Phylogenetic lineages in Vanguerieae (Rubiaceae) associated with *Burkholderia* bacteria in sub-Saharan Africa[1]

Brecht Verstraete[2,6], Steven Janssens[2,3], Benny Lemaire[2,4], Erik Smets[2,5], and Steven Dessein[3]

[2]Plant Conservation and Population Biology, KU Leuven, Leuven, Belgium; [3]National Botanic Garden of Belgium, Meise, Belgium; [4]Department of Biological Sciences, University of Cape Town, Rondebosch, South Africa; and [5]Naturalis Biodiversity Center, Leiden University, Leiden, Netherlands

- *Premise of the study:* It is well known that mutualistic bacteria can provide substantial benefits to their host plants. However, 'how,' 'why,' and the possible applications of such an interaction are only second to the questions 'who is involved?', and 'where does it occur?'. In the coffee family (Rubiaceae), certain species closely interact with endophytic leaf bacteria that are freely distributed among the mesophyll cells. This type of interaction was recently discovered in South Africa. Our aim is to document the bacterial diversity associated with Rubiaceae ('who') and to establish the geographic range of the interaction ('where').

- *Methods:* Representatives of the Vanguerieae tribe in Rubiaceae were investigated for the presence of endophytes with special emphasis on the distributional range of the plant-bacteria association by collecting specimens from different African regions.

- *Key results:* The interaction is found in five genera and is restricted to three major host lineages. The endophytic bacteria belong to the genus *Burkholderia* and are part of the plant-associated beneficial and environmental group. Some endophytes are similar to *B. caledonica*, *B. graminis*, *B. phenoliruptrix* or *B. phytofirmans*, while others are classified in OTUs that show no similarity with any previously described *Burkholderia* species of bacteria.

- *Conclusions:* The association is not obligate from the bacterial point of view and is considered a loose and recent interaction, which is demonstrated by the fact that there is no evidence for coevolution. The geographical distribution of the association is restricted by the distributional range of the host plants covering the whole of sub-Saharan Africa.

Key words: bacterial leaf symbiosis; *Burkholderia*; diversity; endophyte; Rubiaceae; sub-Saharan Africa

Relationships between plants and bacteria are widespread and it is commonly accepted that almost all plant species are associated with endophytic microorganisms (Strobel et al., 2004). Plant-bacteria associations can be designated into one of the following categories: (1) a negative interaction in which one partner has a negative impact on the other; (2) a positive interaction, in which both partners derive benefits from the association; and (3) a neutral interaction, in which none of the partners derives a direct benefit from the interaction and where neither is harmed (Singh et al., 2004). Forming a mutualistic relationship with a bacterial partner can have a major influence on the health and growth of host plants; it enhances abiotic stress tolerance, provides resistance against diseases and pathogens, and may aid nutrient availability and uptake (Compant et al., 2010). However, before questions on the function of such interaction can be answered, it is imperative to have identified the partners involved and to know where the association has evolved naturally.

An example of a (putative) positive interaction is found in the plant family Rubiaceae (coffee family) where leaf nodules with bacterial endophytes occur in the genera *Pavetta*, *Psychotria*, and *Sericanthe* (Lemaire et al., 2011b). The occurrence of bacteria in the leaf blades of some species of these genera is already long known, since they form specialized structures, called leaf nodules or bacterial galls, that are macroscopically visible (Zimmermann, 1902; Miller, 1990). The endophytes of these Rubiaceae plants have been identified as *Burkholderia* bacteria (Lemaire et al., 2011a, b, 2012b; Verstraete et al., 2011). The interaction between plant and its nodulated endophyte is host specific, as each nodulating plant species is colonized by its own single *Burkholderia* lineage (Lemaire et al., 2011b). The cultivation of the leaf nodulated bacteria has not been successful, suggesting a high degree of dependency between both partners and/or an obligate association (Lemaire et al., 2011b). This is corroborated by the occurrence of crippled host plants, i.e., bacterium-free plants develop normally, but gradually their growth and cell differentiation ceases, resulting in dwarfed plants (Lemaire et al., 2012a).

Another interaction between Rubiaceae plants and *Burkholderia* bacteria exists, for which it is not yet known whether it constitutes a beneficial or neutral association. A few genera in Vanguerieae have been shown to harbor endophytic bacteria freely among the mesophyll cells of their leaves (Verstraete et al., 2013a). These endophytes differ from the nodulated bacteria

by not forming distinct visible leaf nodules. Using a cultivation-independent approach the endophytes were identified as *Burkholderia*, the same genus of bacteria that is found in other rubiaceous plants (Verstraete et al., 2011, 2013a). In contrast to the nodulated endophytes, the cultivation of a non-nodulated endophyte has been successful, which indicates that the interaction is not obligate for the bacterial partner (Verstraete et al., 2011). Whether associating with a bacterial partner is necessary for the host plant's survival, remains unclear. However, so far, a *Burkholderia* endophyte has been detected in every single specimen that was investigated (Verstraete et al., 2013a).

In contrast to the well-known interaction between legumes and their root symbionts, bacterial leaf symbiosis in Rubiaceae has been studied far less and therefore holds many unanswered questions. Sound scientific research starts with clearly defining the study group, and that is what in most research on leaf symbiosis has been done so far, i.e., both partners of the endosymbiosis are being identified, their phylogenetic relationships are being unraveled, and evolutionary patterns are studied (Lemaire et al., 2011a, b, 2012a, b; Verstraete et al., 2011, 2013a). There are, however, other aspects of leaf endosymbiosis that merit enquiry. Nodulated bacterial leaf symbiosis is known to occur in the (sub)tropical regions of Africa and Asia (Miller, 1990). This observation is based on the range of the host plants, but no detailed information on the geographical distribution of the symbiosis is available. First reported by Van Wyk et al. (1990), the presence of non-nodulated endosymbionts in Rubiaceae was unequivocally demonstrated in 2011, and the studied host plants were predominantly collected in South Africa (Verstraete et al., 2011, 2013a). A few plants from a limited number of other African countries are also shown to harbor endosymbiotic bacteria, but a clear overview of the actual distribution of leaf symbiosis is lacking.

The tribe Vanguerieae with the genera that hold bacterial endophytes has a large distributional area covering sub-Saharan Africa, which makes it an ideal group to screen for the presence of endophytes in different geographical regions. In this study we investigate East African host plants and combine the data with the existing data of Southern Africa and Western Africa. By doing so, we document the *Burkholderia* diversity associated with Rubiaceae host plants and establish whether the interaction is widespread in sub-Saharan Africa.

