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Detection of cultured and uncultured *Burkholderia cepacia* complex bacteria naturally occurring in the maize rhizosphere

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Summary

The species composition of a *Burkholderia cepacia* complex population naturally occurring in the maize rhizosphere was investigated by using both culture-dependent and culture-independent methods. *B. cepacia* complex isolates were recovered from maize root slurry on the two selective media *Pseudomonas cepacia* azelaic acid tryptamine (PCAT) and trypan blue tetracycline (TB-T) and subjected to identification by a combination of restriction fragment length polymorphism (RFLP) analysis and species-specific polymerase chain reaction (PCR) tests of the *recA* gene. DNA extracted directly from root slurry was examined by means of nested PCR to amplify *recA* gene with species-specific *B. cepacia* complex primers and to obtain a library of PCR amplified *recA* genes. Using the culture-dependent method the species *Burkholderia cepacia*, *Burkholderia cenocepacia*, *Burkholderia ambifaria* and *Burkholderia pyrrocinia* were identified, whereas using the culture-independent method also the species *Burkholderia vietnamiensis* was detected. The latter method also allowed us to highlight a higher diversity within the *B. cenocepacia* species. In fact, by using the culture-independent method the species *B. cenocepacia recA* lineages IIIA and IIID besides *B. cenocepacia recA* lineage IIIB were detected. Moreover, higher heterogeneity of *recA* RFLP patterns was observed among clones assigned to the species *B. cenocepacia* than among *B. cenocepacia* isolates from selective media.

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Introduction

Bacteria belonging to the *Burkholderia cepacia* complex have emerged in recent years as both biocontrol and bioremediation agents (Kilbane *et al*., 1983; McLoughlin *et al*., 1992; Bevivino *et al*., 1998; Hebbar *et al*., 1998; Holmes *et al*., 1998; Bevivino *et al*., 2000) and as important opportunistic pathogens, especially for people with cystic fibrosis (CF) (Govan and Deretic, 1996; Govan *et al*., 1996; LiPuma, 1998; Mahenthiralingam *et al*., 2005).

Recent taxonomic studies revealed that *B. cepacia* complex consists of at least nine closely related bacterial species, i.e. *Burkholderia cepacia*, *Burkholderia multivorans*, *Burkholderia cenocepacia*, *Burkholderia stabilis*, *Burkholderia vietnamiensis*, *Burkholderia dolosa*, *Burkholderia ambifaria*, *Burkholderia anthina* and *Burkholderia pyrrocinia* (Gillis *et al*., 1995; Vandamme *et al*., 1997; 2000; 2002; 2003; Coenye *et al*., 2001; Vermis *et al*., 2004). In addition, Vandamme and colleagues (2003), on the basis of *recA* gene polymorphism, described four phylogenetic lineages, i.e. IIIA, IIIB, IIIC and IIID, within the species *B. cenocepacia*. All nine *B. cepacia* complex species have been identified in both natural environment and in CF sputum cultures, but several recent studies indicate that they are unequally represented in these habitats. As far as natural environment is concerned, *B. cepacia*, *B. cenocepacia recA* lineage IIIB, *B. ambifaria* and *B. pyrrocinia* predominate in environmental samples (Fiore *et al*., 2001; Bevivino *et al*., 2002; Dalmastri *et al*., 2003; 2005; Ramette *et al*., 2005), whereas in CF sputum samples *B. cenocepacia recA* lineages IIIA, IIIB and IIID, and *B. multivorans* account for the majority of the *B. cepacia* complex isolates (LiPuma *et al*., 2001; Agodi *et al*., 2001; Speert *et al*., 2002; Manno *et al*., 2004). The wide diffusion in the natural environment of *B. cepacia* complex species raises great concern about natural habitat as a potential 'reservoir' of human pathogenic strains (Coenye and Vandamme, 2003). A recent study, in which various genotyping methods have been used to analyse *B. cepacia* complex strains recovered from environmental and clinical sources, indicated that human pathogenic strains are not necessarily distinct from environmental

ones (LiPuma *et al*., 2002). These findings have important implications for infection control in CF patients, which presently focuses on patient to patient spread (Speert, 2001). Acquisition of *B. cepacia* complex from the natural environment might explain why current control measures have decreased patient to patient spread but not eliminated the incidence of new infection in CF people (Speert *et al*., 2002; Manno *et al*., 2004). Thus, studies regarding the assessment of *B. cepacia* complex in natural habitat are needed to define the risk posed by environmental strains to CF patients.

