequence similarity) to known reference strains previously isolated from various South African legumes (see Table S4 in the supplemental material). The *recA* and *nodA* sequence data of *Bradyrhizobium* symbionts from the Malawian soils were related (97 to 99%) to known African, South American, and European isolates (see Table S4 in the supplemental material).
DISCUSSION
Spatial distribution of root-nodulated *Burkholderia* at a continental scale. The global survey of the *Burkholderia* record revealed various geographic and host-related patterns within the 16S rRNA, *nifH*, *nodA*, and *nodC* gene data sets at a continental scale. Chromosomal 16S rRNA types were highly diverse (Fig. 2A; Table 1) and unrelated to the host subfamily or geographic region, whereas nitrogen fixation and nodulation genes are clearly structured by a geographic and host factor (Fig. 2B to D), with only a few sequence groups identified across continents and legume sub-
The observation of an association of geography, host legume, and nodulation genes, showing two large clusters of highly diverged nodulation gene types, according to the geographic origin and host subfamily, corroborates previous *Burkholderia* studies (12, 21). All of the African distributed rhizobia were clustered in one group and were highly diverged (<75% gene similarity) from the remaining mimosoid-related *Burkholderia* bacteria.

The geographic distribution of the legume host seems to be the key factor explaining the nodulation and nitrogen fixation gene phylogenetic structure at a continental scale, supporting the idea that the rhizobial biogeography largely follows the hosts (22), which represent two distinct legume floras of South African papilionoids and South American mimosoids in the fynbos and Cerrado/Caatinga biomes, respectively (12, 62). Evidence is accumulating that the vast majority of *Mimosa* species native to central Brazil are associated exclusively with *Burkholderia* bacteria (10, 55), whereas in Mexico, which is considered another large center of radiation of the genus, most of the endemic species are not nodulated by beta rhizobia (17) but are specifically associated with Alphaproteobacteria and only a few lineages are able to form inter-actions with *Burkholderia* (11, 63). Distinct nodule occupancies of beta and alpha rhizobia within the native home range of Brazilian and Mexican *Mimosa* species, respectively, can be largely explained by a combination of geographic separation of the various *Mimosa* clades with distinct symbiont preferences and their sub-

**FIG 4** Phylogenetic tree of rhizobial endosymbionts based on *nodA* data. Support values for the BI and ML analyses are shown at the nodes (Bayesian posterior probabilities, bootstrap support values for the ML analysis). Reference strains are in bold.
sequent coevolution with rhizobia in contrasting soil types (e.g., acid versus neutral/alkaline soils) (11).

Conversely, access and availability of rhizobia, because of varied adaptation to edaphic and climatic factors, may be a critical factor governing the dispersal of legumes outside native areas and thereby influence legume biogeographic patterns. The latter may be true for South Africa and Western Australia, which have frequent angiosperm dispersal events in the Cenozoic (64), associ-

FIG 5 Box plots of pairwise genetic distances between recA (A) and nodA (B) sequence data grouped within four spatial distance classes (0 to 200, 201 to 400, 401 to 600, and 601 to 800 km). Box plots represent observations within 95% confidence intervals, and the whiskers extend from the box to the highest and lowest values, excluding outliers, which are shown as circles. The thick line across each box indicates the median.

FIG 6 Phylogenetic tree based on recA sequences of rhizobial isolates sampled from trapping experiments. The closest reference strains obtained from BLASTN searches (see Table S4 in the supplemental material) are included in the analyses. Bayesian support values are shown at the nodes. The geographic distributions of the isolates and reference strains are shown for each taxon. The number of substitutions per site is shown on the phylogram.
ated with similarity of niches (Mediterranean climate, oligotrophic acidic soils), yet legumes are one of the few (large) families that do not exhibit disjunction between the two continents. While the endemic Australian tribes Bossiaeeae and Mirbelieae are largely associated with Bradyrhizobium lineages (65, 66), the tribe Hypocalypeteae, which is endemic to South Africa and resolved as a sister group to the mirbelioids, is strictly associated with Burkholderia bacteria.

