ORIGINAL ARTICLE

Application of *rpoB* sequence similarity analysis, REP-PCR and BOX-PCR for the differentiation of species within the genus *Geobacillus*

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Abstract

Aim: To investigate the applicability of *rpoB* gene, which encodes the β subunit of RNA polymerase, to be used as an alternative to 16S rRNA for sequence similarity analysis in the thermophilic genus *Geobacillus*. Rapid and reproducible repetitive extragenic palindromic fingerprinting techniques (REP- and BOX-polymerase chain reaction) were also used.

Methods and Results: *rpoB* DNA (458 bp) were amplified from 21 *Geobacillus*- and *Bacillus* type strains, producing different BOX- and REP-PCR profiles, in addition to 11 thermophilic isolates of *Geobacillus* and *Bacillus* species from a Santorini volcano habitat. The sequences and the phylogenetic tree of *rpoB* were compared with those obtained from 16S rRNA gene analysis. The results demonstrated between 90–100% (16S rRNA) and 74–100% (*rpoB*) similarity among examined bacteria.

Conclusion: BOX- and REP-PCR can be applied for molecular typing within *Geobacillus* genus. *rpoB* sequence similarity analysis permits a more accurate discrimination of the species within the *Geobacillus* genus than the more commonly used 16S rRNA.

Significance and Impact of the Study: The obtained results suggested that *rpoB* sequence similarity analysis is a powerful tool for discrimination between species within the ecologically and industrially important strains of *Geobacillus* genus.

Introduction

The aerobic, thermophilic genus *Geobacillus* was defined in 2001 by Nazina *et al.* to include thermophilic bacteria belonging to the *Bacillus* genetic group five, with *Geobacillus stearothermophilus* as the type species (McMullan *et al.* 2004). *Geobacillus* species are widely distributed and readily isolated from natural and human-disturbed habitats (Nazina *et al.* 2001; Meintanis *et al.* 2006), with a continually increasing industrial interest for their thermostable gene products (Lama *et al.* 2004; Schallmey *et al.* 2004). Therefore, studying phylogenetic relations and diversity in this novel bacterial genus is not only a taxonomical concern, but also a necessity in order to exploit its biotechnological potential completely.

Sequence comparison of 16S rRNA genes, which is highly conserved throughout prokaryotic organisms, has been most widely used to determine phylogenetic relationships (Woese *et al.* 1990). However, 16S rRNA gene has often proved to be insufficient for resolving phylogenetic relationships between some closely related species (La Duc *et al.* 2004). Additionally, rRNA sequences used alone can be misleading because of the presence of multiple 16S rRNA operons and the occurrence of recombination within the strain (Gürtler and Mayall 2001). In some cases, the use of other genes for phylogenetic inference has become more demanding, as it overcomes the limitation of the 16S rRNA gene in the phylogenetic resolution of genera and species but not strains (Konstantinidis and Tiedje 2005; Chelo *et al.* 2007). Protein encoding genes

have been suggested and used instead of or in supplement to 16S rRNA, because it is believed that a combination of multiple genetic markers can increase the accuracy and reliability of a phylogenetic scheme (Ko *et al.* 2003; Konstantinidis and Tiedje 2005).

According to Zeigler (2003), a gene used as phylogenetic marker must meet specific criteria: (i) wide distribution among bacteria; (ii) uniqueness in genome; (iii) phylogenetically informative size; and (iv) sequence divergence among related species. *rpoB* gene could be a strong candidate as a genome similarity predictor, as this gene encodes for the β subunit of RNA polymerase and has been proposed as an alternative to 16S rRNA for phylogenetic and biodiversity studies (Drancourt and Raoult 2002; Da Mota *et al.* 2004). This gene is common to all bacteria, exists as a single copy in the genome and is a mosaic of conserved and variable sequence domains, which are key features also met to 16S rRNA gene.

The similarity of *Geobacillus* and thermophilic *Bacillus* type strains was studied by means of repetitive extragenic palindromic-polymerase chain reaction (REP-PCR), BOX-PCR, 16S rRNA and *rpoB* gene sequence analysis. Furthermore, phylogenetic relations of 21 type strains and 11 thermophilic isolates of *Geobacillus* and *Bacillus* genus were demonstrated in phylogenetic trees constructed with 16S rRNA and *rpoB* sequences. The effectiveness of these molecular tools for differentiating and grouping strains belonging to the genus *Geobacillus* is discussed.

