Application of a *recA* gene-based identification approach to the maize rhizosphere reveals novel diversity in *Burkholderia* species

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Abstract

Burkholderia species are widely distributed in the natural environment. We evaluated the use of the *recA* gene in a cultivation-independent approach to examine the *Burkholderia* diversity associated with the maize rhizosphere. Two types of *recA* gene library were constructed, one with broad-specificity *recA* primers (BUR1 and BUR2) and a second from the products of nested PCRs using *Burkholderia*-specific primers (BUR3 and BUR4). The broad-specificity primer set provided near full-length *recA* sequences (869 bp) suitable for the creation of robust environmental sequence data sets; however, the nested PCR approach demonstrated the greatest specificity (84%) for detection of *Burkholderia* species *recA* genes. In addition, the screening approach was able to identify *recA* phylotypes matching *Burkholderia cepacia* complex species previously cultivated from the maize samples and discriminate these from other *Burkholderia*. The ecological benefit of *Burkholderia* species cultivated from maize rhizosphere is well documented, however, the fact that the majority of *Burkholderia recA* genes detected in this study (90%) were suggestive of novel taxa indicates that a wealth of potentially important interactions with uncultivated *Burkholderia* species remain unstudied in this habitat.

Introduction

Bacteria from the genus *Burkholderia* occupy multiple niches of major ecological importance. Many *Burkholderia* species have been isolated from soil and have been reported to be closely associated with the plant rhizosphere (Parke & Gurian-Sherman, 2001; Coenye & Vandamme, 2003). Beneficial environmental interactions of *Burkholderia* species include their ability to facilitate both plant protection and growth promotion as biopesticidal agents (Parke & Gurian-Sherman, 2001), the capacity of certain species to fix nitrogen (Minerdi *et al.*, 2001; Caballero-Mellado *et al.*, 2004; Reis *et al.*, 2004), and versatile catabolic capacity that allows degradation of numerous major pollutants such as trichloroethylene and polychlorinated biphenyls (Shields *et al.*, 1991; Goris *et al.*, 2004). In contrast, several *Burkholderia* species may also cause disease in vulnerable humans (Mahenthiralingam *et al.*, 2005), animals and plants (Coenye & Vandamme, 2003). The *Burkholderia* genus currently comprises 34 formally described species (Coenye & Vandamme, 2003), with nine species forming the *Burkholderia cepacia* complex (Mahenthiralingam *et al.*, 2005). *Burkholderia* taxonomy is complex and recent studies have undertaken a polyphasic approach to characterize new species (Coenye & Vandamme, 2003). Studies on environmental *B. cepacia* complex bacteria have involved cultivation of organisms on selective media followed by further identification including molecular analysis of 16S rRNA gene (Salles *et al.*, 2002) or *recA* (Fiore *et al.*, 2001), or both (Miller *et al.*, 2002; Ramette *et al.*, 2005). The *recA* gene has recently been used as a cultivation-independent approach, however, this study was limited to the detection of only *B. cepacia* complex species occurring in the maize rhizosphere because of the specificity of the PCR used (Pirone *et al.*, 2005). Exploration of the diversity of the entire *Burkholderia* genus in environmental samples has, however, been largely limited to 16S rRNA gene analyses (Salles *et al.*, 2002; Coenye & Vandamme, 2003), revealing *Burkholderia* as important endosymbiotic species such as those forming leaf galls (Van Oevelen *et al.*, 2004).

The *recA* gene has been established as a useful target for the identification of the *B. cepacia* complex species; it affords greater discriminatory power than the 16S rRNA gene for differentiation within this closely related group

Table 1. PCR primers used in the study

PCR name	Primer pair (forward and reverse, 5′–3′)	Product size (bp)	Annealing temperature (°C)	Specificity	Reference
BCR1.2	BCR1, TGACCGCCGAGAAGAGCAA BCR2, CTCTTCTTCGTCCATCGCCTC	1043	58	*Burkholderia cepacia* complex only	Mahenthiralingam *et al*. (2000)
BUR1.2	BUR1, GATCGARAAGCAGTTCGGCAA BUR2, TTGTCCTTGCCCTGRCCGAT	869	58	Broad specificity including *Burkholderia* species	Payne *et al*. (2005)
BUR3.4	BUR3, GARAAGCAGTTCGGCAA BUR4, GAGTCGATGACGATCAT	385	57	*Burkholderia* species only	Payne *et al*. (2005)