MATERIALS AND METHODS

For the host plant analysis, 170 specimens of 100 plant species belonging to Vanguerieae were collected, plus three outgroup species (Appendix S1, see Supplemental Data with the online version of this article). We ensured that different African regions were represented in the sampling. All necessary permits for the collection of these plants were obtained and can be consulted at the National Botanic Garden of Belgium. Plant leaves were collected in the field and immediately put on silica gel to allow rapid dehydration and DNA preservation. The leaves, together with the silica gel, were kept in airtight plastic bags. The plant dataset of this study builds further on a previous taxonomic study in Vanguerieae (Verstraete et al., 2013b). The new data were integrated with the previous data and all details can be found in Appendix S1.

Newly discovered endophytic bacteria were added to an existing data matrix (Verstraete et al., 2013a) and analyzed as a whole (see Appendix S2).

Before extraction of the bacterial DNA, the host plant leaves were surface sterilized with 70% ethanol and ultrapure water under sterile conditions. This procedure has been shown to be adequate to remove possible epiphytes (Verstraete et al., 2011, 2013a). The actual extraction of both plant and endophyte DNA was performed using the E.Z.N.A.™ HP Plant DNA Mini Kit (Omega Bio-Tek, Norcross, Georgia, USA) according to the manufacturer's instructions. For the host plant study, the following DNA markers were used: *trnTL*, *trnLF*, *rpl16*, *petD*, and the ITS region. Further information on the amplification and sequencing of these markers can be found in Verstraete et al. (2013b). Phylogenetic analysis of the bacterial endophytes is based on the three genes 16S rDNA, *gyrB*, and *recA*. Bacterial 16S rDNA amplification was performed using the universal forward primers 16SB and a *Burkholderia* specific reverse primer 16S2 (Lemaire et al., 2012b). Amplification primers for *gyrB* and *recA* genes and their respective temperature profiles are based on the protocol in Verstraete et al. (2011).

All obtained sequences were assembled and edited using the DNA analysis software platform Geneious version 5.4.7 (Biomatters Ltd, Auckland, New Zealand, www.geneious.com). A preliminary sequence alignment was performed with MUSCLE under default parameters (Edgar, 2004) as implemented in Geneious, version 5.4.7, followed by manual fine-tuning resulting in an unequivocal alignment. The plant and bacteria alignment can be found as appendices (see Appendices S3 and S4). Gaps were coded for the host plant data matrix (Simmons and Ochoterena, 2000).

The phylogeny of the host plants was estimated using probabilistic methods under the Maximum Likelihood criterion in the CIPRES web portal (Miller et al., 2010). Maximum Likelihood analysis was performed with RAxML version 7.4.4 using GTRCAT for the bootstrapping phase and GTRGAMMA for the final tree inference (Stamatakis et al., 2008). The clade support was assessed using multiparametric bootstrap resampling with 1000 replicates. Phylogenetic trees of the bacterial endophytes were constructed using Maximum Likelihood analyses in PhyML (Guindon et al., 2010). The DNA substitution model GTR+I+G was selected under the Akaike Information Criterion using jModelTest 2.1.3 (Darriba et al., 2012). Nonparametric bootstrap analysis with 1000 iterations was carried out to calculate the relative support for individual clades found in the likelihood analysis.

Bacterial isolates were identified and grouped into different OTUs based on the pairwise identity of their 16S rDNA sequence with a cut-off value of 0.1%.

RESULTS

Phylogenetic analysis of the tribe Vanguerieae (Rubiaceae)— The combined matrix of one nuclear and three plastid DNA markers contains data on 170 representatives of Vanguerieae and 3 outgroup species. The Maximum Likelihood analysis resulted in a robust phylogenetic hypothesis where the major clades of the tribe are well supported. Fig. 1 shows the best-scoring ML tree with the bootstrap values indicated at the nodes. Unsupported nodes (BS < 65%) are collapsed.

The tribe Vanguerieae is a monophyletic and strongly supported group (BS 100%). In accordance with previous research (Lantz and Bremer, 2004; Verstraete et al., 2013b), several major clades in the tribe are strongly supported. The basal clade groups three genera (*Afrocanthium*, *Keetia*, and *Psydrax*) and the dioecious group (consisting of *Bullockia, Peponidium*, and *Pyrostria*; BS 95%). The so-called 'spiny' group is clearly delimitated and contains most *Canthium* species, *Plectroniella armata* (K. Schum.) Robyns, *Pygmaeothamnus chamaedendrum* (Kuntze) Robyns, *Rytigynia bugoyensis* (K. Krause) Verdc., and *Vangueriella spinosa* (Schumach. & Thonn.) Verdc. (BS 100%). As in all previous studies on Vanguerieae, our study supports the *Fadogia/Rytigynia* group as a clear and distinct clade in the tribe (BS 100%). The representatives of this group share a set of unique indels in the investigated DNA regions. The internal resolution of this clade is however low. The genera *Cuviera* s.s. and *Globulostylis*, as recently redefined by Verstraete et al. (2013b), are both supported as being monophyletic (BS 100%), while *Vangueriella* is not. The *Vangueria* group includes the species *Cuviera schliebenii* Verdc., *Cuviera semseii* Verdc., *Pygmaeothamnus zeyheri* (Sond.) Robyns, and *Robynsia glabrata* Hutch., the genus *Multidentia* (BS 73%), the small genus *Vangueriopsis*, and the large monophyletic genus *Vangueria* (BS 100%), the latter which includes the genus *Pachystigma*.
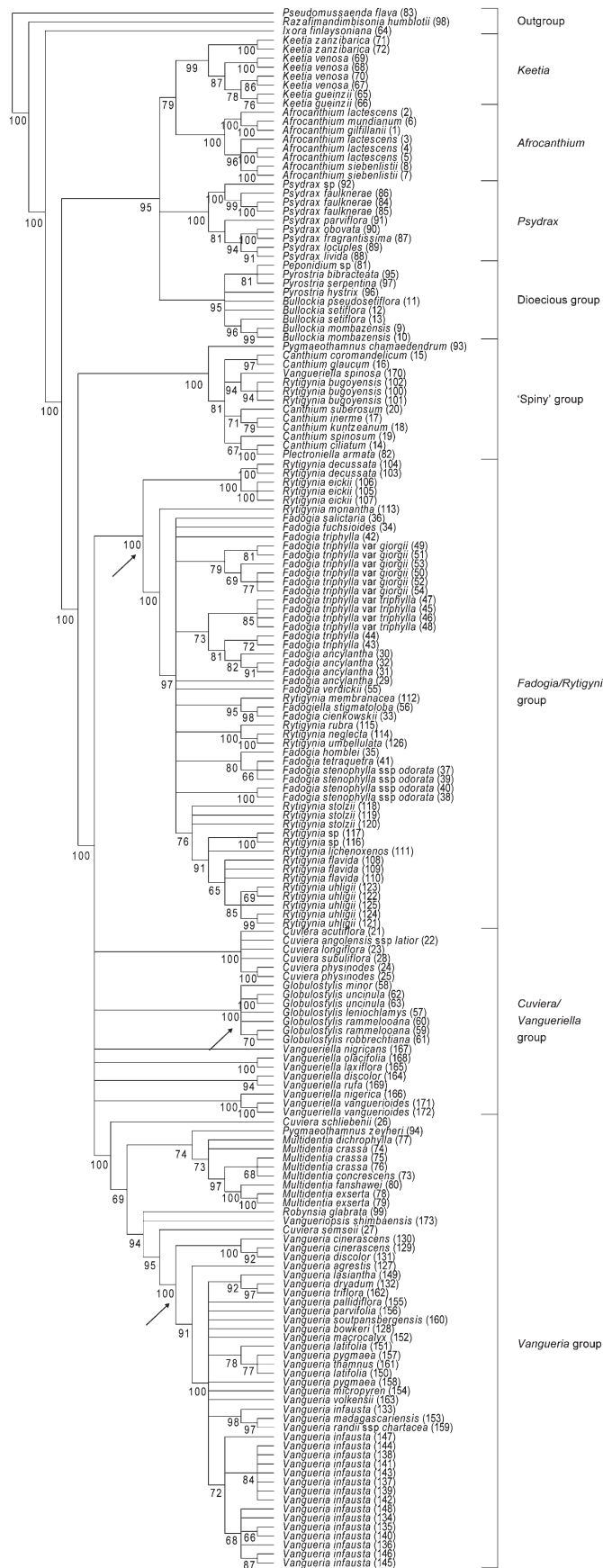
Phylogenetic analysis of the* Burkholderia *bacteria—The presence of endophytic *Burkholderia* bacteria inside the leaves was only detected in five genera of Vanguerieae: *Fadogia*, *Fadogiella*, *Globulostylis*, *Rytigynia*, and *Vangueria*. All other genera within Vanguerieae lack bacterial endophytes in their leaves. Positive or negative results per specimen is indicated in Appendix S1. The dataset with the bacterial sequences contains 186 bacterial isolates: 3 outgroup species, 39 validly described *Burkholderia* species, 17 *Candidatus* species isolated from nodulating plants, and 127 endophytic isolates obtained through a cultivation-independent method. For each taxon, three DNA markers (16S rDNA, *gyrB*, and *recA*) were sequenced and the data matrix is 96% complete (see Appendix S2).

