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Subterranean mammals: reservoirs of infection or overlooked sentinels of anthropogenic environmental soiling?

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Abstract

Global reports of emergent pathogens in humans have intensified efforts to identify wildlife reservoirs. Subterranean mammals, such as bathyergid mole-rats, are largely overlooked, despite their high-level exposure to soil-dwelling microbes. Initial assessment of bathyergid reservoir potential, was determined using a broad-range 16S rRNA PCR approach, which revealed an 83% PCR-positivity for the 234 bathyergid lung samples evaluated. The presence of the *Bacillus cereus* complex, a ubiquitous bacterial assemblage, containing pathogenic and zoonotic species, was confirmed through nucleotide sequencing, prior to group- and species-specific PCR-sequencing. The latter allowed for enhanced placement and prevalence estimations of *Bacillus* in four bathyergid species sampled across a range of transformed landscapes in the Western Cape Province, South Africa. Two novel *Bacillus* strains (1 and 2) identified on the basis of the concatenated 16S rRNA-*gro*EL-*yea*C dataset (2066 nucleotides in length), clustered with *B. mycoides* (ATCC 6462) and *B. weihenstephanensis* (WSBC 10204), within a well-supported monophyletic lineage. The levels of co-infection, evaluated with a *gro*EL strain-specific assay, developed specifically for this purpose, were high (71%). The overall *Bacillus* presence of 17.95% (ranging from 0% for *Georychus capensis* to 45.35% for *Bathyergus suillus*) differed significantly between host species ($\chi^2 = 69.643$; df = 3; $p < 0.05$), being significantly higher in bathyergids sampled near an urban informal settlement ($\chi^2 = 70.245$; df = 3; $p < 0.05$).The results highlight the sentinel potential of soil-dwelling mammals for monitoring anthropogenically-introduced, opportunistic pathogens and the threats they pose to vulnerable communities, particularly in the developing world.

Keywords: Anthropogenic activities, Urban wildlife, Developing countries, Bathyergids, *Bacillus cereus*

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Introduction and purpose

African mole rats (family Bathyergidae) are subterranean rodents that build extensive burrow systems and use roots and tubers as food sources (Berg et al. 2005; Hubálek et al. 2005; Thomas et al. 2009). This role as underground ecosystem engineers, in which a colony of 16, with a total biomass of just 2.2 kg is capable of moving 2.6 metric tons of soil, within a two-week period (Jarvis et al. 1998), underscores the high-level exposure to a broad range of soil-dwelling microbes attained by these subterranean mammals. Thus, opportunistic pathogens occurring naturally in soil or introduced intentionally (through practices such as bioremediation), or accidentally (through the pollution of soil systems, (Baumgardner 2012; Duran Alvarez and Jimenez Cisneros 2014), will become concentrated within bathyergid lungs. In pristine environments, with limited human impact, the microbe-bathyergid associations in the subterranean environment are likely co-evolved and ancient, whereas, in human transformed areas, newly-established microbe-bathyergid interactions likely reflect as high-level infections in these naïve vertebrates.

Microorganisms are significantly influenced by their environment, with many emergent pathogens belonging to soil-associated genera, forming bivalent interactions, growing both in the rhizosphere and opportunistically infecting animal and human hosts (Wilson and Slayers 2003; Berg et al. 2005; Aujoulat et al. 2012). Bathyergids live in close association with the rhizosphere, a nutrient-rich microenvironment connecting sole and plant roots, which is considered a “hotspot” for the evolution of opportunistic bacterial pathogens, because it creates a microclimate where competition, infection and predation between bacteria, fungi, archaea, viruses and protozoa is intense (Berg et al. 2005; Aujoulat et al. 2012; Baumgardner 2012). Selection processes acting in this environment can predispose bacterial species to become pathogenic should they encounter humans or other animals (Berg et al. 2005; Aujoulat et al. 2012).

The recent surge in opportunistic pathogens affecting immune-compromised patients, made more susceptible due to diseases such as HIV, cystic fibrosis or healthcare-associated infections, has raised public health concerns worldwide (Berg et al. 2005; Aujoulat et al. 2012). Up to 30% of people living in and around Cape Town in the Western Cape Province of South Africa are HIV-positive (Kenyon et al. 2013), the majority living in resource-poor settings and relying on less conventional sources of protein, including capture and consumption of Cape dune mole-rats, *Bathyergus suillus* (De Graaff, 1981). It is increasingly recognised that immune-compromised individuals are highly susceptible to pathogenic and innocuous microorganisms, making rapid identification of these organisms important (Comer 2001; Berg et al. 2005; Kenyon et al. 2013).

Concern about the presence of potentially pathogenic bacterial genera present within natural water systems in the Western Cape Province of South Africa was raised in 2012 when 16S rRNA characterisation of the bacterial community structure in the Berg and Plankenburg rivers confirmed the presence of potentially pathogenic *Bacillus cereus* complex (Paulse et al. 2012). As the Berg water system serves as a water resource for various industrial, agricultural, domestic and municipal activities, wastewater contamination would result in spillover of opportunistic pathogens into the surrounding soil systems (Jackson et al. 2009; Paulse et al. 2012; Duran Alvarez and Jimenez Cisneros 2014).

The *Bacillus cereus* complex is a ubiquitous, highly homogeneous assemblage of gram-positive, spore-forming, soil-borne bacterial species of contentious taxonomy (Jensen et al. 2003; Thorsen et al. 2006; Tourasse et al. 2006; Didelot et al. 2009). To date, nine species of medical and economic concern have been recognised, *viz. B. cereus, B. anthracis, B. thuringiensis, B. bombysepticus, B. cytotoxicus, B. toyonensis, B. weihenstephanensis, B. mycoides* and *B. pseudomycoides* (Jensen et al. 2003; Thorsen et al. 2006; Tourasse et al. 2006; Guinebretière et al. 2013; Jiménez et al. 2013; Cheng et al. 2014). Of these, three are of public health concern: (i) *Bacillus cereus*, causing emetic and diuretic food poisoning in humans, is a common soil inhabitant and a well-known food-spoiling organism (Didelot et al. 2009), (ii) *Bacillus anthracis* infects a wide range of mammalian hosts, including humans, causing anthrax, an often fatal haemorrhagic fever and potential bioterrorism agent (Fasanella et al. 2010) and (iii) *Bacillus cytotoxicus*, a thermotolerant species that produces an enterotoxin, known as cytokine K, which results in severe diarrhoeic food-poisoning (Tourasse et al. 2011; Guinebretière et al. 2013).