(Mahenthiralingam *et al*., 2000). We recently expanded the *recA*-based approach to identify the entire *Burkholderia* genus by designing two useful primer sets, BUR1 and BUR2 (Payne *et al*., 2005), which amplify an almost full-length *recA* gene (869 bp) from all *Burkholderia* species. These primers were found not to be genus-specific in that they amplified other betaproteobacterial species. However, they were useful for the generation of primary sequence data from which the specific primers BUR3 and BUR4 were designed and found to produce a 385 bp amplicon only from *Burkholderia* species (Payne *et al*., 2005). Recently, Ramette *et al*. (2005) used a high-throughput cultivation-enrichment approach in conjunction with colony hybridization and PCR using 16S rRNA gene and *recA*-derived probes to examine the extent of cultivable *B. cepacia* complex species diversity present in maize-associated soil samples. In this study, we evaluated the *Burkholderia* species *recA* approach as a cultivation-independent assay performed on the same maize rhizosphere samples from which *B. cepacia* complex species have already been cultivated (Ramette *et al*., 2005).

Materials and methods

Environmental sampling

Maize rhizosphere samples were obtained as described previously (Ramette *et al*., 2005) from the Michigan State University, W. K. Kellogg Biological Station Long-Term Ecological Research (KBS) site (Hickory Corners, MI), and were stored at −20 °C. Total DNA was extracted from 0.5 g of thawed root system samples with the Bio 101® Systems FastDNA® spin kit for soil according to the manufacturer's instructions (Qbiogene, Cambridge, UK). Four replicate DNA extractions were performed on soil aliquots from the root systems of two maize plants (Plant 1 and Plant 3; Ramette *et al*., 2005).

PCR detection of *Burkholderia* species *recA* genes

DNA extracted from the maize rhizosphere samples was subjected to PCR to detect *recA* genes from the *Burkholderia cepacia* complex and other *Burkholderia* species using the primers shown in Table 1. *Burkholderia cepacia* complex *recA* genes were amplified using a PCR with primers BCR1 and BCR 2 (BCR1.2 PCR; Table 1) exactly as described by Mahenthiralingam *et al*. (2000). The PCR methods described by Payne *et al*. (2005) were used to amplify *recA* genes from other *Burkholderia* species. PCRs with primers BUR1 and BUR2 (BUR1.2 PCR; Table1) were used to amplify near full-length *recA* gene products from a broad range of species including *Burkholderia* (Payne *et al*., 2005). Specific PCR of *Burkholderia recA* genes was achieved using PCR with primers BUR3 and BUR4 (BUR3.4 PCR; Table 1) as described (Payne *et al*., 2005). PCR was performed using Qiagen reagents (Qiagen Ltd., Crawley, UK) in 25 μL reactions. Each PCR contained the following: 1 U *Taq* polymerase, 250 μM of each dNTP, 1 × PCR buffer (containing 1.5 mM $MgCl_2$), 10 pmol of each appropriate oligonucleotide primer, and 20 ng of template DNA. Thermal cycling was carried out in a Flexigene thermal cycler (Techgene Ltd., Cambridge, UK) for 30 cycles of 30 s at 94 °C, annealing for 30 s at the appropriate annealing temperature (see Table 1) and extension at 72 °C for 45 s. The following modifications were included: (i) to amplify fragments suitable for cloning, a 20 min final extension time was included in the thermal cycle; (ii) a nested BUR3.4 PCR was performed using a 1000-fold dilution (*c*. 20 ng) of the BUR1.2 product as the template; (iii) a temperature gradient PCR with annealing temperatures between 57 and 64 °C was performed to optimize the BUR3.4 PCR for amplification of high copy number plasmid template DNA in a Dyad DNA Engine thermal cycler (Bio-Rad Laboratories Ltd., Hemel Hempstead, UK). Approximately 2 μL of each PCR product was visualized by agarose gel electrophoresis as described by Mahenthiralingam *et al*. (2000).

Construction and screening of *recA* gene libraries

PCR products from BUR1.2 and BUR3.4 PCRs were cloned with pGEM®-T Easy vector in competent *Escherichia coli* JM109 cells using the manufacturer's protocols (Promega, Southampton, UK). Recombinant clones were picked into

96-well plates containing Luria–Bertani broth (LB) and the antibiotic ampicillin (100 μg mL^{-1}), grown overnight and then stored at −80 °C after the addition of 8% dimethylsulfoxide to each well. Clones from the BUR1.2 library were screened individually for the presence of putative *Burkholderia*-specific *recA* genes using a high throughput 96-well PCR approach as follows: after revival and growth in 96-well plates containing 150 μL LB broth with ampicillin selection, a small amount of each resulting culture was transferred with a 96-point replicator to 5 μL of lysis solution (Ramette *et al.*, 2005) in a 96-well PCR plate and boiled at 99 °C for 10 min.A cocktail of complete BUR3.4 PCR reagents was added to the boiled lysate and subjected to thermal cycling and agarose gel electrophoresis as described above.

