Paraburkholderia strydomiana sp. nov. and *Paraburkholderia steynii* sp. nov.: rhizobial symbionts of the fynbos legume *Hypocalyptus sophoroides*

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Abstract

Twelve nodulating *Paraburkholderia* strains isolated from indigenous South African fynbos legume *Hypocalyptus sophoroides* were investigated to determine their taxonomic status. Genealogical concordance analysis, based on six loci (16S rRNA, *atpD*, *recA*, *rpoB*, *lepA* and *gltB*), revealed that they separate into two consistent and exclusive groups. Average nucleotide identity and DNA-DNA hybridisation comparisons indicated that they were sufficiently divergent from their closest known phylogenetic relatives (*Paraburkholderia caledonica* and *Paraburkholderia terrae*, respectively) to be regarded as novel species. This was also

supported by the results of fatty acid analysis and metabolic characterisation. For these two isolate groups, we accordingly propose the new species *Paraburkholderia strydomiana* sp. nov. with WK1.1f^T (= LMG 28731^T = SARCC1213^T) as its type strain and *Paraburkholderia steynii* sp. nov. with HC1.1ba^T (= LMG 28730^T = SARCC696^T) as its type strain. Our data thus showed that *H. sophoroides* may be considered a promiscuous symbiotic partner due to its ability to associate with multiple species of *Paraburkholderia*.

Keywords

Beta-rhizobia, *Burkholderia*, Genealogical concordance, *Hypocalyptus*, *Paraburkholderia*, South Africa

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA sequences of type strains WK1.1f^T and HC1.1ba^T are HF674688 and HF674712, the *atpD* gene sequences are LN555593 and LN555601, the *rpoB* accession numbers are LN555614 and LN555624 and the *recA* accession numbers are HF544384 and HF544408. Lastly the accession numbers for the *lepA* gene sequences of WK1.1f^T and HC1.1ba^T are LT708273 and LT708274, while the *gltB* accessions are LT708294 and LT708295. The NCBI genome accession numbers for the type strains are MWMK00000000 and MWML00000000.

Introduction

Hypocalyptus sophoroides is a member of the tribe Hypocalypteae in the Papilionoideae subfamily of legumes (Leguminosae) (Schutte and Van Wyk 1998; Legume Phylogeny Working Group 2017). This monogeneric tribe forms part of the so-called "Old world" papilionoid clade and is most closely related to the mirbelioid tribes indigenous to Australia (including Tasmania) and Papua-New Guinea (Van Wyk 2005; Lewis et al. 2005; Legume Phylogeny Working Group 2013). Similar to the other two *Hypocalyptus* species, *Hypocalyptus coluteoides* and *Hypocalyptus oxalidifolius*, the distribution of this perennial shrub or small tree is restricted to the Fynbos biome in the Western Cape Province of South Africa where it mostly grows on rocky sandstone slopes along streams (Stepanova et al. 2013). Symbiotic interactions with nitrogen-fixing bacteria (rhizobia) likely facilitate their survival and growth in these nutrient-poor soils (Yates et al. 2010), because the natural occurrence of root nodules (i.e., plant organs in which rhizobia chemically reduce dinitrogen to ammonium; Masson-Boivin et al. 2009) has been reported for all three species (Sprent 2001; Beukes et al. 2013). *Hypocalyptus* root nodules are elongated, unbranched and indeterminate in shape (see Suppl. Fig. S1 of Beukes et al. 2013), which is similar to what has been reported for their mirbelioid relatives (Sprent 2001; Beukes et al. 2013). A cross-section of a stained *Hypocalyptus sophoroides* nodule (inoculated with *Paraburkholderia tuberum* STM678^{T}) clearly showed the apical meristem, as well as the nitrogen-fixing zone with the *Paraburkholderia*-infected cells interspersed with uninfected cells (Lemaire et al. 2015).

The rhizobia associated with *Hypocalyptus* were originally classified as *Burkholderia* species (Beukes et al. 2013). However, this diverse taxon (i.e., *Burkholderia* sensu lato) has since been split by moving most of the so-called "environmental" and "plant-beneficial-environmental"

species (Suárez-Moreno et al. 2012), as well as symbionts of the fungus *Rhizopus microsporus* (Partida-Martinez et al. 2007), to the new genera *Paraburkholderia* (Sawana et al. 2014), *Caballeronia* (Dobritsa and Samadpour 2016), *Trinickia* and *Mycetohabitans* (Estrada-de los Santos et al. 2018). Accordingly, all the formally described rhizobial and/or endosymbionts of papilionoids from South Africa have been moved to *Paraburkholderia* (Oren and Garrity 2015a, b, 2017). These include *P. tuberum* (Vandamme et al. 2002), *Paraburkholderia sprentiae* (De Meyer et al. 2013a), *Paraburkholderia dilworthii* (De Meyer et al. 2014), *Paraburkholderia rhynchosiae* (De Meyer et al. 2013b), *Paraburkholderia dipogonis* (Sheu et al. 2015), *Paraburkholderia kirstenboschensis* (Steenkamp et al. 2015) and *Paraburkholderia aspalathi* (Mavengere et al. 2014). Because its draft genome apparently lacks *nod* or *nif* loci (Estrada-de los Santos et al. 2018), *P. aspalathi* is likely only present as an endosymbiont.

Of the *Paraburkholderia* species described from South Africa, only *P. kirstenboschensis* included isolates originating from *Hypocalyptus* root nodules (including three isolates from *H. sophoroides*), as well as isolates from other Fynbos legumes (Beukes et al. 2013; Steenkamp et al. 2015). Generally, information regarding nodulation and nitrogen fixation in *Hypocalyptus* species is limited (Sprent 2001). However, two other species originating from South Africa, *P. dipogonis* (Sheu et al. 2015) and *P. tuberum* STM678^T (Vandamme et al. 2002), have recently been shown to be capable of forming effective nodules on *Hypocalyptus* (Lemaire et al. 2015, 2016), while *Paraburkholderia phymatum* STM815^T (isolated from French Guiana) could only form ineffective nodules on *H. sophoroides* (Vandamme et al. 2002; Lemaire et al. 2016). These findings are consistent with the proposal that South Africa and South America represent two distinct centres of diversity for nodulating *Paraburkholderia* (Gyaneshwar et al. 2011). In contrast to *P. phymatum* strains that can nodulate *Mimosa* species (Elliott et al. 2007) and Papilionoideae species such as *Dipogon lignosus* (Liu et al. 2014), South African

Paraburkholderia apparently only nodulate Papilionoideae hosts (Liu et al. 2014) and have not been shown to be capable of effectively nodulating *Mimosa* (de Castro Pires et al. 2018).

An earlier study showed that Hypocalypteae can interact with a range of diverse *Paraburkholderia* isolates (Beukes et al. 2013). Although not representing an extensive survey across the distribution range of individual *Hypocalyptus* species, this study revealed as many as seven distinct *recA*-based genotypes, distributed across the *Paraburkholderia* phylogeny, from the root nodules of *H. sophoroides* (Beukes et al. 2013). The overall objective of the present study was to delineate and characterise the species represented by a set of 12 rhizobial isolates from *H. sophoroides*. Previous work based on 16S rRNA and *recA* phylogenies suggested that this collection of isolates represent two genetically distinct groups (Beukes et al. 2013). Therefore, our specific aims were (i) to delimit the genetically distinct groups within this collection of isolates by using multilocus genealogical concordance analysis (Venter et al. 2017); (ii) to evaluate the overall genetic and phenotypic uniqueness of the delimited groups relative to their closest known relatives; and (iii) to provide descriptions for these groups following the conventions for rhizobial systematics (Graham et al. 1991). To accomplish these aims, the 12 isolates were subjected to a phylogeny-based analysis of genealogical concordance using the gene sequences of six independent loci. The overall support for the delineated groups was then investigated by evaluating various phenotypic properties and genome-based criteria. The exclusive or genealogically concordant groups were then characterised as two new species, named here as *Paraburkholderia strydomiana* sp. nov. and *Paraburkholderia steynii* sp. nov. (see below).