Owing to the high levels of genetic similarity between members of the *B. cereus* complex, a wide range of targets, including plasmid encoded gene regions, such as pXO1 and pXO2 and chromosomal markers, have been reported (Supplementary table 1S). However, all fail to definitively distinguish between species belonging to this monophyletic cluster (Bavykin et al. 2004; Tourasse et al. 2006; Park et al. 2007; Kim et al. 2008; Didelot et al. 2009; Rao et al. 2010; Irenge et al. 2010; Ahmod et al. 2011; Dzieciol et al. 2013). However, it is possible that by using a combination of gene targets, that are more informative than the 16S rRNA gene, that it may be possible to achieve improved phylogenetic placement of *Bacillus* strains present in environmental and clinical specimens. For this reason we selected three gene markers from two published reports for this study, *viz.* a multiplex PCR approach targeting the heat shock operon (*gro*EL) and the gyrase B (*gyr*B) gene regions (Park et al. 2007), and the *yea*C region that is capable of accurately distinguishing *B. anthracis* strains from other *B. cereus* group species (Ahmod et al. 2011).

A reservoir of infection is defined as "one or more epidemiologically connected populations or environments in which the pathogen can be permanently maintained and from which infection is transmitted to the defined population" (Haydon et al. 2002). In the same vein, sentinel surveillance acknowledges that populations of animals can be epidemiologically connected and uses this knowledge for cost-effective disease detection (Halliday et al. 2007). Crucially sentinel surveillance acts as an early-warning system and, in contrast to merely identifying a potential reservoir species, can also provide important information of potential environmental health hazards (Halliday et al. 2007). As underground engineers that live in a range of human-transformed landscapes, bathyergids potentially play a largely overlooked role as reservoirs of infection and / or sentinels for the degree of microbial soiling of environments, through the activities of man. We investigated this potential by screening DNA extracts prepared from lung samples of 234 bathyergids sampled across a range of landscapes in the Western Cape Province of South Africa, initially with a broad-range 16S rRNA approach, and subsequently with two *Bacillus cereus* group-specific and one species-specific assay.

Methods

Study sites, samples and bacterial 16S rRNA characterisation

Lung samples from four mole rat species, belonging to the family Bathyergidae, collected with the required permits and ethical clearance, were evaluated in this study (Table 1). The samples, which were all bi-products from prior studies, comprised of 86 *Bathyergus suillus* (captured as part of a long-term pest control problem), 53 *Georychus capensis*, 45 *Cryptomys hottentotus hottentotus* (Archer et al. 2014) and 50 *Fukomys damarensis* (Streicher et al. 2011). Following euthanasia, individual animals were dissected in a BSL2 cabinet, and lung samples were aseptically collected, and placed in individual, sterilised tubes. DNA extracts were prepared using the Roche High Pure DNA extraction kit (Roche Diagnostics GmbH, Mannheim, Germany) and the manufacturer's prescribed protocol for mammalian tissue. Broad-range PCR amplification with universal 16S rRNA bacterial primers 27F and 1492R (Edwards et al. 1989; Reysenbach et al. 1992; primer set A in Table 2) which yield products of ~1.4 kbp were purified using the Roche High Pure PCR product purification kit (Roche Diagnostics GmbH, Mannheim, Germany). Sanger cycle-sequencing was performed with BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, California), with each of the primers in separate reactions, at an annealing temperature of 56°C.

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TABLE 1 Weight, distribution, soil preference, digging behaviour and sampling information of the four bathyergids species screened for *Bacillus* genome presence

	Bathyergid species			
	Bathyergus suillus	*Georychus capensis*	*Cryptomys hottentotus hottentotus*	*Fukomys damarensis*
Average weight	504-1291g	44-287g	27-84g	131g
Distribution	Endemic to Cape floristic region	South Africa, Namibia, Botswana and Zimbabwe	Restricted to South Africa	Endemic to the western parts of southern Africa (including parts of South Africa and Zambia)
Soil preference	Sandy soils	Loam/clay soils	Loam/clay soils	Loose, arid soils
Sample size	86	53	45	50
Sample site/s	Cape Town airport, Western Cape	Darling, Western Cape	Darling, Western Cape (6); Kamieskroon, Northern Cape (39)	Hotazel, Northern Cape
Sampling Co-ordinates	33°58'10''S 18°35'50''E	33°25'S 18°25'E	33°25'S 18°25'E (Darling) 30°13'S 17°57'E (Kamieskroon)	27°17'S 22°58'E
Habitat type of sampling area	Peri-urban	Semi-natural	Semi-natural	Natural
Sociality	Solitary	Solitary	Social	Eusocial
Evidence of above ground activity	Yes	Yes	Yes	Rare
Average tunnel depth	40-65cm	3-9cm	35cm	21-48cm
Deepest tunnel depth	>2m	N/A	58cm	2.4m
Ethical clearance	AUCC 040702-015	EC118-13	EC005-11	AO27/06

Sources: De Graaff 1981; Skinner and Smithers 1981; Davies and Jarvis 1986; Jarvis and Bennett 1993; Roper et al. 2001; Bennett 2011; Robb et al. 2012; Lövy *et al*. 2014

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TABLE 2 Summary of primers and the optimised reaction conditions used in this study

