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Bacterial Diversity in Some South African Thermal Springs: A Metagenomic Analysis

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ABSTRACT

Hot springs provide a rich source of thermophilic microorganisms, which can be tapped for different biotechnological purposes, such as novel thermophillic microorganisms and enzymes for different industries and applications. Thermal communities are often complex to describe through traditional cultural methods. Total environmental genomic studies (metagenomics) allow rapid characterisation of the microbial diversity. The bacterial diversity in some South African thermal springs was determined using 454 sequencing of the v1-3 and v4-7 variable regions of the 16S rRNA gene sequence. The bacteria diversity was dominated by the genera *Stenotrophomonas, Hydrogenophaga, Flectobacillus, Rheinheimera, Pseudomonas, Zavarzinella, Aquaspirillum* and *Limnobacter*, was detected for the hotsprings. The diversity distinctions could be related to the differences in the thermal spring physicochemical properties. This study gave some picture of the bacterial diversity in some South African hotsprings.

1. INTRODUCTION

Microbial life in extreme environments, including thermal springs, is attracting continual and broad scientific interest. Extreme environments harbor microorganisms that represent the oldest inhabitants on earth, and whose high adaptability has continued to challenge the understanding of biochemistry, biology and evolution. The most recent use of geothermal waters is for their biological wealth, among which are the thermophilic microorganisms. Thermophiles are a group of extremophilic organisms that live in hot environments and are a valuable genetic resource. They contain enzymes that are stable at high temperatures, which make them useful for the pharmaceutical, food processing and chemical industries; they are also for biofuel, biomass and thermo-stable protein production and in metabolic engineering.

Exploitable microbial diversity in the environment is inexhaustive and microorganisms represent the largest reservoir of understudied biodiversity (Sogin et al., 2006). Earlier studies on microbial identification required isolation of a pure culture, but microbiologists later developed molecular based methods for identification of microbial populations without cultivation. New non-culture based approaches have recently been developed that can be used extensively for comprehensive analysis of different communities in a microbial consortia (Petrosino et al., 2009). Metagenomics is the culture-independent genomic analysis of microbial communities. The direct sequence analysis of metagenomic DNA is presently considered the most accurate method for assessing the structure of an environmental microbial community; it does not require selection (e.g. cultivation/enrichment) and greatly reduces technical biases (e.g. PCR amplification).

South Africa is endowed with a number of thermal hot springs (around 87), (Kent, 1949) which often differ in their physical and elemental chemical characteristics (Olivier et al., 2011). Major gaps relating to the description and variation in the diversity of microbes in these hot springs exist. Metagenomic studies of bacterial in hot springs environments in South Africa have not yet been extensively conducted. Tekere et al. (2011 and 2012) reported some of the first analyses for bacterial diversity and phylogenetic in South African thermal springs. This paper presents a metagenomic analysis of bacterial communities in some of the six South African hotsprings

2. MATERIALS AND METHODS

2.1 Sampling

Composite water samples were collected from Die Eiland, Silioam, Souting, Tshipise, Mphephu and Sagole hot water springs for physical, chemical and microbial community studies. The springs are located in the Limpopo Province of South Africa and are of different surface geology. Figure 1 shows the location of some of the South African hotsprings.

Samples were collected at 20 to 50 cm depth around the springs, except for Silioam, where the water was collected from the receiving water pool and the outlet pump (placed on the spring eye). The following water quality parameters were measured *in situ* using the relevant field meters (Mettler Toledo meters, UK): temperature, pH, electrical conductivity (EC), total dissolved solid (TDS) and dissolved oxygen (DO). The water was collected into sterile 2 L bottles and placed into a cooler box for transportation to the laboratory for bacterial diversity studies. Analyses for other physical and chemical parameters were conducted by the Institute for Soil, Climate and Water (Agricultural Research Council), Pretoria using standard methods.

2.2. DNA extraction and 16 S rRNA gene sequence amplification.

The samples were concentrated using both filtration [on cellulose nitrate filters, pore size 1.2 um (Sartorius)] for the clear water, and centrifugation (at 7500 rpm for 10 min) for water with the bulk of the biofilm, and subsequently resuspended in 20 ml of phosphate buffer saline (PBS) (10 mM). Two (2) ml of the samples (both from filtered water and centrifuged biofilm samples) were centrifuged at 7500 rpm for 10 min to collect the pellet. The pellet was resuspended in 1 ml PBS (10 mM) as an additional wash

step. DNA was extracted with the genomic DNA Tissue Mini-Prep Kit (Zymo Research), as per protocol; with an additional DNA wash-step. Cell lysis was followed by purification using modern fast-Spin column purification technology. Five (5) µl of the extracted DNA was run on a 1% agarose gel at 90 V for 30 min to verify the efficiency of the extractions.

