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Combination of genetic tools to discern *Bacillus* species isolated from hot springs in South Africa

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Using phylogenetic analysis of the 16S rRNA gene 43 Gram-positive, spore-forming bacteria of the phylum *Firmicutes* were isolated, cultured and identified from five hot water springs in South Africa. Thirty-nine isolates belonged to the family Bacillaceae, genus *Bacillus* (n = 31) and genus *Anoxybacillus* (n = 8), while four isolates belonged to the family Paenibacillaceae, genus *Brevibacillus*. The majority of isolates fell into the *Bacillus* Bergey's Group A together with *Bacillus subtilis* and *Bacillus licheniformis*. One isolate matched *Bacillus panaciterrae* which has not previously been described as a hot-spring isolate. Three unknown isolates from this study (BLAST <95% match) and three "uncultured *Bacillus*" clones of isolates from hot springs in India, China and Indonesia listed in NCBI Genbank, were included in the analysis. When bioinformatic tools: Basic Local Alignment Search Tool (BLAST), *in silico* amplified rDNA restriction analysis (ARDRA), guanine-cytosine (GC) percentage and phylogenetic analysis are used in combination, but not independently, differentiation between the complex *Bacillus* and closely related species was possible. Identification that relies solely on BLAST of the 16S rRNA sequence can be misleading.

Key words: *Bacillus*, phylogeny, ARDRA, 16S rDNA, South Africa.

INTRODUCTION

Microbes from extreme environments are interesting because they often have unique properties including extremozymes and new drug discovery potential relevant in biotechnology (Gerday, 2002; Jardine et al., 2018). López-López et al. (2013) suggested that the diversity of hot spring environments is not fully appreciated with an estimate of <1% of bacteria in hot springs that are isolated and identified using traditional culture-based methods.

Most commonly used for bacterial identification, is a comparison of the 16S rRNA (ribosomal RNA) gene sequences with known public databases, which offers no information on the physiology and biochemistry. This gene is selected because it has not changed over time and is highly conserved in different bacteria. It is however large enough (1500 bp) to allow for extraction of bioinformatic information (Janda and Abbott, 2007). Although metagenomics reveal the variety of genetic

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diversity within a microbial population, it does not take into account viability and circumstantial contamination. Therefore, any novel bacteria isolated from these unique environmental sites are essential contributions to the current database and general understanding of microbial communities. Furthermore, cultured viable bacteria are critical in understanding the biochemical potential and production of bioactive molecules related to gene expression (Handelsman, 2004).

There are additional tools to differentiate bacterial genera and species. The guanine-cytosine percentage (GC%) of the DNA of bacterial genomes varies with different genera and is useful in bacterial systematics. Also, the GC% has been correlated with the thermostability of a genome and is higher in thermophiles (Wang et al., 2006). Amplified rDNA restriction analysis (ARDRA) allows for a more accurate, rapid and efficient identification compared with the more traditional microbiological and biochemical methods (Rajendhran and Gunasekaran, 2011). A computer-simulated restriction fragment length polymorphism (RFLP) analysis of the polymerase chain reaction (PCR) -amplified 16S rDNA which is the same as ARDRA, is a valid means of identifying unknown organisms (Moyer et al., 1996). The phylogenetic analysis of the 16S rRNA gene allows for maximum discrimination between closely related individual isolates taking into account each base of the entire gene, while ARDRA represents only variations in the restriction enzyme sites.

Worldwide, studies on thermophiles from hot springs have been carried out through both the use of metagenomics (López-López et al., 2013) as well as through isolation and culture-based techniques (Cihan, 2013; Khiyami et al., 2012). Metagenomic analysis has described a predominance of *Proteobacteria* and cyanobacteria in Malaysia (Goh et al., 2011), India (Sharma et al., 2014) and South Africa (Tekere et al., 2011, 2012). However, by isolation and culture, the predominant bacteria are Gram-positive spore-forming *Bacillus* and *Bacillus*-related microorganisms reported in India (Panda et al., 2016), Saudi Arabia (Khiyami et al., 2012), Armenia (Panosyan and Birkeland, 2014) and Jordan (Obeidat et al., 2012).

The classification of the genus *Bacillus* was transformed by major changes where several new genera were proposed (Ludwig et al., 2009). In addition, this is a highly diverse and expanding group, with 25 new genera being described in the past two years (Mandic-Mulec et al., 2015). The relatively new genus *Anoxybacillus*, was established in 2000 and is growing rapidly with six new species being described since 2011 (Mandic-Mulec et al., 2015). Of the 115 endospore-forming *Bacillus* isolates from geothermal regions in Turkey, *Anoxybacillus* was the most abundant, being represented by 53 isolates (Cihan, 2013) suggesting that geothermal environments could be a niche for the discovery of new *Anoxybacillus* species. Because the

genera *Bacillus* and *Anoxybacillus* have been reclassified and novel species are being described at a rapid rate, there may be some incongruence and confusion when comparing the nomenclature of this group from studies prior to the reclassification, and between different studies where the new nomenclature is not taken into account.

In South Africa, more than a third of the 80 hot springs are located in the Limpopo Province. Metagenomic studies of four hot springs revealed only a very low abundance of the phylum *Firmicutes* which includes *Bacillus* and *Bacillus*-related species (Tekere et al., 2011, 2012). Various other phyla were reported but in very small percentages of the total rRNA sequences (<0.2%). The discovery that these hot springs hold a great diversity of bacteria suggests that it may be a resource for potential thermophiles that could have novel biotechnological applications. The aims of this study were to use conventional culture techniques for the isolation of bacteria and to use the 16S rRNA gene sequence analysis for genotypic identification of the isolates. Besides, the sequences were analysed for GC%, ARDRA, and phylogenetic analysis. The use of a combination of tools for identification was investigated.

MATERIALS AND METHODS

Sampling and sampling sites

Water and sediment samples from five hot springs (Tshipise, Siloam, Mphephu, Lekkerus, and Libertas) in the Limpopo Province, South Africa were sampled. Their geographical location with GPS coordinates, average water temperature and pH conditions and local site description have been previously described (Olivier et al., 2011; Jardine et al., 2017).

Isolation of bacteria and determination of optimal growth conditions

Aliquots of 100 mL of water were passed through a 0.22 µm membrane filter and the membrane filters were then placed on the surface of different agar media (Himedia, India): nutrient agar, Actinomycete isolation agar, minimal Luria broth media, potato dextrose agar and cyanobacterial agar for 48 h at 37 and 53°C exactly as described by Jardine et al. (2017). Bacterial isolates from sediment samples were obtained using the streak plate method. Once pure cultures of the isolates were obtained, they were studied for optimal conditions of growth relating to temperature, pH and salinity in order to maintain them in the laboratory.

The optimum pH, temperature and salinity for growth of the bacteria initially isolated at 53°C were determined, by growth in nutrient broth from pH 6 to 10 in intervals of one unit, temperatures between 45 and 70°C in intervals of 5°C, and sodium chloride (NaCl) at concentrations ranging from 0 to 15% w/v, respectively. A bacterial suspension at an optical density (OD) at 600 nm (OD₆₀₀) of approximately 0.3 was made, and 1 mL volumes of nutrient broth were inoculated with 10 µL of the bacterial suspension, incubated under various conditions. The OD at 600 nm was measured using a spectrophotometer (Phillips PU8620 UV/VIS/NIR) to determine whether growth had occurred.

DNA extraction protocol, 16S rRNA gene sequencing and phylogeny

DNA extraction and 16S rRNA gene sequencing have been previously described in Jardine et al. (2017) without any modifications. DNA was extracted by the method described by Dashti et al. (2009), and the 16S rRNA gene was subjected to PCR with universal primers 8F, 27F and 1472R (Galkiewicz and Kellogg, 2008) with the cycling conditions as described by Jardine et al. (2017). The amplicon was Sanger sequenced with Big Dye Terminator 3.1 cycle sequencing kit (ABI) according to the manufacturer's instructions, at the African Centre for DNA Barcoding (ACDB), University of Johannesburg. The resulting consensus sequence of approximately 1400 bp was compared with those in the NCBI database (Genbank) using the Basic Local Alignment Search Tool (BLAST) (McGinnis and Madden, 2004), and EzTaxon-e (Kim et al., 2012). Isolates with a >99% match to the published sequences were identified to the species level, and those with a >97% match were identified to the genus level (Yarza et al., 2014). Alignments were made by CLUSTAL OMEGA (www.ebi.ac.uk), and manually refined using SeaView (Gouy et al., 2010). Statistical confidence in branching points was determined by 1000 bootstrap replicates. Complete and partial sequences from this study were submitted to Genbank. The Genbank accession numbers of the type strains used in the phylogenetic trees are listed in Appendix A.

