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Assessment of a short phylogenetic marker based on comparisons of 3' end 16S rDNA and 5' end 16S-23S ITS nucleotide sequences of the *Bacillus cereus* group

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ABSTRACT

A short phylogenetic marker previously used in the reconstruction of the Order *Bacillales* and the genus *Bacillus* was assessed here at a lower taxa level: species in the *Bacillus cereus* group: *B. anthracis*, *B. cereus*, *B. thuringiensis* and *B. weihenstephanensis*. This maker is 220 bp in length. It is a combination of 150 bp at the 3' end of the 16S rDNA and 70 bp at the 5' end of the 16S-23S ITS sequence. Three additional *Bacillus* species, *B. halodurans*, *B. licheniformis* and *B. subtilis*, and *Clostridium tetani* were included for comparison purposes. A total of eight bacterial species and 12 strains were analyzed. A bootstrapped neighbor-joining tree was inferred from comparative analyses of all allelic sequences of the bacterial species and strains under study. Based on its topology, four major Groups were revealed at the 90% nucleotide sequence identities, Group I to IV. Group I contains all alleles of the *Bacillus cereus* group. Group II contains all alleles of *B. halodurans*. Group III contains all alleles of *B. licheniformis* and *B. subtilis*. Group IV contains all alleles of *Clostridium tetani*. The 220 bp phylogenetic marker used here could resolve different species from different genera. At the genus level, distant species could be distinguished. Very closely-related species, however, were undistinguishable. Species in the *B. cereus* group, most notably *B. cereus*, *B. anthracis* and *B. thuringiensis*, could not be distinguished. After successfully inferring the phylogenies of the Order *Bacillales* and the genus *Bacillus*, we have met the resolving limit of this short phylogenetic marker: *B. cereus*, *B. anthracis* and *B. thuringiensis*.

Keywords: *Bacillus cereus*; 16S rDNA; 16S-23S ITS; Phylogeny

1. INTRODUCTION

The *Bacillus cereus* group comprises six genetically highly related species: *B. cereus* sensu stricto, *B. anthracis*, *B. thuringiensis*, *B. weihenstephanensis, B. mycoides* [1] and *B. pseudomycoides* [2]. They are Gram-positive, rod-shaped, endospore-forming, either obligate or facultative aerobic bacteria [1].

Bacillus cereus is a ubiquitous soil bacterium. It can be a contaminant of a variety of foods: meats, vegetables and dairy products [3,4]. It can cause diarrheal, and emetic food poisoning syndromes [5]. It can also be the etiologic agent of some opportunistic infections [6,7]. *Bacillus anthracis* is the etiologic agent of anthrax, an acute disease in herbivorous mammals, transmissible to other animals, including humans [8]. This species has been studied and developed as a biological weapon [9]. Virulent strains of *B. anthracis* carry two plasmids, pXO1 (181 kb) and pXO2 (96 kb) which may be transmitted to others members of *Bacillus cereus* group [10]. *Bacillus thuringiensis* is an insect pathogen. It is characterized by the synthesis upon sporulation of a parasporal inclusion body. This inclusion body is made of proteins, the δ-endotoxins, which are toxic to several insect larvae [11,12] and other invertebrates [13]. *B. thuringiensis* formulations have been developed for the control of insect pests in agriculture and forestry [14-16] and for the control of insect vectors of human diseases such as malaria, yellow fever, onchocerciasis, etc [17]. *Bacillus weihenstephanensis* is a psychotolerant species characterized by the ability to grow at 7°C and the absence of growth at 43°C. It is also characterized by the presence of specific signature sequences on the 16S rRNA gene (small subunit ribosomal RNA gene) and the *cspA* gene (gene encoding the major cold shock protein) [18]. *B. mycoides* is char-

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acterized by the formation of rhizoid colonies and the absence of motility [19]. *B. pseudomycoides* is phenotypically similar to *B. mycoides* and is distinguished by DNA relatedness and fatty acid composition [2].

The 16S rDNA is the macromolecule of choice in the reconstruction of bacterial phylogenies [20-24]. The 16S rDNA, however, cannot distinguish among species in the *Bacillus cereus* group [25,26]. Genomic approaches have been used in an attempt to elucidate the genetic diversity of three highly closely related species in the *B. cereus* group: *B. cereus*, *B. anthracis* and *B. thuringiensis*. They appear as a single species on the basis of genetic evidence [27].