Reaction code	Primer set used (from 5' to 3')	Genome region targeted	Reference	Primer concentration used	Expected ampilcon size (bp)	Ta used in study
A[1]	27F: AGA GTT TGA TCC TGG CTC AG (F) 1492R: GGC TAC CTT GTT ACG ACT T (R)	16S rRNA	Edwards et al. 1989 Reysenbach et al. 1992	0.4 µM	~1450	56°C
B[2]	BSU063330_F: BIO TTT ACC AGA AGC HCA GCT GC (F) BSU06330_R: GCT AAA AAT TTA ACA TCG TCT GGA (R)	*yea*C	Ahmod et al. 2011 Ahmod et al. 2011	0.4 µM	282-354	55°C
C[3]	BCGSH-1F: GTG CGA ACC CAA TGG GTC TTC (F) BCGSH-1R: CCT TGT TGT ACC ACT TGC TC (R)	*gro*EL	Park et al. 2007 Park et al. 2007	0.5 µM	400	63°C
D[4]	BASH-2F: GGT AGA TTA GCA GAT TGC TCT TCA AAA GA (F) BASH-2R: ACG AGC TTT CTC AAT ATC AAA ATC TCC GC (R)	*gyr*B	Park et al. 2007 Park et al. 2007	0.5 µM	253	63°C
E[4]	BCJH-F: TCA TGA AGA GCC TGT GTA CG (F) BCJH-1R: CGA CGT GTC AAT TCA CGC GC (R)	*gyr*B	Park et al. 2007 Park et al. 2007	0.5 µM	475	63°C
F[5]	BTJH-1F: GCT TAC CAG GGA AAT TGG CAG (F) BTJH-R: ATC AAC GTC GGC GTC GG (R)	*gyr*B	Park et al. 2007 Park et al. 2007	1 µM	299	63°C
G[6]	BMSH-F: TTT TAA GAC TGC TCT AAC ACG TGT AAT (F) BMSH-R: TTC AAT AGC AAA ATC CCC ACC AAT (R)	*gyr*B	Park et al. 2007 Park et al. 2007	0.5 µM	604	63°C
H_1[7]	BsGroEL-F1: CAA GTA GCT GCT ATT TCT GCA (F) BASH-2R: ACG AGC TTT CTC AAT ATC AAA ATC TCC GC (R)	*gro*EL	This study Park et al. 2007	0.4 µM	~260bp	62°C
H_2[7]	BsGroEL-F2: CAA GTA GCT GCT ATT TCT TCG (F) BASH-2R: ACG AGC TTT CTC AAT ATC AAA ATC TCC GC (R)	*gro*EL	This study Park et al. 2007	0.4 µM	~260bp	62°C

Ta : Annealing temperature, [1] Amplifies broad range of bacterial species; [2] Preferentially amplifies *B. cereus* group strains (~350bp), but also targets host genome; [3] Preferentially amplifies *B. cereus* group strains (~400bp), but can result in non-specific amplification; [4] Failed to amplify *B. cereus* group strains in bathyergids; [5] Produces multiple amplicon bands either forming part of host genome or resulting in mixed sequences; [6] Amplifies *B. mycoides* and *B. weihenstephanensis* strains present in bathyergids, [7] Targets specific *B. cereus* complex strains identified in bathyergids.

Evaluation and optimisation of *Bacillus* assays**:**

Bacillus genome detection capabilities of two primer sets (B-C, Table 2) identified through literature review (Table 1S; Park et al. 2007; Ahmod et al. 2011) were evaluated using 19 mole-rat lung extracts, confirmed to be positive for *B. cereus* group species through broad-range 16S amplification and nucleotide sequencing. These 19 positive samples served as controls for optimisation of reaction conditions for clinical specimens, which included primer concentration ranging in intervals from 0.25µM to 1µM and annealing temperature ranging for the two group-specific primer sets (B and C). The four type-specific *gyr*B assays (primer sets D-F, Table 2) were performed using published and adjusted reaction conditions in an attempt to amplify and characterise an additional, potentially phylogenetically informative gene region.

PCR screening and genetic characterisation:

All 234 samples were screened using the optimised reaction conditions for the two group-specific *gro*EL (primer set C, Park et al. 2007) and *yea*C (primer set B, Ahmod et al. 2011) PCR assays. Amplification with both group-specific assays confirmed *B. cereus* complex presence. Samples with conflicting results were subjected to an additional round of screening with both primer sets. In this way, two independent amplifications of either one or both group-specific targets in combination with nucleotide sequencing was used to confirm *Bacillus* presence, and allowed for unequivocal assignment of a positive *B. cereus* status.

All positive samples were subsequently screened using four species-specific primer sets (D-G) that are reported to be specific for strains of *B. anthracis* (D), *B. cereus* (E), *B. thuringiensis* (F) and *B. mycoides* (G) (Table 2; Park et al. 2007). Nucleotide sequences generated using species-specific primer sets were included with the aim of improving *Bacillus* strain assignment to species level.

Finally, as most the 42 positive samples appeared to be co-infected, two primer assays (H_1 and H_2) that target the *gro*EL gene region, were newly designed to confirm the presence of the two *Bacillus* strains identified in the current study, and to obtain accurate estimates of the levels of co-infection. The strategy and steps employed in this study are summarised in Figure 1.