Computer-simulated PCR-RFLP or amplified rDNA restriction analysis (ARDRA)

Computer-simulated PCR-RFLP patterns were generated from the approximately 1400 bp sequence of the 16S rRNA gene (using the computer program RestrictionMapper version 3 "www.Restrictionmapper.org") and restriction enzymes, *Alu1* (15 sites), *Taq1* (18 sites) (Wu et al., 2006), *HaeIII* (24 sites), *Hinf1* (21 sites), *Rsa1* (18 sites) (Wahyudi et al., 2010), *Hph1* (23 sites), *Mbol1* (15 sites) and *Fok1* (14 sites). The presence and absence of the simulated band was used to create a binary data file and the results were present together as a composite. Several bacterial strains from published data were included in the study, to determine the phylogenetic groups into which the isolates fell. The SeaView program was used to analyze the binary data, and a distance neighbour-joining tree was created for detection of clusters.

Guanine-cytosine (GC) content (in percentage)

The GC% for the *Firmicutes* group was calculated with the 1400bp 16S rRNA gene fragment (Yamane et al., 2011) using the ENDMEMO GC calculating tool (www.endmemo.com/bio/gcratio) for all the isolates.

Bacterial strains in this study

The 16S rRNA gene sequences of hot spring isolates from South Africa were allocated accession numbers and deposited in Genbank as indicated in Table 1. Strains of *Anoxybacillus* spp. have accession numbers: MF037806, MF037807, MF037808, MF037809, MF037810, MF037811, MF037812 and MF037813. Strains of *B. licheniformis* were assigned numbers: MF037814, MF037815, MF037816, MF037817, MF037818, MF037819, MF037820, MF037821 and MF037822. Accession numbers: MF037827, MF037828, MF037829, MF037830, MF037831, MF037832, MF037833, MF037834, MF037835, MF037836, MF037837, and MF037838 were given to *B. subtilis* strains.

Bacillus spp. has Genbank accession numbers: MF038049, MF038050, MF038051, and MF039084. Single isolates were assigned the following accession numbers: *Bacillus pumilus* MF038052, *Bacillus panaciterrae* MF038053, *Bacillus methylotrophicus* MF038054, *Solibacillus* species MF039085, and *Aneurinibacillus* species MF040218. MF038055, MF038056, MF038057, and MF038058 were numbers allocated to four *Brevibacillus* species. All reference strains used for analyses are listed in Appendix A together with their associated Genbank accession numbers.

RESULTS

Optimal growth conditions for bacterial isolation and growth

The optimal growth conditions for the isolates were determined as the bacteria needed to be grown as inoculum for further experiments and investigations. The average optimal pH was 7, the average optimal temperature was 55°C, and the average optimal salinity was 5%. However, 19% were also able to grow in 10% salinity. These results are available in Jardine (2017).

16S rRNA gene sequencing

The contiguous sequences were compared to two databases, namely Genbank and EzTaxon-e and the highest percentage similarities and accession numbers are listed in Table 1. Values >97% suggest a match to the genus level, while a value of >99% suggests a match to species level (Yarza et al., 2014). Where no PCR product was obtained, the sequencing was not determined (nd), and in some cases, sequencing was incomplete which did not allow for a full consensus sequence to be constructed. Sequences from this study were submitted to Genbank with their relevant accession numbers as listed earlier.

Percentage guanine-cytosine (GC) content

The GC% and accession numbers for 31 *Bacillus* spp., eight *Anoxybacillus* spp., five *Brevibacillus* spp., one *Aneurinibacillus* spp., and reference strains (from Genbank) are listed in Appendix B for the approximately 1400 bp 16S rRNA gene fragment. Based on the GC% of the 16S rRNA sequences for the isolates in this study, they were grouped into four genera (*Anoxybacillus*, *Bacillus*, *Aneurinibacillus*, and *Brevibacillus*). The average and standard deviations for the GC% for these isolates together with reference strains were calculated (Appendix B) and plotted illustrated in Figure 1. The isolates that fell out of one standard deviation range of the average GC% were earmarked as potentially different from the group, that is, isolates 1T, 11T, 14S and 33Li (Appendix B as indicated by*). In all other respects, there was a

Table 1. Identification of hot spring isolates in South African using 16S rDNA sequences.

Isolate No.*	Isolation Temp °C **	GenBank BLAST	GB access No.	EzTaxon-e	EzTaxon-e Access No.	Submit Access No.	GC content***	ARDRA****	Phylogeny
3T	53	<i>Anoxybacillus rupiensis</i> 94%	KJ842629.1	<i>Anoxybacillus rupiensis</i> 99%	AJ879076	MF037806	√ (56.24)	A	<i>Anoxybacillus</i>
4T	53	<i>Anoxybacillus rupiensis</i> 99%	AM988775.1	<i>Anoxybacillus rupiensis</i> 99%	AJ879076	MF037807	√ (56.15)	A	<i>Anoxybacillus</i>
7T	53	<i>Anoxybacillus</i> sp. ATCC 99%	KJ722458.1	<i>Anoxybacillus rupiensis</i> 99%	AJ879076	MF037808	√ (56.72)	A	<i>Anoxybacillus</i>
11T	53	<i>Anoxybacillus</i> sp. 94%	KF254912.1	<i>Anoxybacillus rupiensis</i> 98%	AJ879076	MF037809	X (55.28)	C	<i>Anoxybacillus</i>
13S	53	<i>Anoxybacillus</i> sp. 99%	FN432807.1	<i>Anoxybacillus rupiensis</i> 98%	AJ879076	MF037810	√ (56.51)	A	<i>Anoxybacillus</i>
17S	53	<i>Anoxybacillus flavithermus</i> 99%	KF039883.1	<i>Anoxybacillus mongoliensis</i> 98%	EF654664	MF037811	√ (56.22)	C	<i>Anoxybacillus</i>
19S	53	<i>Anoxybacillus</i> sp. 99%	KP221933.1	<i>Anoxybacillus rupiensis</i> 99%	AJ879076	MF037812	√ (56.33)	A	<i>Anoxybacillus</i>
43T	53 ^s	<i>Anoxybacillus flavithermus</i> 99%	KC503890.1	<i>Anoxybacillus flavithermus</i> 99%	AVGH01000041	MF037813	√ (56.26)	C	<i>Anoxybacillus</i>
2T	53	<i>Bacillus licheniformis</i> 99%	HM631844.1	<i>Bacillus licheniformis</i> ATCC 98%	AE017333	MF037814	√ (55.4)	B	<i>Bacillus</i> group A
6T	53	<i>Bacillus licheniformis</i> 99%	KJ729823.1	<i>Bacillus licheniformis</i> ATCC 99.8%	AE017333	MF037815	√ (55.43)	C	<i>Bacillus</i> group A
8T	53	<i>Bacillus</i> sp. 98%	GU132507.1	<i>Bacillus licheniformis</i> ATCC 97%	AE017333	MF037816	√ (55.05)	B	<i>Bacillus</i> group A
10T	53	<i>Bacillus licheniformis</i> 97%	HM631844.1	<i>Bacillus licheniformis</i> ATCC 98%	AE017333	MF037817	√ (55.0)	B	<i>Bacillus</i> group A
20S	53	<i>Bacillus subtilis</i> 99%	KC634086.1	<i>Bacillus licheniformis</i> ATCC 99.8%	AE017333	MF037818	√ (55.6)	C	<i>Bacillus</i> group A
28M	53	<i>Bacillus licheniformis</i> 99%	GQ340513.1	<i>Bacillus licheniformis</i> ATCC 99.8%	AE017333	MF037819	√ (54.84)	C	<i>Bacillus</i> group A
30M	53	<i>Bacillus licheniformis</i> 99%	KJ526854.1	<i>Bacillus licheniformis</i> ATCC 99%	AE017333	MF037820	√ (55.15)	B	<i>Bacillus</i> group A
39T	37-53	<i>Bacillus licheniformis</i> 99%	KF879197.1	<i>Bacillus licheniformis</i> ATCC 99.7%	AE017333	MF037821	√ (54.52)	C	<i>Bacillus</i> group A
74T	37	<i>Bacillus licheniformis</i> 99%	JN366749.1	<i>Bacillus sonorensis</i> 99.6%	AYTN01000016	MF037822	√ (54.97)	B	<i>Bacillus</i> group A
12S	53	<i>Bacillus subtilis</i> 99%	KC634086.1	<i>Bacillus subtilis</i> 99%	AMXN01000021	MF037827	√ (54.48)	C	<i>Bacillus</i> group A
14S	53	<i>Bacillus</i> sp. 98%	CP11051.1	<i>Brevibacterium halotolerans</i> 97%	AM747812	MF037828	X (54.23)	B	<i>Bacillus</i> group A
21M	53	<i>Bacillus subtilis</i> 99%	JN585712.1	<i>Bacillus subtilis</i> 99.7%	AMXN01000021	MF037829	√ (55.04)	B	<i>Bacillus</i> group A
22M	53	<i>Bacillus methylotrophicus</i> 99%	JQ765577.1	<i>Bacillus tequilensis</i> 99%	AYTO01000043	MF037830	√ (54.64)	nd	<i>Bacillus</i> group A
33Li	53	<i>Bacillus subtilis</i> 98%	KC182058.1	<i>Bacillus subtilis</i> 99.7%	AMXN01000021	MF037831	X (54.21)	C	<i>Bacillus</i> group A
40Le	37-53	<i>Bacillus subtilis</i> 99%	KP249695.1	<i>Bacillus tequilensis</i> 99.6%	AYTO01000043	MF037832	√ (54.57)	B	<i>Bacillus</i> group A
41Li	37-53	<i>B. licheniformis</i> 97%	KC429774.1	<i>Bacillus subtilis</i> 96%	AMXN01000021	MF037833	√ (54.69)	B	<i>Bacillus</i> group A
47Li	53 ^s	<i>Bacillus subtilis</i> 99%	KP249695.1	<i>Bacillus subtilis</i> 99.9%	AMXN01000021	MF037834	√ (54.53)	C	<i>Bacillus</i> group A
48Li	53 ^s	<i>Bacillus subtilis</i> 99%	NR_118486.1	<i>Bacillus subtilis</i> 99.9%	AMXN01000021	MF037835	√ (54.86)	B	<i>Bacillus</i> group A
54T	37	<i>Bacillus subtilis</i> 99%	HM753614.1	<i>Bacillus subtilis</i> 98.9%	CP002905	MF037836	√ (54.69)	B	<i>Bacillus</i> group A
78S	37	<i>Bacillus</i> sp. 99%	KF984420.1	<i>Bacillus subtilis</i> 99%	ABQL01000001	MF037837	√ (54.45)	C	<i>Bacillus</i> group A
83Li	37	<i>Bacillus subtilis</i> 97%	KF533727.1	<i>Bacillus subtilis</i> 96%	ABQL01000001	MF037838	√ (55.44)	B	<i>Bacillus</i> group A
1T	53	<i>Bacillus subtilis</i> 96%	HM367735.1	<i>Bacillus subtilis</i> 96%	AMXN01000021	MF038049	X (52.86)	C	<i>Bacillus</i> group A
15S	53	<i>Bacillus licheniformis</i> 94%	HM055609.1	<i>Bacillus licheniformis</i> 94%	AE017333	MF038050	√ (55.11)	B	<i>B. licheniformis</i>
18S	53	<i>Bacillus</i> sp. 96%	GU132507.1	<i>Bacillus licheniformis</i> 95%	AE017333	MF038051	√ (54.71)	B	<i>B. licheniformis</i>
52M	53 ^s	<i>Brevibacillus agric</i> 94%	JN812211.1	<i>Brevibacillus agri</i> 94%	D78454	MF039084	√ (55.09)	C	<i>Bacillus</i> group A
24M	53	<i>Bacillus pumilus</i> 99%	KJ526891.1	<i>Bacillus aerophilus</i> 99.8%	AJ831844	MF038052	√ (54.8)	A	<i>Bacillus</i> group A