Nucleotide sequence determination

Plasmid DNA from selected clones was prepared using the Wizard® *Plus* SV Minipreps (Promega) as instructed by the manufacturer. Both strands of the *recA* clones were sequenced directly with the M13F and M13R primers as described (Mahenthiralingam *et al.*, 2000). Sequencing reactions were prepared using Applied Biosystems Big Dye Terminator ready reaction mix version 3.1 and analysed using an ABI-PRISM 3100 Genetic Analyser capillary electrophoresis system running Performance Optimized Polymer 6 (POP-6) in accordance with the manufacturer's instructions (Applied Biosystems, Foster City, CA). Raw sequences were aligned, and a consensus sequence was derived using the CAP contig assembly program within the BioEdit software (Hall, 1999). Putative sequence identity was determined using the Basic Local Alignment Search Tool (BLAST; www.ncbi.nlm.nih.gov). A total of 47 representative *recA* sequences were deposited in GenBank under accession numbers DQ076251 through DQ076297 and the aligned sequence set is available from ftp://cepacia.bios.cf.ac.uk/pub/.

Phylogenetic analysis

A number of different phylogenetic methods had been evaluated in previous studies including neighbour-joining, maximum-likelihood and maximum-parsimony analyses (Mahenthiralingam *et al.*, 2000; Payne *et al.*, 2005). The following phylogenetic scheme was found to be straightforward to apply and provided excellent correlation to the current species distribution in the *Burkholderia* genus (Mahenthiralingam *et al.*, 2000; Vermis *et al.*, 2002; Baldwin *et al.*, 2005; Payne *et al.*, 2005). Multiple nucleotide sequence alignments spanning 385 nucleotides of the *recA* gene were constructed using CLUSTAL W (Thompson *et al.*, 1994). Phylogenetic and molecular evolutionary analyses were conducted using genetic distance-based neighbour-joining algorithms within MEGA version 2.1 (http://www.megasoftware.net/). Gaps and missing data were completely deleted by MEGA2.1 before trees were constructed using the Jukes and Cantor (1969) substitution model with random sequence input order and 1000 data sets examined by bootstrapping. Trees were rooted with the *Bordetella pertussis recA* gene as an outgroup (accession number X53457). To check for the presence of chimeric *recA* genes, sequences were divided into two fragments of equal length and phylogenetic analysis performed with each half. The novel alignment of the majority of CL1.2 and CL3.4 clones was not due to the presence of chimeric *recA* sequences as clustering remained consistent in trees constructed from each half of the sequence.

Results and discussion

The *recA*-based PCR approaches developed in a previous study (Payne *et al.*, 2005) enabled the identification and taxonomic assignment of *Burkholderia* species using DNA extracted from cultivated isolates. To test their efficacy as primers for the investigation of *Burkholderia* diversity in the natural environment, we applied them as a cultivation-independent approach to soil from the root systems of maize plants known to be positive for cultivated members of the *Burkholderia cepacia* complex (Ramette *et al.*, 2005). Amplification of putative *recA* genes using the broad-range BUR1.2 PCR demonstrated that three of four DNA extractions from Plant 1, and 4 of four extractions from Plant 3 were positive for the expected 869 bp PCR product. Testing of the same samples with a BCR1.2 PCR (Table 1; Mahenthiralingam *et al.*, 2000) demonstrated that only two of four extractions from Plant 3 were positive for *B. cepacia* complex-specific *recA* genes. None of the maize rhizosphere samples were positive for the *Burkholderia*-specific BUR3.4 PCR when it was applied directly to the extracted DNA. Given the success of the BUR1.2 and BCR1.2 PCRs, the failure of the BUR3.4 PCR to amplify the soil-extracted DNA is difficult to explain; lack of amplification may have occurred for a number of reasons. The BUR3.4 PCR used 17-mer primers that were shorter than the 19–21 base primers used in the successful *recA*-specific PCRs (Table 1). In combination with the annealing temperature of 57 °C required to obtain the correct 385 bp product from the BUR3.4 PCR, this may have produced conditions which were too stringent to facilitate amplification of limited number of *Burkholderia recA* genes present in the soil samples. In addition, the target site of primer BUR4 was within a central region of the gene that may have a reduced capacity for amplification in comparison with the other *recA*-specific oligonucleotides which were designed to prime at each end of the 1 kb gene. The samples from Plant 3 were selected for further study because: (i) the positive results of the BCR1.2 PCR indicated that they contained DNA from