So far, most studies regarding the presence and distribution of the different *B. cepacia* complex species in natural habitats are referred to maize rhizosphere environment (Balandreau *et al*., 2001; Fiore *et al*., 2001; Bevivino *et al*., 2002; Dalmastri *et al*., 2003; Ramette *et al*., 2005). Moreover, these reports are based on culture-dependent techniques, which may prove unsuitable to provide a reliable picture of the prevalence and species composition of the *B. cepacia* complex in natural environment. In fact, it is acknowledged that methods using direct amplification and analysis of DNA allow more comprehensive detection of soil microbial bacteria than cultivation (Dunbar *et al*., 1999; McCaig *et al*., 2001). Indeed, Miller and colleagues (2002), found that culture-based methods may underestimate environmental *B. cepacia* complex populations because they were able to detect the presence of *B. cepacia* complex in a greater number of samples by using the growth-independent approach than by cultivation. However, these authors did not provide information on the presence of the single species but only on some *B. cepacia* complex's species groups because they used 16S rRNA as target gene, whose nucleotide sequence variation is not sufficient to identify all current species within the complex (Bauernfeind *et al*., 1999; LiPuma *et al*., 1999; Segonds *et al*., 1999).

In this study we applied a growth-independent method based on the *recA* gene polymorphism, that enables the differentiation of the *B. cepacia* complex from closely related bacteria and its sorting into species (Mahenthiralingam *et al*., 2000) in order to assess the presence of the different *B. cepacia* complex species in the maize rhizosphere. Results were compared with those obtained by cultivation-dependent methods.

Results

Isolation and identification of B. cepacia *complex isolates*

A total of 850 colonies was randomly picked up from *Pseudomonas cepacia* azelaic acid tryptamine (PCAT) (432) and trypan blue tetracycline (TB-T) (418) plates and streaked for purity on the same media from which they came. Among the 850 isolates originally selected, 114 did not survive to isolation, i.e. 69 and 45 from PCAT and TB-T media respectively. The isolates were designated as MDIII followed by P or T, based on which media they came from, and by a progressive number of isolation.

Screening polymerase chain reaction (PCR) amplification with specific primers for *B. cepacia* complex, BCR1 and BCR2, revealed that 276 out of 363 (76%) and 343 out of 373 (92%) PCAT and TB-T isolates, respectively, belong to *B. cepacia* complex.

All *B. cepacia* complex isolates were subjected both to restriction analysis of the amplified *recA* gene with the enzyme HaeIII and to species-specific PCR assays. Restriction fragment length polymorphism (RFLP) patterns were compared with those obtained in *B. cepacia* complex reference strains (Mahenthiralingam *et al*., 2000; McDowell *et al*., 2001; Seo and Tsuchiya, 2004). The characteristic RFLP pattern resulting from the digestion with HaeIII and the amplification with species-specific primers allowed us to assign each *B. cepacia* complex isolate to the respective species. As shown in Table 1, not all *B. cepacia* complex species were found within the two groups of isolates from the two media. In fact, only four of the nine species belonging to the *B. cepacia* complex were found among both PCAT and TB-T isolates, i.e. *B. cepacia*, *B. cenocepacia recA* lineage IIIB, *B. ambifaria* and *B. pyrrocinia.* The *B. cepacia* complex isolates assigned to *B. cepacia* from both PCAT and TB-T media showed three patterns designated as E, K and D. All these isolates gave positive amplification with specific primers for *B. cepacia*. The PCAT and TB-T iso-

Table 1. *RecA* RFLP patterns shown by *B. cepacia* complex isolates and clones recovered from maize rhizosphere.

Burkholderia cepacia complex species	*recA* RFLP	PCAT isolates	TB-T isolates	Clones
B. cepacia	E	4	19	–
	K	1	11	4
	D	2	23	1
B. cenocepacia IIIB	AD	186	159	54
	J	13	26	1
	I	4	13	1
	c1	–	–	1[a]
	c2	–	–	3[a]
	c3	–	–	3[a]
B. cenocepacia IIID	U	–	–	29
	c4	–	–	1[b]
B. ambifaria	N	57	69	1[c]
B. pyrrocinia	Se13	9	23	1

a. These clones were assigned to *B. cenocepacia* IIIB by means of *recA* sequencing.
b. This clone was assigned to *B. cenocepacia* IIID by means of *recA* sequencing.
c. *RecA* sequence of this clone clustered closely to *B. cenocepacia* IIIB reference strains (see Fig. 2).

lates assigned to *B. cenocepacia* IIIB showed three patterns, AD, I and J with a prevalence of pattern AD (Table 1). Among the 345 *B. cenocepacia* IIIB isolates, showing the pattern AD, 113 (33%) did not give the specific amplicon with the species-specific primers. All *B. cepacia* complex isolates assigned to *B. ambifaria* recovered from both media showed the pattern designated as N. All these isolates gave positive amplification with specific primers for *B. ambifaria*. As shown in Table 1, all the PCAT and TB-T isolates assigned to *B. pyrrocinia* gave the RFLP pattern designated as Se13 and positive amplification with specific primers of *B. cepacia*, as already observed for bacteria belonging to this species (Vermis *et al*., 2002).