Two functionally distinct clusters are found within the genus *Burkholderia*, i.e., a first group holding the well-known pathogenic species and the second holding plant-associated or environmental species (Fig. 2). All the endophytic bacteria found within the leaves of representatives of Vanguerieae are plant-associated leaf endophytes and are part of the plant-associated beneficial and environmental (PBE) group. The identification of the isolates and the allocation to different OTUs is based on their 16S rDNA sequence similarity. BLAST searches against the nr database of GenBank assigned the endophytes to the genus *Burkholderia*. A species name was allocated when their 16S rDNA sequence showed a similarity larger than 99% with a previously described species. The endophytes of *Rytigynia umbellulata* (Hiern) Robyns are very similar to *B. phytofirmans* (Fig. 2, OTU 5). The endophytes of *Rytigynia* sp., *Vangueria infausta* Burch., *V. randii* S. Moore, and *V. madagascariensis* J.F. Gmel. have a 16S rDNA sequence that is similar to *B. phenoliruptrix* (Fig. 2, OTU 7). The endophytic bacteria of 20 different host plant species from four different genera are very similar to *B. caledonica* based on identical or highly similar 16S rDNA sequences (Fig. 2, OTU 8). The endophyte found in *V. volkensii* K. Schum. has a 16S rDNA sequence that is 99.3% similar to *B. graminis* (Fig. 2, OTU 12). The endophytes of 14 other host plant species do not show a high similarity with any existing *Burkholderia* species and are therefore placed in new OTUs. The numbering of the OTUs refers to and builds further on Verstraete et al. (2013a). OTU 11 is noteworthy since this group is very well delineated and is found near the clade of legume-associated *Burkholderia*. The closest relative to OTU 11 is *B. kururiensis* subsp. *thiooxydans* (98.6%). The endophytes in OTU 13 are closely related to—and perhaps belong to—*B. graminis* (pairwise identity of 98.8%). OTUs 1, 2, 9, and 10 are part of the *B. glathei* clade that was formerly known for the leaf nodulated endophytes, however, the host plants of these four OTUs do not form nodules in their leaves to harbor their endophytes.

Distribution of non-nodulated bacterial endosymbiosis—The actual occurrence of endophytic *Burkholderia* bacteria in leaves of Vanguerieae representatives is based on the geographical data present on the herbarium labels of the studied specimens. The individual latitude and longitude coordinates can be found

Fig. 1. Maximum Likelihood tree showing the phylogenetic relationships within Vanguerieae (Rubiaceae). The major clades are always retrieved and are well supported. Bootstrap values are indicated below the branches and numbers between brackets refer to Appendix S1 (see Supplemental Data with the online version of this article). Non-nodulated leaf endophytes are found in three distinct clades of the tribe (arrows).

in Appendix S5. Distribution data of the genera *Fadogia*, *Fadogiella*, *Globulostylis*, *Rytigynia*, and *Vangueria* are obtained from the World Checklist of Rubiaceae (Govaerts et al., 2013). The interaction is demonstrated in six sub-Saharan African countries: Burundi, Cameroon, Gabon, South Africa, Tanzania, and Zambia (Fig. 3, dots). Besides the actual distribution based on latitude and longitude coordinates, the potential total distributional area can be derived from the distribution range of the host plants and this comprises the whole of sub-Saharan Africa (Fig. 3, grayed area).

DISCUSSION

Major clades in Vanguerieae and presence of non-nodulated endophytes—So far, in every molecular study dealing with the relationships within Vanguerieae (Lantz et al., 2002; Lantz and Bremer, 2004; Verstraete et al., 2013a, b; this study), the same major clades are retrieved and are well supported (Fig. 1).

The existence of a basal clade consisting of the genera *Afrocanthium*, *Keetia*, *Psydrax*, and the dioecious group is indicated in Verstraete et al. (2013a), but the monophyly of the individual groups had been demonstrated earlier (Lantz et al., 2002; Lantz and Bremer, 2004; Razafimandimbison et al., 2009). The presence of this basal clade is corroborated by Kainulainen et al. (2013). In a previous analysis based on a portion of the current dataset, the genus *Keetia* is sister to the remainder of the basal clade and *Afrocanthium* is sister to the dioecious group (Verstraete et al., 2013b, their Fig. 1.). However, with the inclusion of several additional taxa in this study, these two genera are shown to be sister to each other, which is also found in Kainulainen et al. (2013). When we investigated the species in this basal clade for endophytes, none of them are found to harbor *Burkholderia* bacteria in their leaves (see specimen list in Appendix S1).