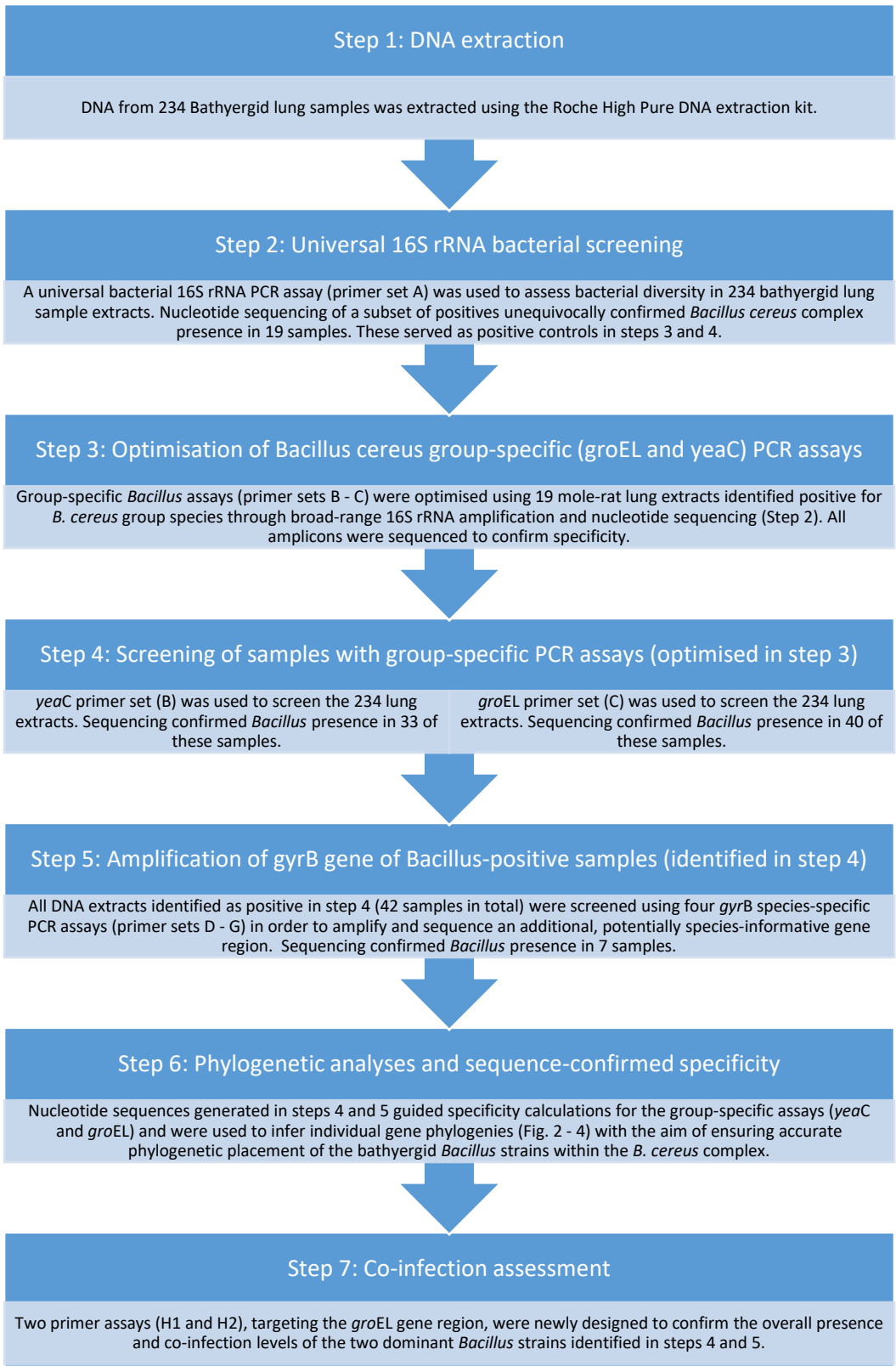

Fig 1: Flow diagram summarising the methodological approach used in this study. Primer codes refer to those detailed in Table 2.

PCR amplification and purification:

All PCRs were run on an ABI 2720 thermal cycler (Applied Biosystems, Foster City, California; Table 2) in a final reaction volume of 40µl, containing 1 x Dream*Taq* Buffer, 0.2 µM dNTPs (Fermentas), 1.5 U of Dream *Taq* (Thermo Fisher Scientific, USA), primers at reaction-specific concentrations (summarised in Table 2) and 3 µl of template DNA.

Touchdown PCRs with an initial denaturation at 96°C for 12s, annealing at determined optimal annealing temperatures (Table 2) for 30s, elongation at 70°C (with time dependent on the size of amplicon targeted) and a final elongation at 70°C for 1min, were performed for all primer sets. PCR product sizes were estimated by 1.5% agarose gel electrophoresis against a DNA molecular weight marker (Fermentas, Waltham, Massachusetts, USA). Products of the correct size were purified either directly from the tube or by gel-slice purification using the Roche High Pure PCR product purification kit (Roche Diagnostics GmbH, Mannheim, Germany).

Nucleotide sequencing and phylogenetic analysis:

Purified DNA was cycle sequenced using BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, California) with each of the PCR primers, in separate reactions, according to the manufacturer specifications and at the final annealing temperature specified for each assay in Table 2. Sequence chromatograms viewed in the Chromas program in MEGA 6, were edited and sense and antisense strands were aligned to generate sequence contigs. The resulting sequences were used in nucleotide Blast (Altschul *et al.* 1990) searches against Genbank (www.ncbi.nlm.nih.gov/blast) to identify the closest sequence matches in this public database.

The 16S rRNA, *gro*EL and *yea*C sequences generated in this study were complemented with reference sequence data and aligned with ClustalW in Mega 6 (Tamura et al. 2013). *Paenibacillus mucilaginosus,* a sister taxon to the genus *Bacillus,* was included for outgroup purposes. Each of the three gene regions were initially individually analysed using uncorrected p-distances and the Neighbor-Joining algorithm (Saitou & Nei 1987) in Mega 6 (Figure 2). Individual datasets were subsequently combined to form a concatenated dataset, 2066 nt in length and comprising of 21 taxa (Figure 3). This 21 taxon dataset was used to identify the best-fit model of sequence evolution under the (Bayesian Information Criterion (BIC) in Mega 6 (Tamura et al. 2013). Minimum Evolution (ME) and Maximum Likelihood (ML) analyses were performed in Mega6 (Nei and Kumar 2000; Tamura et al. 2013) and PhyML version 3.1 (Guindon et al. 2010), respectively. Bayesian Inference (BI) performed in MrBayes (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003) was stopped after 1 000 000 generations. Trees were sampled every 10^{th} iteration and MCMC Trace Analysis Tool version

1.6.0 (Rambaut et al. 2014) was used to confirm a 25% burn in. Two independent MCMC runs, each with one cold and three heated chains, were performed.

Differences in *Bacillus* prevalence between host species, sexes and level of urbanisation of the sampling locality (Table 1) were analysed using Pearson Chi-square tests in IBM SPSS (Version 23.0. Armonk, NY: IBM Corp.). All statistical analyses were performed on a sample size of 234.

Specificity and sensitivity calculations:

Samples were considered positive, if an amplicon of the expected size was obtained. To calculate the sensitivity of an assay the number of true positives (TP) was divided by the sum of the true positives (TP) and the false negatives (FN): TP/(TP+FN). To calculate the specificity of an assay the number of true negatives (TN) was divided by the sum of the true negatives (TN) and the false positives (FP): TN/(TN+FP) (Altman & Bland 1994).

Results

Broad-range PCR confirmation of *Bacillus* genome presence:

Screening of lung samples with broad range 16S rRNA primers (primer set A, Table 2) produced an amplicon of the expected size in 194 (83 %) samples. A subset of these (n=73) selected for purification and nucleotide sequencing produced unambiguous sequences. Nucleotide blast searches revealed high levels of bacterial diversity, with *Bacillus* being the most prevalent genus, occurring in 19 of the 73 (26%) samples sequenced.

Evaluation of *Bacillus cereus* group- and species-specific primer sets:

Screening of the 234 samples with group-specific assays (B and C) revealed that *B. cereus* group strains were present in 39 of the *B. suillus* samples, two of the *C. h. hottentotus* samples and one of the *F. damarensis* samples, and absent from all 53 *G. capensis* samples. The number of sequence-confirmed *Bacillus* positives obtained with this two-gene screening approach was 42, corresponding to an overall bathyergid *Bacillus* prevalence of 17.95%. This value of 42 represented the number of true positives, guiding individual PCR assay sensitivity and specificity calculations.