B. cepacia complex species and (ii) previous cultivation-based analysis had demonstrated that moderate numbers of these bacteria (6.5 $\log_{10}$ CFU per g soil) were recovered from the rhizosphere of this plant (Ramette *et al.*, 2005). Also, by fulfilling the latter criteria, the sample provided a suitable test of discriminatory power of the *Burkholderia* genus *recA* identification approach (Payne *et al.*, 2005) and its ability to distinguish members of the *B. cepacia* complex from other members of this genus.

To estimate *Burkholderia* diversity in the DNA extracted from Plant 3, a two-stage *recA* gene library screening strategy was applied as follows. In the first stage, products from a BUR1.2 PCR (Table 1) were cloned to construct the *recA* library, CL1.2, and clones from this library were screened for the presence of putative *Burkholderia*-specific sequences using a high throughput 96-well BUR3.4 PCR (Table 1). A total of 47 putative *Burkholderia recA*-positive clones were identified from 384 clones screened and 42 of these were successfully sequenced. Eleven (26%) of the sequenced 869 bp *recA* clones were found to be representative of *Burkholderia* species *recA* genes using BLAST analysis. In addition, 28 clones from the CL1.2 library which were negative for amplification with the BUR3.4 PCR were selected at random and analysed to determine the background of non-*Burkholderia* species associated with the sample. None of these were *Burkholderia recA* sequences, but were most closely related to *Betaproteobacteria* such as *Herbaspirillum* spp., *Rubrivivax* spp., and *Dechloromonas* spp., and *Gammaproteobacteria* such as *Legionella* spp., *Xylella* spp., and *Methylococcus* spp. (data not shown). In the second stage screen, a nested PCR with the *Burkholderia*-specific BUR3.4 primers (Table 1) was performed on the BUR1.2 PCR products described above. The resulting 385 bp products were subcloned to create the library CL3.4 and 60 clones were subjected to nucleotide sequence analysis. Forty-four of the CL3.4 clones produced good sequence data and 37 (84%) sequences were found to be representative of *Burkholderia* species. In total, 114 *recA* sequences were determined from the maize rhizosphere sample.

To determine the prevalence of *Burkholderia* DNA associated with maize rhizosphere, the *recA* clone library sequences were subjected to phylogenetic analysis. The tree topology observed correlated well with previous studies (Mahenthiralingam *et al.*, 2000; Payne *et al.*, 2005; Ramette *et al.*, 2005) separating *Burkholderia* species from other genera, clearly defining the *B. cepacia* complex and discriminating the species within this group (Fig. 1). Screening *recA* gene library CL1.2 was the least sensitive means of detecting *Burkholderia* species, resulting in a final detection specificity of 26%. Eleven of the *Burkholderia* species *recA* sequences present in library CL1.2 clustered within the genus and were associated with three novel groups, only distantly related to known cultivated species, (Groups 1, 2, and 4, Fig. 1; bootstrap values $> 50\%$). Only the *Burkholderia phenazinium recA* sequence clustered with these novel *Burkholderia recA* sequences and all of the other cultivated species within the genus fell outside the cluster. Of the remaining CL1.2 *recA* sequences, one was distantly related to *Burkholderia glathei* (clone 4c11; Fig. 1) and three identical CL1.2 *recA* sequences clustered closely with *Burkholderia gladioli* (Group 6; Fig. 1).

The *recA* gene library CL3.4 derived from the nested BUR3.4 PCR (Table 1) proved a more effective means of detecting *Burkholderia* within the maize soil sample, with 84% of the determined *recA* sequences falling within the genus. The majority of CL3.4 clones (35) clustered within novel phylogenetic groups, including those already defined by CL1.2 (Groups 1, 2, and 4; Fig. 1) as well as two further unique clusters (Groups 3 and 5; Fig. 1). Of the 11 BUR3.4 clones within Group 1, three were identical and eight were very closely related (the sequence difference across the entire group was 0.7%). The sequence variation across the four Group 2 BUR3.4 clones was 1%. Group 3 sequences were composed of six closely related clones (2.4% variation across the group) and were part of a novel cluster that included two discrete CL3.4-derived clones (1b1 and 2a7; Fig. 1). Groups 4 and 5 both contained a total of four closely related clones and the sequence variation within each of the latter groups was identical at 1.4%. The Group 5 sequences were distantly related to *B. glathei*. Only two of the CL3.4 clones, 1c5 and 2a1, were assigned to *B. cepacia* complex species, *Burkholderia ambifaria* and *Burkholderia pyrrocinia,* respectively (Fig. 1).