In a previous study, a 220 bp marker was developed and used to infer the phylogeny of species in the genus *Bacillus* and closely-related genera [28]. This marker was a combination of the last 150 bp at the 3' end of the 16S rDNA and the first 70 bp at the 5' end of the 16S-23S rDNA internal transcribed spacer (ITS). More recently, we assessed the usefulness of the 220 bp marker at a higher taxonomic level, the Order *Bacillales* [29]. This marker showed several advantages over the use of 16S rDNA sequences or the generation of extensive phenotypic and genotypic data in phylogenetic analyses. First, the 150 bp at the 3' end of the 16S rDNA allowed discrimination among distantly related species. Owing to its higher rate of nucleotide substitutions, the 70 bp at the 5' end of the 16S-23S rDNA (ITS) added discriminating power among closely related species from same genus and closely related genera from same family. Because of its higher percentage of nucleotide sequence divergence than the 16S rDNA, the 220 bp marker could better discriminate among closely related *Bacillus* [28] and *Bacillales* [29] species. Second, the method was simple, rapid, suited to large screening programs and easily accessible to most laboratories. Third, the marker also revealed species which appeared misassigned and for which additional characterization appeared warranted.

In the current study, we further analyze the resolving power of this short marker in inferring phylogenies at a much lower taxa level: the *Bacillus cereus* group.

2. MATERIALS AND METHODS

2.1. Bacterial Species and Strains

Four species in the *Bacillus cereus* group: *B. anthracis*, *B. cereus*, *B. thuringiensis* and *B. weihenstephanensis* were analyzed. Three additional *Bacillus* species, *B. halodurans*, *B. licheniformis* and *B. subtilis*, and *Clostridium tetani* were included for comparison purposes. A total of eight bacterial species and 12 strains were analyzed (**Table 1**). They were selected on the basis that their complete genome sequences were freely available in GenBank at the National Center for Biotechnology Information (NCBI) completed microbial genomes database (http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi,August 2009). *Bacillus mycoides* and *B. pseudomycoides* were not included because their complete genome sequences have not been determined.

Table 1. Bacterial species used in this study.

Genera	Species	Strain	GenBank accession no.
Bacillus	*anthracis*	Ames	AE016879
		Ames Ancestor	AE017334.2
		Sterne	AE017225.1
	cereus	ATCC 14579	AE016877.1
		ATCC 10987	AE017194.1
		E33L	CP000001.1
	thuringiensis serovar *konkukian*	97-27	AE017355.1
	weihenstephanensis	KBAB4	NC_010184.1
	halodurans	C-125	BA000004.3
	licheniformis	ATCC 14580	AE017333.1
	subtilis subsp. *subtilis*	168	AL009126.3
Clostridium	*tetani*	E88	AE015927.1

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2.2. Sequences

The 16S rDNA and 16S-23S ITS for the 12 bacterial species and strains were retrieved from GenBank, for a total of 129 allelic sequences. The last 150 bp at the 3' end of 16S rDNA and the first 70 bp at the 5' end of 16S-23S ITS were merged into a single 220 bp sequence for each of the 129 alleles under study as described before [28]. This 220 bp sequence will be used as a phylogenetic marker for the 12 bacterial species and strains under study.

2.3. Phylogenetic Analyses

All 129 allelic sequences were aligned using ClustalW [30] (data not shown). A neighbor-joining tree was constructed [31], based on the alignment of the 129 alleles of the 220 bp sequence. The tree was bootstrapped using 1,000 random samples. The neighbor-joining tree was drawn and printed with Tree Explorer, all components of the Molecular Evolutionary Genetics Analysis (MEGA, version 3.1) software package [32].

3. RESULTS AND DISCUSSIONS

In a previous study, a 220 bp sequence was developed as a DNA marker and used to infer the phylogeny of species in the Gram-positive genus *Bacillus* and closely-related genera [28]. This marker was a combination of the last 150 bp at the 3' end of the 16S rDNA and the first 70 bp at the 5' end of the 16S-23S rDNA internal transcribed spacer (ITS). More recently, we assessed the usefulness of the 220 bp marker by extending its analyses at a higher taxonomic level, the Gram-positive Order *Bacillales* [29]. In parallel, a similar marker was used to infer the phylogeny of the Gram-negative Class γ-proteobacteria [33]. In the current study, we further analyze the resolving power of this marker in inferring the phylogeny at a much lower taxa level: the *Bacillus cereus* group.

