Vol.2, No.9, 990-997 (2010)
http://dx.doi.org/10.4236/ns.2010.29121

Natural Science

Phylogeny of the Order *Bacillales* inferred from 3' 16S rDNA and 5' 16S-23S ITS nucleotide sequences

Sabarimatou Yakoubou[1,2], Dong Xu[1], Jean-Charles Côté[1]*

[1]Agriculture and Agri-Food Canada, Research Centre, Gouin Blvd, St-Jean-sur-Richelieu, Canada; *Corresponding Author: Jean-Charles.Cote@agr.gc.ca
[2]Département des Sciences Biologiques, Université du Québec à Montréal, Succ."Centre-Ville" Montréal, Canada

Received 16 April 2010; revised 20 May 2010; accepted 25 May 2010.

ABSTRACT

A short 220 bp sequence was used to study the taxonomic organization of the bacterial Order *Bacillales*. The nucleotide sequences of the 3' end of the 16S rDNA and the 16S-23S Internal transcribed spacer (ITS) were determined for 32 *Bacillales* species and strains. The data for 40 additional *Bacillales* species and strains were retrieved directly from Genbank. Together, these 72 *Bacillales* species and strains encompassed eight families and 21 genera. The 220 bp sequence used here covers a conserved 150 bp sequence located at the 3' end of the 16S rDNA and a conserved 70 bp sequence located at the 5' end of the 16S-23S ITS. A neighbor-joining phylogenetic tree was inferred from comparative analyses of all 72 nucleotide sequences. Eight major Groups were revealed. Each Group was sub-divided into sub-groups and branches. In general, the neighbor-joining tree presented here is in agreement with the currently accepted phylogeny of the Order *Bacillales* based on phenotypic and genotypic data. The use of this 220 bp sequence for phylogenetic analyses presents several advantages over the use of the entire 16S rRNA genes or the generation of extensive phenotypic and genotypic data. This 220 bp sequence contains 150 bp at the 3' end of the 16S rDNA which allows discrimination among distantly related species and 70 bp at the 5' end of the 16S-23S ITS which, owing to its higher percentage of nucleotide sequence divergence, adds discriminating power among closely related species from same genus and closely related genera from same family. The method is simple, rapid, suited to large screening programs and easily accessible to most laboratories.

Keywords: *Bacillales*; 16S rDNA; 16S-23S ITS; Phylogeny

1. INTRODUCTION

In the 1st Edition of Bergey's Manual of Systematic Bacteriology (1986), the genus *Bacillus*, a member of the Class *Bacilli*, encompassed the Gram-positive, rod-shaped, endospore-forming, either obligate or facultative aerobe bacteria [1]. A total of 34 species and 26 additional *species incertae sedis* were described. The genus was highly heterogeneous, exhibiting a wide range of nutritional requirements, physiological and metabolic diversity and DNA base composition. In the following two decades, numerical classifications [2], 16S rDNA sequence alignments [3], characterizations at the genotypic and phenotypic levels of selected *Bacillus* species, all led to the creation of several new genera (briefly reviewed in Xu and Côté [4]).

In 2003, we developed a method for the identification, classification and phylogenetic analyses of *Bacillus* species and species from closely related genera [4]. The method was simple, rapid, suited to large screening programmes and easily accessible to most laboratories. In summary, the method relied on comparisons of a 220 bp nucleotide sequence marker among *Bacillus* species. This 220 bp marker was a combination of a 150 bp sequence at the 3' end of the 16S rRNA gene and a 70 bp sequence at the 5' end of the 16S-23S ITS sequence. In our original study, a total of 40 species was analyzed. We showed that the phylogeny inferred from the 220 bp marker was in agreement with then current classifications based on phenetic and molecular data, with exceptions. It revealed species and genera which appeared misassigned and for which additional characterization appeared warranted.

In the 2nd Edition of Bergey's Manual of Systematic Bacteriology [5], a new taxonomy of the Class *Bacilli* is

presented. It is the result of phylogenetic and principal-component analyses of comprehensive datasets of 16S rDNA sequences [5,6]. The *Bacillus* genus belongs to the Order *Bacillales*. The Order *Bacillales* contains nine families: *Alicyclobacillaceae*, *Bacillaceae*, *Listeriaceae*, *Paenibacillaceae*, *Pasteuriaceae*, *Planococcaceae*, *Sporolactobacillaceae*, *Staphylococcaceae* and *Thermoactinomycetaceae*. All nine families contain a total of 51 genera.

