Identification, origin, and evolution of leaf nodulating symbionts of *Sericanthe* (Rubiaceae)

Benny Lemaire[1*], Elmar Robbrecht[2], Braam van Wyk[3], Sandra Van Oevelen[4], Brecht Verstraete[1], Els Prinsen[4], Erik Smets[1,5,6], and Steven Dessein[2]

[1]*Laboratory of Plant Systematics, K.U.Leuven, Kasteelpark Arenberg 31, PO Box 2437, BE-3001 Leuven, Belgium*
[2]*National Botanic Garden of Belgium, Domein van Bouchout, BE-1860 Meise, Belgium*
[3]*H.G.W.J. Schweickerdt Herbarium, University of Pretoria, Pretoria 0002, South Africa*
[4]*Laboratory of Plant Biochemistry and Physiology, University of Antwerp, Universiteitsplein 1, BE-2610 Antwerp, Belgium*
[5]*Netherlands Centre for Biodiversity Naturalis, PO Box 9517, 2300 RA Leiden, the Netherlands*
[5]*National Herbarium of the Netherlands, Leiden University, PO Box 9514, 2300 RA Leiden, the Netherlands*

* For correspondence. E-mail: Benny.Lemaire@bio.kuleuven.be; Tel.: 0032-16-32-86-36; Fax: 0032-16-32-19-55

Bacterial leaf symbiosis is an intimate association between bacteria and plants in which endosymbionts are housed within leaf nodules. This phenomenon has been reported in three genera of Rubiaceae (*Pavetta*, *Psychotria*, and *Sericanthe*), but the bacterial partner has only been identified in *Psychotria* and *Pavetta*. Here we report the identification of symbiotic bacteria in two leaf nodulating *Sericanthe* species. Using 16S rRNA data and common housekeeping genetic markers (*recA* and *gyrB*) we studied the phylogenetic relationships of bacterial endosymbionts in Rubiaceae. Endosymbionts of leaf nodulating Rubiaceae were found to be closely related and were placed as a monophyletic group within the genus *Burkholderia* (β-Proteobacteria). The phylogenetic analyses revealed a pattern of strict host specificity and placed the two investigated endosymbionts at two distinct positions in the topology of the tree, suggesting at least two different evolutionary origins. The degree of sequence divergence between the *Sericanthe* endosymbionts and their relatives was large enough to propose the *Sericanthe* endosymbionts as new species ('*Candidatus* Burkholderia andongensis' and '*Candidatus* Burkholderia petitii'). In a second part of this study, the pylogenetic relationships among nodulating and non-nodulating *Sericanthe* species were investigated using sequence data from six chloroplast regions (*rps16*, *trnG*, *trnL-trnF*, *petD*, *petA-psbJ*, and *atpI-atpH*). Overall, genetic variation among the plastid markers was insufficient to enable phylogenetic estimation. However, our results could not rule out the possibility that bacterial leaf symbiosis originated once in a common ancestor of the *Sericanthe* species.

Keywords: *Burkholderia*, endosymbionts, bacterial leaf nodulation, *Sericanthe*, Rubiaceae

About 500 species of Rubiaceae are known to house bacterial endosymbionts within internal cavities in the leaf lamina, referred to as bacterial leaf nodules or leaf galls (Miller, 1990). Endosymbionts are persistent and obligate associates of the host plants and are required for the successful development and reproduction of their hosts (Gordon, 1963; Miller, 1990). However, knowledge about the exact benefits conferred by these endosymbionts is still incomplete. Many studies have proposed that the endophytes of nodulated species are involved in the production of phytohormones (reviewed in Miller, 1990). From the endosymbiont's perspective, the colonization of internal plant tissues may provide a stable, uniform, and protective environment.

Leaf nodulated plant species are limited to three distantly related genera: *Pa v e t t a* L., *Psychotria* L., and *Sericanthe* Robbr. These three genera of the Rubiaceae family have no close phylogenetic affinity and belong to distant alliances. *Psychotria* is a member of the subfamily Rubioideae and belongs to the tribe Psychotrieae. *Pavetta* and *Sericanthe* belong to the subfamily Cinchonoideae and have been placed in the tribes Pavetteae and Coffeeae, respectively (Robbrecht and Manen, 2006).

Morphological observations of bacterial endosymbionts have been conducted in all rubiaceous genera (*Pavetta* and *Psychotria* in Miller, 1990; *Sericanthe* in Van Hove, 1972, referred to by the author as '*Neorosea*'). Still, attempts to cultivate and characterize these leaf nodulating bacteria associated have been

unsuccessful to date. Molecular sequencing analyses however now enable the identification of uncultivable endosymbionts. Indeed, the taxonomic position of the endosymbionts of *Pavetta* and *Psychotria* has been recently clarified (Van Oevelen *et al*., 2001, 2002, 2004; Lemaire *et al*., 2011). These studies have demonstrated that every nodulating species hosts its own unique *Burkholderia* endosymbiont. In contrast, the bacterial leaf endosymbionts within the genus *Sericanthe* remain unknown.

The genus *Sericanthe* is composed mostly by shrubs that occupy rain forests, woodlands, savannas and (sub)montane habitats in Southern and Western Africa. Many *Sericanthe* species have a very restricted distribution, as reflected by their rarity and infrequent collection (Robbrecht, 1978a). For a complete description of the geographical distribution of all *Sericanthe* species see Robbrecht (1978b).