Materials and methods

Bacterial isolates, growth conditions and DNA extraction

A set of 12 isolates was examined in this study (isolates WK1.1a, WK1.1c, WK1.1d, WK1.1f^T, WK1.1h, WK1.1i, WK1.1j, WK1.1k, WK1.1m, HC1.1a3, HC1.1ba^T and HC1.1bb). Of these, two isolates (WK1.1a and WK1.1c) were taken directly from root nodules of *H. sophoroides* plants that grew along the Old du Toit's Kloof Pass in the Western Cape Province of South Africa (Beukes et al. 2013). The remaining ten isolates originated from the root nodules of individual plants that were grown, under glasshouse conditions, in soil collected from the same location (Beukes et al. 2013). The nodulation ability of these isolates was demonstrated previously (Beukes et al. 2013).

For comparative purposes, the type strain for *Paraburkholderia hospita* was obtained from the Belgian Coordinated Collections of Microorganisms (BCCM; Universiteit Gent, Belgium). All isolates were routinely grown at 28 ^oC on either Yeast Mannitol Agar (YMA) or Tryptone Yeast Extract Agar (TYA) enriched with $CaCl_2.2H_2O$ to reduce the production of exopolysaccharides. All the isolates were preserved on beads or in 20% glycerol at -70 ^oC as part of the University of Pretoria's Rhizobium Culture Collection. The type strains (WK1.1f^T and HC1.1ba^T) of the proposed novel species have been deposited in the BCCM (with numbers LMG 28731^T and LMG 28730^T, respectively) and in the South African Rhizobium Culture Collection (SARCC, Pretoria, South Africa; with numbers SARCC1213^T and SARCC696^T, respectively).

High-quality DNA was prepared from 48 hour-old Tryptone Soy Agar (TSA; Merck) grown cultures. This was accomplished using the method described by Pitcher et al. (1989) with some modifications (Steenkamp et al. 2015). The extracted DNAs were used in all subsequent PCRs and DNA-DNA hybridisation (DDH) experiments, as well as for whole genome sequencing (see below).

Phylogenetic analysis

DNA sequence information for six independent gene regions was utilised in this study. The sequences for two of these (16S rRNA and *recA*) were available from previous work (Beukes et al. 2013), while those for four additional genes were determined during this study. The latter included *atpD* (encoding ATP synthase subunit beta), *rpoB* (encoding RNA polymerase subunit beta), *gltB* (encoding glutamate synthase subunit alpha) and *lepA* (encoding translation elongation factor 4).

For *rpoB* and *atpD*, PCR amplification and sequencing were performed as described by Steenkamp et al. (2015). For amplifying portions of the *gltB* and *lepA* genes, primer set gltBF (5' CTG CAT CAT GAT GCG CAA GTG 3') and gltBR (5' CTT GCC GCG GAA RTC GTT GG 3') (Spilker et al. 2009), and primer set lepA-F2 (5' TGG TTC GAC AAC TAC GTC GG 3') and lepA-R (5' ATC AGC ATG TCG ACC TTC AC 3') (Estrada-de los Santos et al. 2013) were used. Each 25 µl PCR reaction contained 50 - 100 ng genomic DNA, 10 mM of each primer, 0.1 U/µl Super-Therm *Taq* DNA polymerase (Southern Cross, Biotechnology, SA) and reaction buffer, 25 mM $MgCl_2$ and lastly 2.5 mM of each of the four dNTPs. PCR cycle conditions were as follows: initial denaturation at 95 ^{o}C for 5 min, followed by 30 cycles of denaturation at 95 ^{o}C for 30 s, annealing at 55 ^{o}C for 30 s and elongation at 72 ^{o}C for 1 min 30

s finishing with a final elongation step of 10 min at 72 ºC. The *gltB* and *lepA* amplicons were purified and sequenced in both directions using the original PCR primers as described before (Steenkamp et al. 2015).

Electropherograms for the respective forward and reverse sequences for *atpD*, *rpoB*, *lepA* and *gltB* were analysed and assembled into individual contigs using ChromasLite v2.01 (Technelysium, Queensland, Australia) and BioEdit v7.0.5.3 (Hall 1999). The *atpD*, *recA*, *rpoB*, *lepA* and *gltB* sequences determined in this study have been submitted to the European Nucleotide Archive (https://www.ebi.ac.uk) (see Supplementary Table S1). Also, all of the DNA sequences used in this study were compared to those in the nucleotide database of the National Center for Biotechnology Information (NCBI; https://www.ncbi.nlm.nih.gov; Benson et al. 2017) using blastn (Altschul et al. 1990).

Individual datasets were generated for all six loci. In addition to the sequences for the 12 isolates from *H. sophoroides* and *P. hospita*, these datasets included the corresponding sequences from the type strains of other known species as per the List of Prokaryotic Names with Standing in Nomenclature (LPSN; www.bacterio.net) (Euzéby 1997; Parte 2013). For the latter, only species which have sequences for all five protein-coding gene regions were included in these analyses. We also analysed broader or extended 16S rRNA and *recA* datasets to incorporate newly described species for which these were the only sequence information available. For outgroup purposes, the various gene datasets included the type strains of three *Caballeronia* species (i.e., *Caballeronia fortuita* LMG 29320^{T}; *Caballeronia temeraria* LMG 29319^{T} and *Caballeronia catudaia* LMG 29318^{T}) previously described from South African soil (Peeters et al. 2016).

The 16S rRNA sequences were aligned with MAFFT (Multiple Alignment using Fast Fourier Transformation; http://mafft.cbrc.jp/alignment/server/) using the Q-INS-I strategy to account for secondary structure (Katoh and Toh 2008). The sequences for the respective protein-coding genes were manually aligned in BioEdit by making use of the inferred amino acid sequences. SequenceMatrix (Vaidya et al. 2011) was used to generate a concatenated dataset consisting of the aligned sequences for the five protein-coding genes.

Maximum likelihood (ML) and maximum parsimony (MP) trees were generated using each individual dataset as well as the concatenated dataset. ML trees were inferred using PhyML v3.1 (Guindon and Gascuel 2003) and the best-fit models as indicated by jModelTest v2.1.7 (Guindon and Gascuel 2003; Darriba et al. 2012). In these analyses, all the datasets included a proportion of invariable sites and gamma correction to account for among site variation. The *recA* and *atpD* datasets utilised the TIM2 transition substitution model (Darriba and Posada 2014), the *gltB* dataset utilised the TIM1 transition substitution model, the *rpoB*, *lepA* and concatenated datasets utilised the General Time Reversible (GTR) model (Tavare 1986), while the 16S rRNA datasets used the Tamura and Nei (1993) model. MP trees were inferred with MEGA 6 (Tamura et al. 2013) using Tree-Bisection-Regrafting (TBR; Nei and Kumar 2000) at a search level of 1 in which the initial trees were obtained by the random addition of sequences for which there were 1000 replicates. For the MP analysis, only positions with < 5% alignment gaps, missing data or ambiguous bases were used, while branch lengths for the most parsimonious trees were calculated using the average pathway method (Nei and Kumar 2000). For both ML and MP analyses, branch support was estimated using nonparametric bootstrap analysis of 1000 pseudoreplicates (Felsenstein 1985).

Sequences for the common symbiotic loci, *nifH* and *nodA* (encoding dinitrogenase reductase and an acyltransferase, respectively), for the *Paraburkholderia* isolates associated with indigenous fynbos legumes were obtained during a previous study (Beukes et al. 2013). We included only those for isolates associated with *H. sophoroides* here. These protein-coding regions were aligned as described above, after which maximum-likelihood analyses were performed. The *nifH* dataset used the TIM3 transitional substitution model (Darriba and Posada 2014) and the *nodA* dataset used the Hasegawa-Kishino-Yano (HKY) model (Hasegawa et al. 1985). Only the HKY analysis included a proportion of invariable sites and gamma correction for among site rate variation. Branch support was estimated using bootstrap analysis of 1000 pseudoreplicates.