Today, we further assess the usefulness of the 220 bp marker by extending its analyses beyond the genus *Bacillus*, to a higher taxa level, the Order *Bacillales*. Whereas our first study focused on species from the genus *Bacillus* and species from closely related genera [4], we report here the phylogenetic analyses of 72 species and strains from eight *Bacillales* families and 21 genera.

2. MATERIALS AND METHODS

2.1. Bacterial Strains and Culture Conditions

A total of 72 strains of the Order *Bacillales* were used in this study. These encompassed eight families, 21 genera and 67 species. A total of 31 *Bacillales* strains were obtained from the "Deutsche Sammlung von Mikroorganismen und Zellkulturen" (DSMZ) GmbH, Braunschweig, Germany and were grown according to the DSMZ guidelines (http://www.dsmz.de/microorganisms/media_list.php). *Jeotgalibacillus alimentarius* was obtained from the "Czech Collection of Microorganisms" (CCM), Masaryk University, Brno, Czech Republic and grown according to the CCM guidelines (http://www.sci.muni.cz/ccm/index.html). The nucleotide sequences of the 40 remaining strains were retrieved directly from GenBank. All bacterial strains and their sources are listed in **Table 1**.

Escherichia coli strain TOP10 (Invitrogen Inc., Burlington, ON, Canada) was used for cloning PCR fragments. Strain TOP10 was cultured on Luria-Bertani (LB) agar plates to select transformants or in LB broth to multiply the cells, with shaking at 180-200 rpm at 37℃, 1 h.

2.2. DNA Extraction

Bacterial cells were washed with TESS buffer [10 mM Tris/HCl (pH 8.0), 1 mM Na_2EDTA, 0.1 M NaCl and 0.1% Sarkosyl (*N*-lauroylsarcosine)] and resuspended in TE buffer [10 mM Tris/HCl (pH 8.0), 1 mM Na_2EDTA]. Cells were lysed with 10 mg/ml lysozyme and 0.1% SDS. The subsequent phenol/chloroform extractions and ethanol precipitation were carried out as described by Sambrook and Russel [7].

Recombinant plasmid from *E. coli* strain TOP10 was isolated using the alkaline-lysis method [8] with some modifications as described elsewhere [4].

2.3. Amplification of the 3' End 16S rDNA and the 16S-23S ITS Region

A pair of primers: L516SF (5'-TCGCTAGTAATCGCGG ATCAGC-3') and L523SR (5'-GCATATCGGTGTTAG TCCCGTCC-3'), Reference [4] was used for the amplification of the 3' end of 16S rDNA, the 16S-23S ITS region and the 5' end of 23S rDNA. Amplification was performed in a Thermal Cycler 9600 (Perkin Elmer, Waltham, MA, USA) and the reaction mixtures contained 50 ng template DNA, 0.25 µM each primer, 200 µM dNTP, 1.5 mM $MgCl_2$ and 1.25 U *Taq* DNA polymerase (QIAGEN Inc. Mississauga, ON, Canada) in a final volume of 50 µl. PCR was performed under the following conditions: 45 s at 95℃ and then 30 cycles of 15 s at 94℃, 30 s at 53℃ and 90 s at 72℃. Amplification products were visualized on agarose gels.

2.4. Cloning and Sequencing Methods

The amplified DNAs were cloned into a pCRII-TOPO cloning vector using the TOPO TA cloning kit (Invitrogen, Inc.), following the manufacturer's instructions. *Escherichia coli* strain TOP10 transformants were selected on LB agar plates containing kanamycin (50 µg/ml), 5-bromo-4-chloro-3-indolyl-beta-D-galacto-pyranoside (X-Gal) (40 µg/ml). Multiple clones were submitted for futher analyses for each *Bacillales* species. The recombinant plasmids were isolated using the modified alkaline-lysis method, digested with EcoRI and visualized on agarose gels to confirm the presence of an inserted fragment.

The nucleotide sequences of cloned fragments were determined by the dideoxynucleotide chain termination method [9], using a capillary array automated DNA sequencer (ABI 3730xl DNA analyzer; Applied Biosystems, Foster City, CA, USA). The sequences of both strands were determined.

2.5. Sequence Analysis

The 3' end of the 16S rDNA and the 16S-23S ITS of the 32 *Bacillales* species and strains sequenced in this study were used for analysis. Forty sequences publicly available from GenBank were added for comparison purposes to cover a wider range of *Bacillales* families and genera, for a total of 72 *Bacillales* species and strains. A neighbor-joining tree was constructed [10] based on the alignment of the 72 3' end of the 16S rDNA and the 5' end of the 16S-23S ITS sequences.