A bootstrapped neighbor-joining phylogenetic tree was inferred from comparative analyses of the 220 bp marker from the 129 alleles from the bacterial species and strains under study (**Figure 1**). Four major Groups were revealed based on the topology of the neighbor-joining tree at the 90% nucleotide sequence identities, Group I to IV. Group I contains all alleles of the species in the *Bacillus cereus* group. Group II contains all alleles of *B. halodurans*. Group III contains all alleles of *B. licheniformis* and *B. subtilis*. Group IV contains all alleles of *Clostridium tetani*. Based on nucleotide sequence identities, sub-groups and branches can be revealed. Group I can be sub-divided into three sub-groups at the 95% nucleotide sequence identities. Sub-group I-1 encompasses 27 alleles from the *B. anthracis* strains, 36 alleles from the *B. cereus* strains, 11 alleles from *B. thuringiensis* and one allele from *B. weihenstephanensis*. Sub-group I-2 encompasses six alleles from *B. anthracis*, two alleles from *B. cereus* and one allele from *B. thuringiensis*. Sub-group I-3 encompasses 12 alleles from *B. weihenstephanensis*. A branch corresponding to an allele from *B. weihenstephanensis* is present between sub-groups I-2 and I-3. Group II contains all eight alleles from *B. halodurans*. They show at least 20% nucleotide sequence divergences with alleles from the other *Bacillus* species. Group III contains all alleles from *B. licheniformis* and *B. subtilis*. It can be sub-divided into two sub-groups at the 95% nucleotide sequence identities. Sub-group III-1 encompasses all seven alleles from *B. licheniformis*. Sub-group III-2 encompasses all ten alleles from *B. subtilis*. Group IV contains all six alleles from *Clostridium tetani*. These alleles show at least 26% nucleotide sequence divergence with alleles from species and strains in the genus *Bacillus*.

In accordance with our previous work on the Order *Bacillales*, the 220 bp sequence used as a phylogenetic marker was able to group alleles from same species for *B. halodurans*, *B. licheniformis*, *B. subtilis*, and *Clostridium tetani*, respectively. However, this 220 bp sequence could not group most alleles from same species, exclusive of alleles from others, for the *B. cereus* group. Sub-group I-1 is heterogeneous. It contains alleles from all four species from the *B. cereus* group. The close proximity of *B. cereus*, *B. anthracis* and *B. thuringiensis* is in agreement with previous works based on whole-genome DNA hybridization [34], pulsed-field gel electrophoresis (PFGE) [35], multilocus enzyme electrophoresis (MEE) [36], amplified fragment length polymorphism (AFLP) fingerprinting [37] and multilocus sequence typing (MLST) [38,39], which showed that all three species are genetically highly related. They appear as a single species on the basis of genetic evidence [27]. Sub-groups I-2 is more homogeneous. It mostly contains alleles from *B. anthracis*. Sub-groups I-3 is homogeneous. It only contains alleles from *B. weihenstephanensis*. As shown earlier, on the genus *Bacillus* [28] and the Order *Bacillales* [29], this 220 bp sequence contains 150 bp at the 3' end of 16S rDNA which allowed discrimination among distantly related species and 70 bp at the 5' end of 16S-23S ITS which, owing to its higher percentage of nucleotide sequence divergence, added resolving power among closely related species.

Here, species in the *B. cereus* group, most notably *B. cereus*, *B. anthracis* and *B. thuringiensis*, are too closely related to be discriminated with the 220 bp sequence previously used as a phylogenetic marker. Our work, however, has shown that the alleles in sub-group I-3 could distinguish *B. weihenstephanensis* from all other species.