A second well-delineated group within the tribe is the 'spiny' group. The monophyly of this group has been demonstrated since the first phylogenetic study on Vanguerieae (Lantz et al., 2002). Although new names for the species *Plectroniella armata*, *Rytigynia bugoyensis*, and *Vangueriella spinosa* have been proposed to form a coherent *Canthium* group (Lantz and Bremer, 2004), the synonymization has not yet been accepted in the World Checklist of Rubiaceae (Govaerts et al., 2013). However, our findings are in agreement with Lantz and Bremer (2004). The particular association between the Vanguerieae and *Burkholderia* is not found in this group.

The large *Fadogia/Rytigynia* group (including the genus *Fadogiella*) is molecularly well defined because they share a set of unique gene sequences that sets this group well apart from the rest of the Vanguerieae tribe. In contrast to the group as a whole, the internal resolution is quite low, and as a consequence the species level relationships are still uncertain. When different specimens of the same species are included in the analysis, they are found near each other, indicating good species circumscriptions. For *Fadogia triphylla* Baker, the different varieties can be distinguished molecularly and *Fadogia triphylla* var. *giorgii* (De Wild.) Verdc. and var. *triphylla* are monophyletic (Fig. 1). The specimens (43) and (44) certainly belong to the species *F. triphylla*, but they cannot be placed into one of the varieties. The different varieties are easily distinguishable morphologically and the distinction is now also supported by molecular data. All investigated specimens of *F. triphylla* have bacterial leaf endophytes, but their identity is quite different (OTU 8 vs. OTU 11 in Fig. 2; but see

under next subtitle). The combination of all these observations indicates that the taxonomic status of the varieties within the species *F. triphylla* could be evaluated. All specimens of the *Fadogia/Rytigynia* group that were investigated for the bacterial leaf symbiosis show an association with *Burkholderia* bacteria.

In the *Cuviera/Vangueriella* group, the two genera *Cuviera* s.s. and *Globulostylis* are strongly supported in contrast to *Vangueriella* sect. *Vangueriella*, which is unresolved. This is due to the low genetic variability in the four DNA markers used in this study. A previous study using six markers was able to show the monophyly of *Vangueriella* sect. *Vangueriella*, but it was also demonstrated that *Vangueriella* sect. *Stenosepalae* is seemingly different and belongs to the 'spiny' group (Verstraete et al., 2013b). Concerning bacterial leaf symbiosis, neither section of *Vangueriella* nor the genus *Cuviera* is associated with *Burkholderia* bacteria. *Globulostylis* on the other hand is the exception in this group, since all species contain endophytic bacteria in their leaves.

The final group within the Vanguerieae is the *Vangueria* group, which comprises the genera *Vangueria*, *Multidentia*, and *Vangueriopsis*, the monospecific *Robynsia glabrata*, two species excluded from *Cuviera* (viz. *C. schliebenii* and *C. semseii*), and the species *Pygmaeothamnus zeyheri*. Taxonomic realignments are desirable in this group but are not within the scope of this study. The presence of endophytic *Burkholderia* bacteria has only been demonstrated in the large genus *Vangueria*; all other genera in this group lack them.

Endophytic bacteria in the leaves are present in three clades of Vanguerieae, as is evident from Fig. 1, i.e., the *Fadogia/Rytigynia* group, the genus *Globulostylis*, and the genus *Vangueria*. In total five genera are involved (*Fadogia*, *Fadogiella*, *Globulostylis*, *Rytigynia*, and *Vangueria*) and the presence of endophytes is consistent at the generic level, since all of the species investigated so far have bacteria in their leaves. The observed pattern could be explained in two ways: (1) either this particular interaction appeared three times independently and is quite recent, or (2) it is the result of a single evolutionary event in the past but has been lost in a few groups. Since the non-nodulated endophytes can be cultivated outside their hosts (Verstraete et al., 2011), it is assumed that the interaction is not obligate (at least not for the bacteria) and that it is a loose and recent association. Based on this, the option of three independent origins could be favored; however, the ecological trigger for this interaction is not yet known. At this stage neither hypothesis can thus be refuted.

***Phylogenetic relationships within the genus* Burkholderia**—The latest reviews of the taxonomy of *Burkholderia* show the presence of two distinct groups, i.e., one cluster comprises human, animal, and plant pathogens, while the second cluster contains nonpathogenic species associated with plants and/or the environment (Gyaneshwar et al., 2011; Suárez-Moreno et al., 2012; Estrada-de los Santos et al., 2013). However, this claim is not entirely correct since pathogenic species can also have a positive influence on plant growth (e.g., *B. vietnamensis*) and species that fall in the beneficial group can be plant pathogens (e.g., *B. caryophylli*). In a previous study we enlarged the plant-associated beneficial and environmental (PBE) group by including the *B. glathei* clade, which comprises both nodulated and non-nodulated endophytes of rubiaceous host plants (Verstraete et al., 2013a). This is corroborated by the findings of Estrada-de los Santos et al. (2013), who also include *B. glathei* in their group A, which is the clade with the plant-associated species. All bacterial endophytes discovered in Rubiaceae host plants so far are part of the PBE group and the endophytes in

