'*Candidatus Burkholderia calva*' and '*Candidatus Burkholderia nigropunctata*' as leaf gall endosymbionts of African *Psychotria*

Sandra Van Oevelen,¹ Rupert De Wachter,² Peter Vandamme,³ Elmar Robbrecht^{1,4} and Els Prinsen¹

Correspondence

Sandra Van Oevelen
sandra.vanoevelen@ua.ac.be

^{1,2}Laboratory of Plant Biochemistry and Physiology, Department of Biology¹, and Professor Emeritus, Laboratory of Molecular Biology, Department of Biochemistry², Antwerp University, Campus Drie Eiken, Universiteitsplein 1, B-2610 Antwerp, Belgium

³Laboratory of Microbiology, Faculty of Sciences, Ghent University, Ledeganckstraat, B-9000 Ghent, Belgium

⁴National Botanic Garden, Domein van Bouchout, B-1860 Meise, Belgium

Phylogenetic 16S rRNA gene analysis was used to assign the bacterial leaf-nodulating endosymbionts of two tropical African *Psychotria* species to the genus *Burkholderia*. The microsymbionts of the different *Psychotria* hosts were recognized as distinct and novel species of *Burkholderia* on the basis of relatively low intersequence similarities and sufficiently large evolutionary distances when compared with each other and their closest validly named neighbours. The obligate endosymbiotic nature of the bacteria prevented their *in vitro* cultivation and the deposition of type strains to culture collections. Therefore, the provisional status *Candidatus* is assigned to the bacterial partners of *Psychotria calva* and *Psychotria nigropunctata*, with the proposal of the names '*Candidatus Burkholderia calva*' and '*Candidatus Burkholderia nigropunctata*', respectively.

Bacterial leaf nodulation is a widespread event among African Rubiaceae (angiosperms), with representatives in *Psychotria*, *Pavetta* and *Sericanthe* (Robbrecht, 1988), three genera placed in three different tribes of the family. The most visible aspect of this atypical symbiosis is the establishment of galls in the leaves of the host plant, harbouring the bacterial microsymbiont. The presence of the symbiotic partner is a prerequisite for normal development and survival of the plant (Gordon, 1963). Due to its obligate and cyclic nature, bacterial leaf symbiosis cannot be classified together with any other known type of plant–bacterium interaction.

The role these bacteria play in the development of their host is crucial. Their isolation, cultivation and identification are essential as a first step towards revealing the underlying mechanisms that support this intimate form of co-existence. In a previous study, sequence analysis of the small-subunit (16S) rRNA gene was used to identify the endophyte

associated with *Psychotria kirkii* as a novel *Burkholderia* species. Due to its uncultured nature the provisional status *Candidatus* was proposed for this bacterial endosymbiont, i.e. '*Candidatus Burkholderia kirkii*' (Van Oevelen *et al.*, 2002a). In the current study, similar methods were used to identify the bacterial microsymbionts associated with two further members of the genus *Psychotria*.

Bacteria-rich galls were isolated from *Psychotria calva* Hiern (no. 19620512) and *Psychotria nigropunctata* Hiern (no. 19750521). *Psychotria lucens* var. *lucens* (no. 19610404), a species which is always devoid of bacterial galls (Petit, 1964), was used as an overall negative control. Acquisition numbers refer to the living collection of the National Botanic Garden of Belgium. All methods were as previously described (Van Oevelen *et al.*, 2002a).

DNA extractions were performed using the DNeasy Plant Mini Kit (QIAGEN) according to the manufacturer's instructions. Amplification of the 16S rRNA genes was performed and amplified fragments were cloned into a pGEM-Teasy vector. All clones were subjected to an *EcoRI* digest and partially sequenced, to allow selection of clones containing bacterial 16S rRNA genes. We selected seven and three 'bacterial' clones from *P. calva* and *P. nigropunctata*, respectively. Comparison of the partial sequences (minimum

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The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of '*Candidatus Burkholderia calva*' and '*Candidatus Burkholderia nigropunctata*' are AY277697 and AY277698, respectively.

600 bp) of all the bacterial 16S rRNA genes obtained from a single plant species showed that no differences occurred among them. This was shown for both plants involved, indicating that each species was hosting only a single bacterial strain in its leaf galls.

The complete 16S rRNA gene sequences for the symbionts were determined for each of the plant species. As the bacterial presence is limited to leaf galls and the stem apical region, we considered gall-free samples taken from in between galls as negative controls. If the obtained 16S rRNA genes belonged to contaminating bacteria they would not only be present in the leaf galls but also in any other part of the leaf, including the negative control samples. However, the absence of bacteria in gall-free samples was confirmed for both plant species, which proved that the retrieved 16S rRNA gene sequences represented the bacterial symbionts rather than any contaminants. Likewise, the gall-free species *P. lucens* var. *lucens* was shown to lack any bacterial DNA, as was expected. Additional proof came from the analysis of the apical region. Bacterial 16S rRNA genes obtained from the stem apex of *P. calva* and *P. nigropunctata* were sequenced. Comparisons revealed them to be identical to the bacterial 16S rRNA genes obtained from the leaf galls of the respective species. This indicates the presence of the same bacterial symbiont in both the stem apex and leaf galls. Sequences were submitted to the SSU rRNA database (Wuyts *et al.*, 2004) and aligned with their closest relatives using DCSE software (De Rijk & De Wachter, 1993). Distance matrices were calculated using the Jukes & Cantor (1969) substitution model. Phylogenetic trees were constructed according to the neighbour-joining method (Saitou & Nei, 1987), using the TREECON program (Van de Peer & De Wachter, 1994). Bootstrap values were used as a measure of reliability (Felsenstein, 1985).