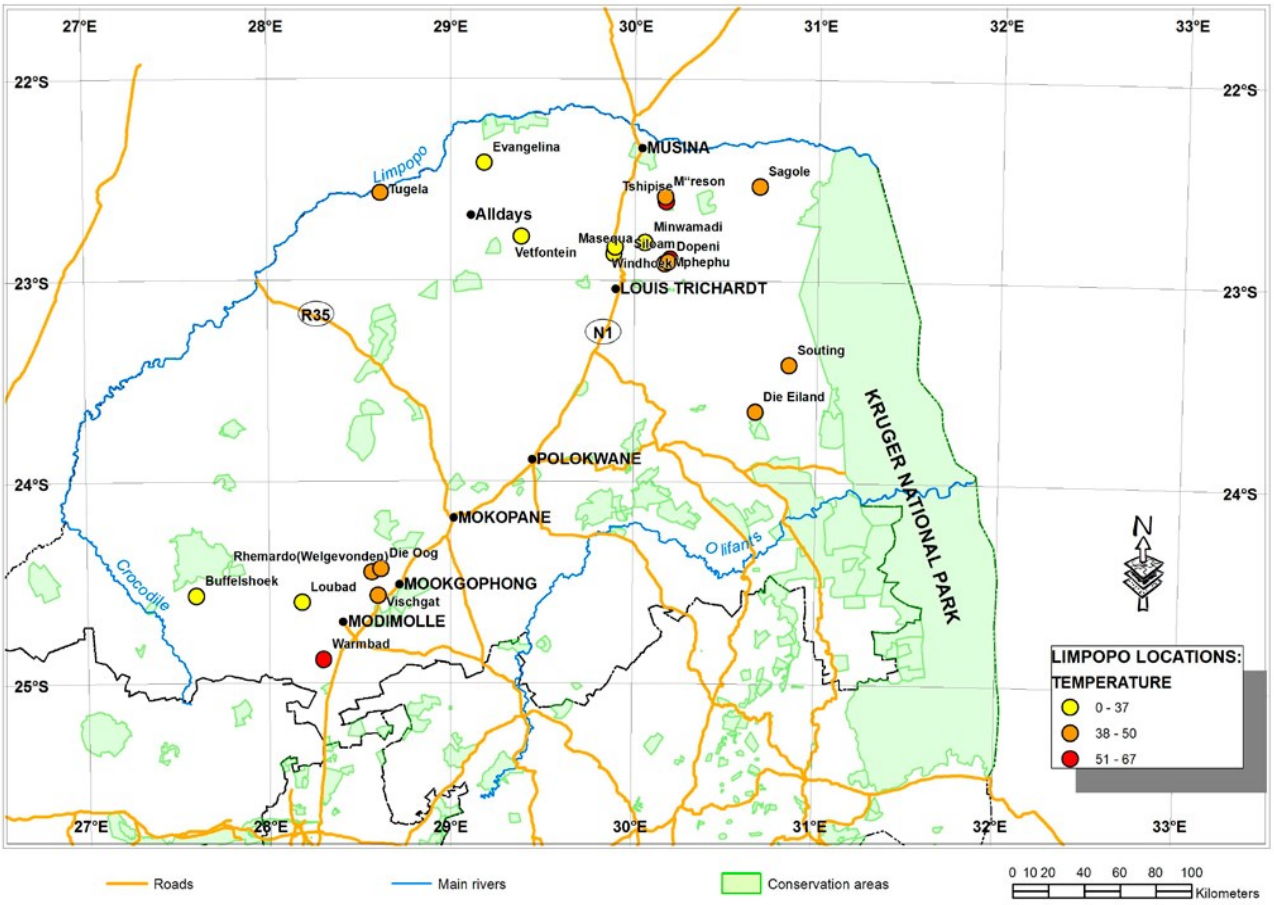

Figure 1: Location of thermal springs in Limpopo Province, South Africa Adapted from: Kent (1949) and Boekstein (1998)

