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► **To cite this version:**

Bram Danneels, Monique Blignaut, Guillaume Marti, Simon Sieber, Peter Vandamme, et al.. Cyclitol secondary metabolism is a central feature of Burkholderia leaf symbionts. 2024. hal-04685355

HAL Id: hal-04685355

<https://hal.inrae.fr/hal-04685355v1>

Preprint submitted on 3 Sep 2024

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1 TITLE : Cyclitol secondary metabolism is a central feature of *Burkholderia* leaf symbionts

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23 *Abstract*

24 The symbioses between plants of the Rubiaceae and Primulaceae families with *Burkholderia*
25 bacteria represent unique and intimate plant-bacterial relationships. Many of these
26 interactions have been identified through PCR-dependent typing methods, but there is little
27 information available about their functional and ecological roles. We assembled seventeen
28 new endophyte genomes representing endophytes from thirteen plant species, including
29 those of two previously unknown associations. Genomes of leaf endophytes belonging to
30 *Burkholderia s.l.* show extensive signs of genome reduction, albeit to varying degrees. Except
31 for one endophyte, none of the bacterial symbionts could be isolated on standard
32 microbiological media. Despite their taxonomic diversity, all endophyte genomes contained
33 gene clusters linked to the production of specialized metabolites, including genes linked to
34 cyclitol sugar analog metabolism and in one instance non-ribosomal peptide synthesis. These
35 genes and gene clusters are unique within *Burkholderia s.l.* and are likely horizontally
36 acquired. We propose that the acquisition of secondary metabolite gene clusters through
37 horizontal gene transfer is a prerequisite for the evolution of a stable association between
38 these endophytes and their hosts.

39 *Introduction*

40 Interactions with microbes play an important part in the evolution and ecological success of
41 plants. For example, mycorrhizal associations are present in a vast majority of land plants,
42 and the association with nitrogen-fixing bacteria provided legumes with an important
43 evolutionary advantage (Brundrett, 1991; van Rhijn and Vanderleyden, 1995; Vessey *et al.*,
44 2005; Smith and Read, 2008). Nevertheless, microbes may also be harmful for plants as
45 microbial pathogen interactions are responsible for major crop losses (Dangl and Jones,
46 2001; McCann, 2020). Many plant-microbe interactions only occur temporarily: contacts
47 between microbes and the host are often limited to a sub-population or a specific
48 developmental phase of the host. However, in some associations microbes are transferred
49 from parents to offspring in a process called vertical transmission, resulting in permanent
50 associations with high potential for co-evolution (Gundel *et al.*, 2017). While vertically-
51 transmitted microbes are common in the animal kingdom, they have been more rarely
52 described in plants (Fisher *et al.*, 2017).

53 A particular case of vertically transmitted microbes in plants are the bacterial leaf
54 endophytes found in three different plant families: the monocot Dioscoreaceae, and the
55 dicot Rubiaceae and Primulaceae. In the genera *Psychotria*, *Pavetta*, *Sericanthe* (Rubiaceae)
56 and *Ardisia* (Primulaceae) this association may manifest in the form of conspicuous leaf
57 nodules that house extracellular symbiotic bacteria (Miller, 1990; Van Oevelen *et al.*, 2002;
58 Lemaire, Robbrecht, *et al.*, 2011; Lemaire, Van Oevelen, *et al.*, 2012; Ku and Hu, 2014). In
59 some of these systems, the symbiont was detected in seeds, indicating that they can be
60 transmitted vertically (Miller and Donnelly, 1987; Sinnesael *et al.*, 2018). Molecular analysis
61 of the leaf nodules revealed that all endophytes are members of the *Burkholderia sensu lato*,
62 more specifically to the newly defined *Caballeronia* genus (Van Oevelen *et al.*, 2002; Ku and
63 Hu, 2014). Similar leaf endophytes, also belonging to the *Burkholderiaceae*, are present in
64 Rubiaceae species that do not form leaf nodules, including some *Psychotria* species (Lemaire,
65 Lachenaud, *et al.*, 2012; Verstraete *et al.*, 2013). To date, only one symbiont of Rubiaceae
66 and Primulaceae has been cultivated: the endophyte of *Fadogia homblei*, which has been
67 identified as *Paraburkholderia caledonica* (Verstraete *et al.*, 2011). Interestingly, members of
68 *P. caledonica* are also commonly isolated from the rhizosphere or soil and have been
69 detected in leaves of some *Vangueria* species (Verstraete *et al.*, 2014).

70 Speculations about possible functions of these leaf symbioses have long remained
71 unsubstantiated because efforts to isolate leaf nodule bacteria or to culture bacteria-free
72 plants were unsuccessful (Miller, 1990). Recently, sequencing and assembly of leaf symbiont
73 genomes of several *Psychotria*, *Pavetta* or *Ardisia* species allowed new hypotheses about the
74 ecological function of leaf symbiosis. Leaf symbiotic *Burkholderia* of *Ardisia crenata*, are
75 responsible for the production of FR900359, a cyclic depsipeptide with potent bioactive and
76 insecticidal properties (Fujioka *et al.*, 1988; Carlier *et al.*, 2016). Similarly, analysis of the
77 genome of *Ca. Burkholderia kirkii* (*Ca. B. kirkii*), the leaf symbiont of *Psychotria kirkii*,
78 revealed a prominent role of secondary metabolism (Carlier and Eberl, 2012). In this species,
79 two biosynthetic gene clusters harboured on a plasmid encode two homologs of a 2-*epi*-5-
80 *epi*-valiolone synthase (EEVS). EEVS are generally required for the production of cyclitol
81 sugar analogs, a family of bioactive natural products with diverse targets (Mahmud, 2003,
82 2009). *Ca. B. kirkii* is likely involved in the synthesis of two cyclitol metabolites: kirkamide, a
83 C₇N aminocyclitol with insecticidal properties, and streptol glucoside, a derivative of valienol

84 with broad allelopathic activities (Sieber *et al.*, 2015; Georgiou *et al.*, 2021). Similar gene
85 clusters containing putative EEVS were also detected in the genomes of other *Psychotria* and
86 a *Pavetta* leaf symbionts (Pinto-Carbó *et al.*, 2016), further highlighting the importance of
87 cyclitol compounds in these leaf symbioses.

88 C₇ cyclitols are a group of natural products derived from the pentose phosphate pathway
89 intermediate sedoheptulose-7-phosphate (SH7P) (Mahmud, 2003). Proteins of the sugar
90 phosphate cyclase family are key enzymes in the synthesis of C₇ cyclitols. Enzymes of this
91 family catalyse the cyclization of sugar compounds, an important step in primary and
92 secondary metabolism (Wu *et al.*, 2007). Within this family, three main categories of
93 enzymes use SH7P as a substrate: desmethyl-4-deoxygadusol synthase (DDGS), 2-*epi*-
94 valiolone synthase (EVS) and 2-*epi*-5-*epi*-valiolone synthase (EEVS), of which EEVS is the only
95 known enzyme involved in C₇N aminocyclitol synthesis (Osborn *et al.*, 2017). EEVS were
96 originally only found in bacteria, where they catalyse the first step in the biosynthesis of C₇N
97 aminocyclitol secondary metabolites (Mahmud, 2003; Sieber *et al.*, 2015). More recently,
98 EEVS homologs have been discovered in some Eukaryotes such as fish, reptiles, and birds as
99 well (Osborn *et al.*, 2015, 2017).

100 A second common feature of the leaf endophytes in Rubiaceae and Primulaceae is their
101 reduced genomes. Leaf nodule *Burkholderia* symbionts of Rubiaceae and Primulaceae
102 typically have smaller genomes than free-living relatives, as well as a lower coding capacity
103 (Pinto-Carbó *et al.*, 2016). This reductive genome evolution is thought to be a result of
104 increased genetic drift sustained in bacteria that are strictly host-associated, which leads to
105 fixation of deleterious and/or neutral mutations and eventually to the loss of genes
106 (Pettersson and Berg, 2007). This process is best documented in obligate insect symbionts
107 such as *Buchnera* and *Serratia*, endosymbionts of aphids, or in *Sodalis*-allied symbionts of
108 several insect groups (Shigenobu *et al.*, 2000; Toh *et al.*, 2006; Manzano-Marín *et al.*, 2018).
109 Some of these symbionts have extremely small genomes and may present an extensive
110 nucleotide bias towards adenosine and thymine (AT-bias) (Moran *et al.*, 2008). The process
111 of genome reduction has multiple stages: first, recently host-restricted symbionts begin
112 accumulating pseudogenes and insertion elements (McCutcheon and Moran, 2012; Lo *et al.*,
113 2016; Manzano-Marín and Latorre, 2016). Non-coding and selfish elements eventually get
114 purged from the genomes over subsequent generations, which together with the general

115 deletional bias in bacteria results in a decrease in genome size (Mira *et al.*, 2001). This
116 ultimately leads to symbionts with tiny genomes, with only a handful of essential genes
117 necessary for survival or performing their role in the symbiosis. This process has been well
118 documented in the leaf nodule symbionts of *Psychotria*, *Pavetta* and *Ardisia* species, but
119 little is known about the genomes and functions of endophytes in species that do not form
120 leaf nodules, notably Rubiaceae species of the *Vangueria* and *Fadogia* genera.

121 Here, we performed a comparative study of Rubiaceae and Primulaceae leaf endophytes
122 from leaf nodulating and non-nodulating plant species using genomes assembled from
123 shotgun metagenome sequencing data as well as isolates. We constructed a dataset of 26
124 leaf symbiont genomes (of which 17 new genomes from this study) from 22 plant species in
125 5 genera. All leaf symbionts show signs of genome reduction, in varying degree, and
126 horizontal acquisition of secondary metabolite clusters is a universal phenomenon in these
127 bacteria.

128 *Material and Methods*

129 *Sample collection and DNA extraction*

130 Leaves of Rubiaceae and Primulaceae species were freshly collected from different locations
131 in South Africa or requested from the living collection of botanical gardens (Table S1).
132 Attempts to isolate the endophytes were made for all fresh samples collected in South Africa
133 (Table S1). Leaf tissue was surface sterilized using 70% ethanol, followed by manual grinding
134 of the tissue in 0.4% NaCl. Supernatants were plated on 10% tryptic soy agar medium (TSA,
135 Sigma) and R2A medium (Oxoid) and incubated at room temperature for 3 days or longer
136 until colonies appeared. Single colonies were picked and passaged twice on TSA medium.
137 Isolates were identified by PCR and partial sequencing of the 16S rRNA gene using the pA/pH
138 primer pair (5'-AGAGTTTGATCCTGGCTCAG and 5'-AAGGAGGTGATCCAGCCGCA) (Edwards *et al.*
139 *et al.*, 1989). PCR products were sequenced using the Sanger method at Eurofins Genomics
140 (Ebersberg, Germany).