Bordetella avium (1)
Bordetella pertussis (2)
Ralstonia pickettii (3)
Burkholderia gladioli pv gladioli (14)
Burkholderia plantarii (29)
Burkholderia glumae (16)
Burkholderia oklahomensis (24)
Burkholderia mallei (20)
Burkholderia dolosa (11)
Burkholderia vietnamiensis (41)
Burkholderia ubonensis (40)
Burkholderia cenocepacia (9)
Burkholderia cepacia (10)
Burkholderia anthina (5)
Burkholderia ambifaria (4)
Burkholderia stabilis (35)
Burkholderia pyrrocinia (30)
Burkholderia glathei (15)
Candidatus Burkholderia mamillata (50)
Candidatus Burkholderia virens (59)
Candidatus Burkholderia crenata (45)
Candidatus Burkholderia andongensis (43)
Candidatus Burkholderia rigidae (53)
Candidatus Burkholderia hispidae (48)
Endophyte of Globulostylis uncinula (96) — OTU 2
Endophyte of Globulostylis uncinula (95)
Endophyte of Globulostylis minor (91)
Endophyte of Globulostylis leriochlamys (90)
Endophyte of Globulostylis rammelooana (92)
Endophyte of Globulostylis robbrechtiana (94) — OTU 1
Endophyte of Globulostylis robbrechtiana (93)
Candidatus Burkholderia nigropunctata (51)
Candidatus Burkholderia calva (44)
Endophyte of Rytigynia decussata (97) — OTU 9
Endophyte of Rytigynia decussata (98)
Endophyte of Rytigynia eickii (101)
Endophyte of Rytigynia eickii (100) — OTU 10
Endophyte of Rytigynia eickii (99)
Candidatus Burkholderia petitii (52)
Candidatus Burkholderia kirkii (49)
Candidatus Burkholderia harborii (46)
Candidatus Burkholderia harborii (47)
Candidatus Burkholderia schumannianae (54)
Candidatus Burkholderia schumannianae (55)
Candidatus Burkholderia schumannianae (56)
Candidatus Burkholderia schumannianae (57)
Candidatus Burkholderia schumannianae (58)
Burkholderia kururiensis (19)
Endophyte of Fadogia triphylla var triphylla (86)
Endophyte of Fadogia triphylla var triphylla (85)
Endophyte of Fadogia triphylla var triphylla (87) — OTU 11
Endophyte of Fadogia triphylla var triphylla (84)
Endophyte of Fadogia triphylla (77)
Burkholderia tropica (38)
Burkholderia sacchari (32)
Burkholderia silvatlantica (33)
Burkholderia nodosa (23)
Burkholderia mimosarum (22)
Burkholderia ferrariae (12)
Burkholderia sprentiae (34)
Burkholderia tuberum (39)
Burkholderia phymatum (27)
Burkholderia caribensis (8)
Burkholderia terrae (36)
Burkholderia hospita (18)
Burkholderia megapolitana (21)
Burkholderia terricola (37)
Endophyte of Vangueria cinerascens (130) — OTU 3
Endophyte of Vangueria cinerascens (129)
Endophyte of Vangueria cinerascens (131)
Endophyte of Vangueria triflora (185)
Endophyte of Vangueria lasiantha (161) — OTU 4
Endophyte of Vangueria lasiantha (160)
Endophyte of Vangueria dryadum (132)
Burkholderia phenazinium (6)
Burkholderia bryophila (6)
Burkholderia xenovorans (42)
Burkholderia fungorum (13)
Burkholderia phytofirmans (28)
Endophyte of Rytigynia umbellulata (127) — OTU 5
Endophyte of Rytigynia umbellulata (126)
Endophyte of Rytigynia umbellulata (125) — B. phytofirmans
Endophyte of Rytigynia umbellulata (124)
Endophyte of Rytigynia umbellulata (128)
Endophyte of Vangueria soutpansbergensis (180)
Endophyte of Vangueria parvifolia (171)
Burkholderia phenoliruptrix (26)
Endophyte of Vangueria infausta (155)
Endophyte of Vangueria infausta (154)
Endophyte of Vangueria infausta (152)
Endophyte of Vangueria infausta (149)
Endophyte of Vangueria infausta (148)
Endophyte of Vangueria infausta (151)
Endophyte of Vangueria infausta (145)
Endophyte of Vangueria infausta (159)
Endophyte of Vangueria infausta (131)
Endophyte of Vangueria infausta (147)
Endophyte of Vangueria infausta (146)
Endophyte of Vangueria infausta (150)
Endophyte of Vangueria infausta (133) — OTU 7
Endophyte of Vangueria infausta (134) — B. phenoliruptrix
Endophyte of Vangueria infausta (135)
Endophyte of Vangueria infausta (136)
Endophyte of Vangueria randii ssp chartacea (179)
Endophyte of Vangueria infausta (144)
Endophyte of Vangueria infausta (142)
Endophyte of Vangueria infausta (141)
Endophyte of Vangueria infausta (139)
Endophyte of Vangueria infausta (137)
Endophyte of Vangueria madagascariensis (168)
Endophyte of Vangueria infausta (140)
Endophyte of Vangueria infausta (138)
Endophyte of Vangueria infausta (143)
Endophyte of Vangueria infausta (156)
Endophyte of Vangueria infausta (153)
Endophyte of Rytigynia sp (114)
Endophyte of Rytigynia sp (113)
Endophyte of Vangueria macrocalyx (167) — OTU 6
Endophyte of Vangueria macrocalyx (165)
Endophyte of Vangueria macrocalyx (166)
Burkholderia graminis (17)
Endophyte of Vangueria volkensii (186) — OTU 12 B. graminis
Endophyte of Rytigynia uhligii (123)
Endophyte of Rytigynia uhligii (118)
Endophyte of Rytigynia uhligii (121)
Endophyte of Rytigynia uhligii (122) — OTU 13
Endophyte of Rytigynia uhligii (120)
Endophyte of Rytigynia uhligii (119)
Burkholderia rhynchosiae (31)
Burkholderia caledonica (7)
Endophyte of Rytigynia rubra (112)
Endophyte of Fadogia tetraquetra (76)
Endophyte of Rytigynia flavida (104)
Endophyte of Rytigynia flavida (102)
Endophyte of Rytigynia flavida (103)
Endophyte of Fadogia homblei (65)
Endophyte of Fadogia homblei (66)
Endophyte of Fadogia homblei (67)
Endophyte of Fadogia homblei (71)
Endophyte of Fadogia homblei (70)
Endophyte of Fadogia homblei (69)
Endophyte of Fadogia homblei (72)
Endophyte of Fadogia homblei (68)
Endophyte of Vangueria latifolia (162)
Endophyte of Vangueria latifolia (164)
Endophyte of Vangueria latifolia (163)
Endophyte of Vangueria thamnus (184)
Endophyte of Vangueria thamnus (183)
Endophyte of Vangueria thamnus (182)
Endophyte of Vangueria thamnus (181)
Endophyte of Vangueria pygmaea (173)
Endophyte of Vangueria pygmaea (172)
Endophyte of Vangueria pygmaea (176)
Endophyte of Vangueria pygmaea (177) — OTU 8
Endophyte of Vangueria pygmaea (175) — B. caledonica
Endophyte of Vangueria pygmaea (174)
Endophyte of Fadogia triphylla var giorgii (81)
Endophyte of Fadogia triphylla var giorgii (80)
Endophyte of Fadogia triphylla var giorgii (79)
Endophyte of Fadogia triphylla var giorgii (78)
Endophyte of Fadogia triphylla var giorgii (82)
Endophyte of Fadogia triphylla var giorgii (83)
Endophyte of Rytigynia monantha (107)
Endophyte of Rytigynia lichenoxenos (105)
Endophyte of Fadogia ancylantha (60)
Endophyte of Fadogia cienkowskyi (63)
Endophyte of Rytigynia stolzii (117)
Endophyte of Rytigynia stolzii (116)
Endophyte of Rytigynia stolzii (115)
Endophyte of Rytigynia neglecta (110)
Endophyte of Rytigynia neglecta (108)
Endophyte of Rytigynia neglecta (109)
Endophyte of Rytigynia neglecta (111)
Endophyte of Rytigynia membranacea (106)
Endophyte of Vangueria pygmaea (178)
Endophyte of Fadogia fuchsioides (64)
Endophyte of Fadogia stenophylla ssp odorata (73)
Endophyte of Fadogia stenophylla ssp odorata (74)
Endophyte of Fadogia stenophylla ssp odorata (75)
Endophyte of Fadogiella stigmatoloba (88)
Endophyte of Fadogiella stigmatoloba (89)
Endophyte of Fadogia ancylantha (62)
Endophyte of Fadogia ancylantha (61)
Endophyte of Vangueria micropyren (170)
Endophyte of Vangueria micropyren (169)
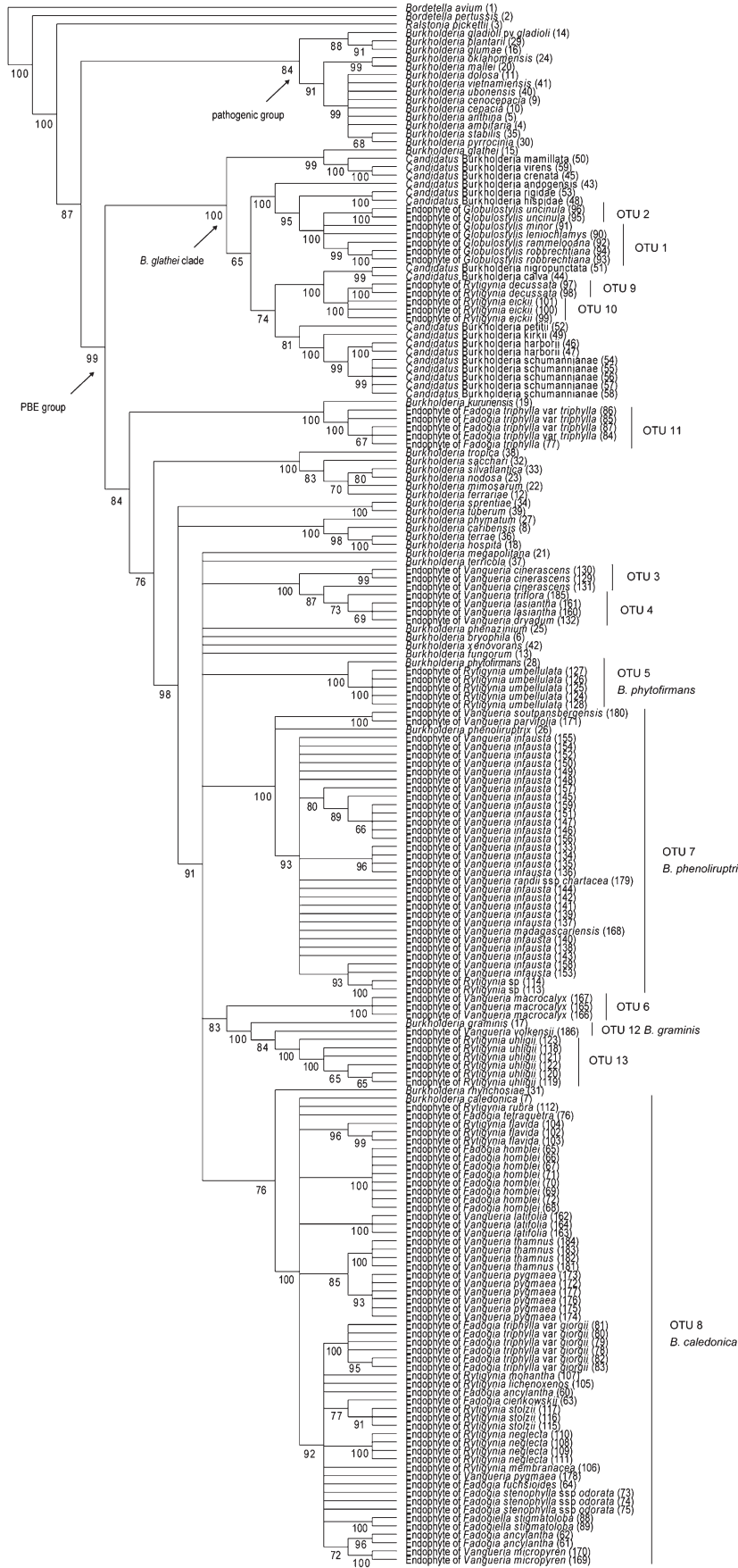
pathogenic group