Colonization of maize root system by B. cepacia *complex and total culturable bacteria*

Burkholderia cepacia complex and total culturable bacteria on maize root system were enumerated by plating sample dilutions on PCAT, TB-T and TSA media. The mean recovery of total culturable bacteria on TSA medium was 7.95 ± 0.31 $\log_{10}$ colony-forming units (cfu) g^{-1} of root system. *B. cepacia* complex population density data obtained by counting colonies on PCAT and TB-T plates were corrected on the basis of specific *B. cepacia* complex *recA* amplification results, so that the 76% and 92% of colonies obtained from PCAT and TB-T media, respectively, were ascribed to *B. cepacia* complex. According to these data, the mean *B. cepacia* complex recovery was 7.1 ± 0.2 and 6.3 ± 0.2 $\log_{10}$ cfu g^{-1} of root system in PCAT and TB-T respectively. *B. cepacia* complex bacteria recovered from maize root system were 14% and 1.12% of total culturable microflora on PCAT and TB-T respectively. Predominant *B. cepacia* complex species were *B. cenocepacia* and *B. ambifaria* in both PCAT and TB-T media (Table 2). Within each species, some genotipically identical isolates could occur because colonies were randomly picked up from PCAT and TB-T plates. However, it is conceivable that the number of genotipically identical isolates should be quite low due to the high genetic polymorphism observed in *B. cepacia* complex populations which naturally colonize the maize rhizosphere (Dalmastri *et al*., 2003).

Table 2. Percentage of species of *B. cepacia* complex recovered using different culture media.

	% from PCAT	% from TB-T
B. cepacia	2.5	15.5
B. cenocepacia IIIB	73.6	57.7
B. ambifaria	20.6	20.1
B. pyrrocinia	3.3	6.7

Root slurry nested PCR assays

Bacterial genomic DNA was successfully obtained from root slurry samples by using the SV Total RNA Isolation System Kit, as revealed by positive amplification of the 16S rDNA gene with the universal primers P0 and P6 for eubacteria. The nested PCR amplification of the root slurry extracted DNA with species-specific primers revealed the presence of the following *B. cepacia* complex species: *B. cenocepacia recA* lineages IIIA and IIIB, *B. vietnamiensis* and *B. ambifaria.* Moreover, a positive amplification with primers that react with both *B. cepacia* and *B. pyrrocinia* has been observed.

Cloning and RFLP assays

The primer sets BCR0/BCR2 and BCR1/BCR2 were used in the semi-nested PCR to amplify *recA* gene from root slurry DNA samples. The primer set BCR0/BCR2 gave the expected PCR product of 1114 bp on all 22 *B. cepacia* complex strains tested. Polymerase chain reaction with primers BCR0 and BCR2 failed to amplify *recA* gene from the following species: *Escherichia coli*, *Pseudomonas aeruginosa*, *Burkholderia tropica* and *Burkholderia gladioli*.

Polymerase chain reaction with primers BCR1/BCR2 failed to amplify PCR products from all the 14 reference strains of the *Burkholderia* species listed in *Experimental procedures*.

One hundred clones, each containing a vector with a 1043 bp insert, representing *recA* gene of the different *B. cepacia* complex species, were generated from the four root slurry samples. The *recA* sequence of each clone was subjected to RFLP analysis with the restriction enzyme HaeIII to determine the species status. As shown in Table 1, the RFLP pattern analysis allowed us to detect the presence of the following species: *B. cepacia*, *B. cenocepacia recA* lineages IIIB and IIID, *B. ambifaria* and *B. pyrrocinia*. Moreover, clones were found giving patterns designated as c1, c2, c3 and c4, that were not identical to those of reference strains (Fig. 1). Most clones with RFLP patterns reported in literature gave positive amplification with *recA* primers of the respective species; only one clone with pattern N resulted negative to the amplification with specific primers for *B. ambifaria* but gave positive amplification with specific primers for *B. cenocepacia* IIIB, and one clone with pattern K was negative to the amplification with specific primers for *B. cepacia*. Clones showing the patterns c1 and c2 resulted positive to the amplification with species-specific primers for *B. cenocepacia* IIIB, whereas clones with patterns c3 and c4 were negative to the amplification with all the specific primers of the *B. cepacia* complex species. Representative clones showing the RFLP patterns c1, c2, c3, c4 and U, as well