141 DNA was extracted from whole leaf samples as follows. Whole leaves were ground in liquid
142 nitrogen using a mortar and pestle. Total DNA was extracted using the protocol of Inglis *et al.*
143 *et al.* (Inglis *et al.*, 2018). Total DNA from a *Fadogia homblei* isolate was extracted following
144 Wilson (Wilson, 2001). Sequencing library preparation and 2x150 paired-end metagenome

145 sequencing was performed by the Oxford Wellcome Centre for Human Genetics or by
146 Novogene Europe (Cambridge, UK) using the Illumina NovaSeq 6000. Sequencing reads were
147 classified using Kraken v2.1.2 against a custom database comprising complete prokaryotic
148 and plastid genome sequences deposited NCBI RefSeq (accessed 4/4/2021), and visualised
149 using KronaTools v2.7.1 (Ondov *et al.*, 2011; Wood *et al.*, 2019).

150 *Bacterial genome assembly*

151 Sequencing reads were trimmed and filtered using fastp v0.21.0 with default settings (Chen
152 *et al.*, 2018). Overlapping paired-end reads were merged using NGmerge with default
153 settings (Gaspar, 2018). For sequencing reads derived from new leaf samples, metagenome
154 assemblies were created using metaSPAdes v3.15 on default settings but including the
155 merged reads (Nurk *et al.*, 2017). Metagenomes were binned using Autometa, using a
156 minimal contig length of 500 bp, taxonomy filtering (-m) and maximum-likelihood
157 recruitment (using the -r option)(Miller *et al.*, 2019). Genome bins identified as *Caballeronia*,
158 *Paraburkholderia*, or *Burkholderia* by Autometa were further assembled by mapping the
159 original reads to these bins using smalt v0.7.6 (Ponsting and Ning, 2010). Mapped reads
160 were extracted using samtools v1.9 (Li *et al.*, 2009) and reassembled using SPAdes v3.15
161 (Bankevich *et al.*, 2012) in default settings but using the --careful option, and binned again
162 using Autometa. Contigs likely derived from eukaryotic contamination were removed after
163 identification by blastn searches (e-value < 1e⁻⁶) against the NCBI nucleotide database
164 (accessed January 2021) (Camacho *et al.*, 2009). Per-contig coverage information was
165 calculated using samtools and contigs with less than 10% or more than 500% of the average
166 coverage were manually investigated, and sequences likely derived from other bacterial or
167 eukaryotic genomes were removed. Genome assembly for reads derived from isolates were
168 assembled using Skesa v2.4.0 using default settings (Souvorov *et al.*, 2018). Assembly
169 statistics were compiled using Quast v5.1.0 (Gurevich *et al.*, 2013).

170 To provide a more homogenous dataset for comparative genomics, Illumina read data for six
171 previously published Rubiaceae symbionts, and the symbionts of *Ardisia crenata* and
172 *Fadogia homblei* were re-assembled as above but using the published draft genomes as
173 trusted contigs for both metaSPAdes and SPAdes assemblies (Table S2). The resulting
174 assemblies were compared to the published assemblies using dotplots created by MUMmer
175 (Marçais *et al.*, 2018). Genome assemblies of the symbionts of *Psychotria kirkii* (Carlier and

176 Eberl, 2012; Carlier *et al.*, 2013) and *Psychotria punctata* (Pinto-Carbó *et al.*, 2016) were
177 downloaded from Genbank (Table S2). To assess whether the (re-)assembled genomes
178 represent new species, genomes were analysed using TYGS (Type Strain Genome Server)
179 (Meier-Kolthoff and Göker, 2019), and NCBI Blastn-based Average Nucleotide Identities (ANI)
180 values calculated using the JSpecies web server (Richter *et al.*, 2016) and the pyANI python
181 package (<https://github.com/widdowquinn/pyani>).

182 *Genome annotation and pseudogene prediction*

183 Assembled genomes were annotated using the online RASTtk pipeline (Brettin *et al.*, 2015),
184 using GenemarkS as gene predictor, and locus tags were added using the Artemis software
185 v18.1.0 (Carver *et al.*, 2012). Prediction of pseudogenes was performed using an updated
186 version of the pseudogene prediction pipeline previously used for leaf symbionts (Carlier *et al.*,
187 2013). Briefly, orthologs of predicted proteins sequences of each genome in a dataset of
188 published *Burkholderia* genomes (Table S3) were determined using Orthofinder v2.5.2
189 (Emms and Kelly, 2019) with default settings. The nucleotide sequences of each gene,
190 including 200bp flanking regions, were aligned to the highest scoring sequence in each
191 orthogroup using TFASTY v3.6 (Pearson, 2000). Genes were considered as pseudogenes if
192 the alignment spanned over 50% of the query protein and the query protein contained a
193 frameshift, or a nonsense mutation resulting in an uninterrupted alignment shorter than
194 80% of the target sequence. Moreover, ORFs were classified as non-functional if at least one
195 of the following criteria was true: amino acid sequence shorter than 50 residues which did
196 not cluster in an orthogroup, and sequence without any significant blastx hit against the
197 reference database (e-value cut off = 0.001); proteins without predicted orthologs in the
198 *Burkholderia* dataset, but which showed a blastx hit against the reference set in an
199 alternative reading frame; and finally proteins without any hit in the *Burkholderia* genome
200 database or in the NCBI nr database. Blastx and blastp searches were performed using
201 DIAMOND v2 (Buchfink *et al.*, 2021). For the genomes of the symbionts of *P. kirkii* and *P.*
202 *punctata* the original gene and pseudogene predictions were used. Insertion elements in
203 both newly assembled and re-assembled genomes were predicted using ISEScan v1.7.2.3
204 with default settings (Xie and Tang, 2017).

205 *Phylogenetic analysis*

206 16S rRNA sequences were extracted from the endophyte (meta)genomes using Barrnap v0.9
207 (<https://github.com/tseemann/barrnap>). For genomes where no complete 16S rRNA could
208 be detected, reads were mapped to the 16S rRNA gene of the closest relative with a
209 complete 16S rRNA sequence. These reads were assembled using default SPAdes (Prjibelski
210 *et al.*, 2020) using the --careful option. Near complete (>95%) 16S rRNA sequences could be
211 extracted using these methods, except for the hypothetical endophyte of *Pavetta revoluta*.
212 The 16S rRNA sequences were identified using the EzBiocloud 16S rRNA identification service
213 (<https://www.ezbiocloud.net/identify>). Phylogenetic analysis of the leaf endophytes and
214 *Burkholderia s.l.* genomes was performed using the UBCG pipeline v3.0 (Na *et al.*, 2018). The
215 pipeline was run using the default settings, except for the gap-cutoff (-f 80). The resulting
216 superalignment of 92 core genes was used for maximum-likelihood phylogenetic analysis
217 using RAxML, using the GTRGAMMA evolution model, and performing 100 bootstrap
218 replications (Stamatakis, 2014). Plastid reference alignments were created using Realphy
219 v1.12 using standard settings and the *Coffea arabica* chloroplast genome (NCBI accession
220 NC_008535.1) as reference (Bertels *et al.*, 2014). Published chloroplast genomes of *Ardisia*
221 *mamillata* (NCBI accession MN136062), *Psychotria kirkii* (NCBI accession KY378696), *Pavetta*
222 *abyssinica* (NCBI accession KY378673), *Pavetta schumanniana* (NCBI Accession MN851271),
223 and *Vangueria infausta* (NCBI accession MN851269) were also included in the alignment.
224 Phylogenetic trees were constructed using PhyML v3.3.3 with automatic model selection,
225 and 1000 bootstrap replicates (Guindon *et al.*, 2010). For plant species with uncertain
226 taxonomic identification, seven plant markers were extracted by blastn searches against the
227 metagenome: ITS, nad4, rbcL and rpl16 of *Pavetta abyssinica* (NCBI accessions MK607930.1,
228 KY492180.1, Z68863.1, and KY378673.1), matK from *Pavetta indica* (NCBI accession
229 KJ815920.1), petD from *Pavetta bidentata* (NCBI accession JN054223.1), and trnTF from
230 *Pavetta sansibarica* (NCBI accession KM592134.1).

231 Core-genome phylogenies of symbiont genomes were constructed by individually aligning
232 the protein sequences of all single-copy core genes using MUSCLE, back-translating to their
233 nucleotide sequence using T-Coffee v13.45 (Di Tommaso *et al.*, 2011), and concatenating
234 into one superalignment. Maximum-likelihood phylogenetic analysis was performed using
235 RAxML, using the GTRGAMMA evolution model, 100 bootstrap replicates, and using

236 partitioning to allow the model parameters to differ between genes. Phylogenetic trees
237 were visualised and edited using iTOL (Letunic and Bork, 2019).

238 *Comparative genomics*

239 Ortholog prediction between leaf symbiont genomes and a selection of reference genomes
240 of the *Burkholderia*, *Paraburkholderia* and *Caballeronia* genera (BPC-set; selected using NCBI
241 datasets tool (<https://www.ncbi.nlm.nih.gov/datasets/genomes>); Table S3) was performed
242 using Orthofinder v2.5.2 using default settings (Emms and Kelly, 2019). Core genome overlap
243 was visualised in Venn diagrams using InteractiVenn (Heberle *et al.*, 2015). Non-essential
244 core genes were identified by blastp searches against the database of essential genes
245 (DEG)(Zhang, 2004), identifying as putative essential genes ORFs with significant matches in
246 the database (e-value < $1e^{-6}$). Standardised functional annotation was performed using
247 eggNOG-mapper v2.1.2 (Huerta-Cepas *et al.*, 2019; Cantalapiedra *et al.*, 2021). Enrichment
248 of protein families in leaf symbiont genomes was determined by comparing the proportion
249 of members of leaf symbionts and the BPC-set in orthogroups. Enriched KEGG pathways
250 were identified by comparing the average per-genome counts of genes in every pathway
251 between leaf symbiont genomes and genomes from the BPC-set. Presence of motility and
252 secretion system clusters was investigated using the TXSScan models implemented in
253 MacSyFinder (Abby *et al.*, 2014, 2016). Homologues of the *Ca. B. kirkii* putative 2-*epi*-5-*epi*-
254 valiolone synthase (EEVS) were identified by blastp searches against the proteomes of the
255 leaf symbiont genomes (e-value cut-off: $1e^{-6}$). Putative EEVS genes were searched against
256 the SwissProt database, and functional assignment was done by transferring the information
257 from the closest match within the sugar phosphate cyclase superfamily (Schneider *et al.*,
258 2004; Osborn *et al.*, 2017). Contigs containing these genes were identified and extracted
259 using Artemis, and aligned using Mauve (López-Fernández *et al.*, 2015). Gene phylogenies
260 were constructed by creating protein alignments using MUSCLE followed by phylogenetic
261 tree construction using FastTree (Price *et al.*, 2009), including the protein sequences of three
262 closely related proteins in other species, determined by blastp searches against the RefSeq
263 protein database (accessed July 2021).