This is the first report on BOX-PCR, REP-PCR and *rpoB* gene application as phylogenetic analysis tools in *Geobacillus* indigenous isolates.

Materials and methods

Bacterial strains

The bacteria used in this study had been isolated from a wide variety of sources, including geothermal water and soils, composted organic matter and temperate habitats. The *Geobacillus* and thermophilic *Bacillus* type strains used in this study were obtained from DSMZ. They included *Geobacillus thermocatenulatus* DSM730, *Geobacillus thermoleovorans* DSM5366, *Geobacillus stearothermophilus* DSM22, *Geobacillus lituanicus* DSM15325, *Geobacillus thermoleovorans subsp. stromboliensis* DSM15392, *Geobacillus kaustophilus* DSM7263, *Bacillus caldolyticus* DSM405, *Bacillus caldotenax* DSM406, *Bacillus caldovelox* DSM411, *Geobacillus gargensis* DSM15378, *Geobacillus jurassicus* DSM15726, *Bacillus thermoclocae* DSM5250, *Geobacillus uzenensis* DSM13551, *Geobacillus subterraneus* DSM13552, *Geobacillus thermodenitrificans* DSM465, *Geobacillus caldoproteolyticus* DSM15730, *Geobacillus tepidamans* DSM16325, *Geobacillus toebii* DSM14590, *Geobacillus pallidus* DSM3670, *Bacillus licheniformis* DSM13 and *Geobacillus vulcani* DSM13174.

The collection was supplemented with 11 environmental *Bacillus* and *Geobacillus* strains. The environmental strains corresponded to 11 different BOX-PCR and REP-PCR genomic fingerprints deriving from a total of 200 isolates. Bacterial isolates were retrieved from volcanic soil, seawater and sediment from two bays near the active volcano of Santorini at Nea Kameni island (Santorini, Greece, 25°25′N, 36°25′E), as described by Meintanis *et al.* (2006). The phylogenetic relatedness of the isolates is presented in Table 1.

BOX- and REP-PCR analysis

Bacteria were harvested from 1-ml cultures in nutrient broth (NB) (Sharlau SA, Spain) after 48 h of incubation at 60°C and at 200 rev min^{-1}. DNA extraction was performed following the protocol of Haught *et al.* (1994). DNA concentration was determined with a spectrofluorimeter (Hitachi U1100) and adjusted to 40 ng μl^{-1}. The BOX element (BOX1A) was amplified using the BOXA1R primer 5′-CTACGGCAAGGCGACGCTGACG-3′ (Versalovic *et al.* 1994). REP1R (5′-IIIIICGICGICATCIGGC-3′) and REP2I (5′-ICGICTTATTCIGGCCTAC-3′) primers were used for REP-PCR (Versalovic *et al.* 1991). PCR and electrophoresis conditions were adjusted according to Rademaker and de Bruijn (1997). The PCR reaction for each isolate was repeated thrice for reproducibility.

Amplification of *rpoB* gene fragments

Consensus *rpoB* PCR primers were designed after the alignment of *rpoB* genes of *Bacillus licheniformis* [AF172323], *Bacillus soronensis* [AJ586416], *Bcillus subtilis* [AJ586566], *Bcillus halodurans* [AP001507], *Bcillus cereus* [AE016998], *Oceanobacillus iheyensis* [AP004953] and *Listeria monocytogenes* [AL591974]. PCR amplification of a 458-bp fragment of the *rpoB* gene, corresponding to nucleotides 31–488 of *B. licheniformis* [AF172323] *rpoB* gene, was performed using the forward primer *rpoB*F 5′-GCGAAGTGTTAGAATTACC-3′ and the reverse *rpoB*R 5′- TCGTATTCTAACCATGCGCC-3′.