The tree was bootstrapped using 1,000 random samples of sites from the alignment, all using CLUSTAL W software [11] at the DNA Data Bank of Japan (DDBJ) (http://clustalw.ddbj.nig.ac.jp/top-e.html), with the Ki-

Table 1. *Bacillale* families, genera, species and strains used to construct the bootstrapped neighbor-joining tree inferred from the 220 bp sequence, and shown in **Figure 3**.

Families, genera and species	Source/Strain	GenBank accession no. This study*	GenBank accession no. Retrieved**	Groups	Sub-groups
Alicyclobacillaceae					
Alicyclobacillus					
acidocaldarius subsp.*acidocaldarius*	DSM 446	EU723605		V	
acidocaldarius subsp. *rittmannii*	DSM 11297	EU723607		V	
acidoterrestris	DSM 3922	EU723608		V	
cycloheptanicus	DSM 4006	EU723610		V	
herbarius	DSM 13609	EU723613			Ungrouped
hesperidum	DSM 12489	EU723615		V	
Bacillaceae					
Amphibacillus					
fermentum	DSM 13869	EU723600		VIII	Ungrouped
tropicus	DSM 13870	EU723602		VIII	iii
xylanus	DSM 6626	EU723603		VIII	iii
Anoxybacillus					
flavithermus	WK1		CP000922	VIII	Ungrouped
Bacillus					
clausii			AP006627	VI	i
subtilis	168		AL009126	VI	i
thuringiensis	Al Hakam		CP000485	VI	ii
thuringiensis var. *konkukian*	97-27		AE017355	VI	ii
weihenstephanensis	KBAB4		CP000903	VI	ii
Filobacillus					
milensis	DSM 13259	EU723621		Ungrouped	
Geobacillus					
caldoxylosilyticus	DSM 12041	EU723625		I	
kaustophilus	HTA426		BA000043	I	
kaustophilus	DSM 7263	EU723629		I	
stearothermophilus	DSM 22	EU723631		I	
subterraneus	DSM 13552	EU723636		I	
thermocatenulatus	DSM 730	EU723638		I	
thermodenitrificans	NG80-2		CP000557	I	
thermodenitrificans	DSM 465	EU723643		I	
thermoglucosidasius	DSM 2542	EU723644		I	
thermoleovorans	DSM 5366	EU723648		I	
uzenensis	DSM 13551	EU723651		I	
Gracilibacillus					
dipsosauri	DSM 11125	EU723655		VIII	
halotolerans	DSM 11805	EU723657		VIII	ii
Halobacillus					
sp.	SA-Hb6		AB367166	VIII	i
Oceanobacillus					
iheyensis	HTE831		BA000028	VIII	i
Virgibacillus					
marismortui	DSM 12325	EU723664		VIII	ii
pantothenticus	DSM 26	EU723672		VIII	ii
proomii	DSM 13055	EU723675		VIII	ii
salexigens	DSM 11483	EU723666		VIII	ii
Listeriaceae					
Listeria					
inoccua	Clip11262		AL592022	VI	iii
monocytogenes	EGD-e		AL591824	VI	iii
elshimeri	SLCC5334		AM263198	VI	iii
Paenibacillaceae					
Aneurinibacillus					
aneurinilyticus	DSM 5562	EU723616		III	i
migulanus	DSM 2895	EU723617		III	i
thermoaerophilus	DSM 10154	EU723618		III	i
Brevibacillus					
agri	ATCC 51360		AF478091	III	ii
borstelensis	ATCC 51668		AF478093	III	ii
brevis	ATCC8246		AY478094	III	ii

Table 1. Continued.

Families, genera and species	Source/Strain	GenBank accession no. This study*	GenBank accession no. Retrieved**	Groups	Sub-groups
brevis	NBRC10059		AP008955	III	ii
chosinensis	ATCC 51359		AF478095	III	ii
parabrevis	ATCC 8186		AF478097	III	ii
formosus	ATCC51669		AF478096	III	ii
Paenibacillaceae					
Paenibacillus					
alginolyticus	ATCC 51185		AF478104	IV	
alvei	ATCC 6344		AF478098	IV	
chondroitinus	ATCC 51184		AF478105	IV	
larvae	ATCC 9545		AF487106	IV	
lautus	ATCC 43898		AF487100	IV	
lentimorbus	ATCC 8244		AY763503	IV	
macerans	ATCC 43899		AF478101	IV	
pabuli	ATCC 14707		AF478102	IV	
popilliae	KLN 3		CP001656	IV	
sp	JDR-2		DQ062684	IV	
validus	ATCC 43897		AF478103	IV	
Pasteuriaceae					
Pasteuria					
ramosa	P5		AY762091	II	
penetrans	CJ-1		AY123968	II	
Planococcaceae					
Jeotgalibacillus					
alimentarius	CCM 7134	EU723660		VI	
Marinibacillus					
marinus	DSM 1297	EU723661		VI	
Ureibacillus					
terrenus	DSM 12654	EU723667		VI	iv
thermosphaericus	DSM 10633	EU723670		VI	iv
Sporolactobacillaceae					
Sporolactobacillus					
terrae	M-116		D16289.1	Ungrouped	
Staphylococcaceae					
Staphylococcus					
aureus	RF122		AJ938182	VII	
arnosus	TM300		AM295250	VII	
epidermidis	RP62A		CP000029	VII	
haemolyticus	JCSC1435		AP006716	VII	
aprophyticus	ATCC 15305		AP008934	VII	
Macrococcus					
caseolyticus	JCSC5402		AP009484	VII	