264 The data generated in this study have been deposited in the European Nucleotide Archive
265 (ENA) at EMBL-EBI under accession number PRJEB52430
266 (<https://www.ebi.ac.uk/ena/browser/view/PRJEB52430>).

267 *Results*

268 *Detection and identification of leaf endophytes*

269 To gain insight into potential association of various Primulaceae and Rubiaceae species with
270 *Burkholderia s.l.* endosymbionts, we collected samples from 16 Rubiaceae (1 *Fadogia* sp., 5
271 *Pavetta* spp., 2 *Psychotria* spp., and 8 *Vangueria* spp.) and 3 Primulaceae (3 *Ardisia* spp.)
272 species (Table S1). We extracted DNA from entire leaves and submitted the samples to
273 shotgun sequencing without pre-processing of the samples to remove host or organellar
274 DNA. We found evidence for endophytic *Burkholderia* in 14 out of 19 species investigated
275 (Table S1). In these samples, the proportion of sequencing reads identified as
276 *Burkholderiaceae* ranged from 5% to 57% of the total, except for the *Pavetta revoluta*
277 sample (0.4%) and 1 of 2 *Vangueria infausta* samples (0.9%). Analysis of 16S rRNA sequences
278 revealed 100% pairwise identity over 1529 bp suggesting that the same endophyte species
279 was present in both *V. infausta* samples. In *Pavetta revoluta*, the closest relative of the leaf
280 endophyte based on 16S rRNA sequence similarity was *Caballeronia calidae* (98.89% identity
281 over 808 bp; Table S1). Of the nine species with significant amounts of *Burkholderia* s.l. reads
282 and for which isolation attempts were made (Table S1), only the endophyte of *Fadogia*
283 *homblei* could be cultured (isolate R-82532). Leaf samples of four species (*Psychotria*
284 *capensis*, *Psychotria zombamontana*, *Pavetta ternifolia*, and *Pavetta capensis*) contained low
285 amounts of bacterial DNA (<2% of reads), and likely do not have stable symbiotic endophyte
286 associations. Seven percent of the reads obtained from the *Pavetta indica* sample were
287 classified as bacterial, but with a diverse range of taxa present indicating possible
288 contamination with surface bacteria (Figure S1). Plastid phylogenies indicated that samples
289 attributed to *Pavetta capensis* and *Pavetta indica* did not cluster with other *Pavetta* species
290 (Figure S2). Analysis of genetic markers revealed that our *Pavetta indica* sample was likely a
291 misidentified *Ixora* sp. Analysis of *Pavetta capensis* marker genes revealed the specimen is
292 likely part of the Apocynaceae plant family, with a 100% identity match against the *rbcl*
293 sequence of *Pleiocarpa mutica*. These samples were not taken into account in further
294 analyses.

295 Analysis of the 16S rRNA sequences extracted from metagenome-assembled genomes
296 (MAGs) identified all leaf endophytes as *Burkholderia s.l.* (Table S1). Phylogenetic analysis
297 shows that all endophytes of *Psychotria*, *Pavetta*, and *Ardisia* cluster within the genus

298 *Caballeronia*, while the endophytes of *Vangueria* and *Fadogia* belong to the
299 *Paraburkholderia* genus (Figure 1A). All endophytes of *Ardisia* are closely related to each
300 other and form a clade with *Caballeronia udeis* and *Caballeronia sordidicola*. Based on the
301 commonly used ANI (95-96%) cut-off, these endophytes are separate species from *C. udeis*
302 and *C. sordidicola* (ANI <94%; 16S rRNA sequence identity <98.4). The endophytes of *Ardisia*
303 *crenata* and *Ardisia virens* are very closely related and belong to the same species: *Ca.*
304 *Burkholderia crenata* (ANI >99%; 16S rRNA sequence identity 99.8%) (Table S4). Similarly,
305 the endophytes of *Ardisia cornudentata* and *Ardisia mamillata* belong to the same species
306 (ANI = 95.56%), which we tentatively named *Ca. Burkholderia ardisicola* (species epithet
307 from *Ardisia*, the genus of the host species, and the Latin suffix -*cola* (from L. n. *incola*),
308 dweller, see species description in Supplementary Information). Endophytes of *Psychotria*
309 and *Pavetta* are scattered across the *Caballeronia* phylogeny, but all are taxonomically
310 distinct from free-living species (Figure 1A; ANI <93% with closest non-endophyte relatives).
311 Each of these endophytes also represents a distinct bacterial species with pairwise Average
312 Nucleotide Identity (ANI) values below the commonly accepted species threshold of 95-96%,
313 except for *Ca. P. schumanniana* and *Ca. B. kirkii* whose genomes share 95.65% ANI (Table
314 S4). The endophytes of *Vangueria* and *Fadogia* form three distinct lineages of
315 *Paraburkholderia*. The endophytes of *Vangueria dryadum* and *Vangueria macrocalyx* are
316 nearly identical (ANI >99.9%; identical 16S rRNA), but do not belong to any known
317 *Paraburkholderia* species (ANI <83% with closest relative *Paraburkholderia* species). We
318 tentatively assigned these bacteria to a new species which we named *Ca. Paraburkholderia*
319 *dryadicola* (from a Dryad, borrowed from the species epithet of one of the host species, and
320 Latin suffix -*cola*, see species description in Supplementary Information). Similarly, the
321 endophytes of *V. infausta*, *V. esculenta*, *V. madagascariensis*, *V. randii*, and *V.*
322 *soutpansbergensis* cluster together with *Paraburkholderia phenoliruptrix* (Figure 1A). While
323 the endophyte of *Vangueria soutpansbergensis* forms a separate species (named here *Ca.*
324 *Paraburkholderia soutpansbergensis*; ANI <95% with *P. phenoliruptrix*) the other endophytes
325 fall within the species boundaries of *P. phenoliruptrix*. (ANI 95-96% between these
326 endophytes and *P. phenoliruptrix*). Lastly, the endophytes of *Fadogia homblei* and *Vangueria*
327 *pygmaea* showed identical 16S rRNA sequences, and clustered with *Paraburkholderia*
328 *caledonica*, *P. strydomiana*, and *P. dilworthii* (Figure 1A). Similarly high ANI values (>97.5%)
329 and 16S rRNA sequence similarity (>99.7%) ambiguously fall within the species boundaries of

330 both *P. caledonica* and *P. strydomiana*. Because endophytes of *F. homblei* were previously
331 classified as *P. caledonica* (Verstraete *et al.*, 2011, 2014), we propose classifying the
332 endophytes of *F. homblei* and *V. pygmaea* as members of *P. caledonica*, and consider *P.*
333 *strydomiana* a later heterotypic synonym of *P. caledonica*.

334 Phylogenetic analysis based on the core genomes of endophytes indicates a general lack of
335 congruence with the host plant phylogeny (Figure S3). Endophytes of *Ardisia* are
336 monophyletic within the *Caballeronia* genus and follow the host phylogeny. In contrast,
337 endophytes of *Pavetta* are not monophyletic and are nested within the *Psychotria*
338 endophytes. Similarly, the *Fadogia homblei* endophyte clusters with endophytes of
339 *Vangueria*.

340 *Leaf endophyte genomes show signs of genome reduction.*

341 We could assemble nearly complete bacterial genomes for all samples where we detected
342 *Burkholderia* endophytes, except for those of the *Pavetta revoluta* and one *Vangueria*
343 *infausta* sample with too few bacterial reads. Binning analysis grouped endophyte
344 sequences in a single bin per sample, with high completeness (>95%) and purity (>97%).
345 Most assemblies ranged between 3.5 and 5 Mbp in size, with 2 outliers: 2.58 Mbp for *Ca. B.*
346 *crenata* Avir, and 8.92 Mbp for *P. caledonica* R-49542 (Table 1). The %G+C of all genomes fell
347 in the range of 59-64 %G+C, which is within the range of free-living *Paraburkholderia* and
348 *Caballeronia* genomes (Vandamme *et al.*, 2017). All genomes showed signs of ongoing
349 genome reduction. Because of rampant null or frameshift mutations, a large proportion of
350 predicted CDS code for non-functional proteins. As a result, coding capacity is low for all
351 endophyte genomes varying between 83% in *P. caledonica* R-49542 and 40% in *Ca. B.*
352 *ardisicola* Acor (Figure 1B, Table 1). In addition, insertion sequence (IS) elements make up a
353 large amount of the genomes: 1.97% of the assembly size on average, but up to almost 10%
354 in some symbionts of *Psychotria* (Table 1). Reassembly of previously investigated
355 endophytes of *Psychotria* and *Pavetta* yielded genomes of similar size to the original
356 assemblies, except for *Ca. Burkholderia schumanniana*. The original genome assembly size
357 was estimated at 2.4 Mbp, while our reassembly counted 3.62 Mbp. A dot plot between
358 both assemblies indicated that the size discrepancy is not solely due to differential
359 resolution of repeated elements (Figure S4). Thus, our new assembly includes 1.2 Mbp of
360 genome sequence that was missed in the original assembly.

361 *Burkholderia* leaf endophytes in Rubiaceae and Primulaceae shared a core genome of 607
362 genes (Figure S5). Even within specific phylogenetic lineages the core genomes were small:
363 774 genes in endophytes belonging to the *Caballeronia* symbionts of *Psychotria* and *Pavetta*,
364 1001 genes in endophytes of *Caballeronia* symbionts of *Ardisia*, and 1199 in
365 *Paraburkholderia* endophytes of *Fadogia* and *Vangueria*. This corresponds to 29.5%, 52.4%,
366 and 28.4% of the average functional proteome for each species cluster, respectively. Only 28
367 proteins of the total core genome did not show significant similarity with proteins from the
368 database of essential genes (Table S5). Eleven of these proteins have unknown functions and
369 a five are membrane-related. Fifteen genes of the endophyte core genome did not have
370 orthologs in >95% of related *Burkholderia*, *Caballeronia*, and *Paraburkholderia* genomes
371 (Table S6). No COG category was specifically enriched in this set of proteins.