In *Sericanthe*, leaf nodules have been reported in 11 or 12 out of 17 species (Robbrecht, 1978a). This small nodulating genus contrasts with the more specious genera *Pavetta* and *Psychotria*, which contain approximately 350 and 80 nodulating species, respectively. Bacterial leaf galls of nodulated *Sericanthe* species are always located on the abaxial side of the leaf and are hardly visible from the adaxial side. The shape and distribution of the nodules on the leaves differ substantially among species and range from linear galls along the mid-vein (e.g. *Sericanthe andongensis*) to dot-shaped or branched nodules scattered in the leaf blade (e.g. *Sericanthe petitii*) (Robbrecht, 1978a, 1981). A similar variation in nodule localization and morphology has been reported in *Pavetta* and *Psychotria* (Bremekamp, 1933).

In the present study, we focus on the endosymbiont identification and evolutionary history of bacterial leaf symbiosis in the genus *Sericanthe*. We propose the hypotheses that (1) leaf nodulated *Sericanthe* species accommodate their own specific endosymbionts and that (2) the bacteria-plant interaction is the result of an ancient and single infection event within an ancestral leaf nodulated *Sericanthe* host.

Material and Methods

Taxon sampling

Silica-dried material from *S. andongensis* and *S. petitii* were collected during botanical field expeditions in South Africa, Cameroon and Zambia. Five accessions of *S. andongensis* and two accessions of *S. petitii* were sampled from different regions in the field and were used to identify the bacterial endosymbionts. A detailed list of sampled species, voucher information and localities is given in Table 1. To determine the phylogenetic position of the endosymbionts of *Sericanthe*, we included related bacterial sequences of *Burkholderia* obtained from GenBank (Table 1).

Three additional *Sericanthe* species (i.e. *S. auriculata*, *S. odoratissima*, *S. spec. nov.*), the latter two of which were collected in the field and the first of which was obtained from an herbarium sample at the National Botanic Garden of Belgium (BR), were included in this study to construct the host phylogeny (Table 1).

DNA extraction, amplification, cloning, and sequencing

Before extraction of the bacterial DNA the silica-dried leaves were rinsed with 70% ethanol to avoid bacterial contamination. Total DNA was extracted from silica-dried collections and herbarium specimens (BR) using the modified CTAB protocol of Tel-Zur *et al.* (1999). Bacterial DNA (16S rRNA, *recA* and *gyrB*) and host chloroplast DNA regions (*rps16*, *trnL-trnF*, *trnG*, *petD*, *petA-psbJ*, and *atpI-atpH*) were amplified with the primers listed in Supplementary data Table 1. All amplification reactions were performed using a GeneAmp PCR System 9700 (Applied Biosystems, USA). Each amplification reaction was performed in 25 µl reaction mix containing 1 µl total DNA, 16 µl H_20, 2.5 µl 10× PCR buffer, 0.75 µl 25 µM $MgCl_2$, 1 µl of 20 µM forward and reverse primers, 2.5 µl 2 µM dNTP, and 0.2 µl *Ta q* DNA polymerase. PCR amplification of endosymbiont DNA regions was performed with PCR parameters as described previously (Lemaire *et al*., 2011). Amplification of *rps16*, *trnL-trnF*, *trnG*, *petA-psbJ*, and *atpI-atpH* was carried out under the following conditions: 94°C for 3 min; 30 cycles at 94°C for 60 sec, 52°C for 60 sec, 72°C for 90 sec; final extension at 72°C for 5 min. The amplification parameters for *petD* were 94°C for 3 min; 30 cycles at 94°C for 60 sec, 55°C for 60 sec, 72°C for 90 sec; final extension at 72°C for 5 min. Amplified products were purified using a modification of the Exo/SAP enzyme cleaning protocol (Werle *et al*., 1994).

Amplified 16S rRNA products were cloned into a pGEM-T vector (Promega), according to the manufacturer's instructions, and transformed into JM109 *E. coli* by heat shock. Plasmid purification was obtained with a PureYield™ Plasmid Miniprep System (Promega).

Purified plasmids and PCR products were sent to Macrogen for sequencing (Macrogen Inc., Korea).

Table 1. Taxon accession data with herbarium vouchers, silica-gel collections, (geographical) origins and GenBank accession numbers of leaf nodulated endosymbionts and host plants. Specimens were obtained from the National Botanic Garden of Belgium (BR). Underlined taxa represent accessions that were newly sequenced for this study.