The nodulation genes nodA and nodC are frequently used to understand symbiotic specificities and their evolutionary adaptation to a specific host (67). Because nodulation genes are involved in the synthesis of Nod factors (i.e., rhizobial signaling molecules required for the earliest host responses), they determine host specificity (68–70) and have been frequently shown to align with their Burkholderia host (12, 17, 30). The specificity of the symbiotic association of Burkholderia with mimosoid and papilionoid legumes is clearly demonstrated in one single species, B. tuberum, which has distinct nodulation genes or symbiotic variants and has been ascribed to symbiovars mimosae and papilionoideae, respectively (71, 72). However, a link between nodA types and the legume subfamily is not strictly predictable for all species. Macropodalyria calyptrata (Table Mt, 617) is able to nodulate with both B. tuberum symbiovar papilionoideae (e.g., STM678 T) (31) and symbiovar mimosae strains (e.g., STM4801) (71). Similarly, the mimosoid symbiont B. phymatum STM815T has been isolated from nodules of the papilionoid P. vulgaris, which is known for its wide range of symbiotic partners (33). Apart from the records involving promiscuous host legumes (siratro, P. vulgaris), Burkholderia species and their nodulation genes appear to group and evolve in close concert with their mimosoid and papilionoid hosts. However, evidence is accumulating that, although rhizobial species (e.g., B. tuberum symbiovar papilionoideae) associated with the subfamily Papilionoideae appear incapable of nodulating mimosoid hosts (31), the opposite is the case (12). In addition to common bean (73), diverse papilionoids such as the fynbos species Dipogon lignosus (19) and legumes of the genera Cyclopia and Virgilia (Table 2; see Fig. S2 in the supplemental material) have been demonstrated to form effective nodules with the mimosoid-nodulating B. phyma-

FIG 7 Phylogenetic tree based on nodA sequences of rhizobial isolates sampled from trapping experiments. The closest reference strains obtained from BLASTN searches (see Table S4 in the supplemental material) were included in the analyses. Bayesian support values are shown at the nodes. The geographic distributions of the isolates and reference strains are shown for each taxon. The number of substitutions per site is shown on the phylogram.
TABLE 2 Nodulation of selected fynbos species after inoculation with B. tuberum STM678 or B. phymatum STM815a

<table>
<thead>
<tr>
<th>Tribe and legume species tested</th>
<th>Nodulation of:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B. tuberum STM678</td>
</tr>
<tr>
<td>Crotalarieae</td>
<td></td>
</tr>
<tr>
<td>Aspalathus carnosa Bergius</td>
<td>E</td>
</tr>
<tr>
<td>Lebeckia ambigua E. Mey.</td>
<td>E</td>
</tr>
<tr>
<td>Indigofereae/Hypocalypetee</td>
<td></td>
</tr>
<tr>
<td>Hypocalypus coluteoides (Lam.)</td>
<td>E</td>
</tr>
<tr>
<td>R. Dahlgren</td>
<td></td>
</tr>
<tr>
<td>Hypocalypus sophoroides (P. J. Bergius)</td>
<td>E</td>
</tr>
<tr>
<td>Gall</td>
<td></td>
</tr>
<tr>
<td>Indigofera filifolia Thunb.</td>
<td>E</td>
</tr>
<tr>
<td>Podalyrieae</td>
<td></td>
</tr>
<tr>
<td>Amphithilea ericifolia (L.) Eckl. &amp; Zeyh.</td>
<td>I</td>
</tr>
<tr>
<td>Calpurnia aacea (Aiton) Benth.</td>
<td>None</td>
</tr>
<tr>
<td>Calpurnia glabrata Brummitt</td>
<td>I</td>
</tr>
<tr>
<td>Calpurnia intrusa (W. T. Aiton) E. Mey.</td>
<td>None</td>
</tr>
<tr>
<td>Calpurnia sericea Harv.</td>
<td>I</td>
</tr>
<tr>
<td>Cyclopia subternata Vogel</td>
<td>E</td>
</tr>
<tr>
<td>Cyclopia genistoides (L.) Vent.</td>
<td>E</td>
</tr>
<tr>
<td>Cyclopia intermedia E. Mey.</td>
<td>E</td>
</tr>
<tr>
<td>Liparia laevigata Thunb.</td>
<td>E</td>
</tr>
<tr>
<td>Liparia splendens (Burm. f) Bos &amp; de Wit</td>
<td>E</td>
</tr>
<tr>
<td>Podalyria burchelli DC.</td>
<td>E</td>
</tr>
<tr>
<td>Podalyria calyptrata (Retz.) Willd.</td>
<td>E</td>
</tr>
<tr>
<td>Podalyria canescens E. Mey.</td>
<td>E</td>
</tr>
<tr>
<td>Podalyria leipoldtii L. Bolus</td>
<td>E</td>
</tr>
<tr>
<td>Podalyria myrtillifolia Willd.</td>
<td>E</td>
</tr>
<tr>
<td>Podalyria rotundifolia (P. J. Bergius)</td>
<td>E</td>
</tr>
<tr>
<td>A. L. Schutte</td>
<td></td>
</tr>
<tr>
<td>Podalyria sericea R. Br</td>
<td>E</td>
</tr>
<tr>
<td>Stirtonanthus tayloriorum (L. Bolus)</td>
<td>E</td>
</tr>
<tr>
<td>B.-E. van Wyk &amp; A. L. Schutte</td>
<td>E</td>
</tr>
<tr>
<td>Virgilia oroboides (P. J. Bergius)</td>
<td>E</td>
</tr>
<tr>
<td>T. M. Salter</td>
<td></td>
</tr>
<tr>
<td>Xiphotheca fruticosa (L.) A. L. Schutte &amp; B.-E. van Wyk</td>
<td>E</td>
</tr>
</tbody>
</table>