Determination of *recA* sequences present in an environmental DNA sample using this PCR approach clearly demonstrated the utility of *recA* as a cultivation-independent approach to examine *Burkholderia* diversity. It is an effective means to identify *Burkholderia* species and members of the *B. cepacia* complex in a single screen that would not be possible using methods based on the 16S rRNA gene where limited sequence diversity precludes such discrimination within this group of bacteria. Although amplification with the BUR1.2 PCR was less successful at revealing the presence of *Burkholderia*, the 869 bp product constitutes $> 80\%$ of the full length of the average *recA* gene and hence has utility for the generation of sequence databases not only for *Burkholderia*, but also can target other closely related species. The high copy number of the plasmid template DNA from the CL1.2 library may have accounted for the reduced specificity of the BUR3.4 PCR screening of CL1.2 clones. A temperature gradient PCR (annealing temperatures 57–64 °C) was subsequently performed to optimize the screen, resulting in the positive amplification of *Burkholderia* CL1.2 clones up to 60.5 °C, while non-*Burkholderia* clones from CL1.2 failed above 59 °C (data not shown).

Overall, nested PCR with BUR3 and BUR4 showed much greater specificity for the detection of *Burkholderia* DNA

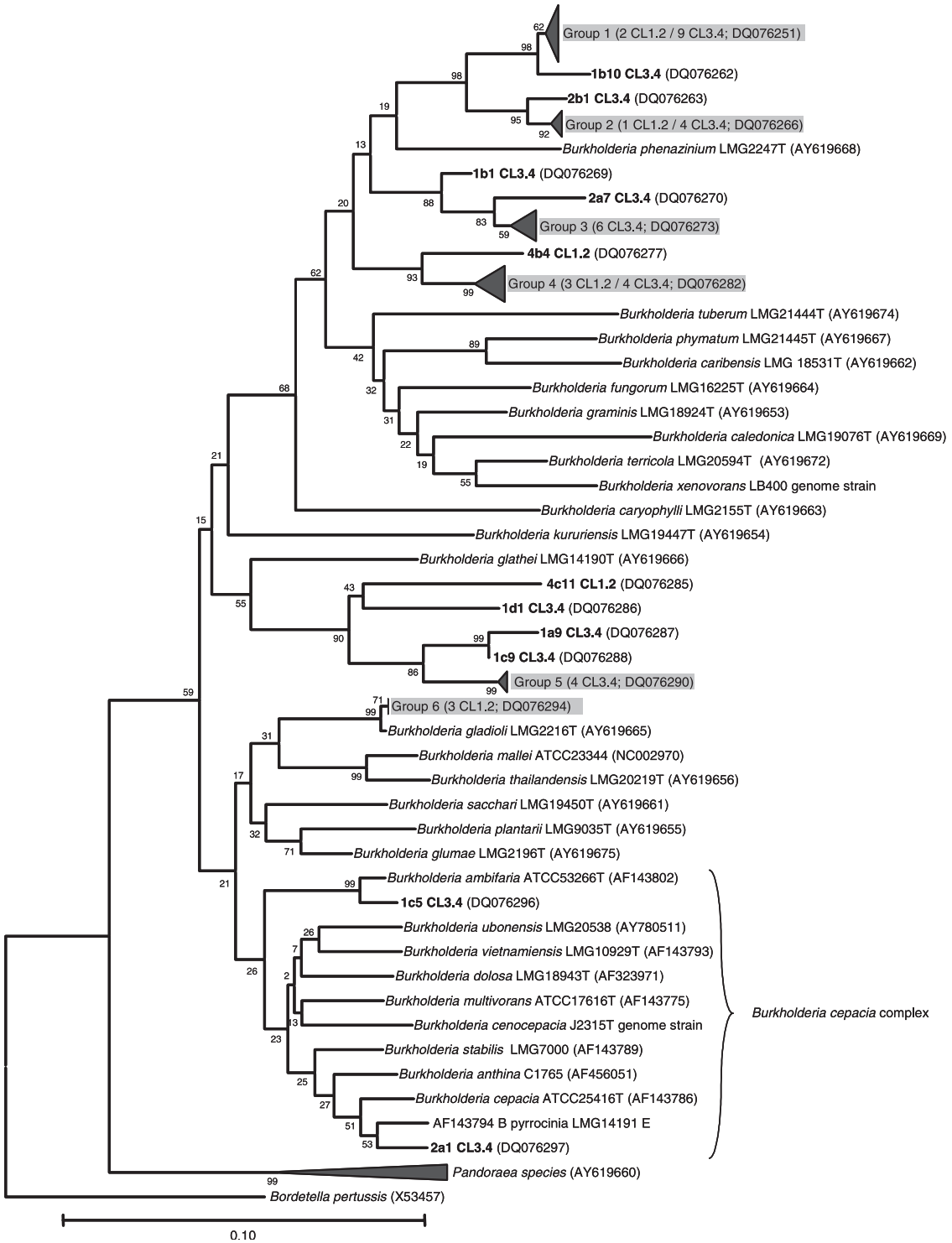

Fig. 1. Phylogenetic analysis of the *Burkholderia recA* sequences obtained from the maize soil environment. Nucleotide sequence alignments of *recA* (385 bases) were constructed and analysed phylogenetically using genetic-distance based neighbour-joining algorithms (Jukes–Cantor matrix model; bootstrapping with 1000 replications; Payne *et al*., 2005). Six clusters (Groups 1–6) containing multiple novel *Burkholderia recA* genes are shown compressed. The clone library from which the sequence originated, the number of sequences in each cluster, and accession number for each representative *recA* gene are shown in parentheses. Bootstrap values and genetic distance scale (number of substitutions per site) are indicated. Species names and strain numbers for reference *Burkholderia recA* genes are shown with accession numbers in parentheses. Three *Pandoraea recA* sequences were included as a closely related genus and the *Burkholderia cepacia* complex group are also indicated.

