

Screening for leaf-associated endophytes in the genus *Psychotria* (Rubiaceae)

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Introduction

Bacterial endophytes are reported from a broad range of host plants including both woody and herbaceous angiosperms (Lodewyckx *et al.*, 2002; Rosenblueth & Martinez-Romero, 2006). In these interactions, the term ‘endophyte’ is used to describe microorganisms that colonize the internal tissue of a healthy plant at a particular moment, without causing visible disease symptoms or negative effects on their host (Schulz & Boyle, 2005; Ryan *et al.*, 2008). The list of known hosts has been increasing progressively year by year, and it is expected that all studied angiosperms accommodate endophytes (Strobel *et al.*, 2004). Most of the studies, however, concern bacteria from the rhizosphere of plants (Berg *et al.*, 2005; Raaijmakers *et al.*, 2008; Compant *et al.*, 2010). Endophytes associated with leaves are substantially less documented, yet a number of examples are known, which can be divided into two categories: nodulating and non-nodulating endophytes.

Nodulating leaf endophytes are housed inside special formed structures within the leaves (i.e. leaf nodules or

Abstract

Burkholderia endophytes were identified within the leaves of non-nodulated members of the genus *Psychotria*. In contrast to leaf-nodulated *Psychotria* species, which are known to accommodate their endosymbionts into specialized endosymbiont-housing structures, non-nodulated species lack bacterial leaf nodules and harbor endosymbionts intercellularly between mesophyll cells. Based on molecular data (*rps16*, *trnG*, and *trnLF*), the phylogenetic reconstruction of the host plants revealed a separate origin of leaf-nodulated and non-nodulated *Psychotria* species. Despite a distinct phylogenetic position of the two host clades, the endophytes of the non-nodulated plants were not placed into a single monophyletic group but were found to be closely related to the leaf-nodulated endosymbionts. The observation of genetically similar endophytes in both nodulated and non-nodulated *Psychotria* lineages suggests that the host plant is playing a crucial role in the induction of leaf nodule formation. Moreover, the concentration of endosymbionts into specialized leaf nodules may be considered as a more derived evolutionary adaptation of the host plant, serving as an interface structure to facilitate metabolic exchange between plant and endosymbiont.

galls), which are clearly visible to the eye. About 500 leaf-nodulated plant species are known to date, placed in three genera of *Rubiaceae* (*Pavetta*, *Psychotria*, and *Sericanthe*) and three genera of *Primulaceae* s.l. (*Amblyanthus*, *Amblyanthopsis*, and *Ardisia*) (Miller, 1990). Recently, much progress has been made in understanding the evolution of this particular bacteria–plant symbiosis (Van Oevelen *et al.*, 2001, 2002, 2004; Lemaire *et al.*, 2011a, b, c, 2012a, b). All nodulated plant species studied so far have a single host-specific endosymbiont adapted to live *in planta* (Lemaire *et al.*, 2011c). Based on sequence analyses, the not yet cultured endosymbionts of all nodulated genera are identified as *Burkholderia* (Van Oevelen *et al.*, 2004; Lemaire *et al.*, 2011a, b, 2012b). In addition to the leaves, the bacteria are also found in the shoot tip to be recruited as an obligate and constant associate for the host plant. In *Psychotria*, for example, the endosymbionts are retained within the host plant during all stages of its life cycle (Miller, 1990; Lemaire *et al.*, 2012a) and are proved to be indispensable for normal plant development and survival (Gordon, 1963; Lemaire *et al.*, 2012a) although, occasionally, the vertically

inherited endosymbionts may be replaced by free-living soil *Burkholderia* when the host plant fails to transfer its original bacteria through the seeds (Lemaire *et al.*, 2011c, 2012a).