Forty of the 234 samples were found to be positive with the *yea*C gene assay (primer set B). Of these positive samples, 33 had an expected amplicon size of ~350 bp, corresponding to an overall PCR prevalence of 14.10%. Three samples had amplicon sizes of ~200 bp, corresponding to a host genome target while the remaining four samples had an amplicon size of ~1000 bp. The calculated sensitivity and specificity values for this primer set were 78.57 % and 100 % respectively. With primer set C, targeting the *gro*EL gene region, 40 samples were PCR-positive.

Sequencing revealed that all 40 were positive for *B. cereus* complex presence, indicating an overall PCR prevalence of 17.09%. Based on these results, primer set C had a sensitivity value of 95.24 %, and a specificity value of 100 %.

All 42 samples confirmed positive for *B. cereus* group by first round genus-specific PCR and nucleotide sequencing, were subjected to species-specific amplification with four primer assays (D – G), targeting the *gyr*B gene region, designed by Park et al. (2007; Table 2). Primer sets D and E, reported to be specific to *B. anthracis* and *B. cereus*, respectively, were negative across all samples, whereas primer set F resulted in multiple bands of the incorrect size, producing either mixed or host genome sequences (Table 2). Seven (16%) of the 42 samples screened using the *B. mycoides* specific primer set (G) were positive and nucleotide sequencing of the expected ~ 600 bp amplicon confirmed that all were *B. cereus* group strains.

Two novel strains belonging to the *B. cereus* complex were identified in the current study and strain-specific PCR assays (H_1 and H_2) were developed to accurately distinguish between these two strains (Table 2). Screening with these strain-specific *gro*EL primer assays H_1 and H_2 revealed high levels of co-infections (71.42% of all positive samples; 74.36% of positive *B. suillus* samples; 100% of positive *C. h. hottentotus* samples and 100% of all positive *F. damarensis* samples), and nucleotide sequencing of the products confirmed that the assays were capable of selectively amplifying each of the targeted strains, making it possible to identify with confidence the number of co-infected samples.

Nucleotide sequence analyses:

Nucleotide sequencing and BlastN results revealed that the *Bacillus* strains present in bathyergids from the western Cape Province had high sequence identity to valid *B. cereus* complex species (Figure 2), for all gene regions evaluated. Sequencing of 16S rRNA, *yea*C and *gro*EL amplicons confirmed the presence of two distinct strains and the presence of mixed infections in bathyergids. *Bacillus* sp. strain 1 in bathyergids was identical to *B. mycoides* (CP009746) and *B. weihenstephanensis* (CP009746), across the *gro*EL and 16S rRNA gene regions, differing from these reference strains by ≥3% across *yea*C (Figure 1). In contrast, *Bacillus* sp. strain 2 was distinct from all *Bacillus* sequence entries presently in the Genbank database, with minimum pairwise p-distances ranging from 1% for 16S rRNA to 4% for *yea*C (Figure 1), on nucleotide level. The phylogenies inferred using the concatenated dataset of the 16S rRNA, *gro*EL and *yea*C gene regions (Figure 2), revealed that the *Bacillus* strains identified in bathyergids clustered within a well-supported *B. cereus* complex clade. In common with previous reports (Helgason et al. 2000; Guinebretière et al. 2008; Tourasse et al. 2011), the phylogenies confirmed that valid members of recognised species did not form monophyletic lineages, but were intermixed with one another (Figure 1).

A significant difference in *Bacillus* occurrence was found between different host species (χ^2 = 69.643; df = 3; $p < 0.05$; Figure 2; Table 4) and between different sampling localities (χ^2 = 70.245; df = 3; $p < 0.05$), while no significant difference was found between different host sexes (χ^2 = 0.741; df = 1; p = 0.389).

Discussion

Continuous human encroachment into natural environments has led to an increased risk of people coming in contact with animals and environments harbouring emerging and re-emerging bacterial pathogens (Comer 2001). Increased human susceptibility, due to diseases such as HIV/AIDS and cystic fibrosis, has amplified the need to understand disease dynamics of opportunistic pathogens (Berg et al. 2005). Therefore, rapid and accurate methods are needed to identify etiological agents present in previously unidentified or overlooked environments and hosts (Shabbir et al. 2015). Due to their subterranean lifestyle, we assessed the role of bathyergid species as reservoirs of infection for opportunistic soil-dwelling bacterial pathogens.

The observation that all *Bacillus* strains, detected in bathyergid lungs, formed part of the *B. cereus* complex (Figure 2), suggests that bathyergids may act as reservoirs of soil-borne pathogens. However, the significantly higher prevalence of *B. cereus* species complex strains in *B. suillus* sampled from a peri-urban setting in close proximity to an informal human settlement suggests a possible role as indicator species for anthropogenic soiling.

Bacillus and bathyergid species both have a close association with specific soil conditions. Therefore, although it could be argued that sampling hosts with different burrowing behaviour in different soil and climatic conditions may have influenced *Bacillus* prevalence in the host species assessed,, Brillard et al. (2015) found that abundance of *B. cereus* complex spores is not affected by soil depths. This indicates that the disparate burrowing behaviour displayed by bathyergid species (Table 1), only plays a limited role in *B. cereus* complex host species exposure.

Differences in climatic conditions and soil structure, between the sampling sites, could provide an alternative explanation for the differences observed. However, a study performed on soil samples obtained from Thailand, Munich (Germany) and the Karwendel mountains (Austria), revealed that species of the *Bacillus cereus* complex have higher diversity in temperate climates, high in temperature fluctuations, compared to those climates with extreme temperatures, showing low fluctuations in mean temperature (Von Stetten et al. 1999). The *B. cereus* complex thus consists of both mesophilic species (with a growth range from above 7˚C to 46˚C) and psychrotolerant species (with a growth range from below 7˚C to 38˚C), with high fluctuation of average temperatures favouring selection for more than one thermal type (Von Stetten et al. 1999; Guinebretière et al. 2010). Thus, although the species occur across a broad range of

rainfall areas (Table 1), the temperate environment inhabited by *B. suillus* would likely only have influenced the diversity of *B. cereus* complex strains that individuals were exposed to.