372 Because secretion of protein effectors is often a feature of endophytic bacteria (Brader *et*
373 *al.*, 2017), we searched for genes encoding various secretion machineries in the genomes of
374 *Burkholderia* endophytes. Flagellar genes, as well as Type III, IV or VI secretion system were
375 not conserved in all leaf endophytes (Figure S6). The most eroded symbionts of *Psychotria*,
376 *Pavetta*, and *Ardisia* lack almost all types of secretion systems, and most also lack a
377 functional flagellar apparatus. Type V secretion systems are present in *Ca. Burkholderia*
378 *ardisicola* Acor, *Ca. B. pumila*, and *Ca. B. humilis*. The genomes of *Paraburkholderia*
379 symbionts of *Vangueria* and *Fadogia* were generally richer in secretions systems, but only
380 T1SS and T2SS are conserved. A Type V secretion system is present in all *Paraburkholderia*
381 endophytes except *Ca. Paraburkholderia dryadicola*. The flagellar apparatus is missing in *Ca.*
382 *P. dryadicola*, *Ca. P. soutpansbergensis*, and *P. phenoliruptrix* Vesc, and is incomplete in
383 some other *P. phenoliruptrix* endophytes. Lastly, only the genomes of *Paraburkholderia*
384 *caledonica* endophytes encode a complete set of core Type VI secretion system proteins.

385 *Genes related to secondary metabolism are enriched in leaf endophytes*

386 We wondered if specific metabolic pathways might be enriched in genomes of leaf
387 symbionts, despite rampant reductive evolution. We assigned KEGG pathway membership
388 for each predicted functional CDS (thus excluding predicted pseudogenes) in leaf symbiont
389 genomes as well as a set of free-living representative *Paraburkholderia* or *Caballeronia*
390 species. The number of genes assigned to a majority of the KEGG pathways (256 pathways in
391 total) was significantly smaller in endophyte genomes compared to their free-living relatives.

392 A small portion (86 pathways) did not differ between leaf symbionts and free-living
393 representatives. Genes belonging to a single pathway were significantly enriched in leaf
394 endophytes: acarbose and validamycin biosynthesis (KEGG pathway map00525). Acarbose
395 and validamycin are aminocyclitols synthesized via *2-epi-5-epi-valiolone synthase* (EEVS).
396 EEVS catalyses the first committed step of C₇N aminocyclitol synthesis^{23,24}, and likely plays a
397 role in the production of kirkamide, a natural C₇N aminocyclitol present in leaves of
398 *Psychotria kirkii* and other nodulated Rubiaceae, as well as streptol and streptol glucoside, 2
399 cyclitols with herbicidal activities (Pinto-Carbó *et al.*, 2016). Indeed, of 10 *Ca. Burkholderia*
400 *kirkii* genes assigned to KEGG pathway map00525, 8 genes were previously hypothesised to
401 play a direct role in the synthesis of C₇N aminocyclitol or derived compounds (Pinto-Carbó *et*
402 *al.*, 2016). Similarly, 7 out of 11 orthogroups most enriched in leaf endophytes were linked to
403 cyclitol synthesis (Table S7). To gain a better understanding of the distribution of cyclitol
404 biosynthetic clusters in leaf endophytes, we searched for homologs of the two *2-epi-5-epi-*
405 *valiolone synthase* (EEVS) genes of *Ca. Burkholderia kirkii* (locus tags BKIR_C149_4878 and
406 BKIR_C48_3593) in the other leaf endophyte genomes. We detected putative EEVS
407 homologs in all but the two genomes of *Ca. B. crenata*. For *Ca. B. crenata* UZHbot9 we have
408 previously shown the genome encodes a non-ribosomal peptide synthase likely responsible
409 for the synthesis of the depsipeptide FR900359 (Fujioka *et al.*, 1988; Carlier *et al.*, 2016;
410 Crüsemann *et al.*, 2018), and these genes were also detected in *Ca. B. crenata* Avir. Because
411 EEVSs are phylogenetically related to 3-dehydroquinate synthases (DHQS), we aligned the
412 putative EEVS sequences retrieved from leaf endophytes to EEVS and DHQS sequences in the
413 Swissprot database. All putative EEVS sequences retrieved from leaf endophytic
414 *Burkholderia* were phylogenetically related to *bona fide* EEVS proteins, but not to
415 dehydroquinate synthase (DHQS) and other sedoheptulose 7-phosphate cyclases. EEVS are
416 otherwise rare in *Burkholderia* s. l., with putative EEVSs present in only 11 out of 5674
417 publicly available *Burkholderiaceae* genomes (excluding leaf symbiotic bacteria) in the NCBI
418 RefSeq database as of June 2022 (Figure S7).

419 *Evolution of cyclitol metabolism in leaf endophytic Burkholderia*

420 Phylogenetic analysis of the endophyte EEVS protein sequences showed the presence of two
421 main clades of *Burkholderia* EEVS homologs, as well as a divergent homolog in the genome
422 of *Ca. B. ardisicola* Acor, and a second divergent homolog in *Ca. P. dryadicola* (Figure 2A).

423 The gene context of these EEVS genes in the different clades reveals that the two main EEVS
424 clades correspond to the two conserved gene clusters previously hypothesized to play a role
425 in kirkamide and streptol glucoside biosynthesis in *Ca. Burkholderia kirkii* (Carlier *et al.*,
426 2013). The gene order of these clusters is very similar in every genome, with a similar
427 genomic context in closely related genomes (Table 2-3). These gene clusters are generally
428 flanked by multiple mobile elements, consistent with acquisition via horizontal gene
429 transfer. Furthermore, the EEVS phylogeny did not follow the species phylogeny, indicating
430 that HGT or gene conversion occurred (Figure 2). For clarity, we named the two main
431 putative cyclitol biosynthetic gene clusters S-cluster (for streptol) and K-cluster (for
432 kirkamide) based on previous biosynthetic hypotheses from *in silico* analysis of the putative
433 cyclitol gene clusters of *P. kirkii* (Figure 2) (Pinto-Carbó *et al.*, 2016). Both K and S-clusters
434 encode a core set of proteins linked to sugar analog biosynthesis: a ROK family protein and a
435 HAD family hydrolase, and both contain aminotransferases (although from different protein
436 families). Two EEVS genes contain nonsense mutations and are likely not functional: the S-
437 cluster EEVS of *Ca. Burkholderia humilis*, and the K-cluster EEVS of *Ca. Burkholderia*
438 *brachyanthoides*. The genome of *Ca. B. humilis* still contains an apparently functional K-
439 cluster EEVS, while the pseudogenized EEVS of *Ca. B. brachyanthoides* is the only homolog in
440 the genome. Interestingly, genes of the K-cluster appear to be exclusive to *Psychotria* and
441 *Pavetta* symbionts, while the S-cluster is more widespread, including in the genomes of
442 *Vangueria* endophytes. Accordingly, we detected kirkamide in leaf extracts of *Psychotria*
443 *kirkii*, but in none of the *Fadogia* or *Vangueria* species we tested (see supplementary
444 methods). We also detected signals that were consistent with streptol/valienol and streptol
445 glucoside by UPLC-QToF-MS in all samples. However, these signals occurred in a noisy part of
446 the chromatogram, and we could not confidently assign these m/z features to streptol or its
447 derivatives (see supplementary methods).

448 The genomes of *Ca. P. soutpansbergensis* and *P. caledonica* R-49542 and R-82532 encoded
449 EEVS homologs of the K-cluster, but the full complement of the genes of the K-cluster is
450 missing (Table 3). In both cases the EEVS gene is flanked by IS elements. Accordingly, we did
451 not detect kirkamide in leaf samples from either *Fadogia homblei* or *P. soutpansbergensis* in
452 our chemical analyses. The genome of *Ca. P. dryadicola* encodes an EEVS that clusters
453 outside of the K- and S-EEVS clusters. Genes with putative functions similar to those of the K-

454 cluster are located in the vicinity of the EEVS in the genome of *Ca. P. dryadicola*:
455 oxidoreductases, an aminotransferase, and an N-acetyltransferase (Table S8). Similarly, *Ca.*
456 *B. ardisicola* Acor contains a second divergent EEVS, in addition to the S-cluster EEVS. This
457 EEVS belongs to a larger gene cluster coding for similar functions also found in the other
458 EEVS-clusters, but contains at least one frameshift mutation and no longer codes for a
459 functional enzyme (Table S8). Lastly, *Ca. B. verschuerenii* contains a second, recently
460 diverged EEVS paralog of the K-cluster. This EEVS is part of a small cluster of genes, with
461 putative functions divergent from those found in the other EEVS-clusters and likely does not
462 play a role in kirkamide synthesis (Table S8).

463

464 *Discussion*

465 *Different evolutionary origins of leaf symbioses in different plant genera*

466 In this work, we investigated the evolution of associations between *Burkholderia s. l.*
467 bacteria and plants of the Rubiaceae and Primulaceae families, and attempted to identify
468 key characteristics of these associations. To this end, we re-analyzed publicly available
469 genome data from previous research, and sequenced and assembled the genomes of an
470 additional 17 leaf endophytes. In addition to leaf endophytes which had been previously
471 detected (Lemaire, Smets, *et al.*, 2011; Verstraete *et al.*, 2011, 2013; Ku and Hu, 2014), we
472 document here the presence of *Burkholderia s.l.* symbionts in *Pavetta hochstetteri* and
473 *Vangueria esculenta*, and possibly *Pavetta revoluta*. In contrast to previous findings
474 (Lemaire, Lachenaud, *et al.*, 2012), we could not detect evidence of leaf endophytes in
475 *Psychotria capensis*, but did confirm the absence of leaf endophytes in *Psychotria*
476 *zombamontana*. Phylogenetic placement of hosts and endophytes are consistent with
477 previous data, except for the placement of *Vangueria macrocalyx* and its endophyte
478 (Lemaire, Lachenaud, *et al.*, 2012; Verstraete *et al.*, 2013). Both chloroplast sequences of *V.*
479 *macrocalyx* and *V. dryadum* and the genomes of their endophytes were near identical while
480 previous research showed a clear phylogenetic difference both between the host species
481 and their endophytes (Verstraete *et al.*, 2013). Blastn analysis of plant genetic markers (ITS,
482 petB, rpl16, trnTF) of both species against the NCBI nr database showed higher identities to
483 markers from *Vangueria dryadum* than to those of *Vangueria macrocalyx*. However, since
484 comparison of the vouchered *V. macrocalyx* specimen to other vouchered *Vangueria*
485 *dryadum* and *V. macrocalyx* by expert botanists clearly separated both species, we decided
486 to consider both species distinct.