The PCR mixture of a final volume of 50 μl, contained 40 ng of template DNA, 10 mmol l^{-1} of KCl, 10 mmol l^{-1} of Tris-HCl (pH 9), 0·1% Triton X-100, 1 U of Promega *Taq* DNA Polymerase, 0·2 mmol l^{-1} of deoxynoucleotide triphosphates, 2·5 mmol l^{-1} of $MgCl_2$, 20 nmol l^{-1} of each of the primers *rpoB*F and *rpoB*R and 2% (v/v) acetamide. After the initial step for 10 min at 94°C, 35 cycles of amplification consisting of denaturation for 1 min at 95°C, annealing of primers for 1 min at 53°C and primer extension for 2 min at 72°C, followed

Table 1 Characterization of the thermophilic bacteria isolates studied in this work

Isolate designation	GenBank accession number	Sequence alignment		Closest phylogenetic relative (GeneBank accession number)
		No. of Nucleotides*	Per cent identity**	
Bacillus sp. T107	DQ642097	1418	99·44%	*Bacillus licheniformis* (AY786999)
Bacillus sp. A49	DQ642096	1401	99·43%	*Bacillus licheniformis* (AY786999)
Geobacillus sp. A60	DQ642095	1430	99·79%	*Geobacillus pallidus* (Z26930)
Geobacillus sp. T38	DQ642094	1465	99·73%	*Geobacillus pallidus* (Z26930)
Geobacillus sp. O83	DQ642093	1480	99·66%	*Geobacillus toebii* (AB116120)
Geobacillus sp. T45	DQ642092	1444	99·93%	*Geobacillus thermodenitrificans* (AB116114)
Geobacillus sp. I112	DQ642091	1429	99·65%	*Geobacillus subterraneus* (AY608957)
Geobacillus sp. S23	DQ642090	1460	100%	*Geobacillus stearothermophilus* (AJ586362)
Geobacillus sp. S9	DQ642089	1561	99·94%	*Bacillus caldotenax* (AY608937)
Geobacillus sp. N60	DQ642088	1561	99·94%	*Bacillus caldotenax* (AY608937)
Geobacillus sp. A83	DQ642087	1500	100%	*Geobacillus vulcani* (AY608940)

*The number of 16S rDNA nucleotides used for the alignment.

**The percentage identity with the 16S rDNA sequence of the closest phylogenetic relative.

by a final extension step for 10 min at 72°C were carried out. The PCR products were purified using Nucleospin Extract® PCR kit (Macherey-Nagel, Germany) and both DNA strands were sequenced commercially (http://www.macrogen.com).

16S rDNA PCR

For the amplification of the region of 16S rDNA of the 11 isolates, primers F27 and R1492 corresponding to nucleotides 8–1510 of the *Escherichia coli* 16S rDNA were used. Primers and PCR conditions were as described previously (Lane 1991). The PCR products were purified using Nucleospin Extract® PCR kit (Macherey-Nagel) and both DNA strands were sequenced commercially (http://www.macrogen.com).

Phylogenetic analysis

The sequence data for both genes were compiled using the ARB software (http://www.arb-home.de) and aligned with sequences obtained from the ARB and GenBank databases using the ARB's FastAligner utility, followed by manual aligning according to the secondary structure. The analyses were performed using the neighbour-joining (NJ) method implemented in PAUP* (Swofford 2003). Bootstrapping was done with 1000 replicates for both the 16S rRNA and *rpoB* data sets. Sequences retrieved from this study have GenBank accession numbers DQ642057–DQ642097.

Results

All type strains available were successfully discriminated with both BOX- and REP-PCR, as shown in Fig. 1. Nineteen type strains produced different banding patterns, while two of these (*Bacillus thermoclocae* DSM5250, *Geobacillus uzenensis* DSM13551), which had 100% *rpo*B sequence similarity, produced the same BOX- and REP-PCR profiles (Fig. 1a,b; lanes 13, 14).

Phylogenetic relationships among the studied *Geobacillus* strains on the basis of comparison of partial *rpoB* and 16S rRNA sequences were inferred by the NJ method (Fig. 2). For the majority of the strains analysed, each phylogram produced similar phylogenetic groups. 16S rRNA gene sequences of the *Geobacillus* and *Bacillus* strains and isolates were analysed and compared with similarity values among 90% and 100%.

rpoB gene fragments were amplified from both strain types and isolates and the nucleotide sequences were determined and compared. An *rpoB* database for *Geobacillus* species was then formed. The strains used in this study showed similarity from 74% to 100% of the *rpo*B sequences analysed, permitting a more accurate discrimination of the species. In particular, 16S rRNA and *rpoB* sequence comparisons were performed, confirming more than 95% similarity for the 65% of the 16S rRNA sequences compared, while the percentage of *rpoB* sequences with more than 95% similarity was only 27·8%. Furthermore, the percentage of sequences with identity higher than 90% was 100% for 16S rRNA and 38·3% for *rpoB*. It is noticeable that 33·1% of the *rpoB* sequences that were assessed in this study had a similarity of less than 80% among them.