*Sequences generated in this study; **Sequences publicly available in GenBank at the onset of this work and retrieved directly.

mura's parameter method [12]. The neighbor-joining phylogenetic tree was drawn using TreeView (version 1.6.6) [13,14].

3. RESULTS

Two primers, one located about 200 nt upstream from the 3' end of the 16S rRNA gene, the other about 80 nt downstream from the 5' end of the 23S rRNA gene (**Figure 1**), were used to amplify the last 200 bp of the 16S rRNA gene and the entire 16S-23S Internal transcribed spacer (ITS) region from 32 *Bacillales* species and strains (**Table 1**). The amplicons ranged in length from 450 to 1,200 bp. The number of amplicons per strain ranged from 1 to 6. A subset of these results is shown in **Figure 2**. Each amplicon was cloned and its nucleotide sequence determined. The homologous DNA sequences from 40 more *Bacillales* species and strains were retrieved directly from GenBank and added in the study (**Table 1**). Together, these 72 *Bacillales* species and strains belong to eight *Bacillales* families and 21 genera.

The last 150 bp located at the 3' end of 16S rDNA and the first 70 bp located at the 5' end of 16S-23S ITS, were combined into a 220 bp sequence. A multiple alignment of these nucleotide sequences from the 72 *Bacillale* species and strains was performed (supplementary data) and a bootstrapped neighbor-joining tree was constructed (**Figure 3**).

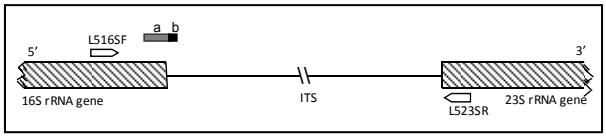

Figure 1. Schematic representation of the 16S and 23S rRNA genes separated by an Internal transcribed spacer (ITS). Orientations and positions of the primers used for amplification, L516SF and L523SR, are shown. The contiguous small grey and black boxes, indicated by the letters "a" and "b" correspond to the last 150 bp at the 3' end of the 16S rRNA gene and the first 70 bp at the 5' end of the ITS, respectively. Together, these boxes correspond to the 220 bp marker used in this study.

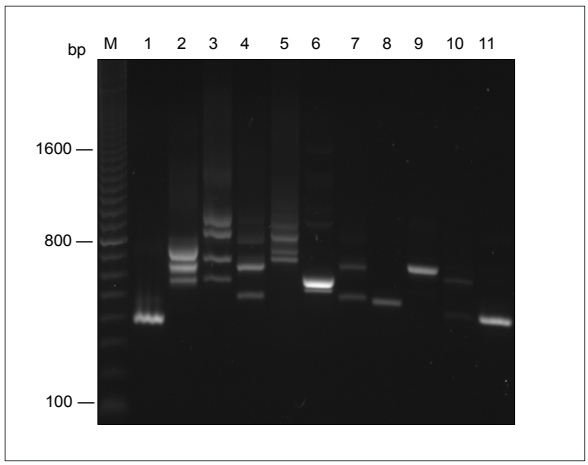

Figure 2. Agarose gel electrophoresis of the amplification products in selected species in the Order *Bacillales* using the L516SF/L523SR primer pair. Lane 1, 100 bp DNA marker; lane 2, *Alicyclobacillus acidocaldarius* subsp. *acidocaldarius*; lane 3, *Alicyclobacillus herbarius*; lane 4, *Geobacillus uzenensis*; lane 5, *Gracilibacillus halodurans*; lane 6, *Geobacillus kaustophilus*; lane 7, *Amphibacillus tropicus*; lane 8, *Virgibacillus proomii*; lane 9, *Virgibacillus salexigens*; lane 10, *Marinibacillus marinus*; lane 11, *Aneurinibacillus aneurinilyticus* ; lane 12, *Filobacillus milensis.*