487 Previous studies showed that Rubiaceae and Primulaceae species with heritable leaf
488 symbionts are monophyletic within their respective genera (Lemaire, Vandamme, *et al.*,
489 2011; Verstraete *et al.*, 2013). Thus, while the transition to a symbiotic state arose
490 separately in multiple plant genera, it likely evolved only once in each plant genus. The only
491 exception is the *Psychotria* genus, where it likely arose twice: once in species forming leaf
492 nodules, and once in species without leaf nodules (Lemaire, Lachenaud, *et al.*, 2012). The
493 repeated emergence of leaf symbiosis is reflected on the microbial side as well. A
494 parsimonious interpretation of whole genome phylogenetic analyses indicates that

495 *Burkholderia* endophytes evolved independently at least 8 times, most probably from
496 ancestors with an environmental lifestyle (Figure 1A). *Caballeronia* endophytes of *Ardisia*
497 seem to have emerged once, with most closely related species commonly isolated from soil
498 (Lim *et al.*, 2003; Vandamme *et al.*, 2013; Uroz and Oger, 2017). As previously reported,
499 symbionts of *Psychotria* and *Pavetta* cluster in 3 distinct phylogenetic groups within the
500 *Caballeronia* genus. Finally, symbionts of *Vangueria* and *Fadogia* belong to 5 distinct clades
501 within the genus *Paraburkholderia*. Apart from *Ca. P. dryadicola* that is without closely
502 related isolates, endophytic *Paraburkholderia* species also cluster together with species
503 commonly isolated from soil (Verstraete *et al.*, 2014; Beukes *et al.*, 2019). High host-
504 specificity is a hallmark of the *Psychotria*, *Pavetta*, and *Ardisia* leaf symbiosis, but this
505 characteristic is not shared in *Vangueria* and *Fadogia*. Based on genome similarity, we
506 identified at least three phylogenetically divergent endophyte species that can infect
507 multiple hosts: *P. caledonica*, *P. phenoliruptrix*, and *Ca. P. dryadicola*. It is also possible that
508 these plants are in the early stages of endophyte capture, where the plant is open to acquire
509 endophytes from the soil, as previously hypothesized for *F. homblei* (Verstraete *et al.*, 2013).
510 Endophytes might later evolve to become host-restricted and vertically transmitted, leading
511 to diversification from their close relatives and forming new species. This could, for example,
512 already be the case for *Ca. P. soutpansbergensis*, which is related to *P. phenoliruptrix* but
513 shows a more divergent genome (ANI <95%). Overall, these results highlight the general
514 plasticity of bacteria in the *Burkholderia s.l.*, as well as the probable frequent occurrence of
515 host-switching or horizontal transfer within leaf symbiotic associations.

516 *Genome reduction is a common trait of leaf endophytes*

517 Bacterial genomes contain a wealth of information yet few leaf endophyte genomes are
518 available. In this study we provide an additional thirteen leaf endophyte genome assemblies
519 among which the first genomes of endophytes from *Vangueria* and *Fadogia*. Aside from the
520 genomes of *P. caledonica* endophytes, all leaf endophyte genomes were small, mostly
521 between 3.5 and 5 Mbp. This is well below the average 6.85 Mbp of the *Burkholderiaceae*
522 family (Carlier *et al.*, 2016; Pinto-Carbó *et al.*, 2016). In addition to their small sizes, the
523 genomes of *Psychotria*, *Pavetta*, and *Ardisia* endophytes show signs of advanced genome
524 reduction. Only 41-70% of these genomes code for functional proteins, compared to an
525 average of about 90% for free-living bacteria (Land *et al.*, 2015). Most of these genomes also

526 contain a high proportion of mobile sequences, up to 9% of the total assembly. Together,
527 this indicates ongoing reductive genome evolution, a process often observed in obligate
528 endosymbiotic bacteria (Moran and Plague, 2004; Bennett and Moran, 2015). Interestingly,
529 the genomes of *Vangueria* and *Fadogia* endophytes, which are not contained in leaf
530 nodules, also show signs of genome erosion: most genomes of *P. phenoliruptrix* endophytes
531 are at or below 5 Mbp in size, with over half of their proteome predicted as non-functional.
532 The genomes of *Ca. P. dryadicola* even approach the level of genome reduction found in
533 most *Psychotria* symbionts. The intermediate genome reduction in endophytes of *Vangueria*
534 and *Fadogia* could be explained by the relatively recent origin of the symbiosis, although leaf
535 symbiosis in *Fadogia* has been estimated to be older than in *Vangueria* (7.6 Mya vs. 3.7 Mya)
536 (Verstraete *et al.*, 2017). Other factors likely contribute to the extent or pace of genome
537 reduction in the endophytes, such as mode of transmission and transmission bottlenecks.
538 The larger genome size and fewer pseudogenes compared to most other leaf endophytes
539 may explain why we could isolate *P. caledonica* endophytes from *F. homblei*, but not other
540 endophytes. We could not identify essential genes or pathways that were consistently
541 missing in the genomes of *Burkholderia* endophytes. It is therefore possible that other
542 endophytic bacteria may be culturable using more complex or tailored culture conditions.

543 *Secondary metabolism as key factor in the evolution of leaf symbiosis*

544 Although leaf symbionts share a similar habitat and all belong to the *Burkholderia s. l.*, their
545 core genome is surprisingly small and consists almost entirely (95%) of genes that are
546 considered essential for cellular life. This poor conservation of accessory functions perhaps
547 reflects the large diversity and possible redundancy of functions encoded in the genomes of
548 *Burkholderia s.l.* that associate with plants. Interestingly, the capacity for production of
549 secondary metabolites is a key common trait of *Burkholderia* leaf endophytes. We previously
550 showed that *Ca. B. crenata* produces FR900359, a cyclic depsipeptide isolated from *A.*
551 *crenata* leaves (Carlier *et al.*, 2016). This non-ribosomal peptide possesses unique
552 pharmacological properties and may contribute to the protection of the host plant against
553 insects (Carlier *et al.*, 2016; Crüsemann *et al.*, 2018). However, our data suggests that the
554 production of cyclitols is widespread in leaf endophytic *Burkholderia*. Indeed, with the
555 exception of *Ca. B. crenata* cited above, we found evidence for the presence of cyclitol
556 biosynthetic pathways in all genomes of leaf endophytic *Burkholderia*. We have previously

557 reported the presence of two gene clusters containing a 2-*epi*-5-*epi*-valiolone synthase
558 (EEVS) in the genomes of *Psychotria* and *Pavetta* symbionts (Pinto-Carbó *et al.*, 2016). These
559 gene clusters are likely responsible for the production of 2 distinct cyclitols: kirkamide, a C₇N
560 aminocyclitol with insecticidal properties which has been detected in several *Psychotria*
561 plants; and streptol-glucoside, a plant-growth inhibitor likewise detected in *Psychotria kirkii*
562 (Sieber *et al.*, 2015; Pinto-Carbó *et al.*, 2016; Hsiao *et al.*, 2019). EEVS from leaf symbionts
563 belong to four phylogenetic clusters, including the two EEVS genes previously detected in
564 *Psychotria* and *Pavetta* symbionts (Pinto-Carbó *et al.*, 2016). Similar to these previously
565 analysed leaf endophyte genomes, the EEVS gene clusters in the newly sequenced genomes
566 are flanked by IS-elements, and their phylogeny is incongruent with the species phylogeny.
567 This indicates that these genes and clusters are likely acquired via horizontal gene transfer.
568 This hypothesis is strengthened by the fact that the closest homologs of the genes in the
569 EEVS clusters are found in genera as diverse as *Pseudomonas*, *Streptomyces*, and
570 *Noviherbaspirillum*, but are rare in the genomes of *Burkholderia s.l.* The presence of the two
571 main EEVS gene clusters (K-cluster and S-cluster) is not strictly linked to the symbiont or host
572 taxonomy. For example, the EEVS of the K-cluster (hypothesised to produce kirkamide) is
573 present in all sequenced symbionts of *Psychotria* and *Pavetta* but also in the endophytes of
574 *F. homblei* and *V. soutpansbergensis*. However, in the latter two, accessory genes of the K-
575 cluster are absent. It is possible that this EEVS interacts with gene products of other
576 secondary metabolite clusters (Osborn *et al.*, 2017). We also noticed that some endophyte
577 genomes contain multiple EEVS genes or gene clusters. This could provide functional
578 redundancy, protecting against the rampant genome erosion present in these genomes. For
579 example, two genes of the S-cluster *Ca. B. hochstetteri* are likely pseudogenes, while the K-
580 cluster gene is still complete. On the other hand, in *Ca. Burkholderia humilis* seven out of ten
581 genes of the S-cluster (including the EEVS) are either missing or non-functional, and the K-
582 cluster is heavily reduced with only four functional genes out of eight (including the EEVS).
583 As one functional EEVS copy remains, it is possible that genes located elsewhere in the
584 genome provide these functions, as kirkamide has previously been detected in extracts of
585 *Psychotria humilis* (Pinto-Carbó *et al.*, 2016). Alternatively, this symbiosis may have reached
586 a “point of no return” where host and symbiont have become dependent on each other and
587 non-performing symbionts can become fixed in the population (Bennett and Moran, 2015).