The PCR reaction was performed on the extracted DNA samples using universal degenerate primers 27F.1 and 1492R as in De Santis et al., 2007 and Tekere et al., 2012. Each PCR reaction contained 5 µl of 10 x Taq Buffer, 2 mM $MgCl_2$, 1.5 U Super-Therm DNA polymerase (Southern Cross), 0.25 mM deoxynucleosides triphosphates (dNTP's), 0.1 µM of each primer, 1 µl of extracted DNA and nuclease free water (NFW) up to the final reaction volume of 50 µl. The PCR cycle started with an initial denaturation step at 94°C for 10 min. This was followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 1 min and extension at 72°C for 1 min, and a final extension at 72°C for 5 min that was then followed by cooling to 4°C. Five (5) µl of the samples were run on a 1% agarose gel at 90 V for 30 min in order to verify amplification. The entire PCR reaction was loaded onto a 1% agarose gel and the correct band size (approximately 1500 bp) was excised. The DNA was recovered from the gel slices by using the GeneJET™ gel extraction kit (Fermentas).

The DNA was subsequently reamplified with two sets of primers in order to amplify two variable regions of the 16S rRNA gene (V1-3 and V4-7). These primers contained the appropriate adaptor and barcode sequences that were necessary for running the samples on the GS-FLX-Titanium (Roche) as described in Tekere et al. (2012). Each PCR reaction was done as previously described, except that the annealing temperature was either 50 or 56°C dependent on the variable region amplified. The entire PCR product was loaded onto a 1% agarose gel and the correct band size (500 to 600 bp) was excised from the gel and subsequently purified as before. The DNA concentrations were quantified by using a Nanodrop spectrophotometer. The samples were pooled at equal concentrations of the filtration and biofilm samples, and V1-3 samples were equal to V4-7 samples. The pooled samples were sequenced on the GS-FLX-Titanium series (Roche) by Inqaba Biotechnology, South Africa.

The resulting data were classified using the Ribosomal Database Project (RDP) Naive Bayesian rRNA Classifier Version 2.2, March 2010, RDP training set 6, based on nomenclatural taxonomy and Bergey's manual with an 80% confidence threshold on the RDP-database. Only sequences that were 50 bp or more were included in the analyses. Data was used to show the phyla, classes, orders, families and genera for both the V1-V3 and V4-V7 regions of all the samples. The information was also used to draw some comparisons between the various samples and variable regions. Sequences were aligned and clustered on the RDP database, where rarefaction curves and Shannon Chao1 indexes were constructed by uploading the respective aligned and clustered files on the RDP-Pyro-pipeline. Both the rarefaction curves and Shannon-Chao1 indexes give an indication of abundance.

3. RESULTS

The determined physical and chemical characteristics of the hot spring water at the thermal springs are shown in Tables 1 and trace elements as reported in Olivier et al., 2011.

Table 1: Some physical and chemical characteristics of the studied hot springs.

Parameter	Hotspring					
	Mphephu	**Tshipise**	**Sagole**	**Siloam**	**Soutini**	**Eiland**
Temperature	**43**°C	**58**°C	**45**°C	**63**°C	**43.9** °C	**41** °C
DO	65.3%	34.7%	9.9%	40.0%	-	-
pH	8.19	8.94	9.24	9.50	7.20	7.63
TDS (ppm)	199.36	460.56	203.76	197.32	-	1028.80
Conduct. (mS/m)	44.00	81.00	39.00	39.00	273.00	330.00
Sodium (mg/l)	44.37	156.31	65.15	66.24	486.00	621.88
Potassium (mg/l)	1.14	4.25	1.10	2.82	15.00	21.79
Calcium (mg/l)	13.73	5.58	1.31	1.38	86.00	53.61
Magnesium (mg/l)	11.25	0.17	0.07	13.33	5.00	9.37
Fluoride (mg/l)	3.16	5.63	1.01	6.11	2.00	2.24
Nitrate (mg/l)	2.12	0.61	0.00	0.00	-	2.69
Chloride (mg/l)	39.38	168.97	47.85	44.35	680.00	982.62
Sulphate (mg/l)	9.26	53.17	18.20	10.44	170.00	143.63
Phosphate (mg/l)	0.00	0.00	0.00	0.00	-	24.86
Carbonate (mg/l)	0.00	6.00	18.00	14.40	-	00
Bicarbonate (mg/l)	151.28	126.88	102.48	107.36	-	-

(-) shows missing result.