Taxon	Strain/Voucher	Origin	GenBank accession number		
			16S rRNA	*recA*	*gyrB*
Burkholderia ambifaria	LMG 19182	Pea, rhizosphere	HQ849072	HQ849130	HQ849186
Burkholderia caribensis	LMG 18531	Vertisol	HQ849077	HQ849135	HQ849190
Burkholderia cepacia	LMG 1223	Allium cepa	HQ849078	JF295011	HQ849191
Burkholderia fungorum	LMG 16225	Phanerochaete chrysosporium, fungus	HQ849081	HQ849138	HQ849194
Burkholderia gladioli	LMG 11626	Poisoned bongkrek	HQ849082	HQ849139	HQ849195
Burkholderia glathei	LMG 14190	Lateritic soil	U96935	AY619666	EU024198
Burkholderia glathei	LMG 14190	Lateritic soil	HQ849084	HQ849141	HQ849197
Burkholderia graminis	LMG 18924	Maize senescent root system	HQ849086	HQ849143	HQ849199
Burkholderia kururiensis	LMG 19447	Aquifer sample	HQ849088	HQ849145	HQ849201
Burkholderia multivorans	LMG 13010	Cystic fibrosis patient	HQ849090	-	HQ849203
Burkholderia oklahomensis	LMG 23618	Soil	HQ849092	HQ849148	HQ849205
Burkholderia plantarii	LMG 9035	Oryza sativa, seedling	HQ849098	HQ849153	HQ849210
Burkholderia stabilis	LMG 14294	Cystic fibrosis, patient	HQ849103	HQ849159	JF295010
Burkholderia tropica	LMG 22274	Sugarcane, roots	HQ849105	HQ849161	HQ849216
Burkholderia tuberum	LMG 21444	Aspalathus carnosa, root nodule	HQ849106	HQ849162	HQ849217
Burkholderia vietnamiensis	LMG 10929	Oryza sativa, rhizosphere soil	HQ849107	HQ849163	HQ849218
Candidatus Burkholderia andongensis	BL 259 (BR)	*Sericanthe andongensis (Hiern) Robbr.*, leaf nodules; South Africa, Louis Trichardt	-	JF916912	JF916907
Candidatus Burkholderia andongensis	BL 271 (BR)	*Sericanthe andongensis (Hiern) Robbr.*, leaf nodules; South Africa, Vhembe	JF916918	JF916913	JF916908
Candidatus Burkholderia andongensis	BL 286 (BR)	*Sericanthe andongensis (Hiern) Robbr.*, leaf nodules; South Africa, Vhembe	JF916919	-	JF916906
Candidatus Burkholderia andongensis	BL 293 (BR)	*Sericanthe andongensis (Hiern) Robbr.*, leaf nodules; South Africa, Tathe Vondo	JF916920	JF916914	JF916909
Candidatus Burkholderia andongensis	SD 1097 (BR)	*Sericanthe andongensis (Hiern) Robbr.*, leaf nodules; Zambia	JF916921	JF916915	JF916905
Candidatus Burkholderia calva	1962-0512 (BR)	*Psychotria calva* Hiern, leaf nodules; Unknown	HQ849116	HQ849172	JF295009
Candidatus Burkholderia calva	1964-0306 (BR)	*Psychotria calva* Hiern, leaf nodules; Ivory Coast	HQ849117	HQ849173	HQ849227
Candidatus Burkholderia hispidae	SD 3176 (BR)	*Pavetta hispida* Hiern, leaf nodules; Cameroon, Ebolowa	HQ849122	HQ849178	HQ849231
Candidatus Burkholderia hispidae	OL 732 (BR)	*Pavetta hispida* Hiern, leaf nodules; Cameroon, Efoulan	HQ849123	HQ849179	HQ849232
Candidatus Burkholderia kirkii	1953-6779 (BR)	*Psychotria kirkii* Hiern, leaf nodules; Unknown	HQ849109	HQ849165	HQ849220
Candidatus Burkholderia kirkii	2000-1946-61 (BR)	*Psychotria kirkii* Hiern, leaf nodules; D.R. Congo, Kantanga	HQ849110	HQ849166	HQ849221
Candidatus Burkholderia nigropunctata	PS 13 (BR)	*Psychotria nigropunctata* Hiern; D.R. Congo, Kisantu	HQ849118	HQ849174	HQ849228
Candidatus Burkholderia nigropunctata	SD 1849 (BR)	*Psychotria nigropunctata* Hiern, leaf nodules; Gabon, Bemboudié	HQ849119	HQ849175	JF295008
Candidatus Burkholderia petitii	SD 1512 (BR)	*Sericanthe aff. petitii (N.Hallé) Robbr.*, leaf nodules; Cameroon, Mbikiliki	JF916923	JF916916	JF916911
Candidatus Burkholderia petitii	OL 658 (BR)	*Sericanthe aff. petitii (N.Hallé) Robbr.*, leaf nodules; Cameroon, Efoulan	JF916922	JF916917	JF916910
Candidatus Burkholderia rigidae	OL 694 (BR)	*Pavetta rigida* Hiern, leaf nodules; Cameroon, Efoulan	HQ849120	HQ849176	HQ849229
Candidatus Burkholderia rigidae	OL 877 (BR)	*Pavetta rigida* Hiern, leaf nodules; Cameroon, Nkolakié	HQ849121	HQ849177	HQ849230
Candidatus Burkholderia schumannianae	SD 1099 (BR)	*Pavetta schumanniana* F.Hoffm.ex K.Schum., leaf nodules; South Africa	HQ849124	HQ849180	HQ849233
Candidatus Burkholderia schumannianae	2001-9442-57 (BR)	*Pavetta schumanniana* F.Hoffm.ex K.Schum., leaf nodules; D.R. Congo	HQ849126	HQ849182	HQ849235
Ralstonia pickettii	12J		NC010678	NC010682	NC010682

Table 1. Continued

Taxon	Strain/Voucher	Origin	GenBank accession number					
			rps16	*trnL-trnF*	*trnG*	*petD*	*petA-psbJ*	*atpI-atpH*
Coffea stenophylla G.Don	1937-0053 (BR)	D.R. Congo	JF916942	JF916964	JF916953	JF916975	JF916931	JF916924
Empogona kirkii Hook.f.	1976-1052 (BR)	Zimbabwe	JF916943	JF916965	JF916954	JF916976	JF916932	JF916925
Sericanthe andongensis (Hiern) Robbr.	SD 1097 (BR)	Zambia	JF916944	JF916966	JF916955	JF916977	JF916933	-
Sericanthe andongensis (Hiern) Robbr.	Chapman 6150 (BR)	Malawi	JF916945	JF916967	JF916956	JF916978	JF916934	-
Sericanthe auriculata (Keay) Robbr.	SD 1467 (BR)	Cameroon	JF916946	JF916968	JF916957	JF916979	JF916935	JF916926
Sericanthe auriculata (Keay) Robbr.	SD 1516 (BR)	Cameroon	JF916947	JF916969	JF916958	JF916980	JF916936	JF916927
Sericanthe odoratissima (K.Schum.) Robbr.	Polhill *et al.* 5007A (BR)	Tanzania	JF916948	JF916970	JF916959	-	JF916937	-
Sericanthe odoratissima (K.Schum.) Robbr.	Salubeni 3135 (BR)	Malawi	JF916949	JF916971	JF916960	JF916981	JF916938	-
Sericanthe petitii (N.Hallé) Robbr.	SD 1512 (BR)	Cameroon	JF916950	JF916972	JF916961	JF916982	JF916939	JF916928
Sericanthe petitii (N.Hallé) Robbr.	OL 658 (BR)	Cameroon	JF916951	JF916973	JF916962	JF916983	JF916940	JF916929
Sericanthe spec. nov.	SD 2608 (BR)	Cameroon	JF916952	JF916974	JF916963	JF916984	JF916941	JF916930