588 The presence of gene clusters coding for specialised secondary metabolites in all leaf
589 symbionts could indicate that secondary metabolite production is either a prerequisite for or
590 a consequence of an endophytic lifestyle. The fact that *P. caledonica* leaf symbionts have
591 EEVS genes of different origin favours the hypothesis that the acquisition of secondary
592 metabolism precedes an endophytic lifestyle. In this case, the ancestor of both endophytes
593 may have acquired differing EEVS genes or EEVS gene clusters through HGT followed by
594 infection of the respective host plants. The lack of EEVS homolog in *Ca. B. crenata* indicates
595 that production of cyclitols is not essential for leaf symbiosis. Interestingly, genomes of the
596 sister species *Ca. B. ardisicola* encode an EEVS and the full S-cluster complement. Since there
597 is strong phylogenetic evidence of co-speciation in the *Burkholderia/Ardisia* association
598 (Lemaire, Smets, *et al.*, 2011; Ku and Hu, 2014), the common ancestor of *Ca. B. ardisicola*
599 and *Ca. B. crenata* possibly possessed both cyclitols and *frs* pathways, and one of these
600 pathways was lost in the lineages leading to contemporary *Ca. B. crenata* and *Ca. B.*
601 *ardisicola*. Alternatively, the genome of the common ancestor of *Ardisia*-associated
602 *Burkholderia* may have encoded cyclitol S-cluster and later acquisition of the *frs* gene cluster
603 in the *Ca. B. crenata* lineage alleviated the requirement of EEVS-related metabolism. The
604 model of horizontal acquisition of secondary functions supports the model of endophyte
605 evolution described by Lemaire *et al* (Lemaire, Vandamme, *et al.*, 2011). Different
606 environmental strains which acquired genes for secondary metabolite production could
607 colonise different host plants in the early open phase of symbiosis. The different
608 phylogenetic endophyte clades observed in the *Burkholderia s.l.* phylogeny could each
609 represent distinct acquisitions of secondary metabolite gene clusters by divergent free-living
610 bacteria followed by colonisation of different host plants. Many *Burkholderia* species
611 associate with eukaryotic hosts, including plants (Eberl and Vandamme, 2016), and many of
612 these associations may be transient in nature. However, useful traits such as synthesis of
613 protective metabolites may help stabilise these relationships, resulting in long-term
614 associations such as leaf symbiosis.

615 **Author contributions:**

616 AC, MM, and BD designed the research. MM identified and collected wild plant specimens
617 from the Pretoria region (South Africa). BD, MB, SS, and AC performed the laboratory

618 experiments and analyses. BD, MM and AC wrote the manuscript with input from all
619 authors.

620 **Acknowledgments**

621 We would like to thank Frédéric De Meyer and Mathijs Deprez for helping with some of the
622 laboratory experiments. We would further like to thank Steven Janssens (Meise Botanic
623 Garden) and Peter Brownless (Royal Botanic Garden Edinburgh) for facilitating the
624 acquisition of plant material for this study. BD and AC would like to thank Klaas Vandepoele,
625 Monica Höfte, Anne Willems and Paul Wilkin for helpful discussion and for proofreading the
626 manuscript. We also thank Aurélien Bailly (University of Zürich, CH) for providing *P. kirkii*
627 samples and help with interpreting mass spectrometry data. Magda Nel of the H.G.W.J.
628 Schweickerdt Herbarium is thanked for her help with plant identification and Mamoalosi
629 Selepe and Sewes Alberts of the Chemistry and Plant and Soil Sciences Departments,
630 respectively (University of Pretoria) for chemical analysis. We also thank Chien-Chi Hsiao and
631 Karl Gademann from University of Zürich (Switzerland) for providing the analytical standards.
632 This work was supported by the Flemish Fonds Wetenschappelijk Onderzoek under grant
633 G017717N to AC. AC also acknowledges support from the French National Research Agency
634 under grant agreement ANR-19-TERC-0004-01 and from the French Laboratory of Excellence
635 project "TULIP" (ANR-10-LABX-41; ANR-11-IDEX-0002-02) and from the French National
636 Infrastructure for Metabolomics and Fluxomics, Grant MetaboHUB-ANR-11-INBS-0010. We
637 thank the Oxford Genomics Centre at the Wellcome Centre for Human Genetics for the
638 collection and preliminary analysis of sequencing data. The Oxford Genomics Centre at the
639 Wellcome Centre for Human Genetics is funded by Wellcome Trust grant reference
640 203141/Z/16/Z. The funders had no role in study design, data collection and analysis,
641 decision to publish, or preparation of the manuscript.

642 **Notes**

643 The authors declare no conflict of interest.

644 *Figure Legends*

645 **Figure 1: Phylogeny of *Burkholderia*, *Caballeronia*, and *Paraburkholderia*, including the leaf endophytes. (A)**
646 UBCG phylogeny of the *Burkholderia* s.l. based on 92 conserved genes. Bootstrap support values based on 100
647 replications are displayed on the branches. Branches with <50% support were collapsed. *Ralstonia*
648 *solanacearum* was used as outgroup to root the tree. Coloured samples in boldface represent the leaf
649 endophytes from Rubiaceae and Primulaceae **(B)** Core genome phylogeny of leaf endophytes based on
650 alignment of 423 single-copy core genes. Bootstrap support values based on 100 replicates are shown on the
651 branches. Samples are colour-coded based on the host genus: Purple – *Ardisia*; Blue – *Psychotria*; Pink –
652 *Pavetta*; Green – *Vangueria*; Orange – *Fadogia*; Black bars represent the coding capacity of the genome (the
653 proportion of the genome coding for functional proteins).

654 **Figure 2: EEVS protein phylogeny and distribution in leaf endophytes. (A)** EEVS protein phylogeny of detected
655 EEVS-genes and their closest relatives. Local support values based on the Shimodaira-Hasegawa test are shown
656 on the branches, and branches with support <50% are collapsed. Coloured samples in boldface are the EEVS
657 homologs found in different leaf endophytes. Colours represent different clusters of similar EEVS genes. K- and
658 S-cluster are named after their putative products (K for Kirkamide, and S for Streptol glucoside). NCBI accession
659 numbers of the close relatives are given next to their species name. The tree is rooted using related 3-
660 dehydroquinate synthase genes (not shown). *The EEVS gene in *Ca. Burkholderia humilis* contains an internal
661 stop codon, creating two EEVS-like pseudogenes. The largest of both was used for the phylogeny. **This EEVS
662 gene of *Ca. Burkholderia verschuerenii* is found outside of the K-cluster. **(B)** Distribution of specialised
663 metabolism in the leaf endophytes. Samples are colour-coded based on the host species: Purple – *Ardisia*; Blue
664 – *Psychotria*; Pink – *Pavetta*; Green – *Vangueria*; Orange – *Fadogia*. Codes next to the species represent
665 presence of specialised metabolite clusters; FR – FR900359 depsipeptide; K – Kirkamide EEVS-cluster; S –
666 Streptol glucoside EEVS-cluster; O – Other EEVS-cluster. K' – Secondary EEVS cluster with EEVS similar to the K-
667 cluster. K* - Only the K-cluster EEVS is present, not the accessory genes.

Tables

Table 1: Genome statistics of newly assembled and re-assembled leaf endophyte genomes. Coding capacity refers to the proportion of the genome that codes for functional proteins.

Endophyte	Host species	Type	Assembly size (Mb)	Contigs	N50 (bp)	%G+C	Annotated genes	Functional genes	Pseudo genes	Coding Capacity (%)	IS elements	IS total length (bp)	Proportion IS (%)
<i>Ca. Burkholderia ardisicola</i> Acor	<i>Ardisia cornudentata</i>	New assembly	3,95	332	19528	59,23	6975	2026	4949	40,30	35	32834	0,83
<i>Ca. Burkholderia crenata</i> UZHbot9	<i>Ardisia crenata</i>	Re-assembly	2,65	607	6399	59,02	3982	1670	2312	54,73	96	69678	2,63
<i>Ca. Burkholderia ardisicola</i> Amam	<i>Ardisia mamillata</i>	New assembly	4,38	333	19687	59,47	7472	2297	5175	40,95	59	48385	1,10
<i>Ca. Burkholderia crenata</i> Avir	<i>Ardisia virens</i>	New assembly	2,58	605	6517	59,05	3839	1648	2191	56,13	63	42190	1,64
<i>Paraburkholderia caledonica</i> R-49542	<i>Fadogia homblei</i>	New assembly	8,92	148	145314	61,59	9185	7695	1490	82,83	96	117852	1,32
<i>Paraburkholderia caledonica</i> R-82532	<i>Fadogia homblei</i>	New assembly	8,71	123	239289	61,53	9054	7353	1701	81,03	77	91097	1,05
<i>Ca. Burkholderia hochstetteri</i>	<i>Pavetta hochstetteri</i>	New assembly	3,50	324	18152	62,51	5453	1823	3630	44,53	29	38062	1,09
<i>Ca. Burkholderia schumanniana</i>	<i>Pavetta schumanniana</i>	Re-assembly	3,62	412	14848	63,47	4938	2453	2485	59,95	69	44239	1,22
<i>Ca. Burkholderia brachyanthoides</i>	<i>Psychotria brachyanthoides</i>	Re-assembly	3,75	648	8356	61,00	6284	2109	4175	46,54	223	149135	3,98
<i>Ca. Burkholderia humilis</i>	<i>Psychotria humilis</i>	Re-assembly	5,32	238	103328	59,60	7828	3264	4564	50,04	64	63278	1,19
<i>Ca. Burkholderia kirkii</i>	<i>Psychotria kirkii</i>	Reference	4,01	203	44916	62,91	6329	2069	4260	45,80	375	353298	8,81
<i>Ca. Burkholderia pumila</i>	<i>Psychotria pumila</i>	Re-assembly	3,70	463	12628	59,13	6835	2192	4643	45,41	195	153499	4,15
<i>Ca. Burkholderia punctata</i>	<i>Psychotria punctata</i>	Reference	3,91	48	100248	64,00	4864	2539	2325	54,61	310	358729	9,17
<i>Ca. Burkholderia umbellata</i>	<i>Psychotria umbellata</i>	Re-assembly	4,22	333	28025	61,30	6967	2306	4661	44,37	91	68761	1,63
<i>Ca. Burkholderia verschuerenii</i>	<i>Psychotria verschuerenii</i>	Re-assembly	6,15	401	27267	62,07	7440	4839	2601	70,21	88	60714	0,99
<i>Ca. Paraburkholderia dryadicola</i> Vdry	<i>Vangueria dryadum</i>	New assembly	4,29	153	50748	61,26	7076	2229	4847	43,21	38	35272	0,82
<i>Paraburkholderia phenoliruptrix</i> Vesc	<i>Vangueria esculenta</i>	New assembly	4,99	180	50333	63,54	6347	3329	3018	59,78	46	54597	1,09
<i>Paraburkholderia phenoliruptrix</i> Vinf	<i>Vangueria infausta</i>	New assembly	5,00	181	49920	63,51	6377	3320	3057	59,29	50	58387	1,17
<i>Ca. Paraburkholderia dryadicola</i> Vmac	<i>Vangueria macrocalyx</i>	New assembly	4,31	150	54987	61,30	7111	2243	4868	43,06	40	37709	0,87