Although there are preferences with respect to soils inhabited, *B. suillus* and *F. damarensis* prefer sandy soil that are less compact compared to the loam clay soils preferred by the both *C. h. hottentotus* and *G. capensis* (De Graaff 1981; Bennett 2011; Robb et al. 2012), little is known about the soil preferences of *B. cereus* complex strains. Strains seemingly prefer alkaline soils, high in calcium and moisture content with high levels of organic matter (Dragon and Rennie 1995; Smith et al. 1999; Jensen et al. 2003; Vilain et al. 2006; Blackburn et al. 2014), but as soil alkalinity and soil nutrient content are heavily affected by a range of factors, including anthropogenically introduced contaminants, further investigation is required into the effect that specific soil conditions will have on bathyergid infection levels (Chesworth 1973; Sparks 1995).

The phylogenetic results confirm previously reported difficulties with *Bacillus cereus* complex species identification (Helgason et al. 2000; Guinebretière et al. 2008; Tourasse et al. 2011), in that members of this complex, that are considered to be valid species, did not form monophyletic lineages in any of the gene trees inferred (Figure 1), and thus, may not constitute monophyletic species (Zwick et al. 2012). The observed paraphyly is likely due to the high degree of similarity displayed between recognised species, highlighting the difficulties in achieving phylogenetic placement of novel strains within the *B. cereus* complex, with confidence. We attempted to address this by characterising three gene regions that should be more informative than the 16S rRNA gene, namely *gro*EL, *yea*C and *gyr*B. In common with previous reports, the individual gene phylogenies did not resolve the taxonomic difficulties associated with this group (Table 1S), however, the concatenated 16S rRNA-groEL-yeaC dataset 2066 nucleotides in length allowed for well-supported placement of the two bathyergid *Bacillus* strains within the complex (Figure 3).

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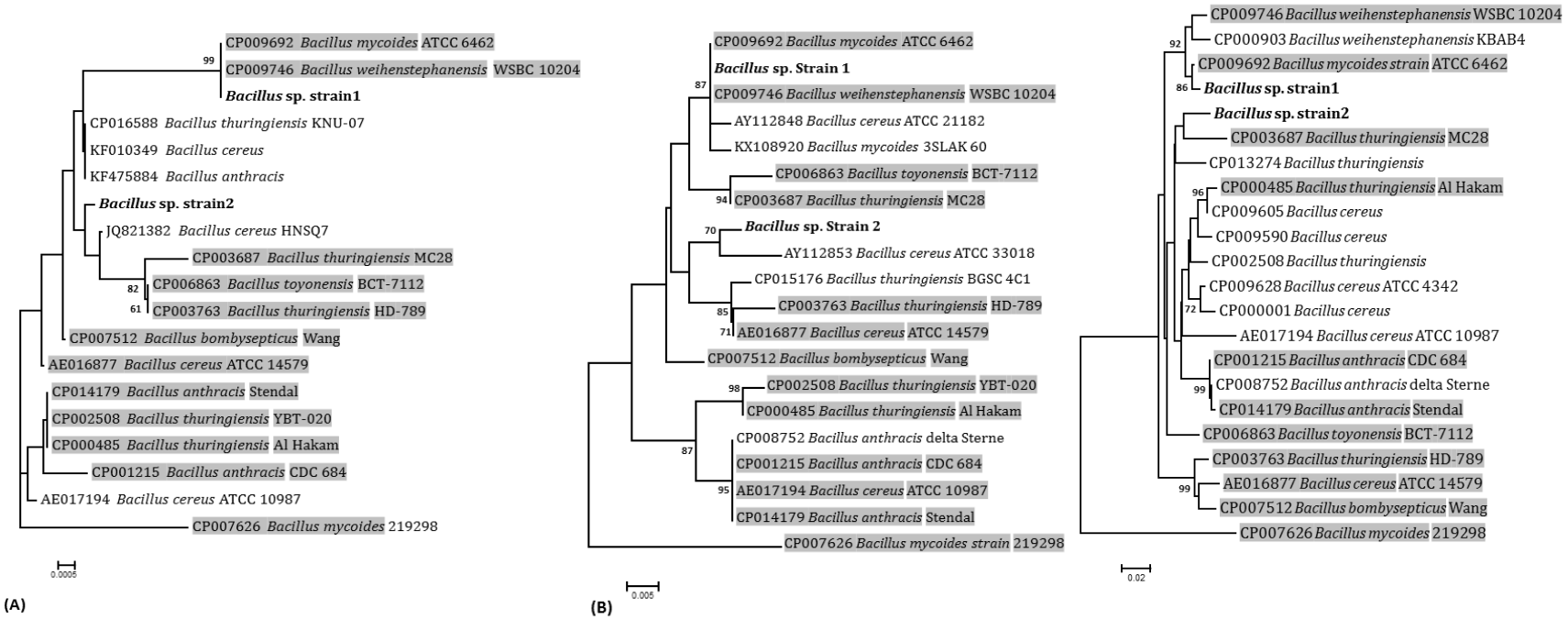

Fig 2 Individual gene trees indicating the *Bacillus cereus* complex subtree, inferred using the neighbour-joining algorithm and [A] an aligned 1437 nt 26 taxon 16S rRNA gene dataset, [B] an aligned 306 nt 26 taxon *gro*EL gene dataset and [C] an aligned 312nt 26 taxon *yea*C gene dataset. Bathyergid *Bacillus* (strains 1 and 2) are indicated in red, reference strains represented in the concatenated gene tree (Fig. 3) are indicated in bold, with the remaining sequences corresponding to the closest matches to strain 1 and strain 2 identified through BlastN searches against the GenBank database. Bootstrap support values > 70% (obtained from 10,000 bootstrap replications of each dataset) are indicated next to relevant nodes of each gene tree.