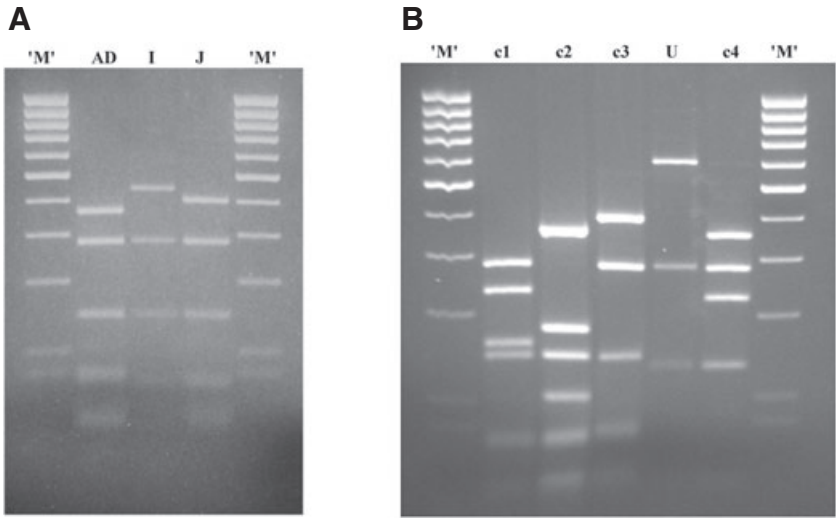

Fig. 1. *RecA* RFLP patterns obtained with the restriction enzyme HaeIII among *B. cenocepacia* isolates (A) and clones (B). The designated RFLP pattern is indicated above each lane. Molecular size standards (100-bp ladder) are in lanes M.

as clones with pattern N and K that were negative to the amplification with species-specific primers were sequenced further to ascertain the correct species. Sequence analysis by using the BLAST program allowed us to assign most of them to *B. cenocepacia*. Phylogenetic analysis indicated that clones showing RFLP patterns designated as c1, c2 and c3, clustered closely with *B. cenocepacia* IIIB strains, clones with pattern K and N that resulted negative to the amplification with the species-specific primers clustered closely with *B. cepacia* and *B. cenocepacia* IIIB strains, respectively, whereas clones with RFLP patterns U and c4 clustered closely with *B. cenocepacia* IIID strains (Fig. 2).

Discussion

Current knowledge on *B. cepacia* complex species distribution in natural environment is based on studies performed on the cultured fraction of naturally occurring bacterial community and on a few habitats (Balandreau *et al*., 2001; Fiore *et al*., 2001; Ramette *et al*., 2005). This study examined the distribution of the *B. cepacia* complex species in the maize rhizosphere, a natural habitat where *B. cepacia* complex is one of the dominant bacterial groups (Di Cello *et al*., 1997; Balandreau *et al*., 2001), by using growth-dependent and growth-independent methods in order to obtain an accurate picture of the species composition of the *B. cepacia* complex population.

The culture-independent method allowed us to assess a greater diversity of the *B. cepacia* complex population naturally occurring in the rhizosphere of maize plants cultivated in a field in Italy than the culture-dependent method. In fact, only by using culture-independent methods we detected the species *B. vietnamiensis*, the phylogenetic *recA* lineages IIIA and IIID of *B. cenocepacia*, and clones showing *recA* RFLP patterns not found among PCAT and TB-T isolates and reference strains. Reference strains of *B. vietnamiensis* and *B. cenocepacia* IIIA and IIID were successfully grown on PCAT and TB-T media (data not shown), ruling out an inability of any of these bacteria to be cultured on these media. Thus, one of the possible explanations of the lack of recovery of bacteria belonging to the species *B. vietnamiensis* and *B. cenocepacia* IIIA and IIID among PCAT and TB-T isolates is that they could be present in a number that is under the detection limit of plating in the media used. Indeed, if the number of a considered bacterium is low, it may be impossible to detect it via plate culture, because it will be outpaced by more numerous or faster-growing bacteria. The lack of recovery by culture-based methods of *B. vietnamiensis* could be partially due to the isolation procedure used. A recent paper, in which both PCAT and semi-solid BAz media have been used to isolate N2-fixing *Burkholderia* spp. from maize rhizosphere, demonstrated that the successful recovery of these bacteria is partially attributed to the semi-selective enrichment using the N-free semi-solid medium (Estrada-de los Santos *et al*., 2001). Moreover, it may be that some species, or some strains, in particular environmental conditions could assume a viable-but-not-culturable (VBNC) state. Bacteria in the VBNC state are thought to be common in soil habitat (McDougald *et al*., 1998). This state of dormancy appears to follow some type of environmental stress, e.g. low temperature, elevated salinity, etc., and may be reversible when the stress is eliminated.