In contrast, non-nodulating leaf endophytes do not form visible nodules and occur between the leaf mesophyll cells. They are undetectable to the eye, and consequently, their distribution among plant species is not well known. Nevertheless, they are, among others, documented in *Vitis vinifera* (West *et al.*, 2010), *Gynura procumbens* (Bhore *et al.*, 2010), *Pinus sylvestris*, *Betula pendula*, *Sorbus aucuparia* (Izumi *et al.*, 2008), *Crocus albiflorus* (Reiter & Sessitsch, 2006), and *Populus alba* (Balestrazzi *et al.*, 2009).

More recently, non-nodulating leaf endophytes have been discovered in several South African *Rubiaceae* species of the genera *Fadogia* and *Vangueria* and have been identified as relatives of *Burkholderia caledonica* (Verstraete *et al.*, 2011). These bacteriophilous plants are known to cause gousiekte, a fatal cardiotoxicosis of ruminants characterized by acute heart failure (Kellarman *et al.*, 2005). In contrast to leaf-nodulated species, the endophytic bacteria colonize the internal leaf tissue between the mesophyll cells and are not restricted to specialized leaf nodules (van Wyk *et al.*, 1990). Finally, different microorganisms were also detected in the leaves of the *Rubiaceae* species *Coffea arabica* L. (Vega *et al.*, 2005).

In light of these recent discoveries, we suspected that non-nodulating leaf endophytes could occur in other genera of *Rubiaceae*, including *Psychotria*. We therefore conducted a screening of endophytes in non-nodulated *Psychotria* species. To yield a picture of the symbiotic origin and to provide novel insights into the endophytic biology of the rubiaceous genus *Psychotria*, a broad phylogenetic study of the host plants including the bacteriophilous species was conducted.

Materials and methods

Leaf morphology

Leaves of the nodulated species *Psychotria kirkii* (accession 2002152647-BR) and the non-nodulated species *Psychotria psychotrioides* (accession Dessein *et al.*, 2639) were prepared for scanning electron microscopy (JEOL JSM-6360; Jeol Ltd, Tokyo, Japan) observation following the critical point drying method described by Lemaire *et al.* (2012b).

Plant material and taxon sampling

Most of the material was collected during a field expedition in Cameroon (2009), but additional samples were obtained from the National Botanic Garden of Belgium

(BR), the herbarium of Wageningen (WAG), and DNA isolation from the study of Andersson (2002). Sampled species, accession numbers, and localities are listed in the Supporting Information, Tables S1 and S2.

DNA extraction, amplification, and sequencing

Prior to DNA extraction, silica-dried leaves were rinsed with 70% ethanol to avoid epiphytic contamination. DNA extraction, PCR amplification, and sequencing were followed as described in Lemaire *et al.* (2012b). Primers and temperature profiles used for the amplification of the bacterial DNA (16S rRNA gene, *recA*, and *gyrB*) and plastid host DNA (*rps16*, *trnG*, and *trnLF*) follow Lemaire *et al.* (2011c). All sequences generated in this study were submitted to GenBank (Tables S1 and S2).

Phylogenetic analyses

Sequences were assembled and edited using the program GENEIOUS v. 5.0.3 (<http://www.geneious.com>). All DNA regions of both endosymbionts and hosts were analyzed separately and combined to evaluate whether the data matrices provided different phylogenetic signals.

Model selection for the Bayesian inference analyses was conducted with MODELTEST v. 3.06 (Posada & Crandall, 1998) under the Akaike Information Criterion (AIC). The general time-reversible model of DNA substitution with gamma-distributed rate variation across invariant sites (GTR+I+G) was selected for 16S rRNA gene, *recA*, and *gyrB* regions. The GTR+G substitution model was chosen for the *rps16*, *trnG*, and *trnLF* datasets. Bayesian analyses were carried out with MRBAYES v. 3.1 (Ronquist & Huelsenbeck, 2003). Four chains (one cold, three heated) initiated from a random starting tree were run simultaneously for four Markov chains, sampling every 1000 generations. The 25% initial trees were discarded as conservative 'burnin.' Convergence of the chains was checked using TRACER v. 1.4 (Rambaut & Drummond, 2007).