B. glathei clade

PBE group

Fig. 3. The potential total distributional area of non-nodulated bacterial leaf symbiosis is based on the combined distributional ranges of the host plants *Fadogia*, *Fadogiella*, *Globulostylis*, *Rytigynia*, and *Vangueria* (grayed area). The actual endosymbiosis is indicated by means of latitude and longitude coordinates (dots). Map adapted from Brummitt (2001).

this study are no exception. Bacterial leaf symbiosis in Rubiaceae was discovered due to the presence of visible bacterial nodules in the leaf blades of certain tropical plants (Miller, 1990). Their identity was established through cultivation-independent studies, which demonstrated that they belong to the genus *Burkholderia* (Lemaire et al., 2011a, b; 2012b). Another type of plant-bacteria interaction was found between Vanguerieae host plants and *Burkholderia* endophytes, i.e., these bacteria are not confined to specialized nodules, instead they are found freely distributed among the mesophyll cells (Verstraete et al., 2011, 2013a). All newly found *Burkholderia* endophytes in this study are associated with Vanguerieae host plants and these do not show any external signs of infection.

The leaf endophytes are identified by 16S rDNA sequence data and are allocated to a bacterial species based on sequence similarity. The endophyte of *R. umbellulata* is similar to

---

←

Fig. 2. Maximum Likelihood tree of the *Burkholderia* endophytes. Bootstrap values are indicated below the branches and numbers between brackets refer to Appendix S2 (see Supplemental Data with the online version of this article). All endophytes belong to the plant-associated beneficial and environmental (PBE) group and are classified into OTUs. Numbering of the OTUs refers to and builds further on information presented in Verstraete et al. (2013a).

B. phytofirmans (Fig. 2, OTU 5). Four host plant species have endophytes that are very similar to be *B. phenoliruptrix* (Fig. 2, OTU 7), while the endophytic bacteria in 20 other plant species are similar to *B. caledonica* (Fig. 2, OTU 8). The 16S rDNA sequences of the endophytes of OTU 11 are 98.1% similar to the type strain of *B. kururiensis*; however, they are 98.6% similar to the type strain of *B. kururiensis* subsp. *thiooxydans* and 99% similar to another strain of this subspecies. Finally, the endophyte in *V. volkensii* is similar to *B. graminis* (Fig. 2, OTU 12). We stress that the allocation to a particular *Burkholderia* species is only based on 16S rDNA sequence similarity and that a comprehensive study of the endophytes should be performed before making a definitive conclusion. The rest of the non-nodulated endophytes are classified in other OTUs that do not correspond to any formally described *Burkholderia* species. For now, we choose to use the term OTU and not establish new species names since a cultivation-independent method for identification was used and other biochemical tests should be performed before validly describing new species. Although many host plant species seem to share a common endophyte, there is some kind of host specificity in the sense that one plant species always has the same endophyte, e.g., all *Rytigynia uhligii* (K. Schum. & K. Krause) Verdc. endophytes belong to OTU 13 (Fig. 2). This observation is consistent for all investigated specimens of one plant species always harbor the same endophyte. This may indicate that the host plant is able to select its preferential endophyte. There is one exception in our analysis, i.e., the endophytes of *Fadogia triphylla* var. *triphylla* are part of OTU 11, while those of *F. triphylla* var. *giorgii* are found in OTU 8. However, when observing the phylogenetic tree of the host plant it is evident that these varieties are phylogenetically distinguishable (Fig. 1). In this case it could be argued that the delineation of this particular plant species is not accurate and should be revised. Combining morphological and molecular data with the presence and identity of endophytic bacteria may prove a powerful tool for plant species delineation.