Table 1. Contd.

32Le	53	<i>Bacillus panaciterrae</i> 99%	NR_041379.1	<i>Bacillus panaciterrae</i> 99%	AB245380	MF038053	√ (54.36)	C	<i>Bacillus</i> group K
77S	37	<i>Bacillus methylotrophicus</i> 99%	KP342210.1	<i>Bacillus methylotrophicus</i> 99.9%	JTKJ01000077	MF038054	√ (54.4)	C	<i>Bacillus</i> group A
73T	37	<i>Solibacillus sylvestris</i> 95%	KF441704.1	<i>Solibacillus sylvestris</i> 95%	AJ006086	MF039085	X (53.91)	C	<i>Solibacillus</i>
86Li	37	<i>Aneurinibacillus migulanus</i> 96%	NR_113764.1	<i>Aneurinibacillus migulanus</i> 96%	X94195	MF040218	√ (56.82)	C	<i>Brevibacillus</i>
16S	53	<i>Brevibacillus</i> sp. 99%	LN681596.1	<i>Brevibacillus formosus</i> 97%	LDCN01000015	MF038055	√ (54.72)	C	<i>Brevibacillus</i>
36Li	53	<i>Brevibacillus</i> sp. 99%	KM403208.1	<i>Brevibacillus agri</i> 99%	D78454	MF038056	√ (55.54)	C	<i>Brevibacillus</i>
70T	37	<i>Brevibacillus</i> sp. 99%	GQ497292.1	<i>Brevibacillus fluminis</i> 99%	EU375457	MF038057	√ (55.3)	C	<i>Brevibacillus</i>
85Li	37	<i>Brevibacillus formosus</i> 97%	KP165013.1	<i>Brevibacillus brevis</i> 98%	AB271756	MF038058	√ (54.25)	C	<i>Brevibacillus</i>
53M	53 ^S	<i>Brevibacillus</i> sp. 92%	GQ497292.1	<i>Brevibacillus fluminis</i> 89%	EU375457	nd	√ (56.73)	C	nd
75S	37	Uncultured bacterium 94%	KJ013386.1	<i>Kocuria sediminis</i> 92%	JF896464	nd	X (57.43)	C	nd

*Locations of hot springs: S (Siloam), T (Tshipise), M (Mphephu), Li (Libertas) and Le (Lekkerrus); **S) Isolation from sediment. Default: isolation from water; ***X) If outlier within the group or (√) if not using GC percentage grouping. ***ARDRA Amplified rDNA restriction analysis.

general match with the GC% and groupings into the four genera.

Figure 1 shows GC% of the family Bacillaceae including genera *Anoxybacillus* and *Bacillus*, the family Paenibacillaceae including the genera *Aneurinibacillus* and *Brevibacillus*, and unclassified *Bacillales* genus *Solibacillus* where the standard deviations of the two genera within the families did not overlap and were therefore different. Therefore, *Aneurinibacillus* could be distinguished from *Brevibacillus*, and similarly, *Anoxybacillus* could be distinguished from *Bacillus* based on GC%.

Computer-simulated amplified (16S rRNA) ribosomal RNA restriction analysis or ARDRA

In this investigation, ARDRA analysis of the eight restriction enzyme patterns was compiled resulting in an accumulative 148 sites which were aligned and analyzed using SeaView (Gouy et al., 2010) and presented as a distance neighbour-joining dendrogram (Figure 2). The three main

groups (A, B, C) are listed in Table 1. Group A included the *Bacillus* reference type strains determined by Bergey's classification (Ludwig et al., 2009) as well as the closely knit group of *Anoxybacillus* reference strain with *Anoxybacillus* spp. from this study. Three uncultured unknown *Bacillus* spp. previously reported from other hot spring studies (uncultured *Bacillus* clones TPB_GMAT_AC4, DGG30 and KSB12) fell into this group A together with isolate 24M. *Bacillus* spp. from this study fell into both groups B and C, however the *Brevibacillus* spp. and the single *Aneurinibacillus* spp. all fell into group C.

Phylogenetic analysis

Phylogenetic analysis of the 16S rRNA gene is commonly used for bacterial identification with greater accuracy than only a BLAST search as it defines the relationship between individual bacteria at every base. It is also more accurate than ARDRA which only provides information at the site of the restriction enzyme activity. A

comparison of the three molecular tools (16S rRNA BLAST search, GC% and ARDRA) and phylogenetic tree analysis shows that there was, in general, good correlation with the grouping of the phylum *Firmicutes* into family Bacillaceae with genera *Bacillus* (n = 31) and *Anoxybacillus* (n = 8), and family Paenibacillaceae genera *Brevibacillus* (n = 3) and *Aneurinibacillus* (n = 1). A neighbour-joining phylogenetic tree of a 914 bp fragment of the 16S rRNA gene sequences between isolates from this study and representative members of type strains of *Anoxybacillus*, *Bacillus*, *Brevibacillus*, and *Aneurinibacillus* is presented in Appendix C supporting the information presented in Table 1.