At first sight, eight major Groups are revealed at the 80% nucleotide sequence identities, Group I to VIII. Group I contains all eleven *Geobacillus* species and strains. *Alicyclobacillus herbarius* forms a single branch between Groups I and II. Group II contains both *Pasteuriaceae* species. Group III contains ten species and strains from two genera of the *Paenibacillaceae* family. Two sub-groups can be revealed. Sub-group i encompasses the three *Aneurinibacillus* species. They share at least 82% nucleotide sequence identities. Sub-group ii encompasses the seven *Brevibacillus* species and strains. They share at least 80% nucleotide sequence identities. Group IV contains all 11 *Paenibacillus* species. Group V contains five of the six species and strains in the *Alicyclobacillaceae* family. *Alicyclobacillus herbarius* is the sixth species, and forms a single branch as explained above, because it shares less than 80% nucleotide sequence identities with members of Group V. Group VI is more heterogenous. It contains 12 species and strains from five genera from three families: the *Planococcaceae, Bacillaceae* and *Listeriaceae*. Four sub-groups and two branches are revealed. *Jeotgalibacillus alimentarus* forms the first branch of Group VI, and belongs to the *Planococcaceae* family. Sub-group i contains two *Bacillus* species of the *Bacillaceae* family, *B. clausii* and *B. subtilis*. *Marinibacillus marinus* forms the second branch of Group VI. It is the second genus of the *Planococcaceae* family. Sub-group ii contains three highly related *Bacillus* species and strains, the two *B. thuringiensis* strains and *B. weihenstephanensis*. Sub-group iii contains all three *Listeria* species of the *Listeriaceae* family. Sub-group iv contains both species of the third *Planococcaceae* genus, *Ureibacillus*. Next, a branch is formed by *Sporolactobacillus terrae*. This genus is the only one known in the *Sporolactobacillaceae* family. This is followed by a branch formed by *Filobacillus milensis*, a member of the *Bacillaceae* family. Group VII contains five *Staphylococcus* and one *Macrococcus* species. Both genera belong to the *Staphylococcaceae* family. Group VIII contains 12 species from six genera, all in the *Bacillaceae* family. Three subgroups and thee branches can be revealed. Sub-group i contains *Oceanobacillus* and *Halobacillus*. They share 87% nucleotide sequence identities. The grouping of *Oceanobacillus* and *Halobacillus* species in sub-group i is in agreement with the work of Lu *et al.*, properties, and genetic data [15]. Sub-group ii contains five species from two genera. It can be further divided into two clusters. The first one contains *Virgibacillus* (*V.*) *pantothenticus*, *V. proomii* and *Gracilibacillus halotolerans*, the second cluster contains *V. marismortui* and *V. salexigens.* The subdivision of all four *Virgibacillus* species into two sub-groups is in agreement with the work of Heyrman *et al*., based on genetic, chemotaxonomic and phenotypic data [16]. Sub-group iii contains two of the three *Amphibacillus* (*Am.*) species, *Am. xylanus* and *Am. tropicus*. Group VIII is completed by three branches: *Gracilibacillus dipsosauri*, *Anoxybacillus flavithermus* and *Amphibacillus fermentum*. All members of Group VIII are halophilic and alkaliphilic bacilli. This grouping is in agreement with the one proposed by Zhilina *et al*., based on physiology and genetic data [17].

When each of the eight major Groups of this neighbor-joining tree are analyzed separately, strains from same species, species from same genus and genera from same family are grouped together. Each Group corresponds to a single *Bacillales* family, exclusive of other families, with one exception, Group VI which contains three fami-