To our knowledge this is the first study which assessed the presence of the *recA* phylogenetic lineages *B. cenocepacia* IIIA and IIID in a natural environment. Indeed, bacteria belonging to these phylogenetic lineages, so far, have been encountered only in clinical samples (Vandamme *et al*., 2003). Since to *B. cenocepacia* IIIA, IIIB and IIID belong the most transmissible

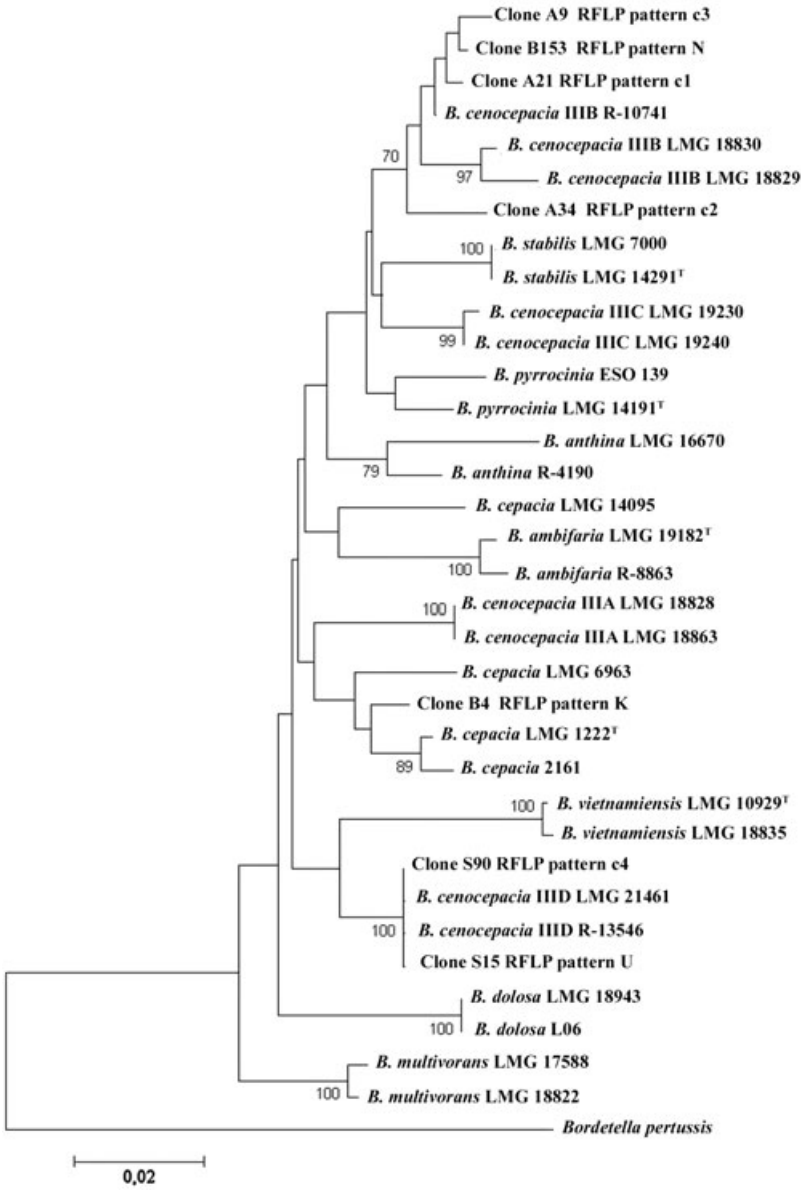

Fig. 2. Phylogenetic tree derived from the *recA* gene sequences analysis of representative strains of each *B. cepacia* complex species, and of clones showing RFLP patterns different from those of reference strains. Two clones with pattern K and N that did not give amplification with the specific primers of the respective species as well as the clone with pattern U were included. The tree was constructed using the Neighbour-Joining method, based on approximately 1043 nucleotides. Genetic distance is shown on the scale and bootstrap analysis (1000 replicates) for node values from 70% are indicated.

and virulent strains (Mahenthiralingam *et al*., 2001; Speert *et al*., 2002; Manno *et al*., 2004), the detection of all these phylogenetic lineages of *B. cenocepacia* in the root system habitat raises great concerns about natural environment as a 'reservoir' of potential pathogenic strains.