a, E, effective nodulation; I, ineffective nodulation (considered if inoculated plants are not greener than uninoculated controls and only few and white nodules are visible). New reports of nodulation are in bold.
b, —, not tested.
c Data from Elliott et al. (31).
d Nodules tested with both B. tuberum STM678 and STM678-GFP.

turn-type symbiont (17, 74), confirming its broad host range and ability to associate with legumes of the mimosoid and papilionoid subfamily.

While symbiosis genes are largely structured according to legume subfamily, 16S rRNA gene clusters are more diverse (Fig. 2A), affiliated with various hosts from different parts of the world (Table 1). A widespread occurrence of Burkholderia strains, especially for 16S rRNA types (Table 1), indicates an intercontinental and global distribution pattern for different Burkholderia species (e.g., B. diazotrophicia, B. mimosarum, B. phymatum, B. sabiae, and B. tuberum). The occurrence and vast diversity of Burkholderia outside Africa and South America are mostly recorded in pan-(sub)tropically distributed Mimosa species (M. pudica, M. pigra,

M. diplopticha). Burkholderia symbionts of these widespread invasive plant species are included in the clustering analyses, and close relationships of nodulation genes with their native distributed relatives support previous observations that rhizobia are cotransported with the seeds or plants from their native habitats to new invasive habitats. In accordance with the cointroduction hypothesis (75), symbionts that have been cointroduced with their hosts or have hitchhiked on introduced material over long distances, bridging geographic barriers between continents, have been evidenced in many studies (15, 19, 76–78). For Burkholderia, a plausible long-distance migration event from South Africa to New Zealand, possibly dispersed across the Australian continent, has been reported in the South African papilionoid D. lignus (tribe Phaseoleae) (20), which is invasive in New Zealand and Australia (19, 20), as revealed by high sequence similarities of the symbiosis genes (nodA sequence clusters 6 and 10 and nodC sequence clusters 9 and 17) between invasive Dipogon populations and native South African relatives from the genera Bolusafra, Crotalaria, Cyclopia, Hypocalypus, Indigofera, Podalyria, and Rhynchosia.

Geographic distribution and specificity of fynbos Burkholderia. While the global Burkholderia diversity was structured for the nodulation genes at the legume subfamily level, an interaction of rhizobia, host legumes, and geographic distribution was not shown at a regional scale, showing widely spread and locally diverse Burkholderia populations in the fynbos. Our results corroborate a previous study demonstrating the widespread occurrence of Burkholderia and the absence of a site sampling effect on the rhizobial diversity of selected Hypocalpytaeae and Podalyrieae species (27, 32). Using geographic distances as a proxy for population connectivity, genetic variation is expected to correlate positively with the sampling site distances. Our study does not show any correlation between genetic variation and geographic distance, suggesting the absence of genetic isolation through high rates of rhizobial dispersal of both chromosomal and symbiosis traits.