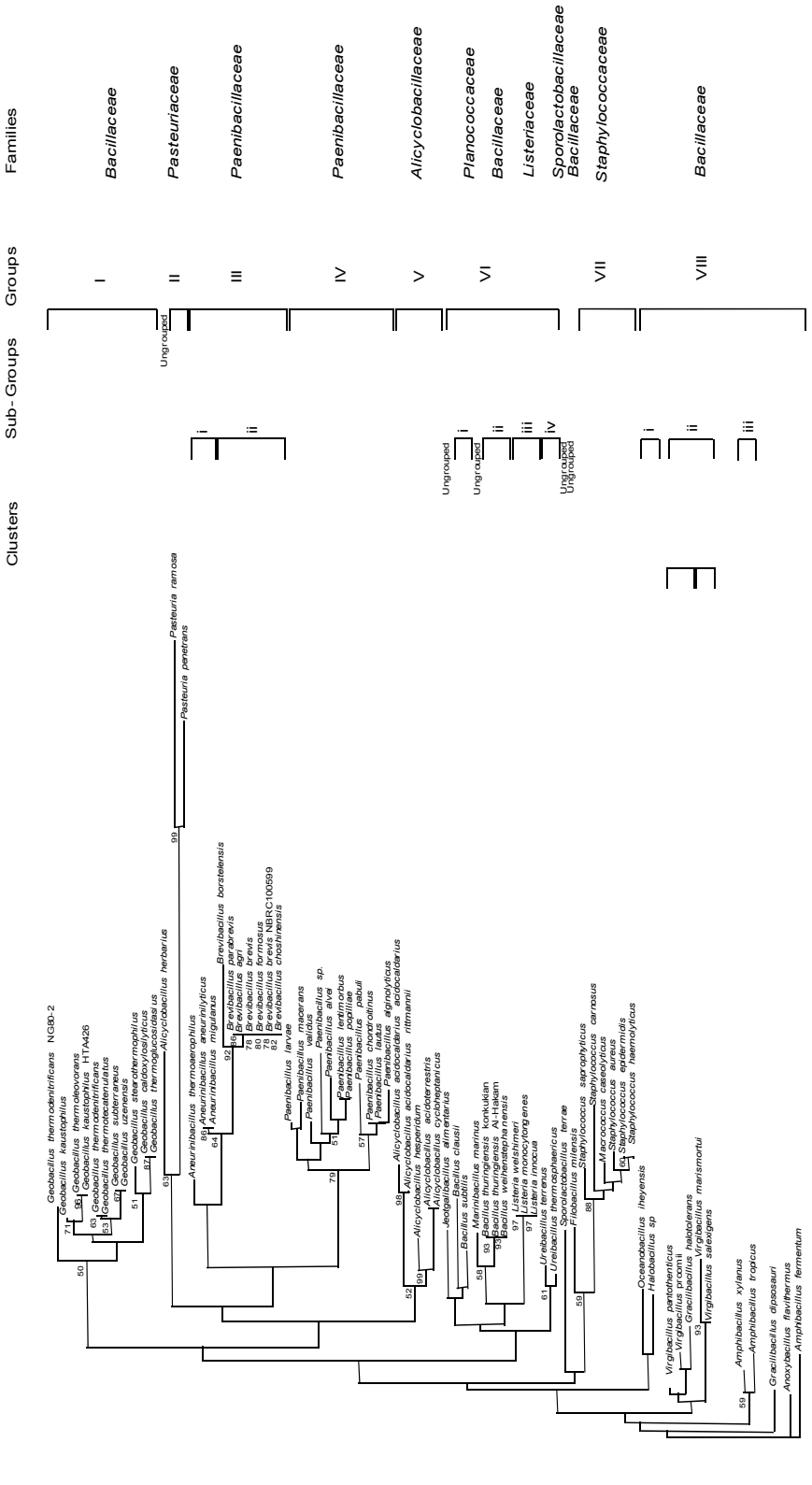

Figure 3. Bootstrapped neighbor-joining tree of 72 *Bacillales* species and strains inferred from the alignment of the 220 bp marker. Major Groups are indicated in capital roman numerals. Sub-groups are indicated in lower case riman numerals. Bootstrap values higher than 50% are indicated (expressed as percentage of 1000 replication). The horizontal bar represents 1% nt difference.

lies. In some cases, Groups were divided into sub-groups which corresponded to genera, with one exception, Group VIII where sub-groups encompassed different genera. The family *Sporolactobacillaceae* forms a branch between Groups VI and VII. Three *Bacillales* families: *Bacillaceae*, *Paenibacillaceae* and *Alicyclobacillaceae*, show some level of heterogeneity. Genera of the *Bacillaceae* family are found into three Groups, Groups I, VI and VIII, and in a branch between Groups VI and VII. Genera of the *Paenibacillaceae* family are found into two Groups, Groups III and IV. Analysis of the *Alicyclobacillaceae* family reveals a different story. Five of the six species and strains are clustered together in Group V. The sixth, *Alicyclobacillus herbarius*, is more distant. It forms a branch between Groups I and II. This is in agreement with the work of Goto *et al.* [18]. Although distinct from all other *Alicyclobacillaceae* based on genomic data, including 16S rDNA sequences, *Alicyclobacillus herbarius* was grouped with the family based on the presence of ω-cycloheptane fatty acids [18].