The interaction between Vanguerieae plants and *Burkholderia* bacteria is not obligate or at least not for these bacteria. In a previous study, the endophytes of *Fadogia homblei* De Wild were successfully cultivated outside their host plant on agar plates (Verstraete et al., 2011). This achievement is not unexpected since the endophytes in OTU 8 belong to the species *B. caledonica* that was originally isolated as free-living soil bacteria (Coenye et al., 2001). Unfortunately, we were not able to cultivate the leaf endophytes of the Vanguerieae plants here because access to fresh plant material is essential. For this study, we only had silica-dried material to our disposal and cultivating endophytes from silica-dried material was not successful. We are, however, convinced that it should be feasible to cultivate the endophytes in OTU 8 outside their host plants. Because multiple host plants share the same endophyte, there is no strict interaction between one endophyte and one host plant, further indicating a loose, non obligate association. However, there are some cautionary notes. There are two partners involved in an interaction, which means the association can be seen from two points of view. The Vanguerieae-*Burkholderia* association can be considered as nonspecific from the point of view of the bacterial partner since the same endophyte is found in different plant species. This providing that the isolates from different plants are really identical and that no lower-level specificity is left undetected. For the plants, however, the association is specific since one plant species only harbors one particular endophyte and so

far not a single representative of the five implicated genera was found to lack endophytes. Whether the plants are really dependent on their endophytes or whether the bacteria are just present without causing a negative impact on their fitness, is not known yet. For now, we assume that the non-nodulated endophytes of Vanguerieae host plants are facultative endophytes from the point of view of the bacteria.

A loose association would imply that coevolution does not occur, but in one case coevolution might explain the observed pattern: the plants *Rytigynia eickii* (K. Schum. & K. Krause) Bullock and *R. decussata* (K. Schum.) Robyns are related to each other (Fig. 1, *Fadogia/Rytigynia* group) and their endophytes are related as well (Fig. 2, OTUs 9 and 10). However, it is not known whether the ancestor of both plant species harbored the ancestor of both endophytes and whether the interaction was specific enough to allow coevolution on a small scale. The pattern is not found elsewhere and the presence of long-term coevolution between non-nodulated leaf endophytes and their host plants is therefore highly unlikely.

Geographical distribution of the Vanguerieae-Burkholderia association—The interaction between two partners is geographically limited to the distributional area of the partner with the smallest range. For this study it is obvious that the geographical pattern of the Vanguerieae-*Burkholderia* association is determined by the distributional range of the host plants. So far all investigated plants of the genera *Fadogia*, *Fadogiella*, *Globulostylis*, *Rytigynia*, and *Vangueria* harbor endophytes in their leaves, but the bacterial partner may be free-living. The geographical data on the herbarium labels of the plants indicates that the association occurs in at least six sub-Saharan countries: Burundi, Cameroon, Gabon, South Africa, Tanzania, and Zambia (Fig. 3, dots). However, if we assume that the interaction is widespread and endophytic *Burkholderia* are present in all representatives of the five Vanguerieae genera, the potential distributional area of the association is much larger and comprises the whole of sub-Saharan Africa. Of course the host plants are confined to certain habitats within this large distributional area: *Globulostylis* and *Rytigynia* are rainforest plants, while *Fadogia*, *Fadogiella*, and *Vangueria* occur in savannah and grassland. Unfortunately, geographical data in the World Checklist of Rubiaceae is only available on the level of countries, but it is clear that political boundaries are not the same as ecological boundaries. Northern Mali, for example, is colored gray because *Fadogia* occurs in Mali according to the database. However, if we take into account that the Sahara Desert covers northern Mali, we can assume that the plants do not grow there. The same goes for Sudan. Given the currently available geographical data, we can only conclude that the Vanguerieae-*Burkholderia* association is geographically restricted to sub-Saharan Africa. However, some nuance is necessary since the plants grow in particular habitats and their environmental requirements have to be taken into account.

In this study we investigated the association between non-nodulated *Burkholderia* endophytes and their Vanguerieae (Rubiaceae) host plants. The interaction is found in five different genera (*Fadogia*, *Fadogiella*, *Globulostylis*, *Rytigynia*, and *Vangueria*) and is restricted to three clades within the plant tribe. The endophytic bacteria all belong to the genus *Burkholderia* and are related to other plant-associated bacteria. Some endophytes are similar to *B. caledonica*, *B. graminis*, *B. phenoliruptrix* or *B. phytofirmans*, while others are classified in different OTUs that show no similarity with any previously described

Burkholderia species. The association is not obligate for the bacterial partner and is considered a loose and recently established interaction, which is demonstrated by the fact that one of the endophytes was cultivated in an earlier study and that no coevolution occurs. The geographical distribution of the association is limited to the distributional range of the host plants and comprises the whole of sub-Saharan Africa.

LITERATURE CITED

BRUMMITT, R. K. 2001. World geographical scheme for recording plant distributions, 2nd ed. Hunt Institute for Botanical Documentation, Carnegie Mellon University, Pittsburgh, Pennsylvania, USA.

COENYE, T., S. LAEVENS, A. WILLEMS, M. OHLÉN, W. HANNANT, J. R. W. GOVAN, M. GILLIS, ET AL. 2001. *Burkholderia fungorum* sp. nov. and *Burkholderia caledonica* sp. nov., two new species isolated from the environment, animals and human clinical samples. *International Journal of Systematic and Evolutionary Microbiology* 51: 1099–1107.

COMPANT, S., M. G. A. VAN DER HEIJDEN, AND A. SESSITSCH. 2010. Climate change effects on beneficial plant-microorganism interactions. *FEMS Microbiology Ecology* 73: 197–214.

DARRIBA, D., G. L. TABOADA, R. DOALLO, AND D. POSADA. 2012. jModelTest 2: More models, new heuristics and parallel computing. *Nature Methods* 9: 772.

EDGAR, R. C. 2004. MUSCLE: Multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research* 32: 1792–1797.