In order to further discern whether the *Bacillus* spp. in the study fell into specific Bergey's groupings (Ludwig et al., 2009), a maximum likelihood phylogenetic tree (% bootstrap values based on 1000 replicates) was drawn with additional reference strains as shown in Figure 3. Isolates 18S and 15S grouped with the reference strains (none Bergey's *Bacillus* A group) and this is consistent with their low match by BLAST of 96

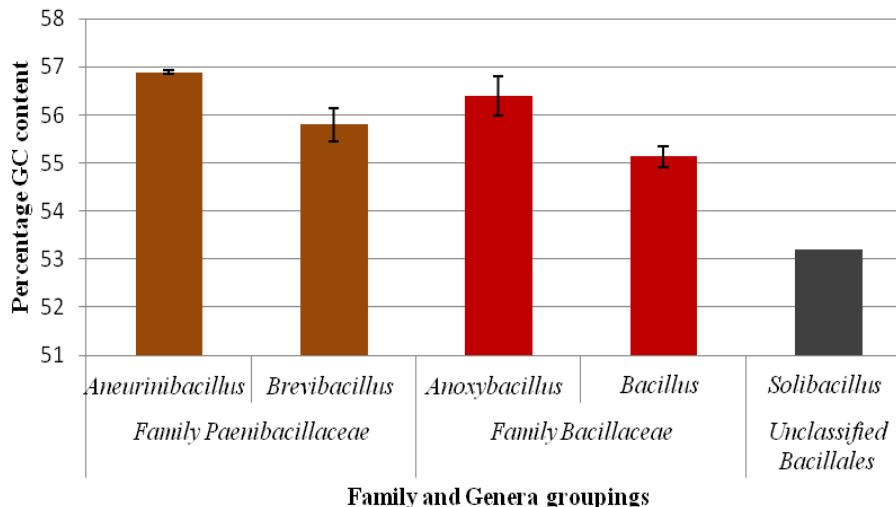


Figure 1. Average GC% of 16S rDNA sequence of isolates from this study and reference type strains of genera *Aneurinibacillus* and *Brevibacillus* (family Paenibacillaceae) and genera *Anoxybacillus* and *Bacillus* (family Bacillaceae) and unclassified genus *Solibacillus* showing that GC% can be used to distinguish between genera.

and 94%, respectively. Isolate 32M was confirmed as closely related to *B. panaciterrae*. Two large groups of isolates contained *B. licheniformis* reference strain, and *B. subtilis* reference strains. Type strains of *Bacillus aerophilous*/*B. pumilus* and *B. methylotrophicus* type strains and the corresponding new isolates from this study (24M and 77S) fell into Bergey's Group A with *B. subtilis*.

Family Paenibacillaceae genus *Brevibacillus*

Paenibacillaceae refers to "nearly *Bacillus*", and phylogenetically, the genus *Brevibacillus* is distinct from the genus *Bacillus* (Xu and Cote, 2003). Four isolates, namely 16S, 36Li, 70T, and 85Li were identified as *Brevibacillus* spp. by BLAST search and confirmed by ARDRA (Figure 2), phylogenetic analysis (Appendix C) and supported by GC% values (Table 1 and Figure 1).

Analysis of unknown isolates

The GC% of four isolates with a <97% BLAST match to published 16S rRNA sequences was significantly different from within their group, that is, isolate 11T within the *Anoxybacillus* group, and isolates 1T, 14S, and 33Li within the *Bacillus* group (Table 1). By ARDRA analysis, the difference was confirmed with 11T that was similar to *Anoxybacillus* spp. by BLAST but fell in ARDRA group C with *Bacillus*/*Brevibacillus* spp. but no further information could be attained with the other three isolates. Therefore, isolates that were not definitively identified by BLAST (<97%) could be further

differentiated by ARDRA. Three isolates (15S, 52M and 73T) were even more poorly matched (<95%) by BLAST.

DISCUSSION

Optimal growth conditions for bacterial isolation and growth

In order to maintain the stock cultures, it was necessary to determine the optimal temperature, pH and salinity conditions for growth. The results showed that most of the bacteria preferred a neutral pH of 7, an incubation temperature ranging between 50 and 55°C, and salinity of 5% NaCl (w/v). The range of temperature was selected because the average temperature of the five hot springs was 52°C. Obeidat et al. (2012) tested eight *Geobacillus* species from hot springs in Jordan with temperatures ranging from 48 to 62°C, and pH between 6 and 7, and found the optimal temperatures to be between 60 and 65°C, and pH 6 to 8. Zhang et al. (2011) reported on two isolates of *Anoxybacillus* with an optimal growth temperature of 55°C and pH of 8. It therefore appears that these spore-forming *Bacillus* and *Bacillus*-related organisms are robust with a tolerance for a wide range of environmental conditions. Extremophiles isolated in this study include the alkaliphilic thermophile *Anoxybacillus flavithermus* with an optimal pH of 10 and a temperature of 50°C (isolate 17S), thermophilic *Anoxybacillus rupiensis* with an optimal temperature of 60°C (isolate 13S), and halotolerant thermophiles of *B. licheniformis* that could grow in 10% (w/v) NaCl (isolates 2T, 6T and 8T)

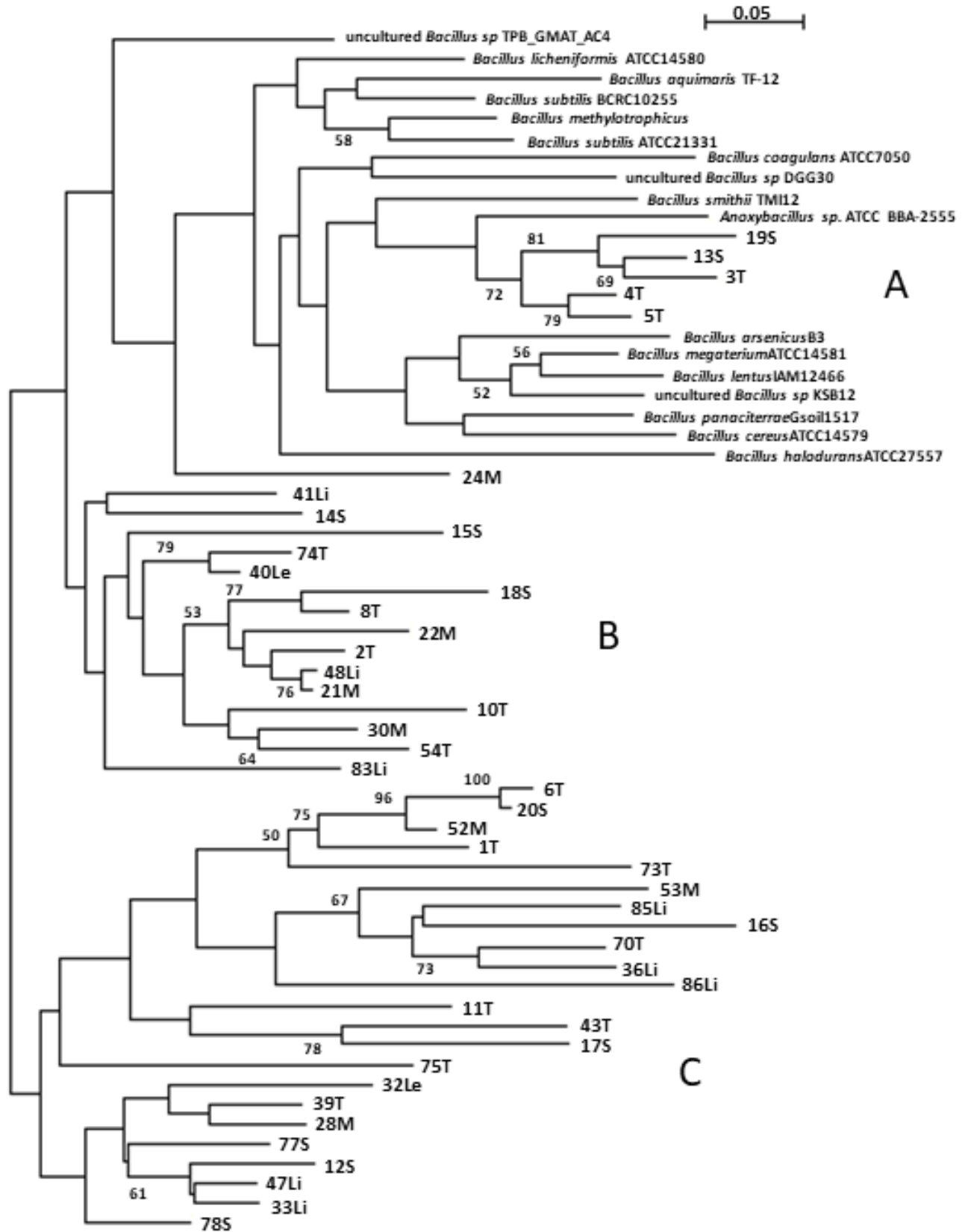


Figure 2. The combined binary data from eight different restriction enzyme digests of the 16S rDNA sequence was used to draw a neighbour-joining distance dendrogram that showed >50% bootstrap of 1000 replicates. Three main clusters (A-C) are indicated in the diagram.