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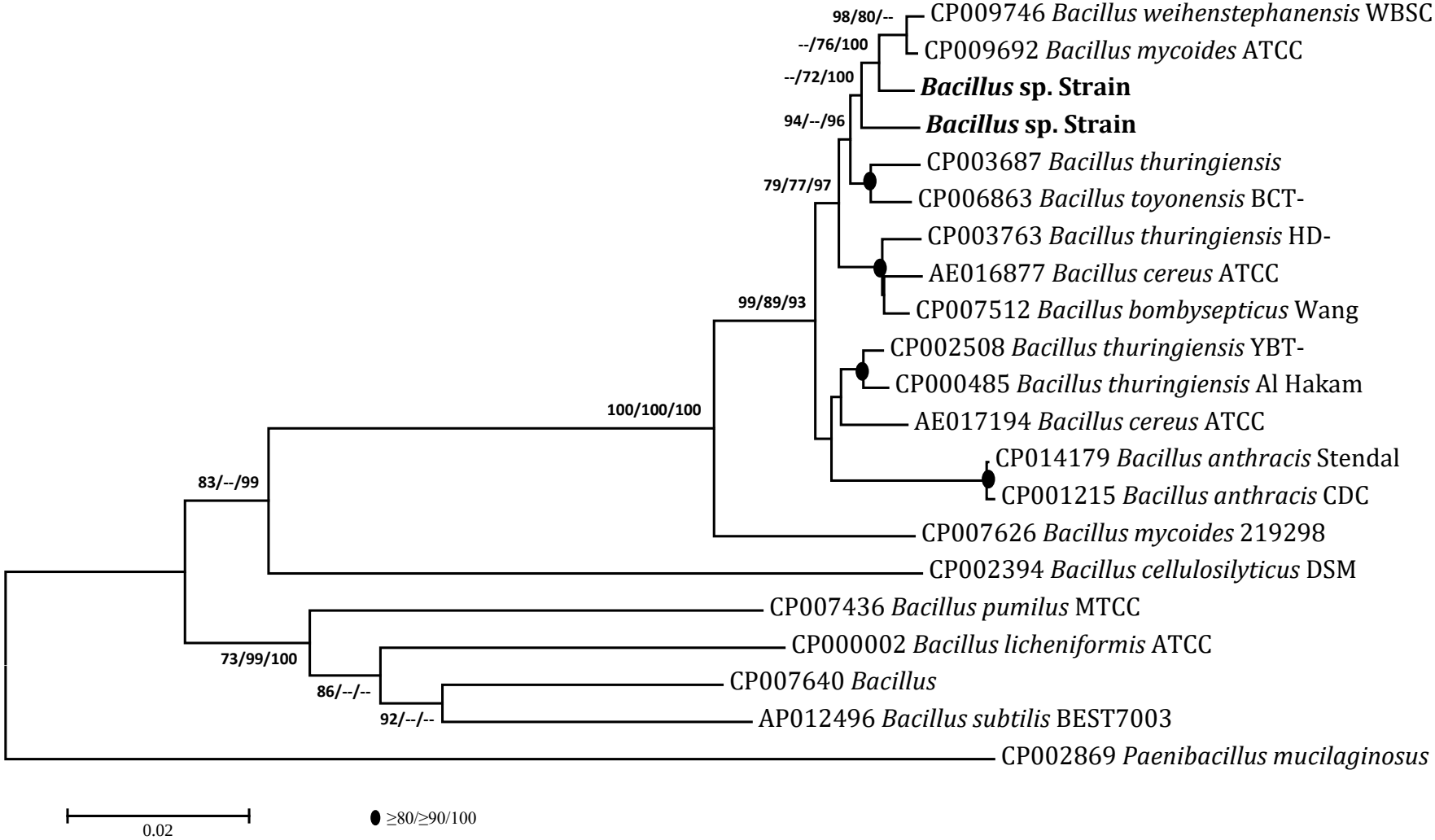

Fig 3 Minimum evolution tree depicting *Bacillus cereus* complex species detected in four bathyergid species (*Bathyergus suillus, Georychus capensis, Cryptomys hottentotus hottentotus* and *Fukomys damarensis*) sampled in the Western Cape Province, South Africa (indicated in bold) with relevant reference sequences, inferred using the combined, aligned 2066 nucleotide dataset comprising the concatenated 16S rRNA, *gro*EL and *yea*C gene regions. Bootstrap support values ≥ 70% from the Minimum evolution analysis (ME; 10 000 bootstrap replicates), and Maximum likelihood analysis (ML; 1000 bootstrap replicates); and posterior support values ≥ 90% from the Bayesian inference (BI; 1 000 000 generations sampled every 10 generations with a 25% burn-in) are indicated as ML/ME/BI next to each node.

In addition to enhanced phylogenetic placement, the assays were selected for their potential utility for detecting *Bacillus cereus* presence in clinical specimens. This was achieved through inclusion of DNA extracted from 19 naturally infected samples, when optimising the published group-specific assays. In particular, the current study confirmed the value of the Park et al. (2007) *gro*EL PCR assay for detecting *B. cereus* complex species, as this assay showed high levels of sensitivity and specificity, relative to the combined two-gene approach. It also emphasised the need to identify alternative primer sets that are better suited to assessing bacterial diversity in small mammals such as bathyergids, as the host genome amplification, which occurred with primer sets B and F, targeting *yea*C and *gyr*B respectively (Table 2), can lead to false positive results. The value of novel test development was demonstrated by the newly-developed PCR assays which selectively amplified each of the *Bacillus* lineages identified in this study, allowing for the determination of levels of co-infection, and confirming the high levels of strain diversity in the temperate environment occupied by *B. suillus*. These optimised protocols hold potential for future studies making use of clinical specimens and environmental samples.

Belowground biodiversity is severely understudied, with the human health implications of interactions between different classes of soil biota, such as microbe-bathyergid interactions, generally being overlooked (Berg et al. 2005; Parker 2010). With continual anthropogenic pollution of once natural environments worldwide, the risk of introducing opportunistic pathogens into transformed ecosystems, particularly in developing southern Africa countries, has increased (Berg et al. 2005; Paulse et al. 2012; Duran Alvarez and Jimenez Cisneros 2014). This, together with climate change, is expected to increase emergent disease incidence, emphasising the need to establish suitable methods for identifying potential reservoirs and routes of transmission (McMichael et al. 2006; Gale et al. 2009; Carlin et al. 2010). Consequently, the 45.35% prevalence of *Bacillus cereus* complex strains in *B. suillus* is significant as this bathyergid species occurs near informal human settlements in which the proportion of immune-compromised individuals is high.

Conclusion

The findings of this study highlight the need for additional research into the roles of anthropogenic pollution, climatic factors and soil structure, on the maintenance and community structure of potentially pathogenic soil microbiota within the environment, underscoring the value of soil-dwelling mammals as early detection systems for opporunistic pathogens and as indicators of environmental soiling.