ESTRADA-DE LOS SANTOS, P., P. VINUESA, L. MARTINEZ-AGUILAR, A. M. HIRSCH, AND J. CABALLERO-MELLADO. 2013. Phylogenetic analysis of *Burkholderia* species by multilocus sequence analysis. *Current Microbiology* 67: 51–60.

GOVAERTS, R., M. RUHSAM, L. ANDERSSON, E. ROBBRECHT, D. BRIDSON, A. DAVIS, I. SCHANZER, AND B. SONKÉ. 2013. World checklist of Rubiaceae. Royal Botanic Gardens, Kew, UK. Website: www.kew.org/wcsp/rubiaceae [accessed October 2013].

GUINDON, S., J. F. DUFAYARD, V. LEFORT, M. ANISIMOVA, W. HORDIJK, AND O. GASCUEL. 2010. New algorithms and methods to estimate maximum-likelihood phylogenies: Assessing the performance of PhyML 3.0. *Systematic Biology* 59: 307–321.

GYANESHWAR, P., A. M. HIRSCH, L. MOULIN, W.-M. CHEN, G. N. ELLIOTT, C. BONTEMPS, P. ESTRADA-DE LOS SANTOS, ET AL. 2011. Legume-nodulating betaproteobacteria: Diversity, host range, and future prospects. *Molecular Plant-Microbe Interactions* 24: 1276–1288.

KAINULAINEN, K., S. G. RAZAFIMANDIMBISON, AND B. BREMER. 2013. Phylogenetic relationships and new tribal delimitations in subfamily Ixoroideae (Rubiaceae). *Botanical Journal of the Linnean Society* 173: 387–406.

LANTZ, H., K. ANDREASEN, AND B. BREMER. 2002. Nuclear rDNA ITS sequence data used to construct the first phylogeny of Vanguerieae (Rubiaceae). *Plant Systematics and Evolution* 230: 173–187.

LANTZ, H., AND B. BREMER. 2004. Phylogeny inferred from morphology and DNA data: Characterizing well-supported groups in Vanguerieae (Rubiaceae). *Botanical Journal of the Linnean Society, Linnean Society of London* 146: 257–283.

LEMAIRE, B., S. JANSSENS, E. SMETS, AND S. DESSEIN. 2012a. Endosymbiont transmission mode in bacterial leaf nodulation as revealed by a population genetic study of *Psychotria leptophylla*. *Applied and Environmental Microbiology* 78: 284–287.

LEMAIRE, B., E. ROBBRECHT, B. VAN WYK, S. VAN OEVELEN, B. VERSTRAETE, E. PRINSEN, E. SMETS, AND S. DESSEIN. 2011a. Identification, origin and evolution of leaf nodulating symbionts of *Sericanthe* (Rubiaceae). *Journal of Microbiology* 49: 935–941.

LEMAIRE, B., P. VANDAMME, V. MERCKX, E. SMETS, AND S. DESSEIN. 2011b. Bacterial leaf symbiosis in angiosperms: Host specificity without co-speciation. *PLoS ONE* 6: e24430.

LEMAIRE, B., S. VAN OEVELEN, P. DE BLOCK, B. VERSTRAETE, E. SMETS, E. PRINSEN, AND S. DESSEIN. 2012b. Identification of the bacterial endosymbionts in leaf nodules of *Pavetta* (Rubiaceae). *International Journal of Systematic and Evolutionary Microbiology* 62: 202–209.

MILLER, I. M. 1990. Bacterial leaf nodule symbiosis. *In* J. A. Callow [ed.], Advances in botanical research, vol. 17, 163-234. Academic Press, San Diego, California, USA.

MILLER, M. A., W. PFEIFFER, AND T. SCHWARTZ. 2010. Creating the CIPRES Science Gateway for inference of large phylogenetic trees. *In* Proceedings of the Gateway Computing Environments Workshop (GCE), New Orleans, Louisiana, 14 Nov. 2010, 1-8.

RAZAFIMANDIMBISON, S. G., H. LANTZ, A. MOULY, AND B. BREMER. 2009. Evolutionary trends, major lineages, and new generic limits in the dioecious group of the tribe Vanguerieae (Rubiaceae): Insights into the evolution of functional dioecy. *Annals of the Missouri Botanical Garden* 96: 161–181.

SIMMONS, M. P., AND H. OCHOTERENA. 2000. Gaps as characters in sequence-based phylogenetic analyses. *Systematic Biology* 49: 369–381.

SINGH, B. K., P. MILLARD, A. S. WHITELY, AND J. C. MURRELL. 2004. Unravelling rhizosphere–microbial interactions: Opportunities and limitations. *Trends in Microbiology* 12: 386–393.

STAMATAKIS, A., P. HOOVER, AND J. ROUGEMONT. 2008. A rapid bootstrap algorithm for the RAxML web servers. *Systematic Biology* 57: 758–771.

STROBEL, G., B. DAISY, U. CASTILLO, AND J. HARPER. 2004. Natural products from endophytic microorganisms. *Journal of Natural Products* 67: 257–268.

SUÁREZ-MORENO, Z. R., J. CABALLERO-MELLADO, B. G. COUTINHO, L. MENDONÇA-PREVIATO, E. K. JAMES, AND V. VENTURIE. 2012. Common features of environmental and potentially beneficial plant-associated *Burkholderia*. *Microbial Ecology* 63: 249–266.

VAN WYK, A. E., P. D. F. KOK, N. L. VAN BERS, AND C. F. VAN DER MERWE. 1990. Non-pathological bacterial symbiosis in *Pachystigma* and *Fadogia* (Rubiaceae): Its evolutionary significance and possible involvement in the aetiology of gousiekte in domestic ruminants. *South African Journal of Science* 86: 93–96.

VERSTRAETE, B., S. JANSSENS, E. SMETS, AND S. DESSEIN. 2013a. Symbiotic β-proteobacteria beyond legumes: *Burkholderia* in Rubiaceae. *PLoS ONE* 8: e55260.

VERSTRAETE, B., O. LACHENAUD, E. SMETS, S. DESSEIN, AND B. SONKÉ. 2013b. Taxonomy and phylogeny of *Cuviera* (Rubiaceae—Vanguerieae) and reinstatement of the genus *Globulostylis* with the description of three new species. *Botanical Journal of the Linnean Society* 173: 407–441.

VERSTRAETE, B., D. VAN ELST, H. STEYN, B. VAN WYK, B. LEMAIRE, E. SMETS, AND S. DESSEIN. 2011. Endophytic bacteria in toxic South African plants: Identification, phylogeny and possible involvement in gousiekte. *PLoS ONE* 6: e19265.

ZIMMERMANN, A. 1902. Über Bakterienknoten in den Blättern einiger Rubiaceen. *Jahrbücher für wissenschaftliche Botanik* 37: 1–11.