4. DISCUSSION

In a 2003 study [4], on *Bacillus* and closely-related genera, a multiple alignment of the 3' end of the 16S rRDA sequence showed that the last 157 nucleotides shared extensive identities among closely related species from same genus. This 157 nucleotide sequence, however, was not conserved among species from different genera. In the same study, a multiple alignment of the 16S-23S Internal transcribed spacer (ITS) sequence showed that the first 70 nucleotides were conserved between alleles of the same strain and between alleles of different strains from same species. This sequence, however, was not conserved among alleles of different species of the same genus. These two sequences, the last 150 bp at the 3' end of the 16S rRNA gene and the first 70 bp at the 5' end of the 16S-23S rRNA ITS, were combined into a single 220 bp marker. This marker was used to infer the phylogeny of *Bacillus* species and species from closely related genera. It could cluster *Bacillus* species and species from closely related genera into taxa akin to genera and could also distinguish closely related species. In this 2003 study, a total of 40 species was analyzed.

In the current study, we further assessed the usefulness of the 220 bp marker at a higher taxonomic level, the Order *Bacillales*. A total of 72 *Bacillales* species and strains from eight *Bacillales* families and 21 genera were covered. The number of *Bacillus* species included in this current study on *Bacillales* was deliberately kept low since this genus had already been covered extensively in our earlier study [4]. The neighbor-joining tree presented here was compared with the revised road map of the Order *Bacillales* shown in the 2nd Edition of Bergey's Manual of Systematic Bacteriology [5]. This revised road map [5] is a consensus phylogenetic tree of the Order *Bacillales*. It is the consensus tree inferred from numerous phylogenetic and principal-component analyses of comprehensive datasets of 16S rDNA sequences [5,6]. Our phylogenetic tree presented here is in agreement with the currently accepted phylogeny of the Order *Bacillales*, based on phenotypic and genotypic data. It is, in general, in agreement with the revised road map of the Order *Bacillales* [5]. In addition, some bacterial species that were not grouped at the genus level in our neighbor-joining tree, exemplified by *Alicyclobacillus herbarius*, were also confirmed by others to be different based on phenotypic and genotypic data [18].

The main discrepancy between our results, obtained with the 220 bp marker, and the revised road map shown in the 2nd Edition of Bergey's Manual of Systematic Bacteriology [5], rests on the grouping of the *Bacillaceae* family. In our study, members of the *Bacillaceae* family are present in three of the eight Groups. In the revised road map [5], two major *Bacillaceae* groups are presented. It is recognized, however, that some species have been misassigned to the *Bacillaceae* family [5]. The revised road map is constructed based on 16S rDNA sequences [5]. Our 220 bp marker contains 150 bp from 16S rDNA and 70 bp from ITS. Owing to its higher rate of nucleotide substitutions, this 70 bp adds discriminating power among species from same genera and genera from same family. As indicated by Ludwig *et al.* [5], and as shown here, the reorganization of the *Bacillaceae* family is still a work in progress.

The use of this 220 bp marker presents several advantages over the use of the entire 16S rRNA gene or the generation of extensive phenotypic and genotypic data in phylogenetic analyses. As shown in an earlier study [4], the method is simple, rapid, suited to large screening programmes and easily accessible to most laboratories. We have shown here that it can group *Bacillales* families and genera in accordance with established phylogenies. Because the 220 bp marker shows a higher percentage of nucleotide sequence divergence than the 16S rRNA gene, it can better discriminate among closely related *Bacillales* species. It can also reveal *Bacillales* species which may appear misassigned and for which additional characterization appear warranted.

In conclusion, in an earlier study [4], a 220 bp marker, based on 3' end of 16S rRDA and 5' end of 16S-23S rRNA ITS, was developed and used to classify species in the *Bacillus* genus and in closely related genera. Here, we showed that this 220 bp marker could be used to reconstruct the phylogeny at a higher taxa level: the Order *Bacillales*. We are planning to follow-up this work by

assessing the resolving power of this marker in reconstructing the phylogeny at a lower taxa level: the *Bacillus cereus* group, sensu lato.

Recently, in parallel, a similar maker was tested and shown to be able to reconstruct the phylogeny of the Class γ-proteobacteria [19].

REFERENCES

[1] Claus, D. and Berkeley, R.C.W. (1986) Genus *Bacillus* Cohn, 1872. In: Sneath, P.H.A., Mair, N.S., Sharpe, M.E. and J.G. Holt., Ed., *Bergey's Manual of Systematic Bacteriology*, The Williams & Wilkins Co., Baltimore, **2**, 1105-1139.

[2] Priest, F.G., Goodfellow, M. and Todd, C. (1988) A numerical classification of the genus *Bacillus*. *Journal of General Microbiology*, **134(7)**, 1847-1882.