Discussion

In this work, the phylogenetic divergence within the thermophilic genus *Geobacillus* was investigated. Rapid and reliable molecular methods that have been previously

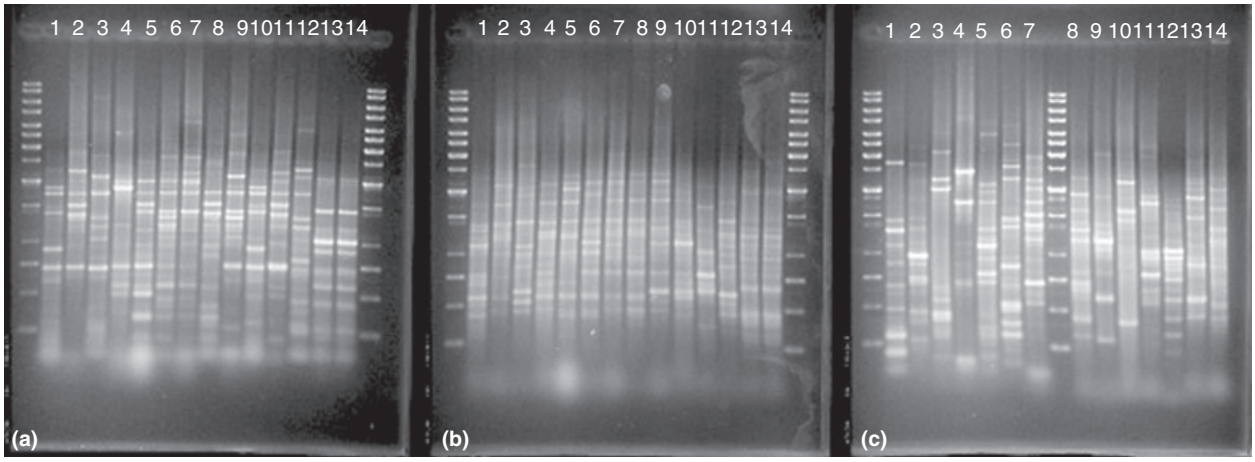

Figure 1 BOX- and REP-polymerase chain reaction (PCR) profiles of the *Geobacillus* type strains. (a) REP-PCR: (1) DSM730; (2) DSM5366; (3) DSM22; (4) DSM15325; (5) DSM15392; (6) DSM7263; (7) DSM405; (8) DSM406; (9) DSM411; (10) DSM15378; (11) DSM15726; (12) DSM5250; (13) DSM13551; (14) DSM13552. (b) BOX-PCR: (1) DSM730; (2) DSM5366; (3) DSM22; (4) DSM15325; (5) DSM15392; (6) DSM7263; (7) DSM405; (8) DSM406; (9) DSM411; (10) DSM15378; (11) DSM15726; (12) DSM5250; (13) DSM13551; (14) DSM13552. (c) 1–7 REP-PCR, 8–14 BOX-PCR: (1, 8); DSM465 (2, 9); DSM15730 (3, 10); DSM16325 (4, 11); DSM14590 (5, 12); DSM3670 (6, 13); DSM13 (7, 14); DSM13174. 1-kb DNA ladder Gene Ruler (Fermentas®) was used in all gels.

applied for the identification and classification in a variety of bacterial genera, BOX- and REP-PCR (Dombek *et al.* 2000; Guillaume-Gentil *et al.* 2002; De Clerck *et al.* 2004), were employed in representatives of the *Geobacillus* genus for the first time in the present study.