<i>Paraburkholderia phenoliruptrix</i> VmadMBG	<i>Vangueria</i> <i>madagascariensis</i>	New assembly	4,77	247	34361	63,48	5912	3214	2698	61,09	45	55093	1,15
<i>Paraburkholderia phenoliruptrix</i> VmadEBG	<i>Vangueria</i> <i>madagascariensis</i>	New assembly	4,76	242	34985	63,48	5901	3212	2689	60,97	45	53173	1,12
<i>Paraburkholderia phenoliruptrix</i> VmadSA	<i>Vangueria</i> <i>madagascariensis</i>	New assembly	5,03	194	50250	63,49	6444	3291	3153	59,22	47	48936	0,97
<i>Paraburkholderia caledonica</i> Vpyg88	<i>Vangueria pygmaea</i>	New assembly	7,44	92	232014	61,89	7426	6194	1232	82,23	54	74083	1,00
<i>Paraburkholderia caledonica</i> Vpyg08	<i>Vangueria pygmaea</i>	New assembly	7,45	106	232088	61,90	7449	6193	1256	82,33	60	79510	1,07
<i>Paraburkholderia phenoliruptrix</i> Vran	<i>Vangueria randii</i>	New assembly	4,98	205	50270	63,33	6379	3294	3085	59,47	59	73129	1,47
<i>Ca. Paraburkholderia</i> <i>soutpansbergensis</i>	<i>Vangueria</i> <i>soutpansbergensis</i>	New assembly	5,18	51	337347	63,12	6801	3259	3542	55,24	35	44578	0,86

Table 2: EEVS S-cluster organisation in endophyte genomes. Genomes of the same host with the same cluster layout are merged. X: Gene present; -: Gene absent; Ψ : Gene predicted to be pseudogene; *: genes present on a different contig than the EEVS gene; Abbreviations: EEVS – 2-*epi*-5-*epi*-valiolone synthase;

	ROK family protein	EEVS	Sugar-nucleotide binding protein	Trehalose-6-phosphate synthase	Aspartate aminotransferase family protein	Alcohol dehydrogenase	HAD family hydrolase	MFS transporter	NTP-transferase	NUDIX hydrolase
Reference accessions	CCD39391	CCD39393	CCD39394	CCD39395	KND54529	CCD39396	CCD39397	CCD39398	CCD39400	CCD39401
<i>Ca. Burkholderia ardisicola</i> Acor	X	X	X	X	X	X	X	X	X	X
<i>Ca. Burkholderia ardisicola</i> Amam	X	X	X	X	X	X	X	X	X	X
<i>Ca. Burkholderia hochstetteri</i>	X	X	X	Ψ	Ψ	X	X	X	X	X
<i>Ca. Burkholderia humilis</i>	X	Ψ	X	Ψ	X	-	-	-	-	Ψ
<i>Ca. Burkholderia kirkii</i>	X	X	X	X	-	X	X	X	Ψ	X
<i>Ca. Burkholderia punctata</i>	X	X	X	X	X	X	X	X	X	-
<i>Ca. Burkholderia schumanniana</i>	X	X	X	X	X	X	-	Ψ^*	X*	X*
<i>Paraburkholderia phenoliruptrix</i> Vesc	X	X	X	X	X	X	X	X	X	Ψ
<i>Paraburkholderia phenoliruptrix</i> Vinf	X	X	X	X	X	X	Ψ	X	X	Ψ
<i>Paraburkholderia phenoliruptrix</i> VmadSA	X	X	X	X	X	X	X	X	X	Ψ
<i>Paraburkholderia phenoliruptrix</i> VmadMBG/VmadBGE	X	X	X	X	X	X	Ψ	X	X	Ψ
<i>Paraburkholderia caledonica</i> Vpyg08/Vpyg88	X	X	X	X	X	X	X	X	X	X
<i>Paraburkholderia phenoliruptrix</i> Vran	X	X	X	X	X	X	X	X	X	X

Table 3: EEVS K-cluster organisation in endophyte genomes. Genomes of the same host with the same cluster layout are merged. X: Gene present; -: Gene absent; Ψ : Gene predicted to be pseudogene; *: protein overlaps with contig end, other genes of the cluster not found on other contigs; Abbreviations: EEVS – 2-*epi*-5-*epi*-valiolone synthase.

	GNAT family N-acetyltransferase	Cupin Domain Containing protein	HAD family hydrolase	Gfo/Idh/MocA family oxidoreductase	6-phospho-beta-glucosidase	DegT/DnrJ/EryC1/StrS family aminotransferase	ROK family protein	EEVS
Reference accessions	CCD36711	CCD36712	CCD36713	CCD36714	CCD36715	CCD36716	CCD6717	CCD36718
<i>Paraburkholderia caledonica</i> R-49542/R-82532	-	-	-	-	-	-	-	X
<i>Ca. Burkholderia brachyanthoides</i>	-	-	-	-	-	-	X/ Ψ *	Ψ
<i>Ca. Burkholderia hochstetteri</i>	X	X	X	X	X	X	X	X
<i>Ca. Burkholderia humilis</i>	-	X	Ψ	X	X	Ψ	X	X
<i>Ca. Burkholderia kirkii</i>	X	X	X	X	X	X	X	X
<i>Ca. Burkholderia pumila</i>	-	X	X	X	X	X	X	X
<i>Ca. Burkholderia punctata</i>	X	X	X	X	X	X	X	X
<i>Ca. Burkholderia schumanniana</i>	X	X	X	X	X	X	X	X
<i>Ca. Burkholderia umbellata</i>	X	X	X	X	X	X	X	X
<i>Ca. Burkholderia verschuerenii</i>	X	X	X	X	X	X	X	X
<i>Ca. Paraburkholderia soutpansbergensis</i>	-	-	-	-	-	-	-	X

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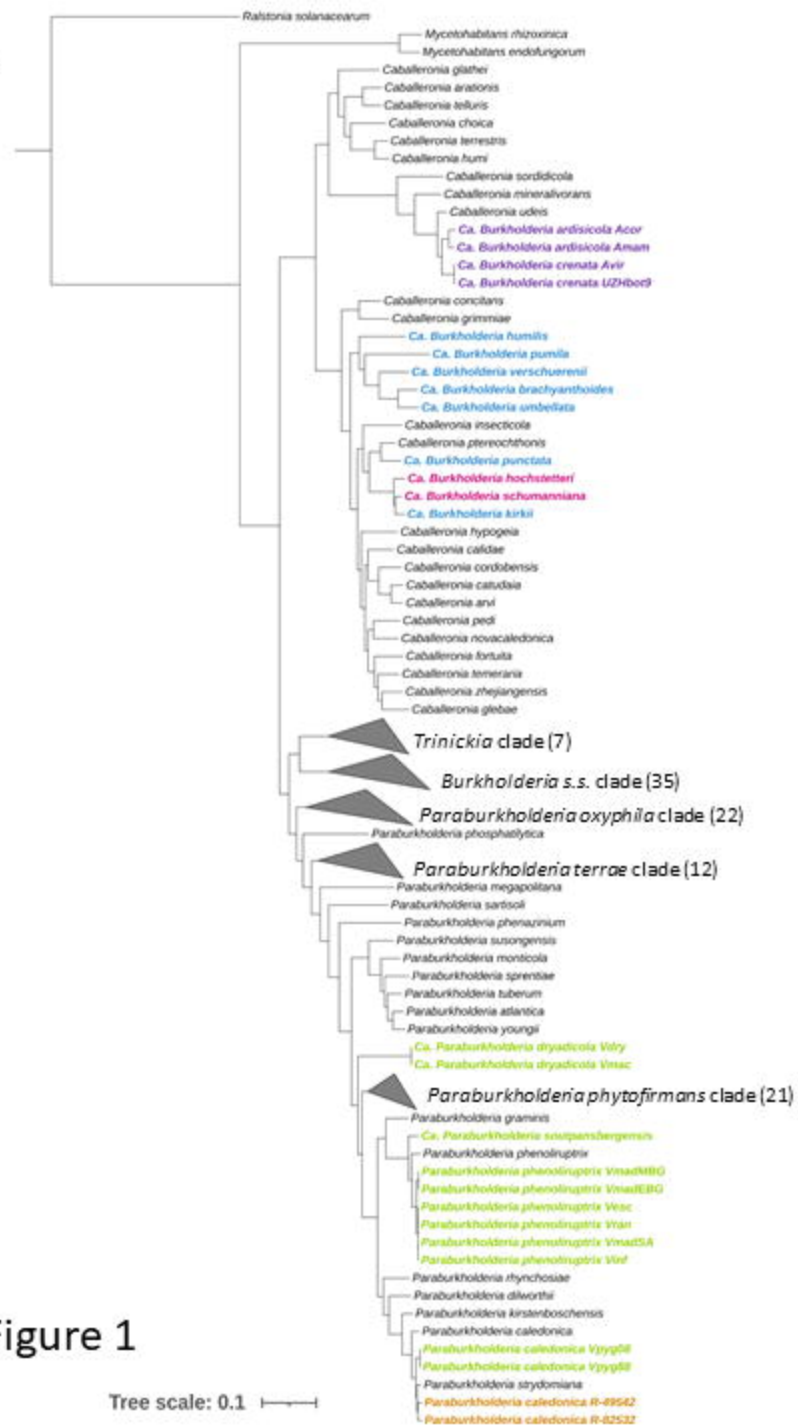
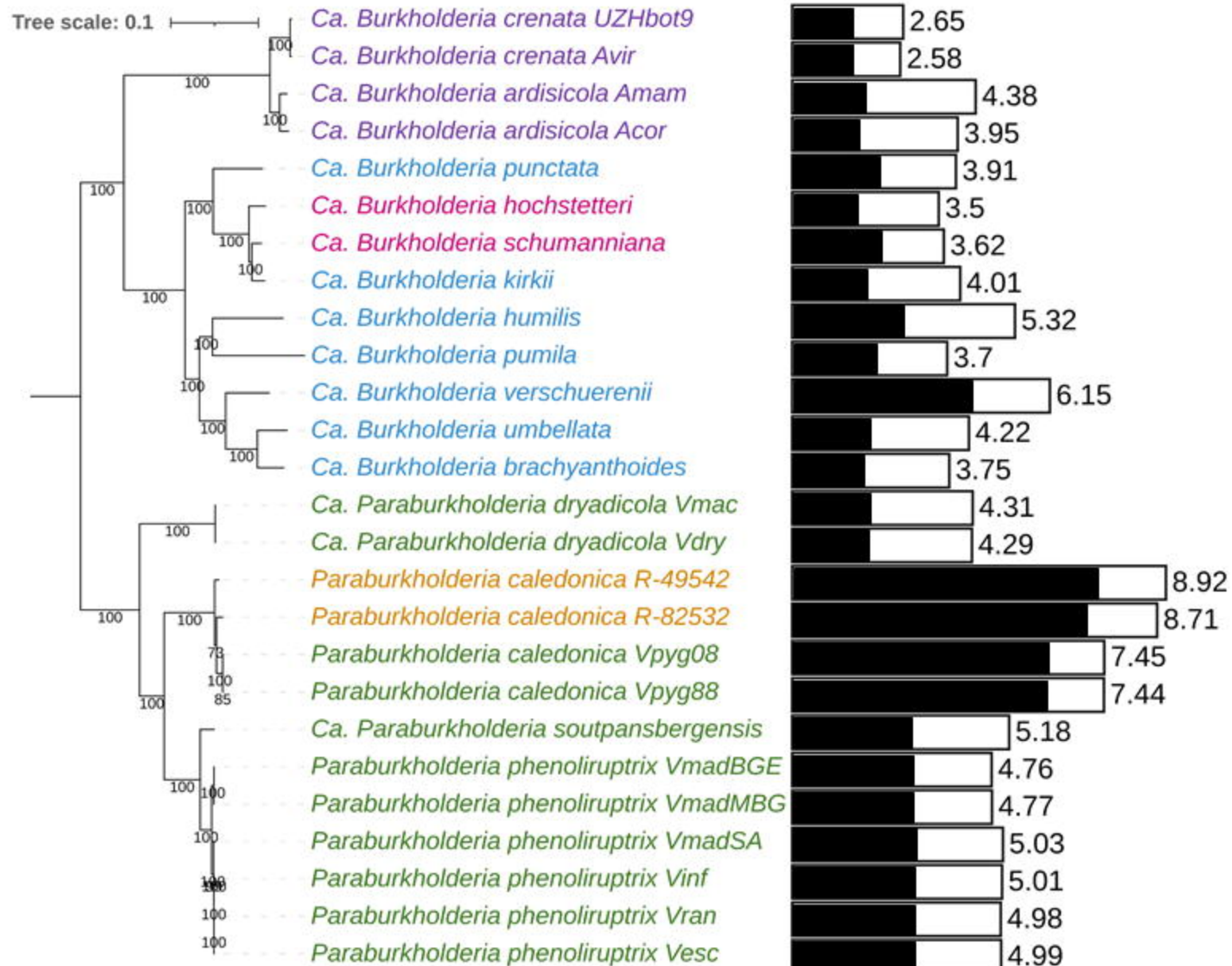
A