Maximum likelihood analyses were carried out using RAXML search algorithm 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.

Results

Host phylogeny

The phylogenetic relationships of 129 species of the *Psychotria* complex were reconstructed with plastid *rps16*,

trnG, and *trnLF* sequence data (Table S1). Representatives of the genera *Rudgea*, *Chassalia*, *Geophila*, *Margaritopsis*, and *Palicourea* were used as out-group (Table S1). The results of Bayesian and likelihood analyses obtained from the three individual host genes (*rps16*, 1000 bp; *trnG*, 726 bp; *trnLF*, 605 bp) were highly concordant. Consequently, the datasets were combined in subsequent analyses to increase phylogenetic resolution. The Bayesian 50% majority rule consensus tree and the maximum likelihood tree of the combined dataset were phylogenetically congruent and are shown in Fig. 1 and in the Fig. S1. Our phylogenetic analyses distinguished several well-defined main clades (labeled as clade 1, clade 2, clade 3, clade 4, and the Pacific clade) that are in agreement with the previous study of Andersson (2002).

All leaf-nodulated *Psychotria* species (49 spp., clade II) were recovered as a monophyletic group with high support values [100% Bayesian posterior probability (BPP)/81% bootstrap support (BS)] and placed as sister group with a newly discovered non-nodulated species (accession Dessein et al. 1769). This sister group relationship is

highly supported (100% BPP/87% BS) with all representatives distributed in Africa.

Clade I (100% BPP/98% BS) comprises African and Neotropical lineages, and no bacterial endophytes were detected in the plants investigated, that is, *Psychotria cart-hagenensis* (accessions BR-19842833, BR-2006012742), *P. lucens* (accessions BR-19610404, BR-19620513), *P. nervosa* (accession BR-2007032959), *P. rufipilis* (accession BR-1995116713), and *P. vogeliana* (accession BR-2009142509).

Clade III (99% BPP/75% BS) contains African, Neotropical, and Asian species. Although the internal resolution is largely poor in clade III, four internal supported groups can be distinguished in relation to their geographical distribution (Fig. 1). In the African subclade A (29 spp., 97% BPP/49% BS), most species were found to accommodate non-nodulated bacterial endophytes (highlighted in gray, Fig 1). In *Psychotria eminiana* (accession FVC 56), *P. auxopoda* (accession BEB 1155), and *P. succulenta* (accessions Dessein et al. 2930, Dessein et al. 2948, Dessein et al. 1314, Dessein et al. 3127), however,