The temperature of the studied hot springs ranged from hot to scalding in temperature (Olivier et al., 2008; Kent, 1949). The temperatures were measured at 58°C for Tshipise, 45 °C Sagole, Siloam 63 °C, Eiland 41 °C, 43.9°C for Souting and 43°C Mphephu. Tshipise is classified as a scalding hot spring (Kent, 1949) and has the highest temperature of these springs. High water temperature exerts selection pressure on microbial species leading to specific flora that survive and tolerate heat stress (Abou-Shanab, 2007). Temperature is one of the most important factors in governing species abundance and distribution (Abou-Shanab, 2007).

The hot springs are all alkaline with pH ranging from 7.2 to 9.5. The TDS was highest at Eiland (1028.8 ppm), followed by Tshipise (460.56 ppm) and lastly 199.36 ppm at Mphephu. High TDS is a reflection of high dissolved chemical ion concentration. Of the spring waters, Soutini has the highest concentrations of sodium, chloride, calcium and sulphate ions; in fact, people from the surrounding area pan for salt around this hot spring.

An analysis of the number of reads obtained for the various samples with the two variable regions (V1-3 and V4-7), ranged from seven to 1046. The very low sequence reads for Die Eiland V1-3 mean further analyses with this sample would give an inaccurate representation of the bacterial community especially when compared to the V4-7 for the same hotspring. The number of usable sequence reads is shown in Table 2.

Table 2: Number of usable sequence reads

Hotspring Name	Die Eiland		Mphephu		Tshipise		Sagole		Silioam		Souting	
Variable region	V1-3	V4-7	V1-3	V4-7	V1-3	V4-7	V1-3	V4-7	V1-3	V4-7	V1-3	V4-7
Number of usable reads	7	720	1117	721	512	120	1046	794	880	568	446	132

The pyrosequencing results showed a vast amount of different bacteria present in the samples and the phyla that were detected are shown in Fig 2a and b. The dominant phyla at all the hotsprings were the Proteobacteria. Other than the Proteobacteria, Mphephu was dominated by the Bacteroidetes as well. Sagole contained a fair amount of cyanobacteria-like sequences which were mainly linked to chloroplast DNA as well as Fusobacteria and Bacteroidetes. Siloam also had Bacteroidetes, Planctomycetes and cyanobacteria-like sequences which were mainly linked to chloroplast DNA. Souting had Bacteroidetes and cyanobacteria-like sequences which were mainly linked to chloroplast DNA. Tshipise was also dominated by cyanobacteria-like sequences which

were mainly linked to chloroplast DNA. Considering that the chloroplast amplicon sequences were not removed, the dominance of the Cyanobacteria in the springs might be an overestimation.