Phylogenetic analyses

Sequences were assembled and edited using the program Geneious 5.0.3 (http://www.geneious.com). A preliminary sequence alignment was created with Muscle (Edgar, 2004) followed by manual adjustments with MacClade 4.04 (Maddison and Maddison, 2001). Molecular data were analyzed using Maximum Likelihood (ML) and Bayesian Inference (BI) criteria, both of which were implemented in the CIPRES web portal (http:// www.phylo.org). ML analyses were performed with RAxML-VI-HPC v2.0 using GTR-GAMMA as the nucleotide substitution model (Stamatakis, 2006). We performed 100 RAxML runs and selected the best ML tree by comparing the likelihood scores. The robustness of the ML tree was calculated with multi-parametric bootstrap resampling and 1000 pseudo-replicates.

Model selection for the Bayesian analysis was conducted with MrModeltest v. 3.06 (Posada and Crandall, 1998) under the Akaike information criterion. For the different datasets, Modeltest selected the following models of evolution: *petD* – GTR; *rps16* – GTR+I; *trnG* – F81; *trnL-trnF* – HKY; *petA-psbJ* – HKY; *atpI-atpH* – GTR; 16S rRNA – GTR+I+G; *recA* – GTR+I+G; *gyrB* – GTR+I+G.

In the combined BI analyses, the concatenated datasets (*petD* + *rps16* + *trnG* + *trnL-trnF* + *petA-psbJ* + *atpI-atpH* and 16S rRNA + *recA* + *gyrB*) were partitioned and the same models were assigned to the separate partitions as selected for the single analyses. Gaps in the chloroplast data were coded according to the simple indel coding method described by Simmons and Ochoterena (2000). Bayesian analyses were conducted with MrBayes 3.1 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003), and four Markov chains were ran simultaneously for five million generations and sampling trees every 100 generations. The 25% initial trees were discarded as conservative "burnin". Convergence of the chains was checked using Tracer v.1.4 (Rambaut and Drummond, 2007).

Morphological observations

Morphological observations of leaf material of *S. andongensis* (accession: Lemaire *et al.* 293) and *S. petitii* (accession: Lachenaud *et al.* 658) were conducted to illustrate the bacterial endosymbionts. Sections through leaf nodules were made with a razor blade and the dissected material was washed repeatedly in 70% ethanol and dehydrated in a 1:1 mixture of ethanol and dimethoxymethan (DMM) for 20 min and in pure DMM for 20 min. After critical-point drying (CPD 030, BAL-TEC AG, Liechtenstein), dried samples were mounted onto aluminium stubs, coated with gold (SPI Module Sputter Coater, Spi Supplies, USA) and observed under a scanning electron microscope (JEOL JSM-6360; Jeol Ltd, Japan).

Results and Discussion

Phylogenetic analyses of the endosymbiont data

The use of 16S rRNA, *recA* and *gyrB* data to infer the phylo-genetic relationships in the genus *Burkholderia* is

quite common and offers high resolution at both high and low taxonomic levels (Payne *et al*., 2005; Tabacchioni *et al*., 2008). These genes have been shown to provide a robust framework to determine the phylogenetic placement of the symbiotic bacteria of leaf nodulating Rubiaceae, as previously described in Lemaire *et al.* (2011). In the present study, a similar approach was used to identify the endosymbionts of nodulating *Sericanthe* species: molecular identification of endosymbionts was first performed by 16S rRNA sequencing and 16S rRNA BLAST searches, and *recA* and *gyrB* genes were used to increase the relative discriminatory power.

Direct sequencing of full-length 16S rRNA from 10 clones per plant species produced consistent results and assigned the endosymbionts of the leaf nodulating *Sericanthe* species (*S. andongensis* and *S. petitii*) in the *Burkholderia* genus. This β-Proteobacteria genus also includes the endosymbionts of the two other nodulating genera of the family, *Psychotria* and *Pavetta*. Amplified *recA* and *gyrB* data with *Burkholderia* specific primers (Supplementary data Table 1) were analyzed in combination with the 16S rRNA data, including 19 nodulated endosymbionts and 14 related *Burkholderia* strains. The phylogenetic analyses of the separate datasets showed similar topologies, except for few terminal branches. The phylogenies produced separately by the three datasets (16S rRNA, *recA* and *gyrB*) are shown in supplementary data Fig. 1.