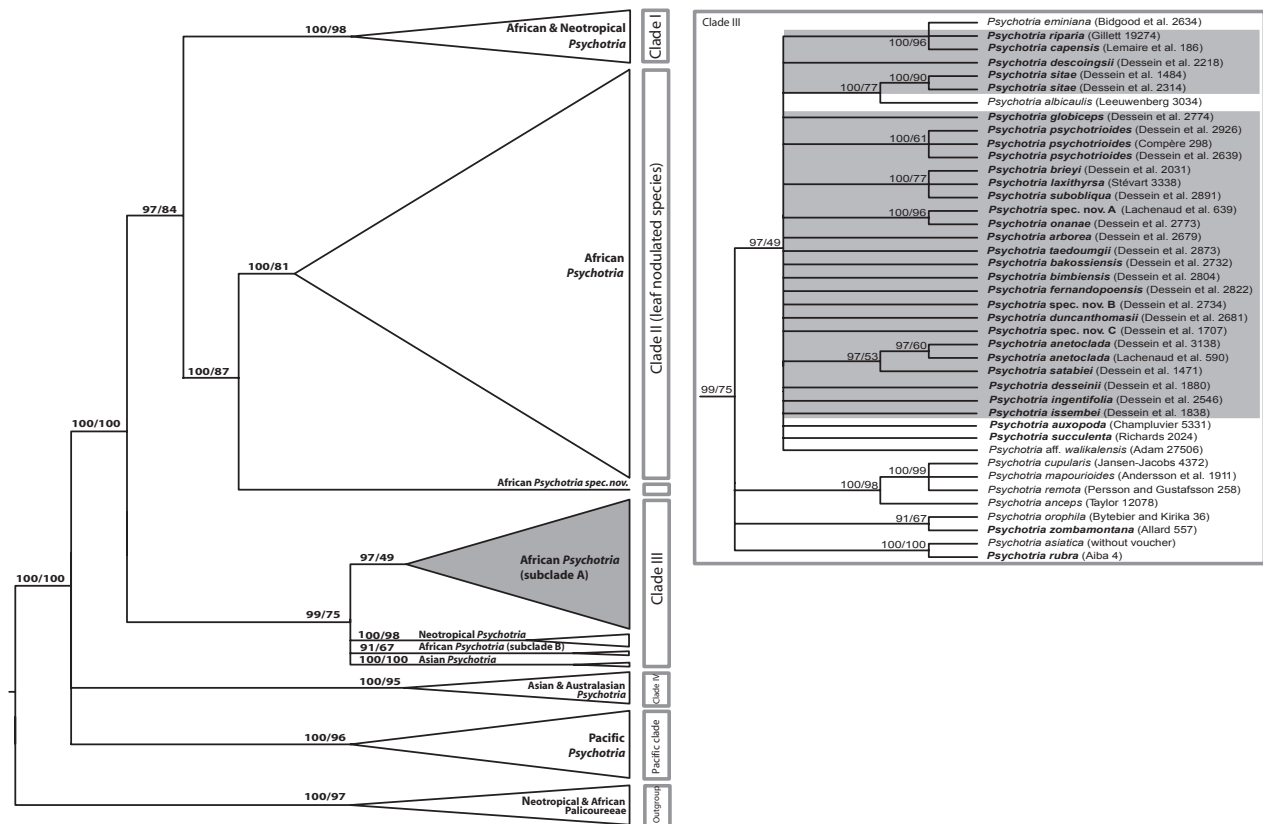


Fig. 1. Schematic representation of the *Psychotria* relationships (left) with detailed phylogenetic relationships of clade III (right). The topologies are based on a three-gene dataset (*rps16*, *trnG*, and *trnLF*). Support values of Bayesian and maximum likelihood analyses are indicated above branches. Leaf-nodulated species belong to clade II, while the bacteriophilous non-nodulated *Psychotria* species (gray shaded) are placed in clade III. Species tested for endophytes are indicated in bold.

endophytes were not detected. In the Asian subclade (100% BPP/100% BS) and African subclade B (91% BPP/67% BS), *Psychotria rubra* (accessions BR-1998153116; BR-2007108337) and *P. zombamontana* (accessions Lemaire *et al.* 118, Lemaire *et al.* 121) were tested for endophytes and no endophytes were detected.

Finally, a major pacific clade (100% BPP/96% BS) was recovered including the myrmecophilous taxa (i.e. *Myrmecodia*, *Myrmephytum*, *Hydnophytum*, and *Squamellaria*; 100% BPP/100% BS). In this group only herbarium material was available, which is not suitable for endophyte screening.