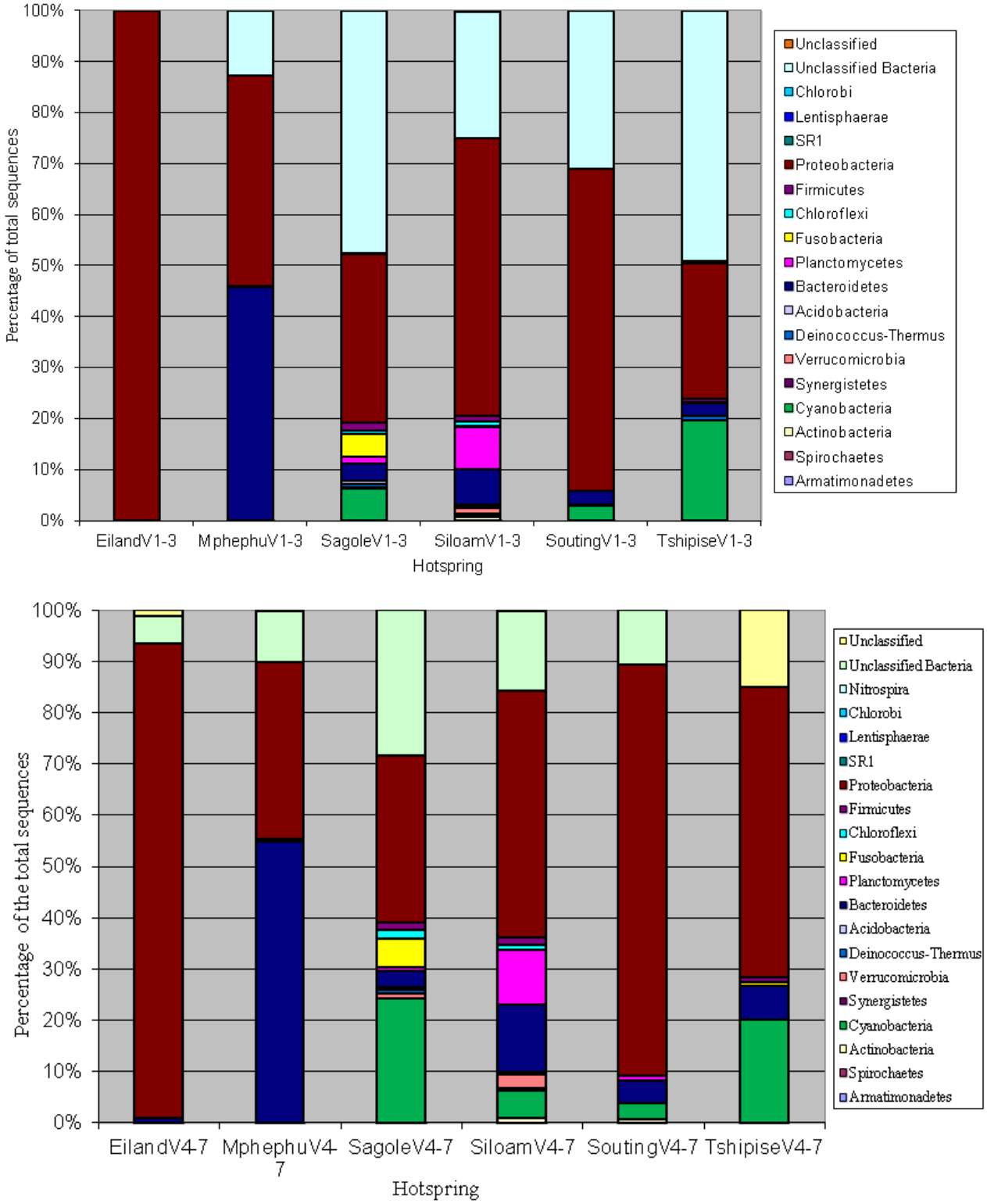

Figure 2 (a(top) and b(bottom)): Abundance and diversity of bacteria phyla detected at the hotsprings using V1-3 (2a) and V4-7 (2b) regions of the 16S rRNA gene sequence.

Figure 3a and b shows stacked column graphs of the classes that were detected with variable region V1-3 (a) and V4-7 (b) respectively. It can be clearly seen that the bacterial composition of the samples differed considerably. The most dominant classes in all the samples were that of the Proteobacteria. The Beta- and Gammaproteobacteria were dominant in all the samples. Other classes that were dominant at Eiland were the Alphaproteobacteria. At Mphephu the Sphingobacteria, Alphaproteobacteria and Flavobacteria were dominant. Sagole contained a fair amount of

cyanobacteria-like sequences which were mainly linked to chloroplast DNA as well as Fusobacteria. Siloam had many Planctomycetacia as well as Sphingobacteria. Both Souting and Tshipise contained many cyanobacteria-like sequences which were mainly linked to chloroplast DNA.