Both the BI and ML analyses produced similar tree topologies and support values (Fig. 1). A well-supported clade (100% Bayesian posterior probability, BPP / 99% bootstrap support, BS) with endosymbionts of leaf nodulating *Psychotria*, *Pavetta*, and *Sericanthe* plants was recovered as sister to *Burkholderia glathei*. All *S. andongensis* endosymbionts were positioned in a clade with maximum support that was sister to the endosymbionts of *Pavetta rigida* and *Pavetta hispida*. The intersequence similarities between the lineages from both clades ranged from 96% (*Candidatus* Burkholderia andongensis vs. *Candidatus* Burkholderia hispidae) to 96.5% (*Candidatus* Burkholderia andongensis vs. *Candidatus* Burkholderia rigidae). The endosymbionts of *S. petitii* (100% BPP / 100% BS) were related to the '*Candidatus* Burkholderia kirkii – *Candidatus* Burkholderia schumannianae' clade. The sequence divergence between both nodulating clades ranged from 94.6% (*Candidatus* Burkholderia petitii vs *Candidatus* Burkholderia kirkii) to 95.0% (*Candidatus* Burkholderia petitii vs *Candidatus* Burkholderia schumannianae). These phylogenetic patterns indicate that endosymbiosis occurred multiple times in Rubiaceae, thus rejecting the hypothesis of a single infection event within the ancestor of extant leaf nodulated *Pavetta*, *Psychotria*, and *Sericanthe* species.

Five different samples of *S. andongensis* and two accessions of *Sericanthe petitii* from different geographical locations were investigated (Table 1). Intraspecific sequence variability among the endosymbiont strains of both species was low (average sequence identity between *S. andongensis* accessions: 16S rRNA - 100%; *recA* - 100%; *gyrB* - 99.9% and *S. petitii* accessions: 16S rRNA - 100%; *recA* - 99.8%; gyrB - 100%), suggesting a stable interaction and high specificity between host and endosymbiont. A similar pattern of host specificity has been documented in *Psychotria* and *Pavetta* (Van Oevelen *et al*., 2001, 2002, 2004; Lemaire *et al*., 2011). The phylogenetic analyses presented in this study show that the evolutionary distances between the *Sericanthe* endosymbionts and their closest relatives were significant compared to the observed intraspecific polymorphism to recognize these endosymbionts as novel *Burkholderia* species. As long as the cultivation of *Sericanthe* endosymbionts is not possible (E. Prinsen 2011, pers. comm.), we propose to record these endosymbionts under a *Candidatus* designation, according to Murray and Stockebrandt (1995). The endosymbionts of *S. andongensis* and *S. petitii* can be described using the specific epithets of their host species as specific epithets for these candidate *Burkholderia* species:

'*Candidatus* Burkholderia andongensis' (andongensis, from the specific epithet of the host plant) (*β*-proteobacteria, genus *Burkholderia*); NC; G-; R; NAS (GenBank nos. JF916921, JF916915, JF916905), oligonucleotide sequence complementary to unique region of 16S rRNA gene 5′-ACTTCGTCCCTAATA ATGGATGGAG-3′, oligonucleotide sequence complementary to unique region of *recA* 5′-CGCGTTCATCGATGCCGAAC ACGCGCTC-3′, oligonucleotide sequence complementary to unique region of *gyrB* gene 5′-TCGCACGGCGTCGTGCAG AACCGTGAAGT-3′; S (*S. andongensis*, leaf galls). Lemaire *et al*. this study.

'*Candidatus* Burkholderia petitii' (petitii, from the specific epithet of the host plant) (*β*-proteobacteria, genus *Burkholderia*); NC; G-; R; NAS (GenBank nos. JF916923, JF916916, JF916911), oligonucleotide sequence complementary to unique region of 16S rRNA gene 5′-GCTTCGGGGTTAATACCCCT GGGG-3′, oligonucleotide sequence complementary to unique region of *recA* 5′-ACGTGCAATACGCCTCGAAGCTTGGC GTGAACGTGCCGGAT-3′, oligonucleotide sequence complementary to unique region of *gyrB* gene 5′-ATGGAGTTC GCGCGTGGAGTCGTGCAGAACCGC-3′; S (*S. petitii*, leaf galls). Lemaire *et al*. this study.