Endophyte identification and phylogeny

Non-nodulated *Burkholderia* endophytes were found in the majority of the *Psychotria* species of the African subclade A of clade III. Endophyte identification was based on BLAST searches of bacterial 16S rRNA gene using the GenBank nucleotide database, as described in Lemaire *et al.* (2012b). Additional data from two molecular markers (*gyrB* and *recA*) were included to improve bacterial identification and phylogenetic analyses. The culture-independent PCR analyses based on 16S rRNA gene, *recA*, and *gyrB* data indicated only one endophyte per plant species. Several replicas from different geographic locations for *Psychotria anetoclada* (13 individuals), *P. sitae* (two individuals), and *P. psychotrioides* (three individuals) were included. In *P. anetoclada* and *P. sitae*, no substantial intraspecific variation was found (*P. anetoclada*: 16S rRNA gene, 99.7%; *recA*, 99.5%; *gyrB*, 99.0%; *P. sitae*: 16S rRNA gene, 99.6%; *recA*, 99.1%; *gyrB*, 99.1%), suggesting a stable interaction and high specificity between endophyte and host. In *P. psychotrioides*, however, the endophyte with accession number Dessein *et al.* 2639 differs substantially from the other two individuals (16S rRNA gene, 97.0%; *recA*, 96.0%; *gyrB*, 97.7%). Between the latter two accessions of *P. psychotrioides* (Dessein *et al.* 2926 and 3093), no pronounced DNA variability within the 16S rRNA gene, *recA*, and *gyrB* regions was observed (16S rRNA gene, 99.9%; *recA*, 99.8%; *gyrB*, 100%).

The DNA sequences of the novel *Psychotria* endophytes were included in a reduced dataset generated from the study of Lemaire *et al.* (2011c) and subjected to detailed molecular phylogenetic analyses. The Bayesian 50% majority rule consensus tree with branch lengths optimized from the three-gene dataset (16S rRNA gene, *recA*, and *gyrB*) is shown in Fig. 2. Overall, Bayesian and maximum likelihood analysis of the combined dataset recovered well-resolved topologies showing no supported incongruencies. Most endophytes of the non-nodulated *Psychotria* species were closely related in one supported

clade (100% BPP/99% BS), nested within the leaf-nodulated endosymbionts. Interestingly, the non-nodulated endophytes of *Psychotria taedoumgii* and *P. psychotrioides* were found at distinct phylogenetic positions, related to *Psychotria* (*Candidatus Burkholderia calva*, *Ca. B. konguensis*, and *Ca. B. recurva*) and *Sericanthe* (*Ca. B. andongensis*) endosymbionts, respectively (Fig. 2).

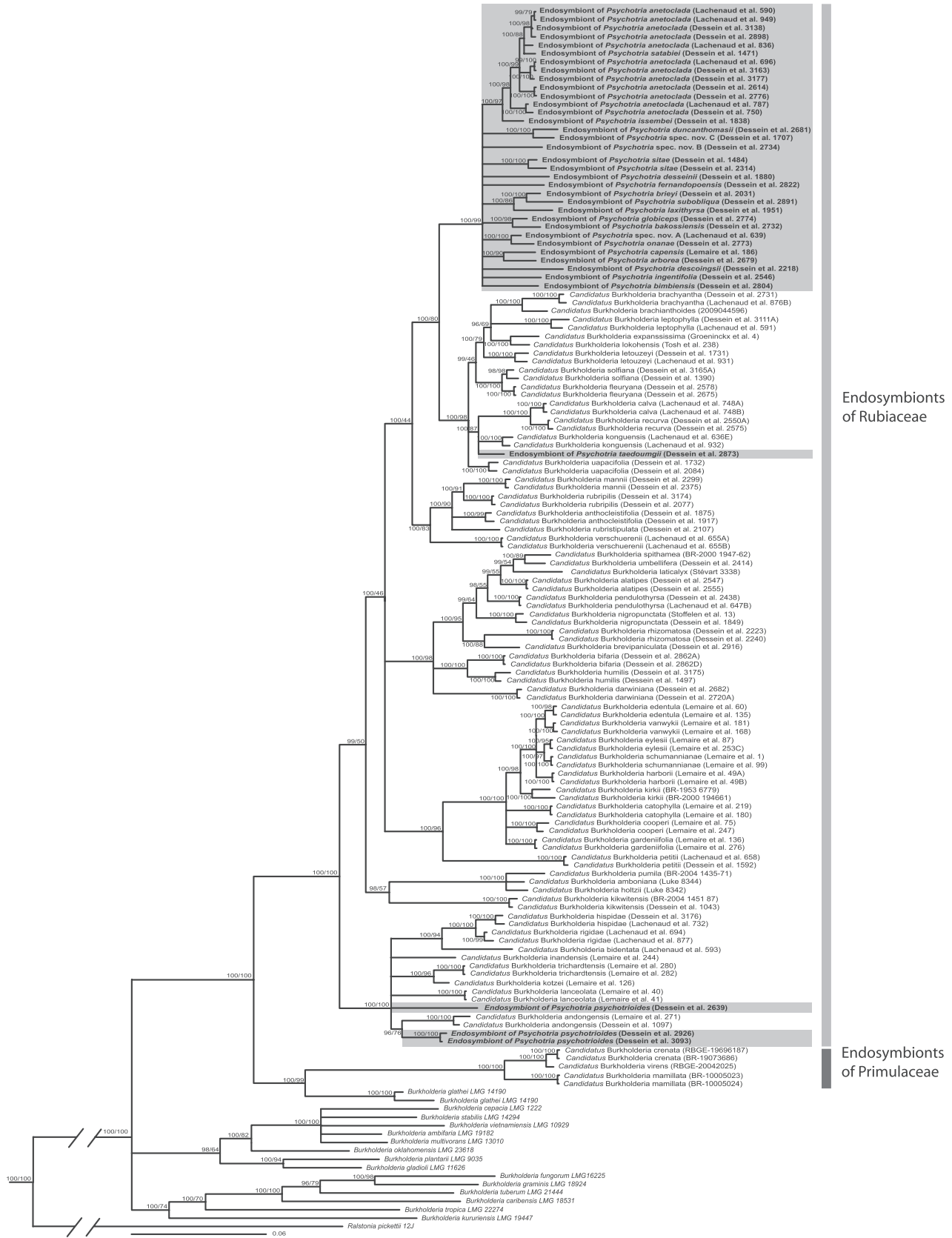
Morphological observation of leaf-associated endosymbionts in *Psychotria*