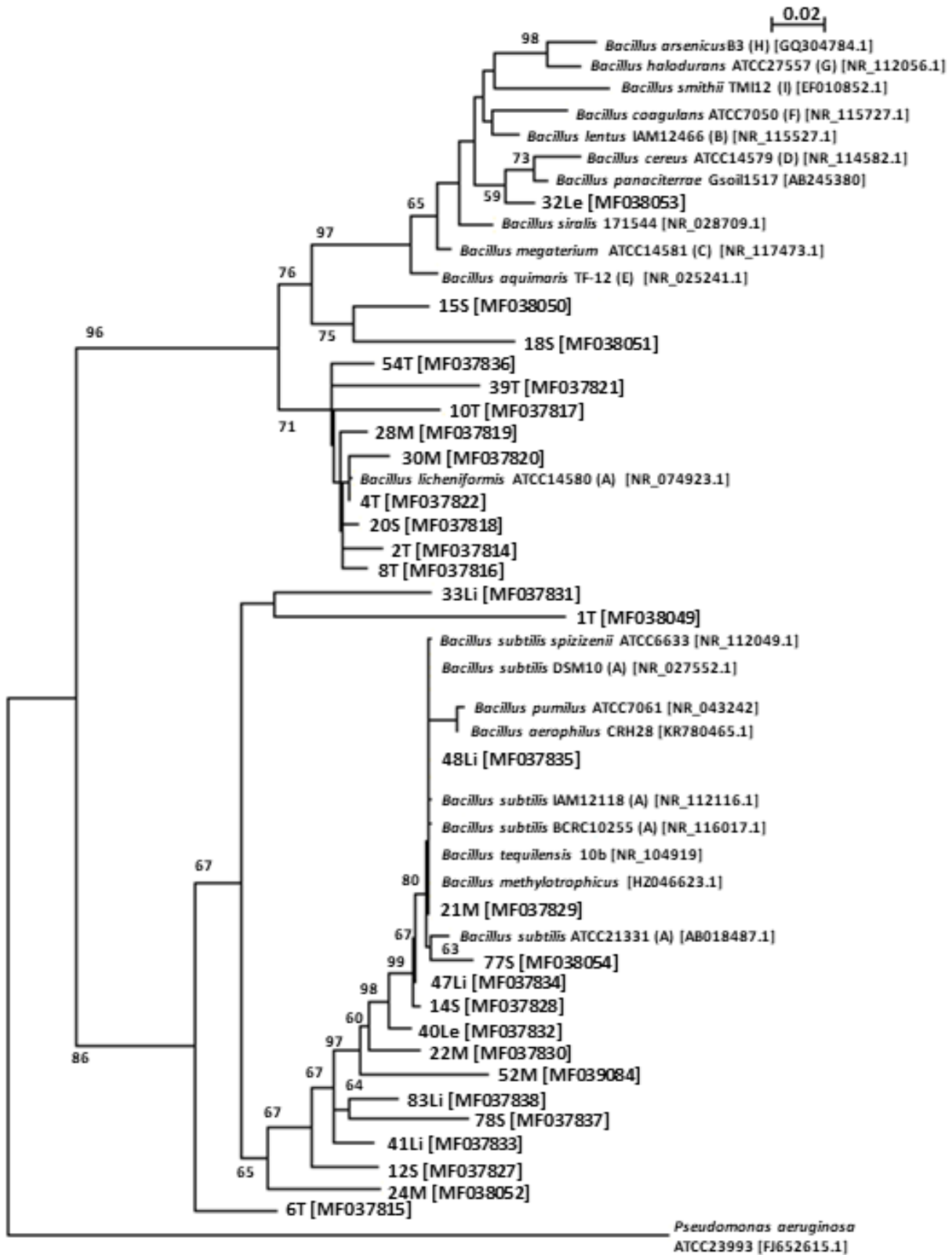


Figure 3. A maximum-likelihood phylogenetic tree of a 914 bp fragment of the 16S rRNA gene sequences of *Bacillus* isolates from this study and representative members of type strains of differently Bergey's groups A-K confirming that most isolates in this study fell into group A (*B. subtilis*/*B. licheniformis*). Bootstrap values (%) are based on 1000 replicates and shown for branches with more than 50% bootstrap support. Bar indicates 0.02 substitutions per 100 nucleotide positions.

(Jardine, 2017).

16S rRNA gene sequencing

Molecular techniques based on genetic sequencing have far surpassed the traditional culture methods to predict biochemical and phenotypic information of a single bacteria or a population. Handelsman (2004) elaborated that culture methods are dependent on environmental and external factors, can be time-consuming, laborious and subject to error. Phenotypic characteristics related to colony morphology, biochemical reactions, serology, pathogenicity and antibiotic resistance can vary considerably, unlike DNA that remains relatively unchanged. The 16S rRNA gene sequence has been used as the gold standard for identification of microorganisms, because it is relatively conserved in all microorganisms, with similarities to allow for PCR with universal primers but enough variability to permit differentiation between species (Rajendhran and Gunasekaran, 2011; Yıldırım et al., 2011). Conventionally, to identify unknown bacterial isolates, the 16S rRNA gene sequences are compared with those in the Genbank database (BLAST), and the closest similarities are listed in Table 1. However, the disadvantage of this tool is that public contributions create the database, and therefore it is possible that unknown sequences may be compared to misidentified or incorrectly named strains. Therefore, to confirm the BLAST results, the sequences were also compared with a more specific 16S rRNA prokaryote gene sequence database using EzTaxon-e (ChunLab USA Inc), a Web-based tool for the identification of prokaryotes (including uncultured prokaryotes). This database is manually curated and quality controlled, and thus less susceptible to be contaminated by false species identifications made by the public and hence, it would be more accurate. For example, in the case of *Bacillus* spp., the results of the BLAST search will not take into account the new reclassification of genera in 2009, and current changes within the group's nomenclature, therefore the results may be erroneous or out of date. Whichever database is used, the cut-off value for the percentage similarity is also critical. Yarza et al. (2014) and López-López et al. (2013) described with statistical proof that >97-98% allows for determination at a species level.

Other investigators have used >97% as a cut-off value (Belduz et al., 2003; Drancourt and Raoult, 2005). When the value is lower than 95%, the result cannot be accurate at the genus or species level.

Percentage guanine-cytosine (GC) content

The GC% of a fragment of DNA or the whole genome refers to the proportion of DNA that is either G-C, but

not A-T, with all the bases present. The G-C bond is stronger than an A-T bond in DNA resulting in a more stable DNA molecule. The GC% of a bacterial genome and the GC% of the stem of the 16S rDNA have been correlated with optimal growth temperatures (Galtier and Lobry, 1997; Wang et al., 2006). Furthermore, the GC% varies among different genera (Muto and Osawa, 1987) which has led to its inclusion as supportive information in the taxonomic classification of bacteria. The GC% of the 16S rRNA gene was included in this study (Figure 1) to establish whether this ratio could be useful in showing which strains were similar and whether it was useful in discriminating between different genera to determine if supportive data could be generated for the discrimination of different bacteria. The results of this study showed that different genera could be distinguished from each other, *Aneurinibacillus* from *Brevibacillus*, and similarly *Anoxybacillus* from *Bacillus* based on GC% providing supportive information but cannot be used in isolation for identification at a genus level.

Computer-simulated amplified ribosomal DNA restriction analysis (ARDRA)

ARDRA is based on the number and size of fragments that are generated when a PCR product of the 16S rRNA gene is digested with a restriction enzyme, and the fragments are separated according to their lengths by agarose gel electrophoresis. The generated pattern can discriminate between species depending on the enzyme used (Rajendhran and Gunasekaran, 2011). The use of computer-simulated fragments is a valid assessment of genotyping (Moyer et al., 1996; Wei et al., 2007; Sklarz et al., 2009), and is even faster and more cost-effective than digesting with the enzyme in the laboratory. In this study, ARDRA was not performed in the laboratory but *in silico*, due to limited resources. Restriction enzymes were selected based on previous investigations of ARDRA on *Bacillus* spp. namely *Rsa1*, *Hinf1* and *HaeIII* (Wahuydi et al., 2010), *Alu1*, *Taq1* and *Rsa1* (Wu et al., 2006), *HaeIII* and *Alu1* (Rai et al., 2015) and *Hph1*, *MbolI* and *Fok1* which resulted in several different fragments when processed by the online tool, Restrictionmapper. In this study, the eight restriction enzymes (*Alu1*, *Taq1*, *HaeIII*, *Hinf1*, *Rsa1*, *Hph1*, *MbolI* and *Fok1*) used independently did not produce informative clustering since each enzyme only revealed information from 15 to 25 sites, and therefore a total of 148 sites from all the restriction enzyme patterns were analysed together. This produced a dendrogram with three main groups (A, B, C) (Figure 2). There was some overlap with the maximum likelihood phylogeny tree (Figure 3) although with a much lower expected resolution. *Anoxybacillus* spp. clustered separately from the *Bacillus* spp. with a convincing

bootstrap value of 72%. *In silico* ARDRA with eight restriction enzymes could not discern the Bergey's groupings of *Bacillus* (A-K) but were useful in demonstration similarities between isolates in this study. This will be discussed further in the identification of isolates that were poorly defined by <95% match with BLAST.