Another informative aspect revealed by this study concerns the greater heterogeneity of *recA* RFLP patterns observed among clones obtained by DNA directly extracted from the root slurry samples than among PCAT and TB-T isolates. Most diversity was found within the species *B. cenocepacia* as revealed by the presence of clones with *recA* RFLP profiles not found among reference strains that were assigned to this species by *recA* gene sequencing. Recently, Ramette and colleagues (2005), observed high polymorphism of *recA* gene also among isolates of *B. ambifaria* recovered from maize rhizosphere using colony hybridization.

In conclusion, the results presented in this paper demonstrate that non-culture-based methods give a more reliable picture of the diversity of the *B. cepacia* complex population naturally occurring on the maize root system than culture-based methods. In fact, by using this approach we were able to detect the species *B. vietnamiensis* besides the species *B. cepacia*, *B. cenocepacia*, *B. ambifaria* and *B. pyrrocinia*, and to highlight a greater diversity within the species *B. cenocepacia,* which is the one comprising the most transmissible and virulent strains. Thus, a growth-independent method is useful to reliably detect the presence of potential pathogenic *B.*

cepacia complex bacteria in environmental samples in order to ascertain the risk posed by some habitats to CF people.

Experimental procedures

Sampling and isolation of B. cepacia *complex*

Zea mays plants (cv. Eleonora) were cultivated in a field located at Dragoni, Caserta, Italy, where maize had been cultivated for 10 years. The soil composition was as follow: sand, 58%; clay, 27%; silt, 15%; organic C, 1.15%; the pH was 5.43. Sixteen maize plants were randomly harvested after 130 days of plant growth. In the same day of the sampling, roots were excised from plants without removing closely adhering soil (rhizospheric soil). The excised roots were randomly grouped in order to obtain four mixtures, named Mix A, Mix B, Mix C and Mix D, each comprising four root systems. Each root system was cut in very small pieces (0.2–0.7 cm) and the four root systems of each mixture were thoroughly mixed within each sample. Five grams of each mixture were placed in a sterile 50 ml plastic tube containing 35 ml of potassium phosphate buffer (PPB 0.02 M pH 6.8) and 50 μl of Tween 80. Each roots suspension was shaken by vortexing for 3 min at maximum speed. Aliquots of each root slurry were immediately frozen at −80°C for later use in the direct DNA extraction. Serial dilutions of the root slurry were plated in triplicate onto the two selective media for *B. cepacia* complex: TB-T (Hagedorn *et al*., 1987) and PCAT (Burbage *et al*., 1982), both amended with 100 μg ml^{-1} of cycloheximide (Sigma) to inhibit fungal growth. Plates were incubated at 28°C for 3 days prior to counting the number of cfu. A total of 850 (90–100 for each mix from PCAT and TB-T, respectively) bacterial colonies were randomly picked up from the same dilution of root slurry sample, i.e. 100-fold dilution from both PCAT and TB-T plates containing approximately 50 to 100 colonies. Isolated colonies were then grown overnight (o/n) in nutrient broth (NB; Difco) medium at 28°C and 200 r.p.m. and stored at −80°C in 30% glycerol until further analysis.

Identification of B. cepacia *complex isolates*

DNA of each bacterial isolate was prepared by lysis of two or three colonies grown o/n on nutrient agar (NA; Difco), according to the procedure described by Vandamme and colleagues (2002). Each bacterial isolate was assigned to the *B. cepacia* complex by means of PCR amplification of *recA* gene with specific primer pairs for *B. cepacia* complex, BCR1 and BCR2 (Table 3), by using the procedure described by Mahenthiralingam and colleagues (2000). The species status of each isolate was determined by RFLP analysis of the *recA* gene, and confirmed by means of PCR of *recA* gene performed with species-specific primers when available (Table 3), according to the procedures previously described (Mahenthiralingam *et al*., 2000; Coenye *et al*., 2001; Vandamme *et al*., 2002; Vermis *et al*., 2004). Amplification was carried out with a Gene Amp PCR System 9700 instrument (Applied Biosystem) and restriction fragments were separated by electrophoresis on 3% agarose gels by standard procedures (Sambrook *et al*., 1989).

Table 3. *RecA* primers pairs used in this study.