The endosymbionts of nodulated and non-nodulated *Psychotria* specimens were observed in *P. kirkii* (Fig. 3a and c) and *P. psychotrioides* (Fig. 3b and d), respectively. A cross-section of a leaf observed using SEM shows a bacterial nodule filling the width of the leaf completely. A layer of flattened cells occludes the bacterial colony (Fig. 3a). The endosymbionts of leaf-nodulated plants are restricted to the leaf nodule structure (Fig. 3c). In non-nodulated lineages, no leaf gall structures are visible (Fig. 3b), and endophytes are dispersed between the mesophyll cells over the whole leaf (Fig. 3d). The length of the bacteria varies between 1 and 2 μm . No flagella structures were observed.

Discussion

This phylogenetic study reports a novel origin of bacterial leaf symbiosis in the genus *Psychotria*. More specific, 22 *Psychotria* species of the non-nodulated clade III (see Fig. 1) were found to house endophytes. This clade is only distantly related to clade II comprising exclusively leaf-nodulated hosts. In the non-nodulated bacteriophilous clade, several host plants were found to lack endophytes, suggesting that the history of leaf symbiosis is characterized either by losses of ancestral infections or by multiple and independent infections of the host plants. The absence of endophytes in a given species was confirmed by different replicas, minimizing the chance of possible undetected infections. Nevertheless, our results indicated that bacterial leaf symbiosis originated in nodulated and non-nodulated *Psychotria* species separately, and at least once in the non-nodulated clade (Fig. 2).

The new non-nodulating endophytes were identified as members of the genus *Burkholderia* (β -Proteobacteria), closely related to the leaf-nodulated endosymbionts of the *Rubiaceae* genera *Psychotria*, *Pavetta*, and *Sericanthe* (Fig. 2). The systematic survey in *Psychotria* provides hereby additional evidence for the prevalence of *Burkholderia* as microorganisms that are highly adapted to establish symbiotic interactions (Compant *et al.*, 2008). *Burkholderia* is a widespread and abundant genus comprising over 60 validly named species with most lineages