Although the usefulness of ARDRA to cluster related bacteria has been described (Rahmani et al., 2006), including *Bacillus* spp. (Rai et al., 2015) from hot springs (Pagaling et al., 2012), soil environments (Wu et al., 2006; Wahyudi et al., 2010), clinical, dairy and industrial settings (Logan et al., 2002), Sklarz et al. (2009) warned that its prediction power to identify clones should be cautioned. In the latter study of computer generated ARDRA of 48 759 sequences from a ribosomal database, they reported that clones could be separated into different genera, but the clusters did not overlap with phylogenetic analysis of sequence data. This was supported and confirmed by this study. In combination with GC%, BLAST and phylogeny, *in silico* ARDRA is a useful tool for identification of bacteria using 16S rDNA, with phylogenetic analysis providing the most discriminating and accurate information. Because *in silico* findings completely reflected experimental results reported in *Lactobacillus* species (*Firmicutes*) (Oztuk and Meterelliyoz, 2015), and the shortage of publications describing computer generated ARDRA in *Bacillus* spp., the following discussions include comparisons with investigations where ARDRA was not simulated.

Phylogenetic analysis

The family Bacillaceae are distinguished by their ability to form heat tolerant endospores, and as a result, they are abundant, robust and well distributed in many environmental niches, including hot springs. The prototype *B. subtilis* was first described in 1872, and prior to the 1990s, the genus *Bacillus* mainly constituted the family Bacillaceae. However, since then, many significant taxonomic changes have occurred, which has resulted in new genera being described and several species formerly '*Bacillus*' now reclassified into other genera. Consequently, a comparison with older published literature revealed that previously named '*Bacillus*' would appear as other genera in later publications. Mandic-Mulec et al. (2015) reviewed this group in great detail. Also, this family is expanding extremely rapidly with 25 new genera described in 2013 and 2014, and a total of 62 genera listed in 2015. The genus *Bacillus* within the family Bacillaceae is the largest group with 226 species described in 2015, and it is expanding rapidly with 38 new species having been described between August 2013 and March 2015.

Some of these species are represented by only one

isolate making verification challenging and increasing the complexity of *Bacillus* phylogeny. This confusion regarding the phylogeny of the genus *Bacillus* was reported by Maughan and Van der Auwera (2011) who observed that phenotypic groupings are not congruent with 16S rRNA groupings because this group is phenotypically so variable. A comparison of publications on *Bacillus* phylogeny in 1991 (Rössler et al., 1991), in 2003 (Xu and Cote, 2003) and 2009 (Ludwig et al., 2009) confirm the exploding evolutionary changes in this group's nomenclature. As a result, the nomenclature and classification of this group are challenging, and difficult to keep up to date.

Therefore, a literature review of *Bacillus* spp. isolated from hot springs will result in different nomenclature used depending on the date of publication. What may have been previously called *Bacillus* could be named '*Geobacillus*' or '*Paenibacillus*' (meaning 'almost *Bacillus*') in later publications introducing incongruence between different studies. A significant proportion of publications on the identification of *Bacillus* spp. from hot springs rely on only one tool of identification, a comparison of 16S rRNA gene sequence to a public database, that is, BLAST (Ghalib et al., 2014; Obeidat et al., 2012) which has its shortcomings as previously mentioned. However, this study will show that other means of genotyping, such as phylogenetic analysis, can disprove conclusions that are based only on the BLAST tool. The consensus of an identification using the 16S rRNA gene sequence on Genbank BLAST, is >97% match (Yarza et al., 2014), and if studies are not stringent in applying this cut-off value, and merely report bacterial identification based on any genetic similarity, this leads to more 'misidentification' within this group.

Family Bacillaceae genus *Anoxybacillus*

As compared to other groups, the *Anoxybacillus* group is relatively new, having been established in 2000. Cihan et al. (2012) suggested that *Anoxybacillus* is the most dominant genus in hot springs. Twelve of the 15 new species of *Anoxybacillus* listed in Appendix A were isolated from hot springs. Thirty-five of the 53 isolates of *Anoxybacillus* from hot springs in Turkey showed uniquely different patterns with ARDRA compared with 12 type species (Cihan, 2013) providing further evidence that new species of *Anoxybacillus* can be found in hot springs and that differentiation from reference strains is discernible by 16S rRNA phylogeny and ARDRA. A BLAST search confirmed that eight isolates from this study were *Anoxybacillus* spp. including *A. flavithermus* and *A. rupiensis*. The neighbour-joining phylogenetic tree grouped the eight isolates, with convincing bootstrap values (Appendix C).

However, the GC% of isolate 11T differed by more

than one standard deviation from published *Anoxybacillus* spp. data (Appendix B), and the other *Anoxybacillus* isolates from this study. Results from ARDRA analysis confirmed that isolate 11T did indeed group separately from the *Anoxybacillus* cluster A (Figure 2), and requires further investigation.

Family Bacillaceae genus *Bacillus*

The majority of the isolates in this study fell within the genus *Bacillus*, more specifically into Bergey's Group A which includes *B. subtilis* and *B. licheniformis*, two very closely related species (Ludwig et al., 2009). These two species are commonly described as isolates from hot springs in many investigations. In order to ensure that the isolates did not fall into other Bergey's groups not represented in the phylogenetic tree of Appendix C, another phylogenetic tree was drawn with only the *Bacillus* isolates from this study and reference type strains from Bergey's Group B (*Bacillus lentus*), Group C (*Bacillus megaterium*), Group D (*Bacillus cereus*), Group E (*Bacillus aquimaris*), Group F (*Bacillus coagulans*), Group G (*Bacillus halodurans*), Group H (*Bacillus arsenicus*), Group I (*Bacillus smithii*) and Group J (*Bacillus panaciterrae*) (Figure 3). The sequences were obtained from published databases as listed in Appendix A. It confirmed that all the isolates in this study clustered with Group A: *B. subtilis*/*B. licheniformis* by phylogeny and that the single isolate 32Le was found not to be Bergey's Group A *Bacillus* spp. but clustered with *B. panaciterrae* as confirmed by the Genbank BLAST result. Isolate 32Le was not differentiated from the rest of the *Bacillus* reference strains with respect to GC% (Appendix B) and ARDRA clustering did not correlate with the phylogeny tree. *B. panaciterrae* is represented by only one type strain (Gsoil1517) isolated from a ginseng field (Ten et al., 2006), and has not been previously reported as an isolate from a hot spring environment. However, only a tentative conclusion can be made that this is the first report of *B. panaciterrae* being isolated from hot springs because there is only one type strain represented in this group and therefore statistically inconclusive. However, its novelty and difference from the other *Bacillus* isolates need to further investigation.

Another example of the complexity of *Bacillus* identification relates to isolate 24M which, with a BLAST search, convincingly matched (99.85%) with *Bacillus aerophilus* and *Bacillus stratosphericus*. However, these reference strains were isolated (Shivaji et al., 2006) from samples of high altitude atmospheric cryotubes. Recently, Branquinho et al. (2015) suggested that the nomenclature of these be dropped from bacterial systematics as *B. aerophilus* and *B. stratosphericus* were not represented in any type-culture collection and that they should be absorbed into

the group of *B. pumilus*. However, Liu et al. (2015) reported that *B. aerophilus* was actually *Bacillus altitudinis*, and *B. stratosphericus* was a *Proteus* spp. This finding is a prime example where a Genbank BLAST result matches up to a nomenclature that is already dubious and questionable. By maximum likelihood phylogeny the placement of 24M is inconclusive and did not cluster with *B. pumilus*. *B. aerophilus* has not been reported as an isolate of hot springs although *B. pumilus* has (Aanniz et al., 2015). One needs to be aware of the fact that a bacterial isolate that has a 99.85% match to 16S rDNA sequences in databases within the public domain can be a different species.

Family Paenibacillaceae genus *Brevibacillus*

Brevibacillus is generally thought to be mesophilic although in this study isolates 16S and 36Li were isolated at 53°C. Isolation of mesophilic *Brevibacillus* from higher temperatures is typical due to the presence of heat-tolerant spores, and *Brevibacillus* spp. has been reported previously from hot springs (Derekova et al., 2007; Cihan et al., 2012).

Even though there are challenges in precise identification of isolates, the aerobic Gram-positive spore-forming bacteria isolated in this study were similar to those reported in other investigations. Narayan et al. (2008) reported that of 104 isolates from hot springs in Fiji, 58% were *A. flavithermus* and 19% were *B. licheniformis*/*Geobacillus stearothermophilus*. *Anoxybacillus*, *Brevibacillus*, *Geobacillus*, and *Bacillus* made up the 76 isolates cultured from hot springs in Turkey (Derekova et al., 2008). Of 115 isolates, Cihan et al. (2012) listed seven genera in hot springs in Turkey which included *Anoxybacillus*, *Brevibacillus*, *Geobacillus*, and *Paenibacillus*. From hot springs in Morocco, Aanniz et al. (2015) found that 97.5% of 240 isolates were *Bacillus* spp. including *B. licheniformis* (n = 119), *B. subtilis* (n = 6) and *B. pumilus* (n = 3).

Analysis of unknown isolates

Unknown isolates in this investigation (15S, 52M and 73T) and three "unknown *Bacillus*" sequences obtained from Genbank that remain as yet unidentified: clone TPB_GMAT_AC4; Genbank HG327138.1 from hot springs in India); clone KSB12; Genbank JX047075.1 from Indonesia and clone DGG30; Genbank AY082370.1 from China were included in the analysis.