In the fynbos region, local environmental variables, rather than spatial dispersal factors, are most likely the major ecological drivers of rhizobial distributions. In a recent study, Lemaire and associates (26) showed that genetic variation of fynbos Burkholderia was correlated with differences in site elevation, a feature also observed in symbionts of South American Mimosa species (10); hence, the indirect effects of temperature and rainfall may play a significant role in forming the rhizobial community structure.

Symbiotic associations of fynbos legumes with Burkholderia have been described in many lineages with various degrees of specificity. In the tribe Podalyrieae, a strong preference for Burkholderia is observed, showing all legume species and genera (except for Calpurnia, which is not endemic to the fynbos; Table 2; see Fig. S2 in the supplemental material) strictly nodulated with Burkholderia (12, 26, 27). Other common plant groups, such as the tribes Crotalarieae and Indigofereae, also contain Burkholderia-philous species, although (closely related) legume lineages within the same tribes and co-occurring in the similar habitats have been recorded with classical alpha-rhizobial lineages (26 and references therein).

In this study, the Burkholderia-legume interaction was further investigated at a finer taxonomic scale. Diverse phylogenetic clusters of Burkholderia strains were observed within native legume genera of the tribes Crotalarieae (Aspalathus, Crotalaria, Lebeckia, Rafnia), Indigofereae (Indigofera), Phaseoleae (Bolusafra, Dipogon, Rhynchosia), and Podalyrieae (Amphithalea, Podalyria, Virgilia), but without a host-specific pattern (Fig. 3 and 4). For
both chromosomal and nodulation genes, with the latter symbiotic genes determining host specificity (68), a relaxed association among genetically similar rhizobia and different legume species, genera, and tribes was demonstrated. The variation of host-Burkholderia interactions corroborates a previous rhizobial screening in selected legume genera of the tribes Hypocalypteae (Hypocalypthus) and Podalyrieae (Cyclopia, Podalyria, Virgilia) (27, 32). In South America, a similar relaxed host-specific interaction has been described for Burkholderia bacteria and their mimosoid hosts (10, 62). The predominance or prevalence of Burkholderia bacteria in both papilionoid and mimosoid legumes, but without a host-specific pattern, indicates that the host genotype has not been a major factor in the local Burkholderia distribution. This observation is in line with the present host range study, showing selected South African papilionoid species able to form effective nodules with the strains B. tuberum STM678 and B. phymatum STM815. B. phymatum, which is found as a common symbiont of Mimosa in French Guiana, Papua New Guinea, India, and China (12, 16, 17, 71), has not been isolated from field nodules collected in the fynbos, yet it is able to nodulate selected papilionoids (Dipogon, Cyclopia, Virgilia). The promiscuous character of the papilionoid-Burkholderia symbiosis has previously been demonstrated in other species of Podalyrieae (12) and Phaseoleae (19, 31, 52).

Although fynbos legumes were generally associated with diverse Burkholderia species, individual root nodules consistently accommodated a single strain. The observation of a single Burkholderia strain per nodule may suggest high selective constraints of the host toward the symbiont. In order to retain a stable and mutualistic interaction, legumes generally hinder the emergence of opportunistic rhizobial strains and select cooperative (i.e., effectively nitrogen-fixing rhizobia) ones over nonbeneficial symbi-
onts (referred to as partner choice) (79, 80) by providing only one beneficial symbiont with ample carbon resources, while an uncooperative nodule occupant is disfavored with host resources (referred to as host sanctions) (81, 82). However, the general observation of a relaxed interaction or accommodation of diverse rhizobial strains per host individual may indicate that the one-nodule–one-strain interaction is a result of high competitiveness for nodule formation among rhizobial strains, rather than to selection by the host plant.