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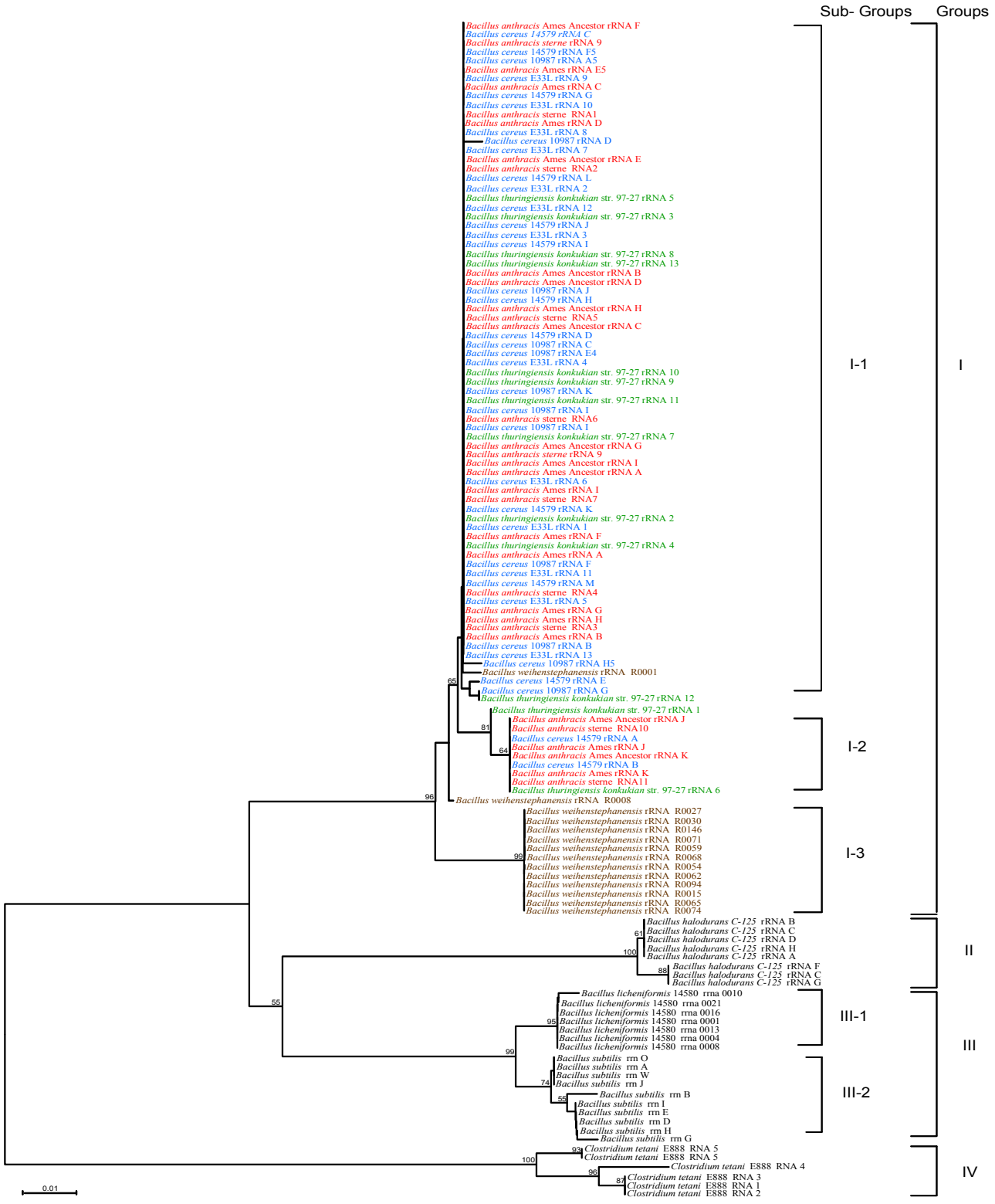

Figure 1. Bootstrapped neighbor-joining tree inferred from comparative alignment of the 220 bp marker from the 129 alleles from the 12 bacterial species and strains under study. Major groups are indicated in capital roman numerals. Sub-groups are indicated in arabic numerals. Bootstrap values higher than 50% are indicated (expressed as percentage of 1000 replication). The horizontal bar represents 1% nt difference. *Bacillus anthracis*, *B. cereus*, *B thuringiensis* and *B. weihenstephanensis*'s alleles are written in red, blue, green and brown, respectively. *B. halodurans, B. licheniformis, B. subtilis* and *Clostridium tetani*'s alleles are written in black ink.

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4. CONCLUSIONS

Previous genetic analyses have shown that *B. cereus*, *B. anthracis* and *B. thuringiensis* should be regarded as a single species. We have shown here that a 220 bp marker, used to reconstruct the phylogeny of the Order *Bacillales* and the family *Bacillaceae*, was unable to discriminate between these three highly-related species. We have reached the limit of the resolving power of the 220 bp sequence as a phylogenetic marker: *B. cereus*, *B. anthracis* and *B. thuringiensis*.

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