within the maize rhizosphere (Fig. 1). The primary rationale for working with maize rhizosphere samples was the fact that they are associated with an abundance of *B. cepacia* complex species (Fiore *et al.*, 2001; Salles *et al.*, 2002; Coenye & Vandamme, 2003). Therefore, the *recA* approach (Payne *et al.*, 2005) can be evaluated against bacteria that have been cultivated from these samples (Ramette *et al.*, 2005). Our cultivation-independent analysis correlated well with the cultivation-based approach performed previously (Ramette *et al.*, 2005), identifying *recA* clones most closely related to *B. ambifaria* and *B. pyrrocinia* (Fig. 1). (Ramette *et al.*, 2005) had cultivated both of these species from soil associated with the root system of Plant 3, and while *B. ambifaria* was recovered from all seven plant samples examined, *B. pyrrocinia* was less prevalent and was only isolated from one other plant in the original survey. The abundance of these two *B. cepacia* complex species within the soil sample was $< 1 \times 10^6$ CFU per g of soil (Ramette *et al.*, 2005), indicating that the sensitivity of the *recA* approach is limited with no *B. cepacia* complex species *recA* genes detected in clone library CL1.2. Only two *recA* genes corresponding to cultivated *B. cepacia* complex species were detected after nested PCR with BUR3 and BUR4.

An exciting finding from this study was that 90% (43 of 48 sequences) of the *recA* genes associated with the maize rhizosphere sample were assigned to putatively novel as yet uncultivated *Burkholderia* species. Clones from three of these novel groups (Groups 1, 2 and 4; Fig. 1) were identified in both the CL1.2 and CL3.4 *recA* gene libraries. The high bootstrap values for these groups indicate that they represent important uncharacterized taxa. Although simultaneous cultivation of non-*B. cepacia* complex *Burkholderia* species was not attempted in this study, the fact that the vast majority of *recA* sequences analysed were not closely related to cultivated *Burkholderia* reference species strongly indicated that uncultivated and even potentially endosymbiotic *Burkholderia* are highly abundant within the maize rhizosphere. We have described for the first time the utility of the *recA* gene as a cultivation-independent means to examine directly the diversity of all *Burkholderia* species. It has the resolution to identify and discriminate between species members of the closely related *B. cepacia* complex, and differentiate them from the rest of genus. In addition, the analysis revealed an abundance of potentially novel *Burkholderia* species associated with the maize soil system which have not been cultivated and whose potential role in the maize rhizosphere is clearly worthy of further analysis.

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