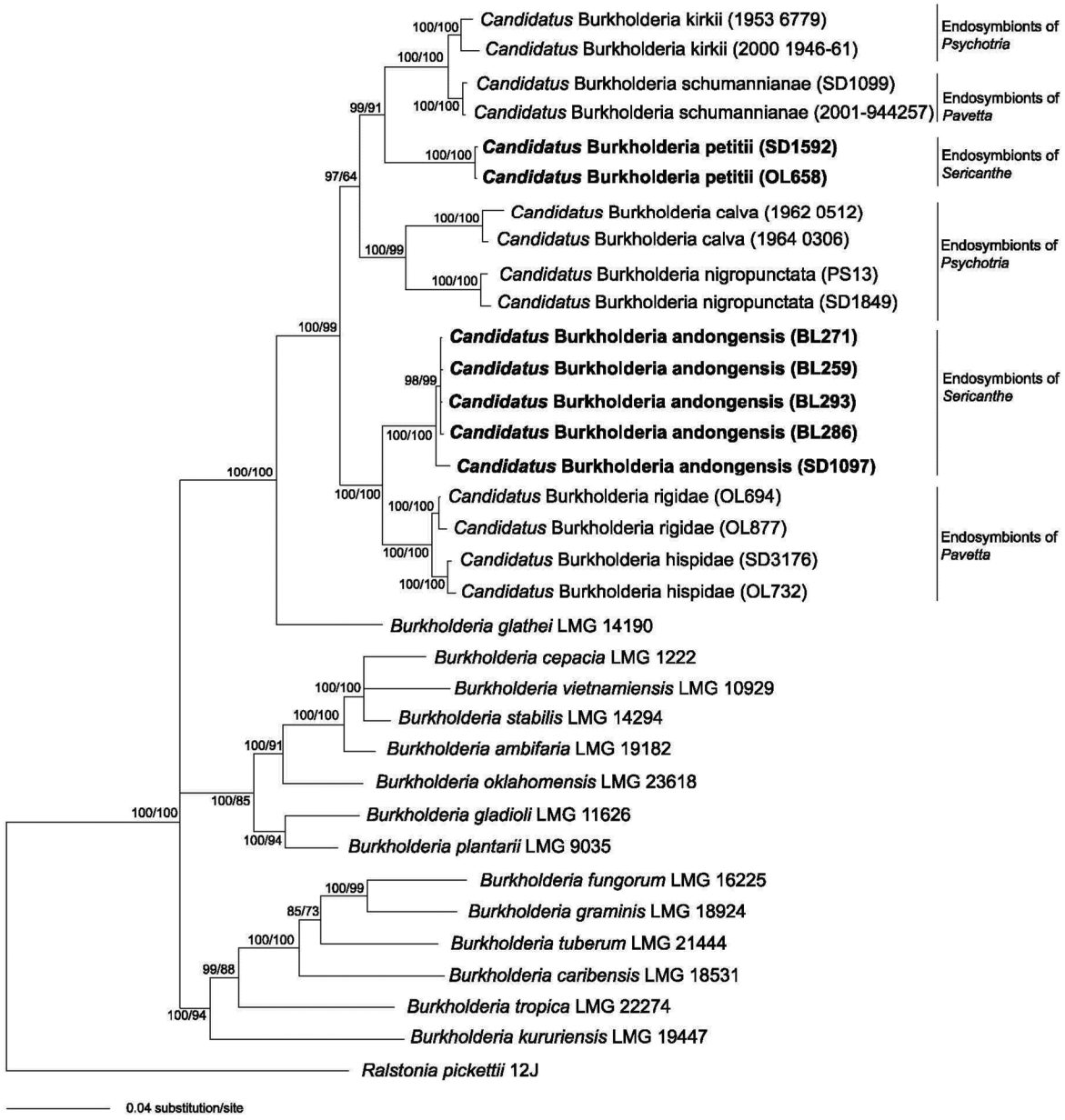

Fig. 1. Phylogenetic tree of bacterial endosymbionts based on 16S rRNA, *recA* and *gyrB* data. Support values for the Bayesian and Maximum Likelihood analyses are given at the nodes (Bayesian posterior probabilities-bootstrap values from the Maximum Likelihood analysis). Branches of leaf nodulating endosymbionts are shown in bold. Names of newly proposed bacterial taxa are shown in bold.

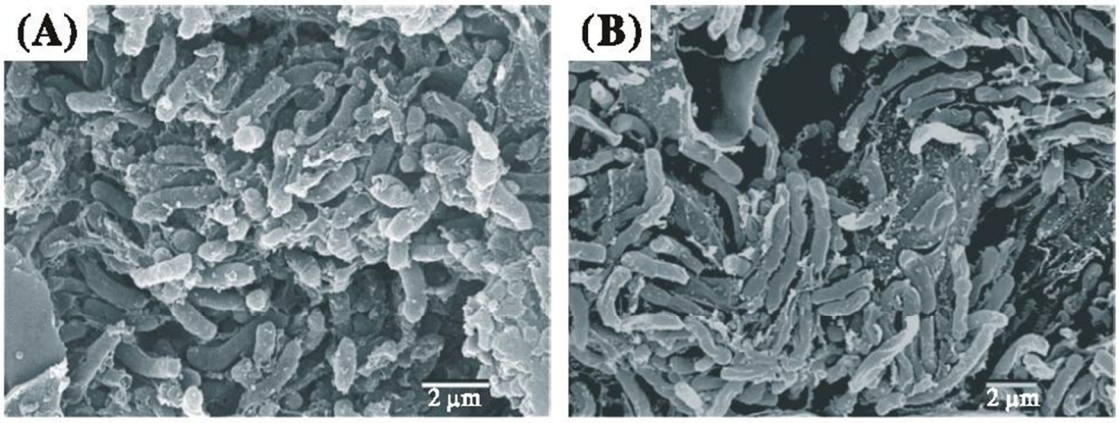

Fig. 2. SEM photographs of leaf nodulating endosymbionts of (A) *S. petitii* and (B) *S. andongensis*. Non-flagellated rod-shaped bacteria with a mean length of 2 µM are visible.

Morphological observations of leaf nodulating endosymbionts

The phylogentic analyses showed that leaf nodulated *Sericanthe* species accommodate a single species-specific endosymbiont. As a result, we were able to use the non-specific scanning electron microscopy to illustrate the endosymbionts in leaf nodule structures.

The bacterial endosymbionts within leaf nodules of *S. andongensis* and *S. petitii* are shown in Figs. 2A and B. Cross SEM sections of leaves were made to illustrate the bacterial morphology and the localization of the endosymbionts within nodules. The endosymbionts were restricted to the leaf gall structures and were clearly visible as rod shaped bacteria with an average length of 2 µm. No flagella were observed. The endosymbionts of *Sericanthe* were similar in size (1-2 µm) and shape (bacterial rods) compared to the symbionts of *Psychotria* and *Pavetta* [see previous observations in the study of Va n Oevelen *et al.* (2004) and Lemaire *et al.* (2011)].