Isolate 15S matched with *B. licheniformis* using BLAST and GC%, but not with phylogenetic analysis where it clustered with 18S supported by a 75% bootstrap value suggesting that it was possibly not a "group A type *Bacillus*". Similarly, isolate 52M also

matched to *Brevibacillus* spp. using BLAST, but did not phylogenetically or by GC% group with the *Brevibacillus* reference strain (Appendix C). ARDRA results suggested it was associated with Bergey's group A *Bacillus* and not *Brevibacillus*. Had the identification of both isolates 15S and 52M exclusively relied on BLAST results, the outcomes could be mis-identification. *Solibacillus*, an undefined member of the family Bacillaceae was included in this study because isolate 73T was similar using BLAST. Its "different" status was confirmed by a lower GC% of 53.91%, but ARDRA did not add any further discerning information. Neighbour-joining phylogenetic analysis placed isolate 73T with *Aneurinibacillus* spp. (Appendix C) and therefore this isolate could not be assigned to any genus with any degree of certainty.

Clone DGG30 from China, had a GC% of 53.2% similar to *Solibacillus* and a standard deviation different to that of *Bacillus*, *Anoxybacillus* and *Brevibacillus*. It clustered with the ARDRA group A suggesting it was not related to Bergey's group A *Bacillus* spp. Clone KSB12 from India was confirmed and grouped with *Bacillus* spp. by GC%; and by ARDRA, clustered with *B. megaterium* and *B. lentus* with a 56% bootstrap value suggesting that it could be related to Bergey's *Bacillus* group B or C. Indonesian isolate Clone TPB_GMAT_AC4 was confirmed to be *Bacillus* spp. by GC% but no further resolution could be obtained about its identification. Thus, published data from 16S rDNA sequences obtained from "uncultured bacteria" can be analyzed retrospectively using a combination of tools.

In conclusion, 43 isolates from Limpopo hot springs were cultured, and by comparison with 16S rDNA sequences in public databases and phylogenetic analysis, grouped into four genera: *Anoxybacillus*, *Bacillus*, *Brevibacillus*, and *Aneurinibacillus*. More specifically, the following species were identified: *A. flavithermus*, *A. rupiensis*, *B. subtilis* and *B. licheniformis*. Singular *Bacillus* spp. that are phylogenetically related to *B. panaciterrae*, *B. pumilus* and *B. methylotrophicus* were also identified; however, these three isolates require further characterization. All, except *B. panaciterrae* have been previously isolated from hot-spring environments. However, when the 16S rRNA gene sequences were analyzed by simulated computer-generated ARDRA using a collection of eight different restriction enzymes, additional discernment of individuals was possible. Therefore, this study shows that the use of a single molecular tool may result in a misrepresentation of *Bacillus* and *Bacillus*-related identification and that, when possible, a combination of tools should be used.

The complexity and problems regarding the *Bacillus* phylogeny were discussed. Only a small portion of the microbial diversity present in hot springs can be cultured, compared with the more comprehensive assessment of microbial diversity obtained using the metagenomic approach. Improved isolation rates could

include the use of different media and different incubation conditions. Three different types of extremophiles with different properties (alkaliphilic, thermophilic and halophilic) as well as three unknowns were isolated, suggests that hot-spring water is a resource for potentially important bacteria useful in biotechnology and as a supply of novel bacteria. Hot springs sites need to be protected, conserved and maintained as valuable indigenous and pristine natural resources.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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APPENDIXES

Appendix A. List of GenBank accession numbers for 16S rRNA sequence data of reference strains and unknown isolates used in this study.

Strain or isolate	Size (bp)	GenBank or NCBI accession number
<i>Aneurinibacillus</i> sp. U33	1451	GenBank: KJ725179.1
<i>Aneurinibacillus danicus</i> NBRB102444	1501	GenBank: AB681786.1
<i>Aneurinibacillus migulanus</i> strain ATCC 9999	1422	NR_115593
<i>Aneurinibacillus tyrosinisolvens</i>	1458	GenBank: AB899818.1
<i>Anoxybacillus</i> sp. ATCC BBA-2555	1548	GenBank: KJ722458.1
<i>Anoxybacillus bogrovensis</i> strain NBIMCC 8427=DSM 17956	1341	NCBI Reference Sequence: NR_115021.1
<i>Anoxybacillus eryuanensis</i> strain E-112	1519	NCBI Reference Sequence: NR_117229.1
<i>Anoxybacillus flavithermus</i> strain WK1	1524	NCBI Reference Sequence: NR_074667.1
<i>Anoxybacillus flavithermus</i> subsp. <i>yunnanensis</i> strain E13	1449	NCBI Reference Sequence: NR_117774.1
<i>Anoxybacillus gonensis</i> strain G2	1382	NCBI Reference Sequence: NR_025667.1
<i>Anoxybacillus kaynarcensis</i> strain D1021	1430	NCBI Reference Sequence: NR_108265.1
<i>Anoxybacillus kestanbolinensis</i> strain K4	1376	GenBank: AY248711.1
<i>Anoxybacillus pushchinoensis</i> strain k-1	1338	NCBI Reference Sequence: NR_037100.1
<i>Anoxybacillus rupiensis</i> strain TSSC-4	1503	GenBank: KC759325.1
<i>Anoxybacillus rupiensis</i> strain TS_04	1463	GenBank: KJ842629.1
<i>Anoxybacillus rupiensis</i> strain TS_01	1463	GenBank: KJ842627.1
<i>Anoxybacillus rupiensis</i> strain A3	1428	GenBank: KC310454.1
<i>Anoxybacillus rupiensis</i> strain JF82	1463	GenBank: KF254911.1
<i>Anoxybacillus rupiensis</i> strain JF83	1510	GenBank: KC849452.1
<i>Anoxybacillus rupiensis</i> strain FZWP-10	1319	GenBank: JX914493.1
<i>Anoxybacillus salavatliensis</i> strain A343	1397	NCBI Reference Sequence: NR_104492.1
<i>Anoxybacillus tengchongensis</i> strain T-11	1519	GenBank: FJ438370.1
<i>Anoxybacillus tepidamans</i> strain R-35643	1507	NCBI Reference Sequence: NR_116985.1
<i>Anoxybacillus thermarum</i> strain DSM 17141	1358	NCBI Reference Sequence: NR_118117.1
<i>Anoxybacillus vitaminiphilus</i> strain 3nP4	1511	NCBI Reference Sequence: NR_108379.1
<i>Anoxybacillus voinovskiensis</i> strain TH13	1506	NCBI Reference Sequence: NR_024818.1
<i>Bacillus aerophilus</i> strain CRh28	1345	GenBank: KR780465.1
<i>Bacillus aquimaris</i> strain TF-12 (E)	1507	NCBI Reference Sequence: NR_025241.1
<i>Bacillus arsenicus</i> strain B3 (H)	1515	GenBank: GQ304784.1
<i>Bacillus cereus</i> strain ATCC 14579 (D)	1482	NCBI Reference Sequence: NR_114582.1
<i>Bacillus coagulans</i> strain ATCC 7050 (F)	1549	NCBI Reference Sequence: NR_115727.1
<i>Bacillus halodurans</i> strain ATCC 27557 (G)	1508	NCBI Reference Sequence: NR_112056.1
<i>Bacillus lentus</i> strain IAM 12466 (B)	1486	NCBI Reference Sequence: NR_115527.1
<i>Bacillus licheniformis</i> strain ATCC 14580	1545	NCBI Reference Sequence: NR_074923.1
<i>Bacillus megaterium</i> strain ATCC 14581 (C)	1495	NCBI Reference Sequence: NR_117473.1
<i>Bacillus methylotrophicus</i>	1510	GenBank: HZ046623.1
<i>Bacillus panaciterrae</i> Gsoil1517	1476	AC AB245380;
<i>Bacillus pumilus</i> strain ATCC 7061	1434	ACCESSION NR_043242
<i>Bacillus siamensis</i> strain IHB B 16121	1516	GenBank: KM817270.1
<i>Bacillus siralis</i> strain 171544	1430	NCBI Reference Sequence: NR_028709.1
<i>Bacillus smithii</i> strain TBMI12 (I)	1453	GenBank: EF010852.1
<i>Bacillus subtilis</i> ATCC 21331	1504	GenBank: AB018487.1
<i>Bacillus subtilis</i> subsp. <i>spizizenii</i> strain ATCC 6633	1507	NCBI Reference Sequence: NR_112049.1
<i>Bacillus subtilis</i> strain BCRC 10255	1468	NCBI Reference Sequence: NR_116017.1
<i>Bacillus subtilis</i> strain IAM 12118	1553	NCBI Reference Sequence: NR_112116.1
<i>Bacillus subtilis</i> strain JCM 1465	1472	NCBI Reference Sequence: NR_113265.1
<i>Bacillus subtilis</i> strain DSM 10	1517	NCBI Reference Sequence: NR_027552.1
<i>Bacillus tequilensis</i> strain 10b	1456	ACCESSION NR_104919

Appendix A. Contd.