Organism(s) detected	Primers	Sequence (5′-to 3′)	Product size (bp)
B. cepacia complex	BCR0[a]	ACAGTGTCTGCATTCGTG	1114
(First run of semi-nested PCR)	BCR2[b]	CTCTTCTTCGTCCATCGCCTC	
B. cepacia complex	BCR1[b]	CTTGACCGCCGAGAAGAGCAA	1043
	BCR2[b]	CTTCTTCGTCCATCGCCTC	
B. cepacia	BCRG11[b]	CAGGTCGTCTCCACGGGT	492
	BCRG12[b]	CACGCCGATCTTCATACGA	
B. multivorans	BCRBM1[b]	CGGCGTCAACGTGCCGGAT	710
	C2-3[c]	CTCGGCTTCGTCGTGGC	
B. cenocepacia IIIA	BCRG3A1[b]	GCTCGACGTTCAATATGCC	378
	BCRG3A2[b]	TCGAGACGCACCGACGAG	
B. cenocepacia IIIB	BCRG3B1[b]	GCTGCAAGTCATCGCTGAA	781
	BCRG3B2[b]	TACGCCATCGGGCATGCT	
B. stabilis	BCRG41[b]	ACCGGCGAGCAGGCGCTT	647
	BCRG42[b]	ACGCCATCGGGCATGGCA	
B. vietnamiensis	BCRBV1[b]	GGGCGACGGCGACGTGAA	378
	BCRBV2[b]	TCGGCCTTCGGCACCAGT	
B. dolosa	BCRBM1[b]	CGGCGTCAACGTGCCGGAT	260
	C6-3[c]	TGATGAAGATCACGAGGCAA	
B. ambifaria	BCRBC1[d]	GTCGGGTAAAACCACGCTG	810
	BCRBC2[d]	ACCGCAGCCGCACCTTCA	
B. anthina	BCRG81[e]	TACGGTCCGGAATCGTCG	473
	BCRG82[e]	CGCACCGACGCATAGAAT	

a. Newly designed primer.
b. Mahenthiralingam and colleagues (2000).
c. Dřvínek and colleagues (2002).
d. Coenye and colleagues (2001).
e. Vandamme and colleagues (2002).

Extraction of DNA from soil sample

Frozen aliquots of root slurry samples were used to provide DNA for a series of PCR assays. DNA was extracted by using the SV Total RNA Isolation System Kit (Promega) according to the manufacturer's instructions. Samples were extracted in triplicate. Extracted DNA was stored in water at –20°C until use. To ascertain whether genomic DNA suitable for PCR amplification was obtained, 2 μl of root slurry-extracted DNA samples were used as template to amplify bacterial 16S rDNA with the universal primers P0 and P6 according to the procedure described by Di Cello and colleagues (1997).

Amplification of B. cepacia *complex* recA *gene by species-specific primers from root slurry-extracted DNA*

DNA extracts were subjected to nested PCRs by using the procedure previously described by Dřvínek and colleagues (2002), with the following modifications. The first PCR round was performed with 2 μl of the DNA extract. The annealing temperature of the first PCR round was 58°C. The second PCR round was carried out with 1 μl of the first-round PCR product. All PCR mixtures were performed in a total volume of 25 μl with 1X PCR Polymed buffer, 0.5 U of *Taq* polymerase (Polymed), 250 μM (each) deoxynucleoside triphosphate, 1.5 mM $MgCl_2$, 20 pmol (each) of primers and amplifications were carried out with a Gene Amp PCR System 9700 instrument (Applied Biosystem). One microlitre of the first-round PCR product was further used for species detection. The PCR set-up was identical to that of the second round; the species specificity of the eight reactions was maintained by the use of respective species-specific primers (Table 3). BSA (Sigma) 0.2 mg ml^{-1} was added to all PCR mixtures to improve the quality and yield of PCR reactions.

Cloning of recA *PCR products*

RecA amplicons of 1043 bp were generated from DNA extracted from root slurry with a semi-nested PCR approach in which the primer set BCR0/BCR2 followed by BCR1/BCR2 was used. The primer BCR0 was designed on the basis of complete *recA* sequences (available within GenBank) of the *B. cepacia* strains JN25 (accession no. D90120) and ATCC 17616 (accession no. U70431) (Table 3). Twenty-two strains representing the nine *B. cepacia* complex species were used to validate the primer set BCR0/BCR2. The list includes reference strains from the Laboratorium voor Microbiologie (LMG) (Ghent University, Ghent, Belgium) collection and strains from the *B. cepacia* complex collection of the ENEA Microbial Ecology Laboratory. The *B. cepacia* complex strains were: *B. cepacia* LMG 1222^T and LMG 18821; *B. multivorans* LMG 18822 and LMG 17588, *B. cenocepacia* LMG 16656, LMG 16654, MVPC1/16, LMG 19230, LMG 19240, FC 7, *B. stabilis* LMG 18888 and LMG 14294, *B. vietnamiensis* LMG 10929^T and LMG 18835, *B. dolosa* LMG 18941 and LMG 18942, *B. ambifaria* LMG 11351 and MCI7, *B. anthina* LMG 16670 and LMG 20980, *B. pyrrocinia* LMG 15958 and MVPC1/13. Negative controls were *Burkholderia tropica* Ppe8^T, *Burkholderia gladioli* ATCC 10248 ^{T}, *Pseudomonas aeruginosa* PAO1 and *Escherichia coli* XL1-blue.