Phylogenetic analyses of hosts

To reconstruct the phylogenetic relationships between nodulated and non-nodulated *Sericanthe* species, 66 sequences were generated including six chloroplast regions (Table 1). Genetic variation among all chloroplast DNA regions was extremely low, ranging from 0.8% to 3.5% of variable sites (Supplementary data Table 2). In contrast, the alignment of the 16S rRNA, *recA* and *gyrB* sequences revealed higher levels of genetic variability. This difference in sequence variability between plants and bacteria is probably linked to different rates of molecular evolution associated with differences in body size, metabolic rate, DNA repair and generation time (Bromham, 2009). The phylogenetic relationships obtained from the six individual plastid markers were analyzed separately, and the resulting tree topologies were phylogenetically consistent. Consequently, the datasets were combined in subsequent analyses to increase phylogenetic resolution. Indels were binary coded and added to data matrices to increase support values. The Bayesian majority rule consensus tree and the Maximum Likelihood tree were congruent and are shown in Fig. 3. Overall, most phylogenetic relationships were resolved with high support values. However, the phylogenetic relationships between the nodulating *Sericanthe* species (showed in bold) and non-nodulating species were not completely resolved, showing a polytomy with members of *S. andongensis*, *S. odoratissima*, *S. petitii*, and *S. auriculata*. All nucleotide positions within the alignment were examined by eye and no single character was informative to resolve this node. Nevertheless, the observed phylogenetic relationships in this study do not rule out the possibility that bacterial endosymbiosis evolved in a parsimonious way, as demonstrated for other nodulated genera, i.e. *Psychotria* (Andersson, 2002) and *Pa v e t t a* (De Block *et al.* unpublished).

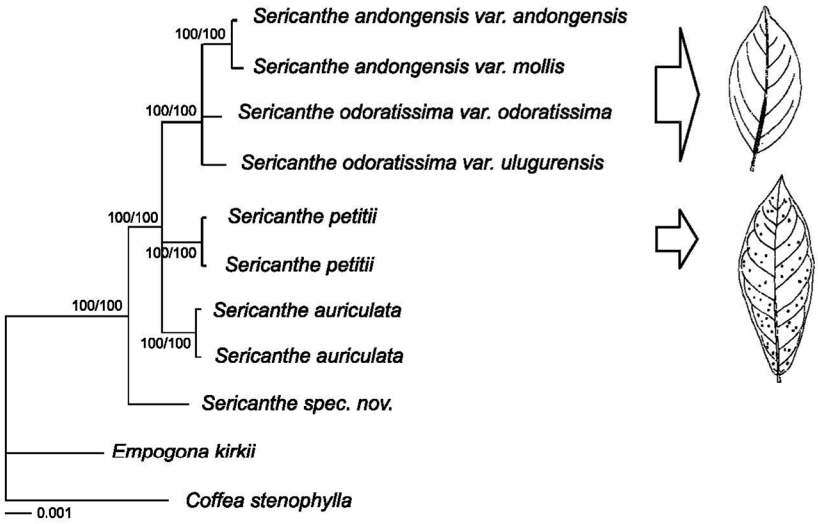

Fig. 3. Phylogenetic tree of hosts based on chloroplast data (*rps16*, *trnG*, *trnL-trnF*, *petD*, *petA-psbJ*, and *atpI-atpH*). Support values for the Bayesian and Maximum Likelihood analyses are given at the nodes (Bayesian posterior probabilities - bootstrap values from the Maximum Likelihood analysis). Branches of leaf nodulated representatives are shown in bold. Leaves with leaf nodules are redrawn from Robbrecht (1978a). Top: leaf galls located along the midvein (*S. andongensis* var. *andongensis*). Bottom: leaf galls dispersed over the leaf blade (*S. petitii*).

Conclusions

The three nodulating genera have no close affinity and have been placed within different tribes and Rubiaceae subfamilies, which could lead to the conclusion that bacterial leaf nodule symbiosis originated independently in these three genera. Surprisingly, our results demonstrate that all endosymbionts of leaf nodulating Rubiaceae are closely related, but that neither the endosymbionts of *Sericanthe* nor the endosymbionts of *Pavetta* or *Psychotria* are monophyletic. These findings contrast with previous results showing that these three nodulating taxa are monophyletic (Andersson, 2002; Davis *et al*., 2007; T o s h *et al*., 2009; De Block *et al*., unpublished). The present results suggest thereby that the history of bacterial leaf symbiosis is characterized by horizontal symbiont transfers and reject the hypothesis of strict co-speciation between plant and bacteria at generic level.

Acknowledgements

The authors thank Elsa van Wyk and Magda Nel for their excellent support at the H.G.W.J. Schweickerdt Herbarium, Department of Plant Science, University of Pretoria (South Africa). We are also grateful to Norbert Hahn, who accompanied us during the expedition in South Africa. We thank the King Léopold III Fund and the Fund for Scientific Research – Flanders (FWO) which provided financial support for fieldwork in South Africa. This work was supported by the Institute for the Promotion of Innovation by Science and Technology in Flanders (IWT Vlaanderen, no. 71488). General financial support was provided by a grant of the Research Program of the Fund for Scientific Research – Flanders (Belgium) (FWO – Vlaanderen, G.0343.09N) and the K.U. Leuven (OT/05/35).