Genome sequences for isolates WK1.1f^T and HC1.1ba^T

The whole genome sequences for isolates WK1.1f^T and HC1.1ba^T were determined. Their respective high-quality DNAs were subjected to whole genome shotgun sequencing at Stellenbosch University's Central Analytical Facilities (CAF; Stellenbosch, South Africa). The Ion Torrent 200-base pair (bp) chemistry (Thermo Fisher Scientific) was used for library preparation, while sequencing with HiQ chemistry on an Ion PI™ Chip (Thermo Fisher Scientific) was used to generate single-ended reads. The reads were filtered and trimmed using FastX Tools v0.0.14, which eliminated short reads (< 150 bases) and removed adaptor sequences and regions of low quality. The remaining sequences were assembled using SPAdes v3.6.0. (Nurk et al. 2013). The statistical report for the assemblies was generated using BBMap (Joint Genome Institute, U.S. Department of Energy).

ANI and DDH

The assembled genomes for WK1.1f^T and HC1.1ba^T were used to determine the pair-wise ANI values between each isolate and its closest known relatives, as determined by the extended *recA* phylogeny. This was done using JSpecies (Richter and Rosselló-Móra 2009), where ANI values were calculated by pair-wise BLAST comparisons of genomes that were in turn artificially sectioned into 1020 base fragments. Only those fragments that were > 30% similar over an aligned region of > 70% of their lengths to the reference were considered homologous. JSpecies then calculated the ANI values across the genomes by averaging the percentage identities obtained for individual homologous fragments.

DDH experiments were performed in-house following the procedure described by Goris et al. (2007), with minor modifications (Steenkamp et al. 2015). These experiments included all 12 of the isolates examined in this study. We also included the type strains of the species closely related to the isolates from *H. sophoroides*, as based upon the extended *recA* phylogeny.

Phenotypic characterisation

Cell morphology, size and motility were determined using Zeiss Stereo and compound microscopes and Auxiovision version 4.8 software. To study growth characteristics, isolates were streaked onto TYA, YMA, TSA and MacConkey Agar without crystal violet (MA; Merck). Incubation was aerobic at 28 ^oC for 3-4 days, except for MA, where isolate growth was evaluated at 29 ^oC and 37 ^oC. Growth of the isolates in Tryptone Yeast Broth (TYB, Oxoid) containing either 0.5% (wt/vol) or 1% (wt/vol) NaCl was evaluated at 28 ^oC. To determine the optimum growth pH, all 12 isolates were grown in a pH range of 4-9 in Yeast Mannitol Broth

at 28 ºC with shaking for 4 days. Catalase activity was determined by flooding a colony with 10 % H_2O_2. Oxidase activity was detected by blotting a loopful of cells on sterile filter paper soaked in 1 % *N,N,N',N'*-tetramethyl ρ-phenylenediamine.

The metabolic profiles for the various isolates were determined using API 20NE strips (bioMérieux), as well as the Biolog GN2 MicroPlate system (Biolog). These tests were performed under standardised conditions and according to the manufacturers' instructions, with incubation at 28 ºC and results recorded after 48 h. Colonies used for inoculating the various test substrates included in these tests were taken from 24 h to 48 h cultures on TSA at 28 ºC.

The proposed type strains (WK1.1f^T and HC1.1ba^T) of each of the novel species delineated here were subjected to whole cell fatty acid analysis. Strains were cultured on TSA following the prescribed MIDI protocol (http://www.midi-inc.com/techNote_101.pdf). The analysis was done by the Food and Environmental Research Agency (Sand Hutton, York, United Kingdom) using an Agilent Technologies 6890 gas chromatograph and the MIDI Sherlock Microbial Identification System version 6.2.

Results

Phylogenetic analysis

BLAST comparison of the 16S rRNA sequences of the 12 isolates from *H. sophoroides* to those in the NCBI nucleotide database showed that they were all most similar to species of the genus *Paraburkholderia*. For example, the 16S rRNA sequences for the 12 isolates shared >

99% identity with *P. caledonica* LMG 19076^T or *P. terrae* LMG 23368^T. To determine the overall relatedness of the 12 *Paraburkholderia* isolates to other members of the genus, a 16S rRNA phylogeny was generated, which included all validly named *Paraburkholderia* species (Fig. 1). This showed that eight isolates (WK1.1f^T, WK1.1k, WK1.1a, WK1.1j, WK1.1i, WK1.1h, WK1.1d and WK1.1m) formed a well-supported monophyletic group that included the type strain of *P. caledonica*. The proposed type strain for this group, WK1.1f^T, shares 100% 16S rRNA sequence similarity when aligned with *P. caledonica* LMG 19076^T. The remaining four isolates (HC1.1ba^T, WK1.1c, HC1.1bb and HC1.1a3) formed a well-supported and exclusive group, closely related to *P. terrae* KMY02^T and *P. hospita* LMG 20598^T. The aligned 16S rRNA sequence similarity between HC1.1ba^T and *P. terrae* KMY02^T is 100% while it shares 98.6% sequence similarity with *P. hospita* LMG 20598^T. Similar overall patterns were observed in the *recA* phylogeny with comparable taxon selection (Suppl. Fig. S1). The two main differences in the *recA* tree were that the first group of eight isolates grouped separately from the type strain for *P. caledonica*, while the second group of four isolates formed a well-supported group that contained the type strain for *P. terrae*. Based on these combined data, and using Nixon and Wheeler's (1990) approach, these two diagnosable groups (i.e., *P. caledonica* plus WK1.1f^T, WK1.1k, WK1.1a, WK1.1j, WK1.1i, WK1.1h, WK1.1d and WK1.1m, and *P. terrae* plus HC1.1ba^T, WK1.1c, HC1.1bb and HC1.1a3) were regarded as putative species hypotheses, the validity of which we interrogated in subsequent analyses.