Phylogenetic analysis, based on their full-length 16S rRNA gene sequences, places the endosymbionts of *P. calva* and *P. nigropunctata* in the genus *Burkholderia*, as was shown earlier for the bacterial partner of *P. kirkii*, '*Candidatus Burkholderia kirkii*' (Van Oevelen *et al.*, 2002a).

Intersequence similarities range from 95.9 to 97.9%. Sequence similarities to the closest cultured neighbour, *Burkholderia* sp. NF100 (Hayatsu *et al.*, 2000), are alike, ranging from 96.5 to 97.9%, whereas similarities shared with the closest validly named species, *Burkholderia glathei* (Viallard *et al.*, 1998), are slightly lower, ranging from 96.2 to 96.5%.

A distance matrix was constructed containing 13 *Burkholderia* species. The evolutionary tree presented in Fig. 1 is based on this analysis. Even though all symbionts cluster closely together, the evolutionary distances separating the different microsymbionts are sufficiently large to recognize the endosymbionts of both *P. calva* and *P. nigropunctata* as distinct and novel *Burkholderia* species. In previous studies we included six different varieties of *P. kirkii*, a geographically widespread species that shows great morphological variation (Van Oevelen *et al.*, 2002a, b). Despite the diverse nature of this species, our phylogenetic analysis showed that the microsymbionts of the six acquisitions of *P. kirkii* that were investigated are very closely related and represent a single *Burkholderia* species. Our current data suggest that all microsymbionts of *Psychotria* originate from a single *Burkholderia* species. They also indicate that bacterial leaf nodulation in *Psychotria* is the result of a single infection event in an ancestor to modern *Psychotria*, after which the closed nature of the symbiotic cycle allowed genetic differentiation during the subsequent co-evolution of plant and bacteria. The inter-isolate distances between microsymbionts of distinct host species are large enough to permit recognition of three different *Burkholderia* species, corresponding to the three investigated *Psychotria* species.

Scanning electron microscopical images of the bacterial endosymbionts are presented in Fig. 2. Cross-sections through the bacterial galls were made from fresh leaves using razor blades. Sections were dehydrated by a treatment in 70% ethanol (30 min) and methylal (2 × 30 min), and subsequently critical-point dried. Dried samples were mounted on aluminium stubs, sputter coated with gold and analysed using a JEOL 6400 scanning electron

Fig. 1. Neighbour-joining tree based on the full 16S rRNA gene sequence of 13 strains of *Burkholderia*. Similarities were adjusted by the Jukes–Cantor model; insertions and deletions were taken into account. Bootstrap values represent percentage support of the nodes based on 1000 resamplings. *Pandoraea norimbergensis* served as outgroup.

Fig. 2. Scanning electron microscopical images showing the bacteria inhabiting the leaf galls of (A) *P. calva* Hiern (acquisition number 19620512) and (B) *P. nigropunctata* Hiern (acquisition number 19750521). Arrows indicate bacteria. Bars, 2 µm.

microscope. Bacterial rods are visible in the leaf galls of *P. calva* (Fig. 2a) and *P. nigropunctata* (Fig. 2b). No flagella are observed.

Despite extensive efforts to grow the endophytes on an elaborate series of liquid and solid culture media, their cultivation has not been successful as yet. This could be either the result of an extreme endosymbiosis in which the microsymbiont has lost its free-living potential through evolution, or the result of the symbiotic requirement for unidentified plant-specific nutrients which are essential for its survival. However, until we succeed in cultivation of the endophytes or additional phenotypic data become available, we assign them the provisional status *Candidatus* as proposed by Murray & Stackebrandt (1995).

Descriptions of '*Candidatus Burkholderia calva*' and '*Candidatus Burkholderia nigropunctata*'

'*Candidatus Burkholderia calva*' (calva, from the specific epithet of the host plant) [(β -Proteobacteria, genus *Burkholderia*); NC; G–; R; NAS (GenBank no. AY277697), oligonucleotide sequence complementary to unique region of 16S rRNA gene 5'-TCGGAACCCTGCTGAAAGGTGGGGTGCTCGAAAGAGAACCGGT-3'; S (*Psychotria calva*, stem apex and leaf galls)]. Van Oevelen *et al.*, this study.

'*Candidatus Burkholderia nigropunctata*' (nigropunctata, from the specific epithet of the host plant) [(β -Proteobacteria, genus *Burkholderia*); NC; G–; R; NAS (GenBank no. AY277698), oligonucleotide sequence complementary to unique region of 16S rRNA gene 5'-CCCTGCTGAGAGGTGGGGTGCTCGGAAGAGAACC-GTC-3'; S (*Psychotria nigropunctata*, stem apex and leaf galls)]. Van Oevelen *et al.*, this study.

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