In order to prove that the primer set BCR1/BCR2 do not work with bacteria closely related to *B. cepacia* complex, 14 strains representing several *Burkholderia* species other than *B. cepacia* complex were tested. The *Burkholderia* strains were: *Burkholderia caribensis* LMG 18531^T, *Burkholderia caryophylli* ATCC 25418^T, *Burkholderia fungorum* LMG 16225, *B. gladioli* ATCC 10248^T, *Burkholderia glathei* ATCC 29195^T, *Burkholderia glumae* ATCC 33617^T, *Burkholderia graminis* C4D1M^T, *Burkholderia kururiensis* Kp23, *Burkholderia phenazinium* LMG 2247, *Burkholderia sacchari* LMG 19450, *Burkholderia terricola* LMG 20594, *Burkholderia thailandensis* ATCC 700348^T, *B. tropica* Ppe8^T and *Burkholderia unamae* MTI-641^T. Semi-nested *recA* PCR amplification was performed as follows: the first PCR round was carried out in a total volume of 25 μl with 1X PCR buffer (Qiagen), 1.5 mM $MgCl_2$, 200 μM (each) deoxynucleoside triphosphate, 20 pmol (each) of primers BCR0 and BCR2, 1 U of HotStarTaq DNA Polymerase (Qiagen), and 2 μl of the DNA extract. After an initial activation step of 15 min at 95°C, the reaction mixture was subjected to the following thermal cycling parameters in a Gene Amp PCR System 9700 instrument (Applied Biosystem): 94°C for 5 min followed by 30 cycles of 30 s at 94°C, 45 s at 58°C, 60 s at 72°C and a final extension of 10 min at 72°C. The second round of the semi-nested PCR was performed in a total volume of 25 μl with 1X PCR buffer (Qiagen), 1.5 mM $MgCl_2$, 200 μM (each) deoxynucleoside triphosphate, 20 pmol (each) of primers BCR1 and BCR2, 1 U of HotStarTaq DNA Polymerase (Qiagen), and 2 μl of the first-round PCR product. The second-round PCR run program was carried out as previously described by Dřvínek and colleagues (2002) preceded by an initial activation step of 15 min at 95°C.

The purified PCR product was used in a blunt-end ligation reaction with EcoRV digested pBluescript vector (Stratagene). Transformation of competent *E. coli* XL1-Blue cells (Bullock *et al*., 1987) was done by the thermic shock method as described by Sambrook and colleagues (1989). Clones were screened for insert on Luria–Bertani broth (LB) medium supplemented with 100 μg ml^{-1} ampicillin (Sigma) by using α-complementation with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal, Eurobio) and isopropyl-β-D-thiogalactopyranoside (IPTG, Ambion). All clones containing the insert of the correct size were cultured in 3 ml of LB for storage at –80°C and then subjected to the identification procedure for *B. cepacia* complex species based on *recA* gene polymorphism as described above.

DNA sequence analysis

The reaction mixtures containing the *recA* gene amplified with primers BCR1 and BCR2 were purified using the MinElute PCR purification kit (Qiagen) according to the supplier's instructions. Sequencing reactions were prepared using Applied Biosystem Big Dye® Terminator sequencing kit version 3.1, according to the manufacturer's instructions and analysed using a 3730 DNA Analyzer Applied Biosystem apparatus. Thermal cycling was performed with a Gene Amp PCR System 9700 instrument (Applied Biosystem) and consisted of 30 cycles of 30 s at 94°C, 30 s at 48°C and 4 min at 60°C. The reactions were carried out at the ENEA Genome Research Facility DNA Sequencing Laboratory (ENEA, C.R.

Casaccia, Italy). Raw sequences from both strands of the PCR products were then aligned, and a consensus sequence was derived using DNASTAR software (DNASTAR). Analyses of sequences were performed with the basic sequence alignment BLAST program run against the nr database [National Center for Biotechnology Information web site (http://www.ncbi.nlm.nih.gov/blast)]. To construct the phylogenetic tree of *recA* sequences, the Neighbour-Joining method Tamura 3-Parameter was used [MEGA v2.1 (http://www.megasoftware.net)].

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