[3] Ash, C., Farrow, A.E., Wallbanks, S. and Collins, M.D. (1991) Phylogenetic heterogeneity of the genus *Bacillus* revealed by comparative analysis of small-subunit-ribosomal RNA sequences. *Letters in Applied Microbiology*, **13(4)**, 202-206.

[4] Xu, D. and Côté, J.-C. (2003) Phylogenetic relationships between *Bacillus* species and related genera inferred from comparison of 3' end 16S rDNA and 5' end 16S-23S ITS nucleotide sequences. *International Journal of Systematic and Evolutionary Microbiology*, **53**, 695-704.

[5] Ludwig, W., Schleifer, K-H. and Whitman, W.B. (2009) Revised road map to the phylum *Firmicutes*. In: De Vos, P., *et al.* Eds., *Bergey's Manual of Systematic Bacteriology*, 2nd Edition, The *Firmicutes*, Springer-Verlag, New York, **3**, 1-17.

[6] Garrity, G.M., Bell, J.A. and Lilburn, T. (2005) The Revised Road Map to the Manual. In: Brenner, D.J., Krieg, N.R., Staley, J.T. and Garrity, G.M., Eds., *Bergey's Manual of Systematic Bacteriology*, 2nd Edition, The *Proteobacteria*, Part A, Introductory Essays. Springer, New York, **2**, 159-220.

[7] Sambrook, J. and Russell, D.W. (2001) Molecular Cloning: A Laboratory Manual. 3rd Edition, Cold Spring Harbor Laboratory Press, N.Y..

[8] Stephen, D., Jones, C. and Schofield, J.P. (1990) A rapid method for isolating high quality plasmid DNA suitable for DNA sequencing. *Nucleic Acids Research*, **18(24)**, 7463-7464

[9] Sanger, F., Nicklen, S. and Coulson, A.R. (1977) DNA sequencing with chain-termination inhibitors. *Proceedings of the National Academy of Sciences*, USA, **74**, 5463-5467.

[10] Saitou, N. and Nei, M. (1987) The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution*, **4(4)**, 406-425.

[11] Thompson, J.D., Higgins, D.G. and Gibson, T.J. (1994) CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. *Nucleic Acids Research*, **22(22)**, 4673-4680.

[12] Kimura, M. (1980) A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution*, **16(2)**, 111-120.

[13] Page, R.D.M. (1996) TreeView: An application to display phylogenetic trees on personal computers. *Computer Application in the Biosciences*, **12(4)**, 357-358.

[14] Page, R.D.M. (2000) TreeView—tree drawing software for Apple Macintosh and Windows. http://taxonomy.zoology.gla.ac.uk/rod/treeview.html

[15] Lu, J., Nogi, Y. and Takami, H., (2001) *Oceanobacillus iheyensis* gen. nov., sp. nov., a deep-sea extremely halotolerant and alkaliphilic species isolated from a depth of 1050 m on the Iheya Ridge. *FEMS Microbiology Letters*, **205(2)**, 291-297.

[16] Heyrman, J., Logan, N.A., Busse, H.J., Balcaen, A., Lebbe, L., *et al*. (2003) *Virgibacillus carmonensis* sp. nov., *Virgibacillus necropolis* sp. nov. and *Virgibacillus picturae* sp. nov., three novel species isolated from deteriorated mural paintings, transfer of the species of the genus *Salibacillus* to *Virgibacillus*, as *Virgibacillus marismortui* comb. nov. and *Virgibacillus salexigens* comb. nov., and emended description of the genus *Virgibacillus*. *International Journal of Systematic and Evolutionary Microbiology,* **53**, Part 2, 501-511.

[17] Zhilina, T.N., Garnova, E.S., Tourova, T.P., Kostrikina, N.A. and Zavarzin, G.A., (2001) *AmAmphibacillus fermentum* sp. nov. and *Amphibacillus tropicus* sp. nov., new alkaliphilic, facultatively anaerobic, saccharolytic bacilli from Lake Magadi. *Mikrobiologiya*, in Russian, **70(6)**, 825-837. English Translation: *Microbiology*, **70(6)**, 711-722.

[18] Goto, K., Matsubara, H., Mochida, K., Matsumura, T., Hara, Y., *et al.* (2002) *Alicyclobacillus herbarius* sp. nov., a novel bacterium containing omega-cycloheptane fatty acids, isolated from herbal tea. *International Journal of Systematic and Evolutionary Microbiology*, **52(1)**, 109-113.

[19] Yakoubou, S. and Côté, J.-C. (2010) Phylogeny of γ-proteobacteria inferred from comparisons of 3' end 16S rRNA gene and 5' end 16S-23S ITS nucleotide sequences. *Natural Science*, **2(6)**, 535-543.