Banding patterns produced from the application of these methods, contained an adequate number of bands according to Rademaker and de Bruijn (1997), indicating that both BOX- and REP-PCR fingerprints are applicable for typing of *Geobacillus* strains. Through comparison of the two methods, we found that the REP-PCR method generated more informative results than BOX-PCR for the studied strains; REP-PCR profiles were more distinct for the different strains, including a higher number of bands. A similar finding has been documented by Dombek *et al.* (2000), who used REP-PCR techniques for the differentiation of *E. coli* strains. DNA fingerprints produced when BOX primer was applied were of higher quality compared with REP-PCR fingerprints produced by the same strains. REP-PCR genomic fingerprint protocols, as well as the random amplified polymorphic DNA RAPD-PCR technique, have been applied in the closely related *Bacillus* genus exhibiting high sensitivity in discrimination at the strain level for mesophilic and thermophillic species (Ronimus *et al.* 1997; Mora *et al.* 1998; Guillaume-Gentil *et al.* 2002).

Phylogenetic trees constructed with the 16S rRNA and *rpoB* gene sequences using the NJ method (Fig. 2) were similar for the studied *Geobacillus* strains, with the *rpoB*-based phylogenetic tree having longer branch length, because of the higher sequence variability of the *rpoB* gene. The same difference was designated by Zeigler (2005) during comparison of 16S rRNA and *recN* deriving phylogenies because of the mutational saturation of the later gene. Although bootstrap values were lower for the *rpoB* tree, both phylogenetic analyses shared satisfactory bootstrap support.

In terms of the 16S rRNA gene sequences of *Geobacillus* and *Bacillus* type strains and isolates, similarity values among 90% and 100% were retrieved, in agreement with Zeigler (2005) who confirmed 16S rRNA gene sequences similarity of *Geobacillus* type strains, higher than 98·5%. Despite the general use of the 16S rRNA gene as framework for modern bacterial classification, it has often been proved to show limited variation for the discrimination of closely related taxa and strains (Nübel *et al.* 1996). On the other hand, protein-coding genes exhibit higher genetic variation, which can be used for the classification of closely related species (Ko *et al.* 2003; Bavykin *et al.* 2004; Konstantinidis and Tiedje 2005; Zeigler 2005).

rpoB gene sequences demonstrated a range of similarity between 74% and 100%, thus permitting a more accurate discrimination of the species. *rpoB* gene similarities could be used to distinguish between closely related strains such as DSM15730 and DSM16325, isolates O83 and DSM14590, isolates S23 and DSM22, which showed sequence similarity over 99% for the 16S rRNA gene. However, the sequence similarities of these strains for *rpoB* gene were 85·9%, 97·5% and 96·5%, respectively for each one of the pairs. Da Mota *et al.* (2004) obtained similar results, as their comparison of the discriminative power of the two genes in *Paenibacillus* group proved that