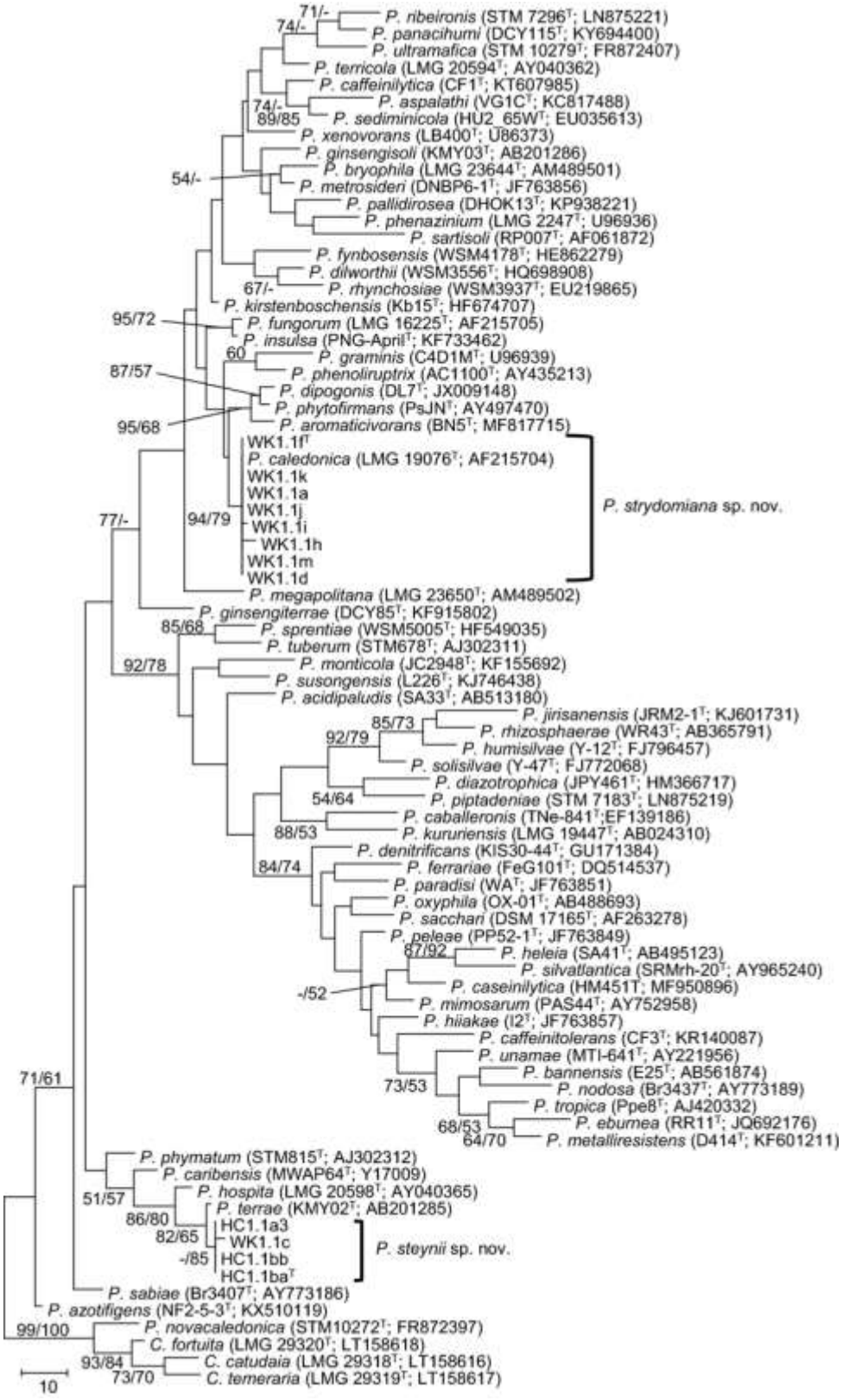

Fig. 1. A maximum parsimony (MP) tree inferred from 16S rRNA sequence analysis of a group of 12 *Paraburkholderia* isolates associated with *Hypocalyptus sophoroides*. The length of the tree is 1001 steps, the consistency index is 0.21, the retention index is 0.68 and the homoplasy index is 0.79. A similar tree was obtained using maximum likelihood (ML) analysis. Statistical support for the groupings are shown in the order MP/ML and only support ≥ 50% are indicated. Information regarding the type strain and GenBank accession numbers for each species is indicated in brackets. Species names which appear in inverted commas are combinations that have not yet been validly published. The scale bar corresponds to 10 character state changes

The 12 *H. sophoroides* isolates, together with *P. caledonica* and *P. terrae* were subjected to genealogical concordance analysis based on the partial sequences of five protein-coding genes. These were *recA* (ca. 850 bp), *atpD* (ca. 1200 bp), *rpoB* (ca. 1000 bp), *gltB* (ca. 582 bp) and *lepA* (ca. 747 bp). Phylogenetic analyses of these individual datasets supported the separation of the 12 *H. sophoroides* isolates into the two groups recovered before (Fig. 2 and Supplementary Figures S2-S7). Across all five of the phylogenies, the eight isolate group remained highly supported (97-100% bootstrap support) and excluded *P. caledonica* LMG 19076^{T} as its closest known relative. The four isolate group was well-supported (95-100% bootstrap support) in the *atpD*, *rpoB*, *gltB* and *lepA* genealogies but, as before, included the type strain of *P. terrae* in the *recA* phylogeny.

Analysis of *nifH* and *nodA* sequences separated the isolates originating from *H. sophoroides* into two clusters (Suppl. Fig. S8A and B). One of these clusters included the eight isolate group observed in the *recA*, *atpD*, *rpoB*, *gltB* and *lepA* genealogies (Fig. 2). The other *nifH*- and *nodA*-based cluster included the previously observed four isolate cluster. Both of the *nifH*- and *nodA*-based clusters also included isolates of *P. kirstenboschensis* and, as yet, unnamed isolates from *H. sophoroides* root nodules. It would therefore appear as if there are at least two lineages of the symbiotic locus responsible for interaction with *H. sophoroides*.