References

Andersson, L. 2002. Relationships and generic circumscriptions in the *Psychotria* complex (Rubiaceae, Psychotrieae). *Syst. Geogr.* Pl. 72, 167-202.
Bremekamp, C.E.B. 1933. The bacteriophilous species of *Psychotria*. *Lond. J. Bot.* 71, 271-281.
Bromham, L. 2009. Why do species vary in their rate of molecular evolution? *Biol. Lett.* 5, 401-404.
Davis, A.P., M. Chester, O. Maurin, and M. Fay. 2007. Searching for the relatives of *Coffea* (Rubiaceae, Ixoroideae): the circumscription and phylogeny of Coffeeae based on plastid sequence data and

morphology. *Am. J. Bot.* 94, 313-329.
Edgar, R.C. 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res.* 32, 1792-1797.
Gordon, J.F. 1963. The nature and distribution within the plant of the bacteria associated with certain leaf-nodulated species of the families Myrsinaceae and Rubiaceae, p. 370. Thesis. Univ. London.
Huelsenbeck, J.P. and F. Ronquist. 2001. MRBAYES: Bayesian inference of phylogeny. *Bioinformatics* 17, 754-755.
Lemaire B., P. De Block, S. Van Oevelen, B. Verstraete, E. Smets, E. Prinsen, and S. Dessein. 2011. Identification of the bacterial endosymbionts in leaf nodulating *Pavetta* species (Rubiaceae). *Int. J. Syst. Evol. Microbiol.* Article in press.
Maddison, D.R. and W. P. Maddison. 2001. MacClade 4: Analysis of phylogeny and character evolution, version 4.01. Sinauer Associates, Sunderland, Massachusetts, USA.
Miller, I.M. 1990. Bacterial leaf nodule symbiosis. *Adv. Bot. Res.* 17, 163-243.
Murray, R.G.E. and E. Stackebrandt. 1995. Taxonomic note: implementation of the provisional status Candidatus for incompletely described prokaryotes. *Int. J. Syst. Bacteriol.* 45, 186-187.
Payne, G.W., P. Vandamme, S.H. Morgan, J.J. LiPuma, T. Coeyne, A.J. Weightman, T.H. Jones, and E. Mahenthiralingam. 2005. Development of *a recA* gene-based identification approach for the entire *Burkholderia* genus. *Appl. Environ. Microbiol.* 71, 3917-3927.
Posada, D. and K.A. Crandall. 1998. Modeltest: testing the model of DNA substitution. *Bioinformatics* 14, 817-818.
1. Rambaut, A. and A.J. Drummond. 2007. Tracer v1http://evolve.zoo. ox.ac.uk/software.html.
Robbrecht, E. 1978a. *Sericanthe*, a new African genus of Rubiaceae (Coffeeae). *Bull. Nat. Plantentuin Belg.* 48, 3-78.
Robbrecht, E. 1978b. Rubiaceae. *Distr. Pl. Afr*. 13: map 406-429.
Robbrecht, E. 1981. Studies in tropical African Rubiaceae (I). *Bull. Nat. Plantentuin Belg.* 51, 165-189.
Robbrecht, E. and J.F. Manen. 2006. The major evolutionary lineages of the coffee family (Rubiaceae, angiosperms). Combined analysis (nDNA and cpDNA) to infer the position of Coptosapelta and Luculia, and supertree construction based on *rbcL*, *rps16*, *trnL-trnF* and *atpB-rbcL* data. A new classification in two subfamilies, Cinchonoideae and Rubioideae. *Syst. Geogr. Pl.* 76, 85-146.
Ronquist, F. and J.P. Huelsenbeck. 2003. MrBayes 3: Bayesian phylo-genetic inference under mixed models. *Bioinformatics* 19, 1572-1574.
Simmons, M.P. and H. Ochoterena. 2000. Gaps as characters in sequence-based phylogenetic analyses. *Syst. Biol*. 49, 369-381.
Stamatakis, A. 2006. RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics* 22, 2688-2690.
Tabacchioni, S., L. Ferri, G. Manno, M. Mentasti, P. Cocchi, S. Campana, N. Ravenni, and *et al*. 2008. Use of the *gyrB* gene to discriminate among species of the *Burkholderia cepacia* complex. *FEMS Microbiol. Lett.* 281, 175-182.
Tel-Zur, N., S. Abbo, D. Mylaboski, and Y. Mizrahi. 1999. Modified CTAB procedure for DNA isolation from epiphytic cacti of the genus *Hylocereus* and *Selenicerus* (Cactaceae). *Plant Mol. Biol. Rep.* 17, 249-254.
Tosh, J., A.P. Davis, S. Dessein, P. De Block, S. Huysmans, M.F. Fay, E. Smets, and E. Robbrecht. 2009. Phylogeny of *Tr i c a l y s i a* (Rubiaceae) and its relationships with allied genera based on plastid DNA data: resurrection of the genus *Empogona. Ann. Mossouri. Bot. Gard.* 96, 194-213.
Va n Hove, C. 1972. Structure and initiation of nodules in the leaves of *Neorosea andongensis* (Hiern) N. Hallé. *Ann. Bot.* 36, 259-262.
Van Oevelen, S., R. De Wachter, P. Vandamme, E. Robbrecht, and E. Prinsen. 2002. Identification of the bacterial endosymbionts in leaf galls of *Psychotria* (Rubiaceae, angiosperms) and proposal of '*Candidatus* Burkholderia kirkii' sp. nov. *Int. J. Syst. Evol. Microbiol.* 52, 2023-2027.
Van Oevelen, S., R. De Wachter, P. Vandamme, E. Robbrecht, and E. Prinsen. 2004. '*Candidatus* Burkholderia calva' and '*Candidatus* Burkholderia nigropunctata' as leaf gall endosymbionts of African *Psychotria*. *Int. J. Syst. Evol. Microbiol.* 54, 2237-2239.
Va n Oevelen, S., E. Prinsen, R. De Wachter, and E. Robbrecht. 2001. The taxonomic value of bacterial symbiont identification in African *Psychotria* (Rubiaceae). *Syst. Geogr. Pl.* 71, 557-563.
Werle, E., C. Schneider, M. Renner, M. Volker, and W. Fiehn. 1994. Convenient single step, one tube purification of PCR products for direct sequencing. *Nucleic Acids Res.* 22, 4354-4355.