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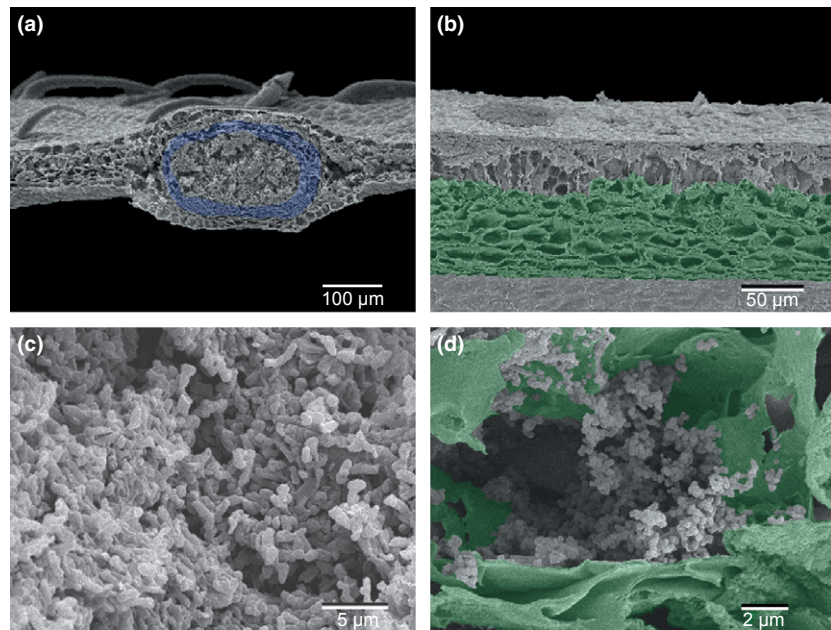


Fig. 3. Scanning electron microscopy images of the leaves and associated endosymbionts of the nodulated *Psychotria kirkii* (a and c) and the non-nodulated *Psychotria psychotrioides* (b and d). (a) A cross-section through a leaf nodule showing the bacterial mass surrounded by the nodule sheath (shaded in blue). (b) A transverse section of a non-nodulated leaf with bacterial endosymbionts located between mesophyll cells (shaded in green). (c) Bacterial endosymbionts are located within the leaf nodule. (d) Endophytes are diffused between mesophyll cells of the host plant.

found in mutualistic associations of insects (Kikuchi *et al.*, 2011), fungi (Partida-Martinez & Hertweck, 2005), and angiosperms (Elliott *et al.*, 2007). For instance, a well-known beneficial *Burkholderia*–plant interaction is the legume–root endosymbiosis where the endosymbionts are able to fix atmospheric nitrogen (Moulin *et al.*, 2001). The remarkable diversity and ability to adapt to various environments and host organisms of *Burkholderia* could be explained by extraordinary genome plasticity enabled by their metabolic versatility and the presence of many insertion sequence elements (Lessie *et al.*, 1996; Miche *et al.*, 2001; Ohtsubo *et al.*, 2005). Therefore, it is expected to find even more symbiotic *Burkholderia* in *Rubiaceae* and other flowering plants.

More important is that the non-nodulated *Burkholderia* endophytes were not recovered as a monophyletic group but placed at different positions intermingled with leaf-nodulating *Burkholderia* species. As a result, the microorganisms involved in the bacterial leaf symbiosis of non-nodulated *Psychotria* species are closely related to the leaf-nodulating endosymbionts (Fig. 2). Although the symbionts are phylogenetically related, their accommodation within leaf tissue differs substantially between nodulated and non-nodulated *Psychotria* species. Our morphological investigations indicate that non-nodulated endophytes are dispersed intercellularly between the mesophyll cells, while the leaf-nodulating endosymbionts are

located and limited within leaf nodules. Leaf nodules initiate and develop from substomatal chambers located under certain precociously formed stomatal pores (Lersten & Horner, 1976). The process of nodule and leaf development occurs in close harmony with their endosymbionts, and it is shown that in the absence of bacteria, the host plant is unable to proceed normal nodule and leaf growth (Miller, 1990; Lemaire *et al.*, 2012a). However, the presence of related *Burkholderia* symbionts in nodulated and non-nodulated *Psychotria* species suggests that leaf nodule organogenesis is mainly orchestrated by the host. Hence, compartmentalization of the endosymbionts in leaf nodules may be a more derived evolutionary feature of the host serving as sophisticated interface to exchange metabolites between the symbiotic partners.