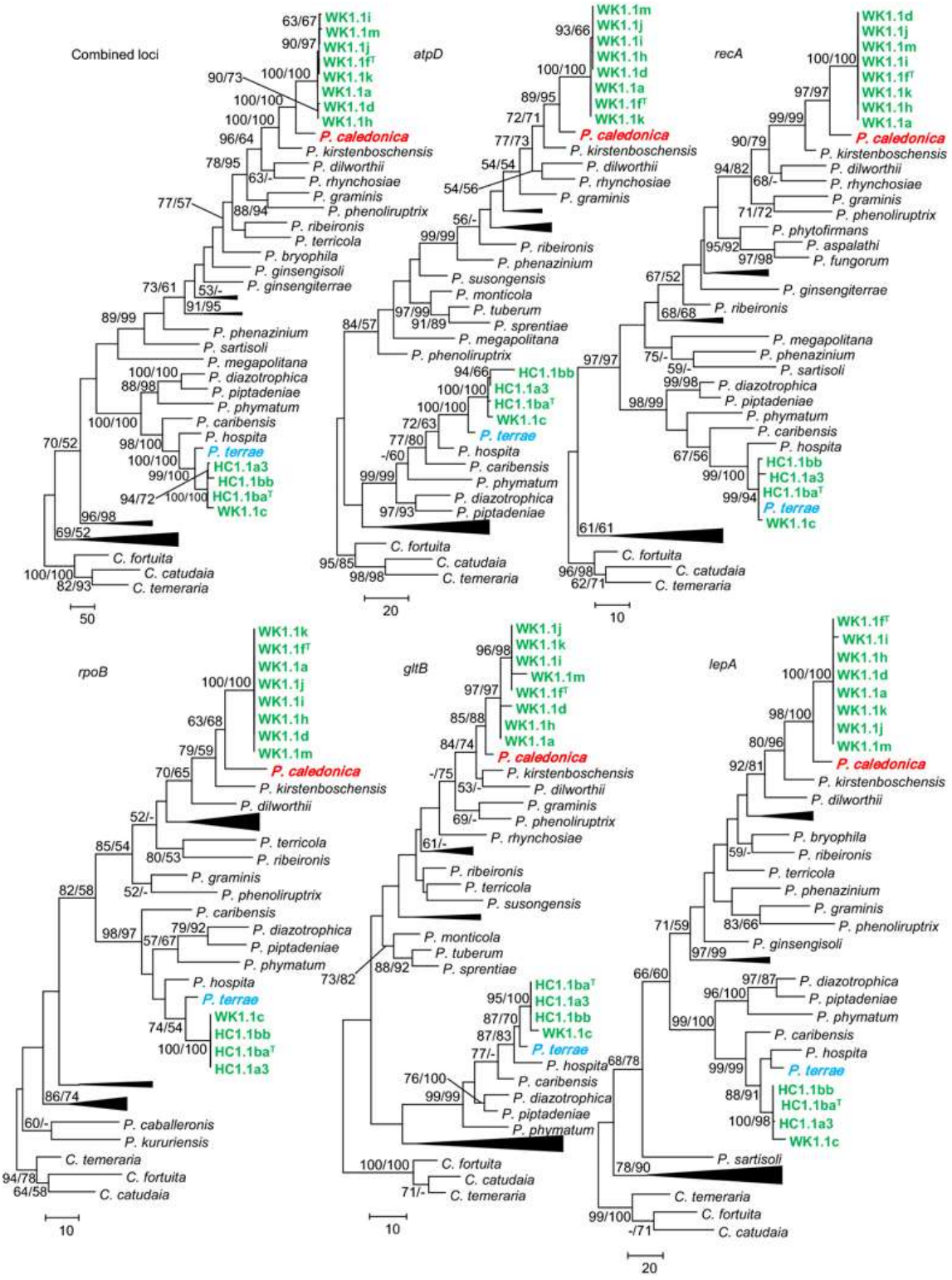

Fig. 2. Results for the genealogical concordance analysis based on five protein-coding genes. The trees were produced with maximum parsimony, although similar groupings were obtained with maximum likelihood analysis. Bootstrap support, based on 1000 pseudoreplicates, are indicated at the branches in the order MP/ML where only support ≥ 50% are indicated. The 12 isolates from *Hypocalyptus sophoroides* are indicated in green, while their close relatives, *P. caledonica* and *P. terrae*, are indicated in red and blue respectively. The scale bar corresponds to 10 character state changes for the *recA*, *rpoB* and *gltB* phylogenies, 20 changes for the *atpD* and *lepA* phylogenies, and 50 changes for the concatenated phylogeny. Three *Caballeronia* species were used as outgroups. For details regarding maximum-likelihood support values, type strain designations and GenBank accession numbers, view Supplementary Figures S2 to S7

Genome sequences of isolates WK1.1f^T and HC1.1ba^T

For strain WK1.1f^T a total of 6,240,372 single-ended reads were generated, which corresponded to 5,789,317 quality-filtered reads with an average length of 190 bases. Assembly of these reads generated a draft genome consisting of 8,397,958 bases across 259 contigs with 120.63 x coverage, N50 of 105,781 and G + C content of 61.6%. Using the Rapid Annotation Subsystem technology (RAST; Aziz et al. 2008) this genome was predicted to contain 8339 protein-coding genes with 55 genes encoding RNAs. Likewise, a total of 7,375,488 single-ended reads were generated for strain HC1.1ba^T. Application of the quality filter resulted in 6,800,907 reads with an average length of 200 bases, which assembled into a draft genome consisting of 11,448,031 bases. This assembly consisted of 1466 contigs at a coverage of 108.72 x, with an N50 of 29,805 bases and G + C content of 61.8%. RAST predicted that the HC1.1ba^T genome contains 11,936 protein-coding genes and 58 genes encoding RNAs. Additionally, to meet the proposed minimum standards for using these genomes for taxonomic purposes (Chun et al. 2018), we also confirmed that their full-length 16S rRNA gene sequences were identical to those determined previously using Sanger sequencing. These draft assemblies are available from NCBI under the accession numbers MWMK00000000 and MWML00000000.

ANI and DDH

Comparison of the draft genome sequence for strain WK1.1f^T (representative of the eight isolate group) with those of the type strains for validly named species closely related to it (i.e., *P. caledonica* NBRC 102488^T, *P. dilworthii* WSM3556^T, *P. rhynchosiae* WSM 3937^T and *P. kirstenboschensis* LMG 28727^T) generated ANI values below the proposed 95-96% threshold

(Richter and Rosselló-Móra 2009; Arahal 2014) for *P. kirstenboschensis*, *P. rhynchosiae* and *P. dilworthii* (Table 1). In the comparison between WK1.1f^T and *P. caledonica* NBRC 102488^T, reciprocal ANI values of 94.97 and 95.51% were obtained, which fall within the proposed cut-off threshold with which to delineate novel species (Richter and Rosselló-Móra 2009; Arahal 2014). Similarly, for the comparisons involving HC1.1ba^T (representative of the four isolate group) (Table 1). ANI values lower than 95-96% were obtained for the comparisons with *P. hospita* LMG 20598^T and *P. caribensis* MWAP64^T, while the comparison between HC1.1ba^T and *P. terrae* NBRC 100964^T yielded reciprocal values of 95.18 and 95.91%. Therefore, most of the comparisons yielded ANI values below the 95-96% cut-off threshold, while the current closest neighbours of each proposed new species had comparison values in the range 95-96% (Richter and Rosselló-Móra 2009; Arahal 2014).

Table 1 Pairwise comparison of the shared ANI percentage between *P. strydomiana* sp. nov. WK1.1f^T (a) and *P. steynii* sp. nov. HC1.1ba^T (b) and their closest relatives which have genome sequences. Reciprocal search results are indicated above and below the diagonal. The proportions of the genomes included in these analyse are indicated in parentheses (see Supplementary Table S3)

	P. strydomiana sp. nov. WK1.1F^T	*P. caledonica* NBRC 102488^T	*P. kirstenboschensis* LMG 28727^T	*P. rhynchosiae* WSM3937^T	*P. dilworthii* WSM3556^T
(a) *P. strydomiana* sp. nov. WK1.1f^T	-	94.97 (83.41)	91.92 (72.20)	86.06 (60.77)	87.75 (67.16)
P. caledonica NBRC 102488^T	95.51 (74.31)	-	92.65 (68.54)	86.27 (58.30)	88.11 (64.39)
P. kirstenboschensis LMG 28727^T	91.84 (68.80)	92.1 (73.42)	-	86.84 (56.64)	88.88 (63.88)
P. rhynchosiae WSM3937^T	86.32 (59.43)	86.22 (64.03)	87.12 (58.13)	-	87.84 (65.71)
P. dilworthii WSM3556^T	88.13 (63.57)	88.08 (67.65)	89.21 (62.67)	87.94 (63.24)	-

	P. steynii sp. nov. HC1.1ba^T	*P. terrae* NBRC 100964^T	*P. hospita* LMG 20598^T	*P. caribensis* MWAP64^T
(b) *P. steynii* sp. nov. HC1.1ba^T	-	95.18 (79.50)	93.45 (69.36)	90.81 (72.78)
P. terrae NBRC 100964^T	95.91 (72.41)	-	94.82 (71.21)	91.69 (74.65)
P. hospita LMG 20598^T	94.14 (71.41)	94.54 (79.86)	-	91.48 (74.61)
P. caribensis MWAP64^T	91.98 (61. 82)	92.09 (69.20)	92.17 (61.91)	-

We complemented the ANI analysis with conventional laboratory DDH experiments (Table 2). Values obtained for comparisons among individuals from the eight isolate group all exceeded 90%, while comparisons with their closest known relative, *P. caledonica* LMG 19076^T, yielded values of < 69% (Table 2). Similarly, for the four isolate group (Table 2), the within-group comparisons produced values of > 94%, while those involving other species (i.e., *P. terrae* LMG 23368^T) had values below the accepted 70% threshold for species (Achtman and Wagner 2008). These values were in the same range as those calculated using digital DDH (Meier-Kolthoff et al. 2013); an average of 66.4% similarity (based on three methodologies) for *P. strydomiana* sp. nov. vs. *P. caledonica*, and an average of 65.2% similarity for *P. steynii* sp. nov. and *P. terrae*.