<i>Brevibacillus agri</i> strain DSM 6348	1487	NCBI Reference Sequence: NR_040983.1
<i>Brevibacillus fluminis</i> strain CJ71	1413	GenBank: EU375457.1
<i>Brevibacillus panacihumi</i> strain DCY35	1473	GenBank: EU383033.1
<i>Pseudomonas aeruginosa</i> strain ATCC 23993	1458	GenBank: FJ652615.1
<i>Solibacillus sylvestris</i> HR3-23	1528	PUBMED; 10319505.
Uncultured <i>Bacillus</i> sp. clone KSB12	1504	GenBank: JX047075.1
Uncultured <i>Bacillus</i> sp. clone TPB_GMAT_AC4	1278	GenBank: HG327138.1
Uncultured <i>Bacillus</i> sp. clone DGG30	1569	GenBank: AY082370.1

The size of the 16S rRNA sequence in base pairs (bp) is also indicated.

Appendix B. Comparison of GC content (%) for the 16S rDNA sequences for reference type strains obtained from GenBank, and isolates in this study.

Isolate	Fragment size	GC content (%)	Genus	Average	SD
<i>Anoxybacillus</i> sp. ATCC BBA-2555	1548	56.65	-	-	-
<i>Anoxybacillus bogrovensis</i> strain NBIMCC 8427=DSM 17956	1341	55.78	-	-	-
<i>Anoxybacillus eryuanensis</i> strain E-112	1519	56.09	-	-	-
<i>Anoxybacillus gonensis</i> strain G2	1382	56.58	-	-	-
<i>Anoxybacillus kaynarcensis</i> strain D1021	1430	55.94	-	-	-
<i>Anoxybacillus kestanbolinensis</i> strain K4	1376	55.6	-	-	-
<i>Anoxybacillus pushchinoensis</i> strain k-1	1338	55.9	-	-	-
<i>Anoxybacillus tengchongensis</i> strain T-11	1519	56.48	-	-	-
<i>Anoxybacillus thermarum</i> strain DSM 17141	1358	56.55	-	-	-
<i>Anoxybacillus vitaminiphilus</i> strain 3nP4	1511	56.78	-	-	-
<i>Anoxybacillus voinovskiensis</i> strain TH13	1506	55.71	-	-	-
<i>Anoxybacillus tepidamans</i> strain R-35643	1507	56.67	-	-	-
<i>Anoxybacillus salavatliensis</i> strain A343	1397	56.4	-	-	-
<i>Anoxybacillus flavithermus</i> WK1 strain WK1	1524	56.56	-	-	-
<i>Anoxybacillus flavithermus</i> subsp. <i>yunnanensis</i> strain E13	1449	56.73	-	-	-
<i>Anoxybacillus rupiensis</i> strain TSSC-4	1503	56.95	-	-	-
<i>Anoxybacillus rupiensis</i> strain TS_04	1463	56.93	-	-	-
<i>Anoxybacillus rupiensis</i> strain TS_01	1463	56.73	-	-	-
<i>Anoxybacillus rupiensis</i> strain A3	1428	56.09	-	-	-
<i>Anoxybacillus rupiensis</i> strain JF82	1463	56.57	-	-	-
<i>Anoxybacillus rupiensis</i> strain JF83	1510	56.76	-	-	-
<i>Anoxybacillus rupiensis</i> strain FZWP-10	1319	56.33	-	56.40	0.41
11T *	1060	55.28	<i>Anoxybacillus</i>	-	-
4T	1407	56.15	<i>Anoxybacillus</i>	-	-
17S	1407	56.22	<i>Anoxybacillus</i>	-	-
3T	1394	56.24	<i>Anoxybacillus</i>	-	-
43T	1390	56.26	<i>Anoxybacillus</i>	-	-
19S	1406	56.33	<i>Anoxybacillus</i>	-	-
13S	1398	56.51	<i>Anoxybacillus</i>	-	-
7T	1421	56.72	<i>Anoxybacillus</i>	56.21	0.50
75S	1393	57.43	Unknown	-	-
<i>Bacillus subtilis</i> ATCC 21331	1504	55.12	-	-	-
<i>Bacillus licheniformis</i> strain ATCC 14580	1545	55.53	-	-	-
<i>Bacillus tequilensis</i> strain 10b	1456	55.22	-	-	-
<i>Bacillus subtilis</i> subsp. <i>spizizenii</i> strain ATCC 6633	1507	55.14	-	-	-
<i>Bacillus subtilis</i> strain BCRC 10255	1468	55.12	-	-	-

Appendix b. Contd.

<i>Bacillus subtilis</i> strain IAM 12118	1553	55.05	-	-	-
<i>Bacillus subtilis</i> strain JCM 1465	1472	55.23	-	-	-
<i>Bacillus subtilis</i> strain DSM 10	1517	55.31	-	-	-
<i>Bacillus pumilus</i> strain ATCC 7061	1434	55.16	-	-	-
<i>Bacillus panaciterrae</i> Gsoil1517	1476	54.74	-	-	-
<i>Bacillus methylotrophicus</i>	1510	55.36	-	-	-
<i>Bacillus siamensis</i> strain IHB B 16121	1516	55.01	-	-	-
<i>Bacillus soralis</i> strain 171544	1430	54.72	-	55.13	0.22
1T *	1417	52.86	<i>Bacillus</i>	-	-
33Li *	1079	54.21	<i>Bacillus</i>	-	-
14S*	1370	54.23	<i>Bacillus</i>	-	-
78S	1001	54.45	<i>Bacillus</i>	-	-
12S	1083	54.48	<i>Bacillus</i>	-	-
39T	1060	54.52	<i>Bacillus</i>	-	-
47Li	1069	54.53	<i>Bacillus</i>	-	-
40Le	1369	54.57	<i>Bacillus</i>	-	-
41Li	1386	54.69	<i>Bacillus</i>	-	-
54T	1379	54.69	<i>Bacillus</i>	-	-
18S	1411	54.71	<i>Bacillus</i>	-	-
28M	1065	54.84	<i>Bacillus</i>	-	-
48Li	1409	54.86	<i>Bacillus</i>	-	-
74T	1388	54.97	<i>Bacillus</i>	-	-
10T	1402	55	<i>Bacillus</i>	-	-
21M	1408	55.04	<i>Bacillus</i>	-	-
8T	1406	55.05	<i>Bacillus</i>	-	-
15S	1379	55.11	<i>Bacillus</i>	-	-
30M	1358	55.15	<i>Bacillus</i>	-	-
2T	1413	55.4	<i>Bacillus</i>	-	-
6T	1380	55.43	<i>Bacillus</i>	-	-
83Li	1396	55.44	<i>Bacillus</i>	54.75	0.54
24M	1303	54.8	<i>Bacillus</i>	-	-
32Le	918	54.36	<i>Bacillus</i>	-	-
77S	1057	54.4	<i>Bacillus</i>	-	-
Uncultured <i>Bacillus</i> sp. clone KSB12	1504	55.053	Unknown	-	-
Uncultured <i>Bacillus</i> sp. clone TPB_GMAT_AC4	1278	54.695	Unknown	-	-
<i>Brevibacillus agri</i> strain DSM 6348	1487	55.68	-	-	-
<i>Brevibacillus fluminis</i> strain CJ71	1413	55.56	-	-	-
<i>Brevibacillus panacihumi</i> strain DCY35	1473	56.2	-	55.8	0.34
16S	1398	54.72	-	-	-
36Li	1399	55.54	-	-	-
52M	1396	55.09	Unknown	-	-
70T	1396	55.3	-	-	-
85Li	1375	54.25	-	54.95	0.58
<i>Aneurinibacillus tyrosinisolvens</i>	1458	56.86	-	-	-
<i>Aneurinibacillus</i> sp. U33	1451	56.92	-	-	-
<i>Aneurinibacillus danicus</i> NBRB102444	1501	56.89	-	56.89	0.03
86Li	1392	56.82	-	56.82	-
53M	1389	56.73	Unknown	-	-
<i>Solibacillus sylvestris</i> HR3-23	1528	53.2	-	53.2	-
Uncultured <i>Bacillus</i> sp. clone DGG30	1569	53.218	Unknown	-	-
73T	1408	53.91	Unknown	-	-

(*) denotes isolates that grouped into a different ARDRA cluster, not *Anoxybacillus* and *Bacillus*.

APPENDIX C



Figure. A neighbour-joining phylogenetic tree of a 914 bp fragment of the 16s rRNA gene sequences between isolates from this study and representative members of type strains of *Anoxybacillus*, *Bacillus*, *Brevibacillus*, and *Aneurinibacillus*. Bootstrap values (%) are based on 100 replicates and shown for branches with more than 50% bootstrap support. Bar indicates 0.02 substitutions per 100 nucleotide positions.