A wide dissemination of *Burkholderia* species observed within leaf-nodulated and non-nodulated *Psychotria* representatives may partly explain the successful diversification of this *Rubiaceae* genus. The genus *Psychotria* is one of the largest genera in flowering plants comprising more than 1800 species worldwide and is often an important component of the undergrowth in tropical rain forests (Hamilton, 1989; Taylor, 1996; Mabberley, 2008; Davis *et al.*, 2009). It is widely accepted that symbiotic mutualisms greatly facilitate and drive host diversification and evolution (Scannerini & Bonfante, 1991; Simon *et al.*, 1993; Ercolin & Reinhardt, 2011). Plants provide an

Fig. 2. Phylogenetic results of bacterial endosymbionts based on 16S rRNA gene, *recA*, and *gyrB* data. Support values of Bayesian and maximum likelihood analyses are given at the nodes (BPP – bootstrap values from the maximum likelihood analysis). Endophytes of non-nodulated *Psychotria* species are shaded in gray.

attractive niche for endophytic bacteria as a source of nutrition and protection against (a)biotic stresses. In return, endophytes promote plant growth, which may be the result of enhanced nutrient uptake (e.g. nitrogen fixation) (Hurek *et al.*, 2002), preventing pathogen infections, stimulating plant defense responses (van Loon *et al.*, 1998), and/or producing phytohormones (Long *et al.*, 2008). The latter type of beneficial contribution to the host is known to play an important role in the leaf-nodulated *Psychotria* hosts (Gordon, 1963; Van Oevelen *et al.*, 2003). Similarly, endophytes of non-nodulated *Psychotria* species may also be responsible for the production of growth substances.

Previous phylogenetic studies dealing with the evolution of bacterial leaf nodulation discovered that every host species was infected by a single specific endosymbiont, which is the result of a vertical transmission mode (Lemaire *et al.*, 2011c). The host life cycle is continuously accompanied by the symbiont and ensures maternal transmission through the seeds (Miller, 1990; Lemaire *et al.*, 2012a). However, a co-speciation analysis and a population genetic study rejected a strict vertical endosymbiont transmission because of occasional external colonization events by soil *Burkholderia* (Lemaire *et al.*, 2011c, 2012a). Nevertheless, reinfections seem to occur in a species-specific manner.

In this study, a similar degree of host specificity in non-nodulated *Psychotria* species was demonstrated (Fig. 2). For different populations of *Psychotria anetoclada* and *P. sitae*, endophyte DNA was identical and considered as the same species (Stackebrandt & Ebers, 2006). In addition, evidence for horizontal symbiont transmission was also detected based on the separate positions of two non-nodulated endophytes (i.e. *P. taedoumgii* and *P. psychotrioides*) among leaf-nodulated representatives.

This study surveyed the presence of non-nodulating endosymbionts in *Psychotria* species that have no visible bacterial nodules. It can be expected that other *Rubiaceae* are receptive for leaf-associated endophytes. Therefore, a broader study on *Rubiaceae* is needed to discover the complete endophyte biodiversity and to understand the biology and evolution of leaf symbiosis in a wider taxonomic context. Ongoing research into leaf symbiosis holds great promise to untangle these questions and to elucidate this neglected aspect of plant evolution.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. *Psychotria* phylogeny based on chloroplast data (*rps16*, *trnG*, *trnLF*).

Table S1. Accession numbers, voucher data and origin of *Psychotria* lineages.

Table S2. Accession numbers, voucher data and origin of bacterial endosymbionts.

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