Table 2 DDH-based percentage DNA similarity amongst *Paraburkholderia* strains belonging to *P. strydomiana* sp. nov. (a) and *P. steynii* sp. nov. (b) and their closest known relatives. The values in brackets indicate the difference between the reciprocal reactions divided by 2

	WK1.1a	WK1.1f^T	WK1.1j	WK1.1m
(a)*P. strydomiana* sp. nov.				
WK1.1a	100			
WK1.1f^T	91.4 (± 8.6)	100		
WK1.1j	98.5 (± 1.6)	92.3 (± 2.5)	100	
WK1.1m		100 (± 0.4)		100
P. caledonica LMG 19076^T		63.7 (± 3.2)	61.2 (± 9.2)	68.6 (± 9.8)

	HC1.1a3	HC1.1ba^T	HC1.1bb
(b) *P. steynii* sp. nov.			
HC1.1a3	100		
HC1.1ba^T	94.4 (± 5.6)	100	
HC1.1bb	99.4 (± 0.6)	95.7 (± 4.3)	100
P. terrae LMG 23368^T		26.0 (± 5.0)	

Phenotypic characterisation

Our results showed that all these bacteria are Gram-negative, motile rods that are 0.6-0.7 µm wide and 1.5-2.0 µm long in the case of eight isolate group, while for the four isolate group cells are 0.7-0.8 µm wide and 2.3-2.7 µm long. Isolates of both groups grow well on YMA,

TYA and TSA at 28 ºC. On all these growth media, colonies of the eight isolate group are creamy white, smooth and round with entire margins. Copious amounts of extracellular polysaccharides were observed on YMA. Colonies of the four isolate group were observed to be less smooth and mucoid on YMA agar and rough on TSA. All these strains grow in TYB containing 0.5 and 1% NaCl. Most of the strains could grow across the whole pH range tested (4-9), although no growth was observed for isolates WK1.1a and WK1.1m at pH 9. The members of the eight isolate group grow on MacConkey agar at 29 ºC and 37 ºC, but no growth was observed for the members of the four isolate group on this medium. All strains were found to be positive for catalase and oxidase. Supplementary Table S2 contains an overview of the results obtained for the various phenotypic tests, including those obtained with the API 20NE and Biolog GN2 MicroPlate systems. The TaxonNumbers for the two species in the Digital Protologue database are TA00804 (*P. steynii* sp. nov.) and TA00795 (*P. strydomiana* sp. nov.), respectively.

A number of phenotypic features were identified that could differentiate the eight and four isolate groups from their near neighbours (Table 3), as determined based upon the extended *recA* phylogeny. The members of the eight isolate group can be differentiated from *P. caledonica* LMG 19076^T and *P. kirstenboschensis* LMG 28727^T by the assimilation of adipic acid and m-inositol, respectively. The members of the four isolate group can be distinguished from *P. terrae* LMG 23368^T by nitrate reduction and the assimilation of adipic acid, m-inositol, citrate and alpha-ketovaleric acid.

Table 3 Phenotypic traits that differentiate *P. strydomiana* sp. nov. and *P. steynii* sp. nov. from the type strains of closely related *Paraburkholderia* species

Phenotypic traits [a]					
	1 [b]	**2**	**3**	**4**	**5**
Isolation source	RN	RN	RN	RH	RH
Nitrate reduction	V	+	V	+	-
Activity of					
Arginine dihydrolase	V	V	+	-	+
Tryptophan deaminase	ND	ND	+	-	-
Urease	-	+			
β-Galactosidase	+	+	V	+	+
Assimilation of					
Adipic acid	+	+	+	-	-
Capric acid	V	+	V	+	+
D-Glucose	+	+	+	+	+
D-Mannitol	+	+	+	+	+
D-Mannose	+	+	+	+	+
l-Arabinose	+	+	+	+	+
m-Inositol	-	-	+	ND	+
2-Aminoethanol	+	+	V	ND	ND
L-Serine	V	+	+	ND	+
a-Ketovaleric acid	V	+	-	ND	-
a-Ketoglutaric acid	V	+	V	ND	ND
N-Acetylglucosamine	+	+	+	+	+
Trisodium citrate	V	+	V	-	-
Growth in O/F medium					
D-Glucose	-	-	-	-	-
DNA G + C content	62.0	62.0	61.8	62.0	62.0

[a] The results of the various properties were coded as follows: RN refers to root nodule; RH refers to rhizosphere; + indicates that all isolates test positive; - indicates that all isolates test negative; V indicates that the results are variable; ND indicates that no data were available.

[b] Strains are numbered as follows: 1. *Paraburkholderia strydomiana* sp. nov. (represented by isolates WK1.1f^T, WK1.1k, WK1.1a, WK1.1j, WK1.1i, WK1.1h, WK1.1d and WK1.1m); 2. *P. steynii* sp. nov. (represented by HC1.1ba^T, WK1.1c, HC1.1bb and HC1.1a3); 3. *P. kirstenboschensis* LMG 28727^T; 4. *P. caledonica* LMG 19076^T; 5. *P. terrae* LMG 23368^T

Fatty acid analysis revealed the presence of $C_{16:0}$ in strains WK1.1f^T and HC1.1ba^T, respectively representing the eight and four isolate groups, which is consistent with what has been reported for members of *Burkholderia* sensu lato (Yabuuchi et al. 1992; Garrity et al. 2005). Both strains WK1.1f^T and HC1.1ba^T were found to contain a small percentage of an unidentified fatty acid with a chain-length value of 10.928 or possibly 12:0 ALDE, which has also been seen in *Paraburkholderia fungorum* and *P. caledonica* (Coenye et al. 2001). Supplementary Table S2 contains details of the major fatty acids identified in the two proposed type strains.

Discussion

We used a six-step polyphasic approach that incorporates the principles of genealogical concordance for delineating putative species hypotheses (Venter et al. 2017) for a group of 12 isolates resulting from a previous study (Beukes et al. 2013). In the previous study two widely used taxonomic markers (16S rRNA and *recA* gene sequences) were used to identify putative species boundaries (Weir et al. 2004; Vinuesa et al. 2005; Shams et al 2013) from a collection of fynbos-associated *Paraburkholderia* symbionts. This allowed delineation of two distinct groups, each containing a known species of *Paraburkholderia* (i.e., *P. caledonica* and *P. terrae*, respectively). Interrogation of these initial species boundaries were accomplished with

steps 2-5 of the Venter et al. (2017) workflow. DNA sequences for multiple independent loci were obtained (step 2), where we specifically used those for protein-coding genes that are commonly employed for taxonomic purposes in *Paraburkholderia* or *Burkholderia* sensu lato (Spilker et al. 2009; Estrada-de los Santos et al. 2013; Steenkamp et al. 2015). The next three steps involved inference and comparison of the five single-gene genealogies to examine the validity of the initially identified species hypotheses. All genealogies showed that the isolates designated here as *P. strydomiana* sp. nov. (i.e., the eight isolate group) represent a unique group that excluded other known species (including *P. caledonica* that was initially thought to be part of this species hypothesis). These genealogies also showed that the four isolate group represents a unique taxon that is distinct from all known species, here designated *P. steynii* sp. nov. The only exception was observed in the *recA* tree, in which *P. terrae* grouped with our *P. steynii* sp. nov. isolates. This was most likely due to the highly conserved nature of *recA* and incomplete lineage sorting subsequent to the divergence of the two species (Galtier and Daubin 2008; Venter et al. 2017), although horizontal gene transfer of this locus between the species cannot be excluded (Venter et al. 2017).

The final step of the Venter et al. (2017) workflow involved testing our species hypotheses for *P. strydomiana* sp. nov. and *P. steynii* sp. nov. with additional biological and genetic information. Both species hypotheses were not contradicted by the results of our DDH experiments and ANI comparisons. For the analyses comparing *P. strydomiana* sp. nov. and *P. steynii* sp. nov. with other known species (including *P. caledonica* and *P. terrae*), DDH similarity values were all below the 70% guideline value for species delineation (Goris et al. 2007; Achtman and Wagner 2008). Consistent with this, ANI values below 95% were obtained in comparisons between the draft genomes of the two proposed new type strains and those of closely related species, although the ANI comparisons between the proposed type strains and

their closest known phylogenetic neighbours were within the cut-off range of 95-96% (Richter and Rosselló-Móra 2009; Arahal 2014); exceptions in ANI cut-off values for different groups have been noted (e.g. Steenkamp et al. 2015; Ciufo et al. 2018; Shapiro 2018) and the high genome similarity could be an indication of recent divergence (Shapiro 2018), between *P. strydomiana* sp. nov. and *P. caledonica*, and *P. steynii* and *P. terrae*.