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Supplementary Files

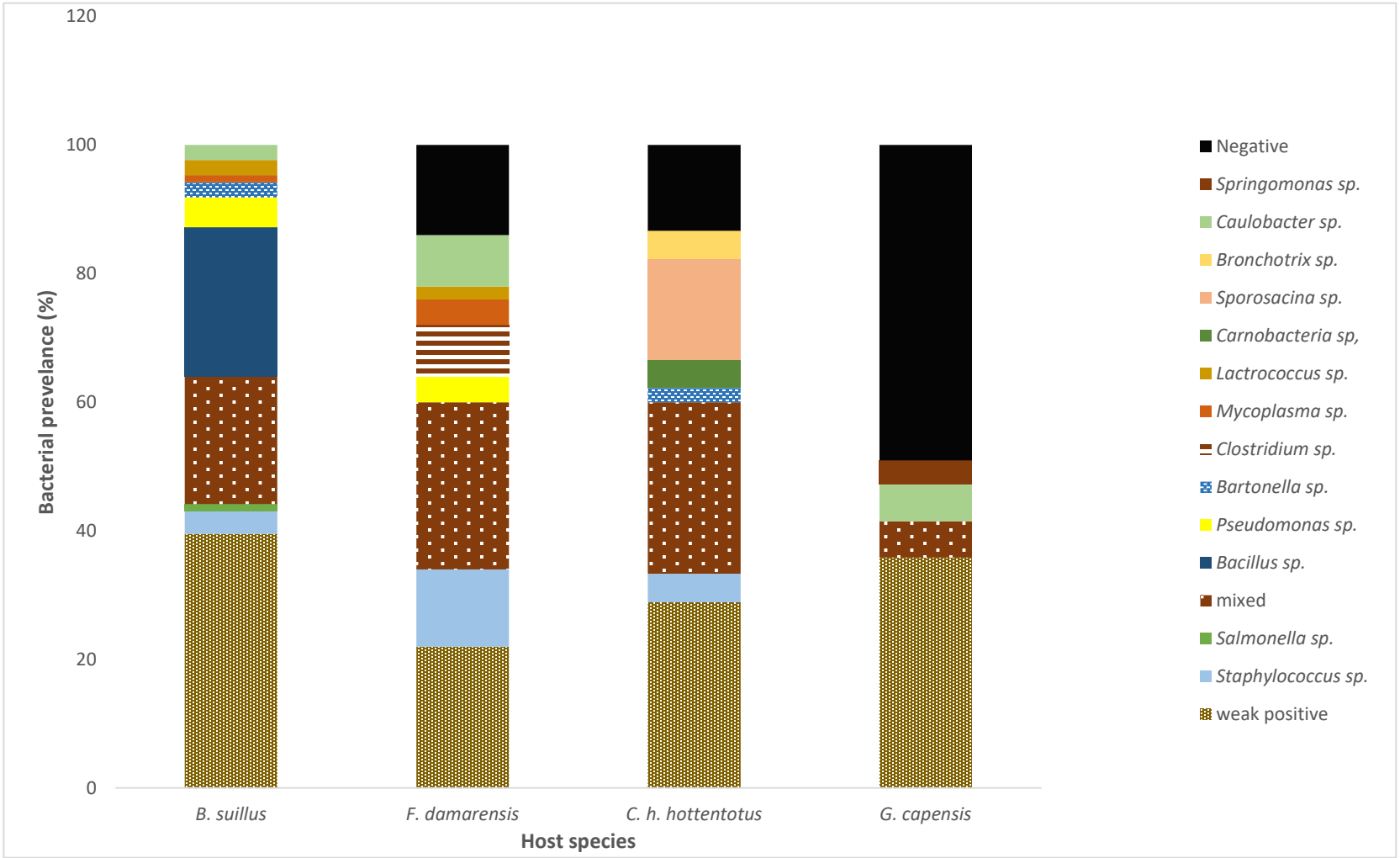

Figure 1S: Composition of the bacterial genera identified by broad-range 16S rRNA PCR and sequencing of lung tissue DNA extracts of four bathyergid species sampled from the Western Cape Province, South Africa. Bathyergid species are indicated below bars.

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TABLE S1: Summary of the 12 methods considered for inclusion in this study

Gene region	Reference	Rationale for gene target and assay inclusion / exclusion
***gro*EL**	Park et al. 2007 Chang et al. 2003	Park et al. (2007) designed a PCR assay targeting a ~400bp amplicon of this gene region. In contrast, Chang et al. (2003) designed a PCR assay targeting a ~600bp amplicon of the *gro*EL gene region. We used the general 'rule of thumb' that diagnostics assays that target a region < 500 bp in size tend to perform better when working with clinical specimens. We therefore selected the PCR assay designed by Park et al. (2007) for our study.
***gyr*B**	Park et al. 2007 Yamamoto & Harayama 1995	Using the *gyrB* gene region as genetic marker to distinguish between different *Bacillus* species produces similar phylogenetic branching patterns as the more laborious, "gold standard" of bacterial classification, DNA:DNA hybridization (La Duc et al. 2004). Unlike the primers reported by Yamamoto and Harayama (1995), the primers of Park et al. (2007) contained fewer degenerate sites and were designed to amplify targets < 600bp in size. The PCR assays of Park et al (2007) were therefore selected.
***yea*C**	Ahmod et al. 2011	These authors designed a PCR assay using a large conserved indel, and reported that the assay was useful for distinguishing *B. anthracis* from other members of the *B. cereus* group.
pXO1/ pXO2	Kolstø et al. 2009	pXO1 and pXO2 are two large plasmids, containing virulence genes (Kolstø et al. 2009), thus allowing for assessment of horizontal transfer of virulence factors. However, as the plasmid genes are easily lost and are not sufficiently informative for distinguishing between different species within the *Bacillus cereus* group (Radnedge et al. 2003), this gene target was not considered.
16S rRNA **23S rRNA** **16S–23S rDNA**	Ash et al., 1991 Ash & Collins 1992 Cherif et al., 2003	Studies using the 16S rRNA, 23S rRNA gene regions as well as the 16S–23S rDNA intergenic spacer region have failed to reveal consistent differences between species belonging to this group (Kim et al. 2008).
rpoB **saspB** **BA813**	Ko et al. 2003 Callahan et al. 2008 Ramisse et al. 1999	Methods using these gene targets have failed to accurately distinguish *B. anthracis* from other species of the *B. cereus* species complex (Rao et al. 2010; Ahmod et al. 2011).
***csa*B**	Zheng et al. 2013	This gene region can differ between different strains of the same species depending on the environment in which these strains have evolved (Zheng et al. 2013) and has not been used in many studies.

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