Figure 1

Tree scale: 0.1

B

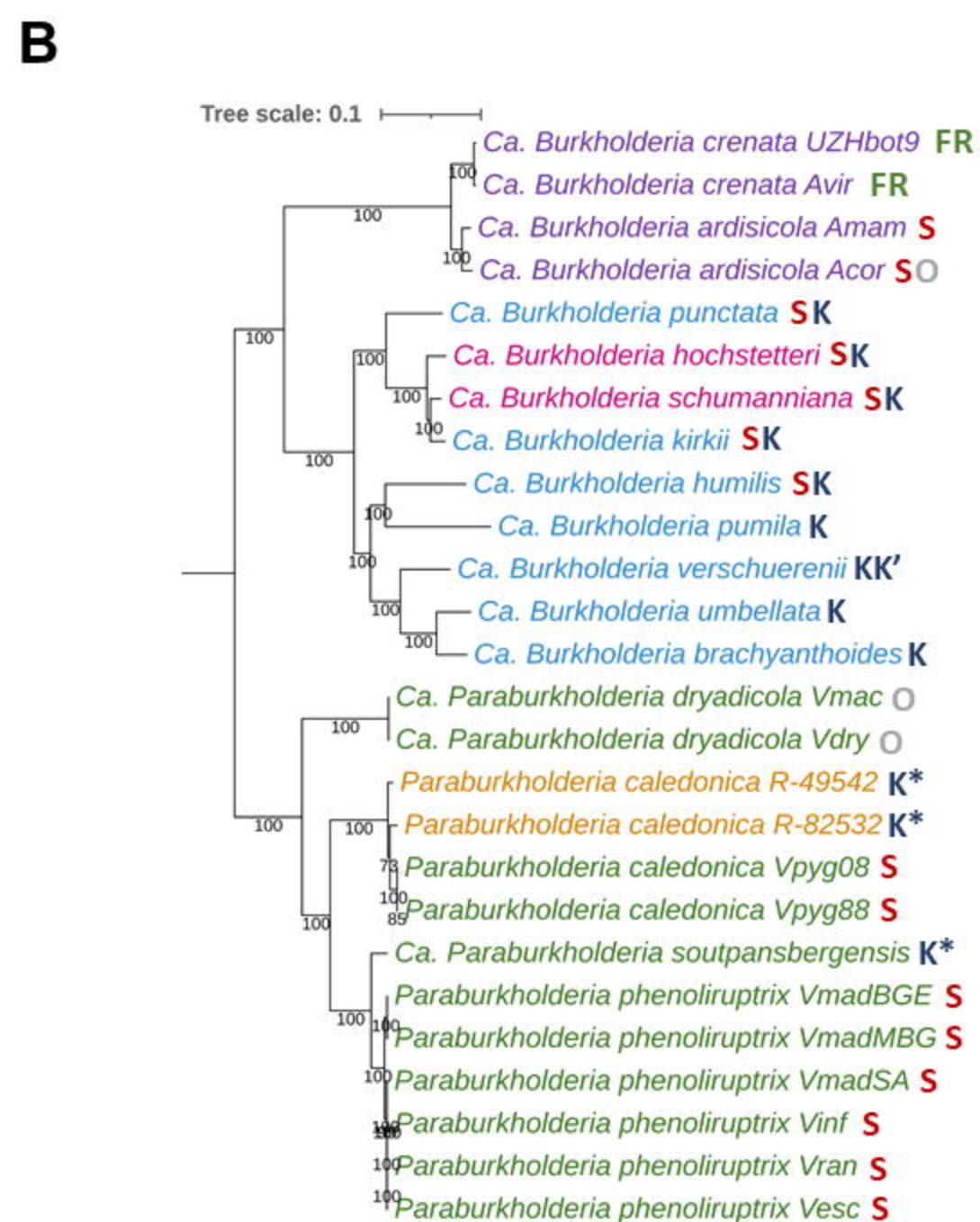
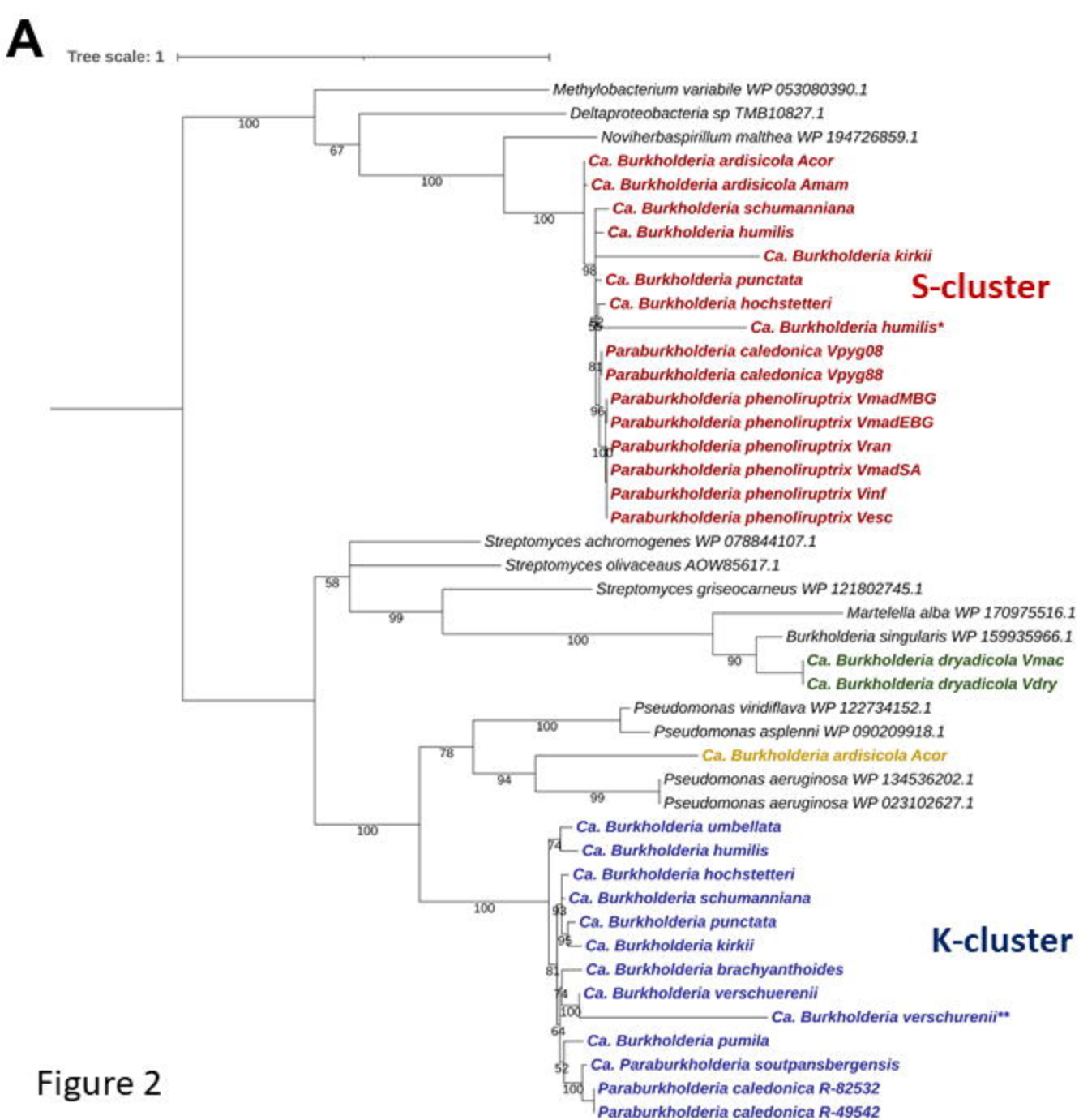


Figure 2