We are aware that some researchers would consider these isolates as subspecies of *P. caledonica* and *P. terrae*, however we would caution against implementing this view due to the significant differences in biology between these isolates and their closest neighbours. *Paraburkholderia strydomiana* sp. nov. and *P. steynii* sp. nov. are both root-nodulating plant symbionts whereas the type strains for *P. caledonica* NBRC 102488^{T} and *P. terrae* NBRC 100964^{T} do not possess this ability. Also, the genomes of *P. caledonica* and *P. terrae* do not contain any of the common nodulation loci *nodABCD* (data not shown; as viewed on GenBank). The only exceptions are *nodI* and *nodJ*, but these genes have functions in both nodulating and non-nodulating bacteria (Aoki et al. 2013).

For the intraspecies comparisons of isolates belonging to *P. strydomiana* sp. nov. and *P. steynii* sp. nov., DDH similarity values exceeding 70% were retrieved. The suitability of these cut-off values has also been shown previously for *Paraburkholderia* species (e.g., De Meyer et al. 2013b, 2014; Baek et al. 2015; Steenkamp et al. 2015; Bournaud et al. 2017). Several biochemical properties also supported the *P. strydomiana* sp. nov. and *P. steynii* sp. nov. species hypotheses. For example, *P. strydomiana* sp. nov. can be differentiated from its near neighbours (*P. caledonica* and *P. kirstenboschensis*) by the assimilation of adipic acid and m-inositol, while *P. steynii* sp. nov. can be differentiated from its near neighbour *P. terrae* by nitrate reduction, the assimilation of adipic acid, m-inositol, citrate and valerate. Taken

together, this study thus presents independent lines of evidence, based on multiple types of genetic and biological data, for supporting *P. strydomiana* sp. nov. and *P. steynii* sp. nov. as novel and stably delimited taxa (Dayrat 2005; Kämpfer and Glaeser 2012).

Based on our multigene phylogeny (see Fig. 2 and Supplementary Figure S7), *P. strydomiana* sp. nov. and *P. steynii* sp. nov. are both related to known rhizobial species. *P. strydomiana* is closely related to other nodulators (*P. kirstenboschensis*, *P. dilworthii* and *P. rhynchosiae*) from South Africa (De Meyer et al. 2013b, 2014; Steenkamp et al. 2015), while *P. steynii* is related to various *Mimosa*-nodulating species (*Paraburkholderia diazotrophica*, *Paraburkholderia piptadeniae*, *P. phymatum* and *Paraburkholderia caribensis* (Vandamme et al. 2002; Elliott et al. 2007; Sheu et al. 2013; Bournaud et al. 2017). However, both *P. strydomiana* sp. nov. and *P. steynii* sp. nov. are closely related to non-nodulators. The closest known relative of *P. strydomiana* is the environmental species *P. caledonica* (Coenye et al. 2001). This species has been reported from rhizosphere soils in the UK, Scotland, the Netherlands and South Africa, as well as leaf endophytes of Rubiaceae from several countries in Sub-Saharan Africa (including South Africa) (Coenye et al. 2001; Verstraete et al. 2014). The closest known relatives of *P. steynii* are *P. terrae* (Yang et al. 2006) and *P. hospita* (Goris et al. 2002). The type strain of *P. terrae* is diazotrophic and was first isolated from forest soil in South Korea (Yang et al. 2006), but the species predominantly receives attention for the strain BS001 that is a symbiont of the fungus *Lyophyllum* sp. strain Karsten, for which it also provides antifungal protection (Nazir et al. 2013; Nazir et al. 2014). *P. hospita* was originally isolated from agricultural soil from Belgium (Goris et al. 2002) and information regarding its biology and distribution is limited.

The majority of the strains defined here as belonging to *P. strydomiana* sp. nov. were originally isolated from *H. sophoroides* nodules resulting from trapping experiments and nodulation was proven on either siratro (*Macroptilium atropurpureum*) or cowpea (*Vigna unguiculata*) (Beukes et al. 2013). Three of the four strains defined here as belonging to *P. steynii* sp. nov. originated from nodules resulting from trapping experiments performed with *H. sophoroides*, while all strains tested positive for nodulation on either cowpea (*Vigna unguiculata*) or siratro (*Macroptilium atropurpureum*) (Beukes et al. 2013). The findings presented in this study thus expand the known range of rhizobia with which *H. sophoroides* associates to three *Paraburkholderia* species, i.e., *P. strydomiana* sp. nov., *P. steynii* sp. nov. and *P. kirstenboschensis*. Based on previous work (Beukes et al. 2013), this legume probably associates with at least two additional *Paraburkholderia* species that still require characterisation. All of these *H. sophoroides* symbionts were obtained from the same collection site at two or three different time points, which suggest that the exploration of more sites could reveal the existence of additional symbionts for this legume. Furthermore, irrespective of their species identities, the *Paraburkholderia* isolates interacting with this plant bear one of at least two forms of the symbiotic *nifH*- and *nodA* locus. This suggests diversity in the locus determining the *H. sophoroides* interaction and that it may be maintained and expressed in multiple genomic backgrounds (i.e., species). As the symbiosis enables legumes to grow under adverse environmental conditions (Thrall et al. 2000, 2005; Klock et al. 2015), it can be suggested that being able to interact with multiple rhizobial partners under the nutrient-poor conditions of the Fynbos biome would be advantageous for *H. sophoroides*. Also, *Paraburkholderia* symbionts of legumes in the Fynbos biome typically do not have many strict host interactions (Lemaire et al. 2015). Further research is, however, required to determine whether *P. strydomiana* and *P. steynii* follow the same pattern and also nodulate legumes other than *H. sophoroides*.

Description of *Paraburkholderia strydomiana* sp. nov.

Paraburkholderia strydomiana (stry.do.mi.a'na. N.L. fem. adj. *strydomiana*, named in honour of Professor Barend (Ben) Wilhelm Strydom (1932-2018) for his contributions towards bacteriology in South Africa).

Cells are Gram-negative, motile rods that are 0.6-0.7 µm wide and 1.5-2.0 µm long. Grows well on Yeast Mannitol Agar, Tryptone Yeast Agar and Tryptone Soy Agar at 28 ºC. Grows in TYB containing 0.5 and 1% NaCl. Most strains can grow in the pH range 4-9. Growth is observed on MacConkey agar at 29 ºC and 37 ºC. Positive for catalase and oxidase. Positive for the assimilation of D-glucose, L-arabinose, D-mannose, D-mannitol, N-acetyl glucosamine, potassium gluconate, capric acid, adipic acid, malic acid, trisodium citrate and phenyl acetic acid. No assimilation of D-maltose and L-tryptophan, while D-glucose fermentation also does not occur. Negative for the liquefaction of gelatin as well as for the hydrolysis of aesculin. Positive for β-glucosidase activity and negative for urease activity. Unable to reduce nitrate to nitrite (given by the negative NO_3 and N_2 potassium nitrate tests). Positive for the oxidation of L-arabinose, D-arabitol, D-fructose, L-fucose, L-rhamnose and D-sorbitol while the oxidation of adonitol, D-cellobiose, α-D-lactose, maltose, D-melibiose, D-raffinose, sucrose and xylitol is negative. The major fatty acids are $C_{16:0}$, $C_{16:1}$ ω7c and/or $C_{16:1}$ ω6c, $C_{17:0}$ cyclo and $C_{18:1}$ ω7c. The DNA G + C content of the type strain is 61.6 % as determined from the draft genome.
The type strain, WK1.1f^T (= LMG 28731^T = SARCC1213^T), was isolated from rhizosphere soil associated with *Hypocalyptus sophoroides* located in Old du Toit's Kloof Pass (Western Cape Province, South Africa) in 2004.

Description of *Paraburkholderia steynii* sp. nov.

Paraburkholderia steynii (ste.y'ni.i. N.L. gen. n. *steynii*, of Steyn, in honour of Professor Pieter Lodewikus Steyn (1936 -), who initiated the research on indigenous nitrogen-fixing bacteria at the University of Pretoria).