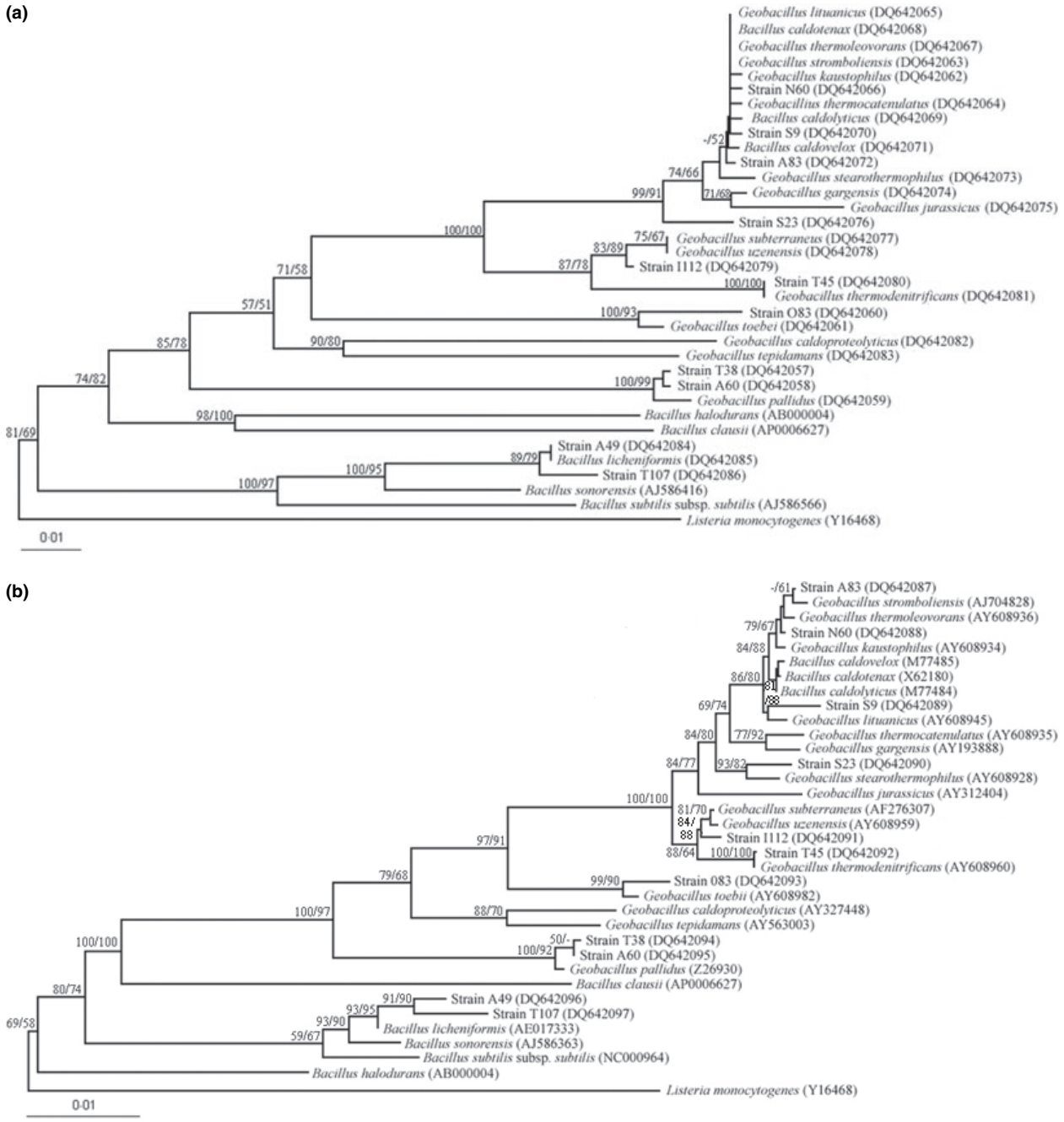

Figure 2 *rpoB*-based (a) and 16S rRNA (b) neighbour joining (NJ) distance tree showing the phylogenetic relationships of thermophilic bacterial isolates and type *Bacillus* and *Geobacillus* species. The 16S rRNA tree is based on *Escherichia coli* positions 8-1510 of the 16S rRNA gene. The *rpoB* tree is based on *Bacillus licheniformis* positions 31-488 of the *rpo*B gene [AF172323]. Bootstrap support values (%) are given at nodes for NJ distance (first value) and maximum parsimony (second) criteria; – indicates value <50%. Both trees were rooted with *Listeria monocytogenes.*

rpoB was 3·1 times more discriminative than 16S rRNA. Additionally, in other studies *rpoB* has also been used successfully as a genotyping approach to overcome obstacles from 16S rRNA gene similarity in phylogenetic studies within *Staphylococcus, Bacillus, Mycobacteria* and *Mycoplasma species* (Kim *et al.* 1999, 2003; Drancourt and Raoult 2002; Ko *et al.* 2003).

In conclusion, our results suggested that *rpoB* can be used as an alternative molecular marker to 16S rRNA gene for successfully resolving phylogenies between closely

related species and subspecies within the genus *Geobacillus*. This practice was also well documented by Zeigler (2005), who demonstrated that 16S rRNA gene is a powerful tool for analysing higher order taxa, illustrating at the same time the complementary discriminative ability of *recN* gene for the genus *Geobacillus*.

During the brief period since its description (Nazina *et al.* 2001), the genus *Geobacillus* and its members have become a significant research focus, as they have considerable potential for applications in biotechnology and bioremediation (Houde *et al.* 2004; Meintanis *et al.* 2006). The data presented here demonstrate that a multiphasic approach comprising REP-PCR genomic fingerprints and *rpoB* gene sequence similarity analysis constitute a suitable molecular approach for the validation and maintenance of taxonomy within the *Geobacillus* genus.

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