**Nodulation of fynbos legumes outside their distribution range.** A legume growing in nonnative soil can only form nodules when naturalized populations of compatible rhizobia are available in the soil. In our inoculation experiment, siratro and *P. pinnata* nodulated in soils collected from South Africa and Malawi, whereas *P. calytrata*, *I. filifolia*, and *D. lignosus* were nodules free in Malawian soil. The inability to form nodules in Malawian soil suggests that these legumes, known to exhibit a strong host preference for *Burkholderia* (26, 27, 83), did not find their specific *Burkholderia* symbionts in the Malawian (savanna) soil, which had a substantially higher pH than the Cape soil. The occurrence and success of *Burkholderia* in South African (fynbos) soils, but also in the South American Cerrado/Caatinga biomes, can be linked with the general ecological adaptation of these symbionts to acidic soil conditions, which may play a prominent role as an ecological driver of rhizobial diversity (21, 29, 30, 35). In Malawi, legume nodulation by *Burkholderia* has never been reported, as far as we know, and further *Burkholderia* surveys in other African soils are needed to provide evidence of a more limited distribution pattern on the African continent, with the fynbos biome reported as a major center of diversity.

The inability of legumes to form a symbiosis with *Burkholderia* in Malawian soils does not necessarily indicate the absence of *Burkholderia* in other regions of Africa (e.g., see the report of *Burkholderia* nodulating the nonnative common bean in Moroccan soil [33]) but may also result from incompatible types of symbiosis genes within local *Burkholderia* communities. In this context, the observation that *M. pudica* is unable to nodulate within *Burkholderia*-rich fynbos soils strongly suggests that the necessary mimosoid-type nodulation genes (which are genetically distinct from the papilionoid-type nodulation genes) do not occur naturally in these soils. The absence of effective rhizobia and their compatible symbiosis genes is a potential barrier to the colonization of novel habitats by the host legumes. For exotic legumes such as *M. pudica*, it appears that the host needs to bring its own native symbionts into the new environment for optimal and successful colonization and distribution (15, 16).

In contrast to legumes with a specific preference for *Burkholderia*, *P. pinnata* was nodulated by *Mesorhizobium* in fynbos soils and by *Bradyrhizobium* in Malawian soil, indicating a more relaxed interaction, albeit one that does not involve beta rhizobia. Although field nodules of this genus have been consistently associated with *Mesorhizobium* in the fynbos (26), *Bradyrhizobium* was also able to nodulate *Psoralea* effectively, probably in the absence of the preferred *Mesorhizobium* symbionts in these savanna soils. The genus *Psoralea* has a center of diversity in the fynbos, but several species occur in montane grasslands in northeastern South Africa, Mozambique, and Swaziland, and two species are naturalized in Australia (48). The current *Mesorhizobium* diversity from fynbos *Psoralea* has been placed in a separate cluster unrelated to known 16S rRNA or nodA gene types from other African localities, suggesting rhizobial strains restricted to the Cape region. The *Bradyrhizobium* isolates from the Malawian soils, however, were closely related to *B. elkanii* and are geographically widespread and able to nodulate a broad range of legumes from different continents (65, 84–87). In a recent study by M. A. Parker (88), a phylogenetic analysis of a broad sampling of *Bradyrhizobium* strains from diverse plant groups provided evidence of a broad host range of most bradyrhizobial lineages, including *B. elkanii*, that are associated with diverse legume tribes.

**Concluding remarks.** *Burkholderia* populations, like many free-living microbes and other (classical) rhizobial groups, are widespread and occur on different continents (except Antarctica and Europe), a phenomenon that can be explained by their capacity for long-distance dispersal. By investigating nodulation genes of publicly available sequence data, rather than taxonomic identities (16S rRNA types), we observed a strong biogeographic relationship that corresponds largely to two main groups of *Burkholderia* with distinct host-related affinities. Indeed, various phylogenetic studies have described taxonomically diverse papilionoid- and mimosoid-associated rhizobia with a geographic structure preserved in the nodulation genes (nodA and nodC), supporting the hypothesis that traits (i.e., nodulation genes) rather than taxon names (i.e., chromosomal genes) are the fundamental units of biogeography (89). In contrast to the global investigation of *Burkholderia*, regionally distributed species in the fynbos did not show any geographic distribution pattern. Within the Cape region, genetic variation of both chromosomal and nodulation genes was unrelated to geographic or host factors, suggesting that nodulating *Burkholderia* bacteria are omnipresent in the fynbos biome and do not constrain the distribution of their native host legumes in terms of compatible symbionts.

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**REFERENCES**


6. Bala A, Murphy P, Gillier KE. 2003. Distribution and diversity of rhizobia nodulating agroforestry legumes in soils from three continents in the trop-


70. Kiers ET, Denison RF. 2008. Sanctions, cooperation, and the stability of