Cells are Gram-negative, motile rods that are 0.7-0.8 µm wide and 2.3-2.7 µm long. Grows well on Yeast Mannitol Agar, Tryptone Yeast Agar and Tryptone Soy Agar at 28 ^{o}C. Grows in TYB containing 0.5 and 1% NaCl, while no growth is observed on MacConkey agar at 29 ^{o}C and 37 ^{o}C. Can grow in the pH range 4-9. Positive for catalase and oxidase. Positive for the assimilation of D-glucose, L-arabinose, D-mannose, D-mannitol, N-acetyl glucosamine, potassium gluconate, capric acid, adipic acid, malic acid, trisodium citrate and phenyl acetic acid. No assimilation of D-maltose, L-tryptophan and no D-glucose fermentation. Negative for the liquefaction of gelatin as well as for the hydrolysis of aesculin. Positive for β-glucosidase and urease activity. Positive in the NO_3 potassium nitrate test but negative for the N_2 potassium nitrate test. Positive for the oxidation of adonitol, D-cellobiose, D-trehalose, L-arabinose, D-arabitol, D-fructose, L-fucose, L-rhamnose and D-sorbitol, while the oxidation of α-D-lactose, maltose, D-melibiose, D-raffinose, sucrose and xylitol is not observed. The major fatty acids are $C_{16:0}$, $C_{16:1}$ ω7c and/or $C_{16:1}$ ω6c, $C_{17:0}$ cyclo, $C_{18:1}$ ω7c and $C_{19:0}$ cyclo ω8c. The DNA G + C content of the type strain is 61.8 % as determined from the draft genome.

The type strain, HC1.1ba^{T} (= LMG 28730^{T} = SARCC696^{T}), was recovered from rhizosphere soil associated with *Hypocalyptus sophoroides* from Old du Toit's Kloof Pass (Western Cape Province, South Africa) in 2006.

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Supplementary Information

Supplementary Table S1. GenBank accession numbers for the sequences generated in this study as well as for the 12 *Paraburkholderia* isolates being investigated

Supplementary Table S2. Metabolic, biochemical and fatty acid properties according to Biolog GN2, API 20NE tests and as determined by gas chromatography. Results include all the strains of the two novel species, except for the fatty acid analysis which was only performed on the respective type strains.

Supplementary Table S3. Percentages of genomes analysed during ANI analyses (number of nucleotides/ number of fragments).

Supplementary Table S4. Comparative genome characteristics of *P. strydomiana* sp. nov., *P. steynii* sp. nov. and their closest phylogenetic neighbours

Supplementary Figure S1. One of nine most parsimonious tree inferred from the analysis of *recA* sequences of a group of 12 *Paraburkholderia* isolates associated with *Hypocalyptus sophoroides*. The length of the tree is 739 steps, the consistency index is 0.30, the retention index is 0.69 and the homoplasy index is 0.70. Statistical support for the groupings are shown in the order MP/ML and only support ≥ 50% are listed. Information regarding the type strain,

GenBank accession number/locus tag for each species is indicated in brackets, while the number accompanied by * is a GOLD Project ID. Species names which appear in inverted commas (‘…’) are combinations which have not yet been validly published. The scale bar corresponds to 10 character state changes.

Supplementary Figure S2. One out of two most parsimonious trees inferred from the analysis of *atpD* sequences of a group of 12 *Paraburkholderia* isolates associated with *Hypocalyptus sophoroides*. The length of the tree is 1076 steps, the consistency index is 0.32, the retention index is 0.70 and the homoplasy index is 0.68. Statistical support for the groupings is shown in the order MP/ML and only support ≥ 50% is listed. Information regarding the type strain, GenBank accession number/locus tag for each species is indicated in brackets, while the number accompanied by * is a GOLD Project ID. Species names which appear in inverted commas (‘…’) are combinations which have not yet been validly published. The scale bar corresponds to 20 character state changes.

Supplementary Figure S3. One of 12 most parsimonious trees inferred after analysing the *recA* sequences of the 12 *Paraburkholderia* isolates associated with *Hypocalyptus sophoroides*. The tree has a length of 793 steps, a consistency index of 0.31, a retention index of 0.69, and homoplasy index of 0.69. Bootstrap support is indicated only when ≥ 50% and appear in the order MP/ML. Listed in brackets is the type strain and GenBank accession number/locus tag for each species, while the number accompanied with * is a GOLD Project ID. Species appearing in inverted commas (‘…’) are combinations which have not yet been validly published. The scale bar corresponds to 10 character state changes.

Supplementary Figure S4. One of two most parsimonious tree resulting from the analysis of *rpoB* sequences from 12 *Paraburkholderia* isolates associated with *Hypocalyptus sophoroides*. The tree length is 830 steps, it has a consistency index of 0.28, a retention index of 0.65 and a homoplasy index of 0.70. Bootstrap support is indicated in the order MP/ML and only support of ≥ 50% is shown. Information regarding the type strain, GenBank accession number/locus tag of each species is listed in brackets, while * denotes a GOLD Project ID. Species names in inverted commas ('…') are combinations which have not yet been validly published. The scale bar corresponds to 10 character state changes.

Supplementary Figure S5. One of three most parsimonious trees resulting from the analysis of *lepA* sequences for the 12 *Paraburkholderia* isolates associated with *Hypocalyptus sophoroides* in this study. This tree is 1502 steps long, has a consistency index of 0.30, a retention index of 0.64, and a homoplasy index of 0.70. Bootstrap support is indicated in the order MP/ML and only support of ≥ 50% are shown. Type strain and GenBank accession number/locus tag for each species is listed in brackets, while * denotes a GOLD Project ID. Species in inverted commas ('…') are combinations awaiting valid publication. The scale bar corresponds to 20 character state changes.

Supplementary Figure S6. The most parsimonious tree inferred from the analysis of the *gltB* sequences of 12 *Paraburkholderia* isolates associated with *Hypocalyptus sophoroides*. The tree has a length of 648, a consistency index of 0.33, a retention index of 0.69 and homoplasy index of 0.67. Statistical support for the groupings are indicated in the order MP/ML and only support of ≥ 50% are shown. Listed in brackets is the type strain and GenBank accession numbers/locus tags for each species, while * indicates a Gold Project ID. Species names in inverted commas

('…') are combinations which are still awaiting valid publication. The scale bar corresponds to 10 character state changes.

Supplementary Figure S7. One of four most parsimonious trees inferred from analysing a five gene concatenated dataset (*atpD* + *recA* + *rpoB* + *gltB* + *lepA*) including the 12 focal *Paraburkholderia* species. The tree length is 5059 steps, has a consistency index of 0.29, a retention index of 0.64 and a homoplasy index of 0.71. Statistical support of ≥ 50% is indicated in the order MP/ML. Listed in the brackets are the type strain number for each species. Species names in inverted commas ('…') are combinations which are still awaiting valid publication. The scale bar corresponds to 50 character state changes.

Supplementary Figure S8. A. Maximum likelihood phylogeny of a segment of the *nifH* locus for all available *H. sophoroides* associated rhizobial isolates. The tree is rooted at the midpoint, while the key indicates to which *Paraburkholderia* species the isolate belongs. Listed in brackets is the GenBank accession number for that isolate. The scale bar indicates the number of nucleotide changes per site. **B.** Maximum likelihood phylogeny of a fragment of the *nodA* locus for all available *H. sophoroides* rhizobial isolates. Listed in brackets are the GenBank accession number for the isolate. The tree is rooted at the midpoint and follows the same key as for part A. The scale bar indicates the number of nucleotide changes per site. The *nifH* sequence for isolate WK1.1k and the *nodA* sequence for WK1.1e were not available.

Conflict of Interest: The authors declare that they have no conflict of interest.

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