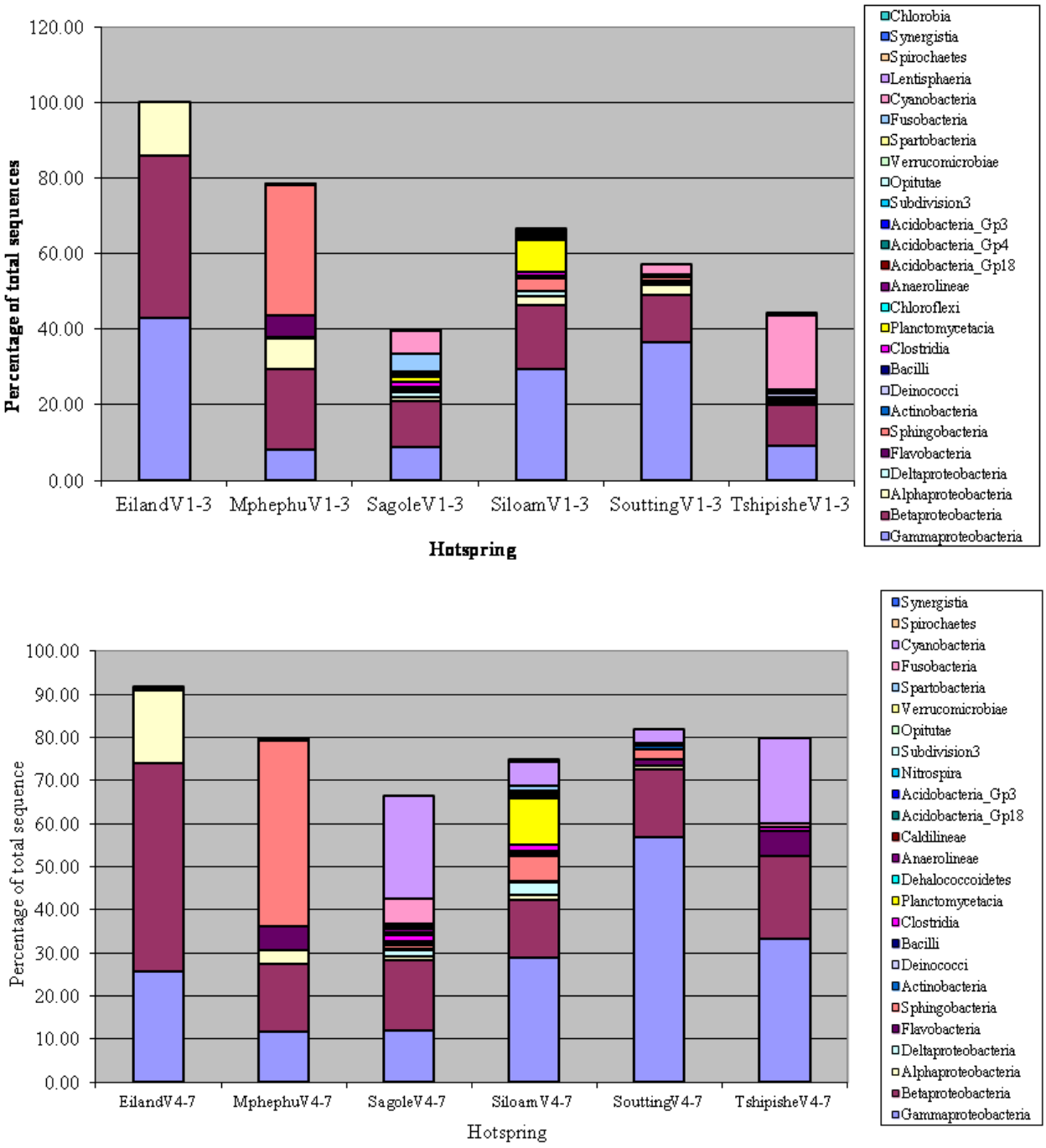

Figure 3 (a(top) and b(bottom)): Abundance and diversity of bacteria classes detected at the hotsprings using V1-3 (3a) and V4-7 (3b) regions of the16S rRNA gene sequence.

Bacterial diversity could be determined and classified differentially using the V1-3 and V4-7 variable regions. A higher average percentage of sequences were classified with the V4-7 than the V1-3. Table 3 shows the average sequences classified into the different ranks by the V1-3 and V4-7. In most cases, sequences obtained from the V4-7 region could be used to classify individuals to a lower taxonomic rank. However, even though region V4-7 was able to group more sequences to a lower taxonomic rank, both regions gave a similar sample distribution when investigating the results presented in the graphs (Figures 2 and 3). Table 4 shows the results of the top 3 most abundant sequences for each hotspring and as determined by either V1-3 or V4-7.

Rarefaction curves were constructed in order to evaluate if all of the diversity within the hot springs were detected. It is believed that a sequence difference of 3% is able to differentiate between species, where a sequence difference of 5% is indicative of different genera and a sequence difference of 10% is used to differentiate at the family/class level. In cases where the full diversity has been detected, the slope of the lines will flatten out. This implies that even if more sequences were obtained, the number of Operational Taxonomic Units (OTU's) detected in the samples would not increase. However, if the slope of the lines does not

flatten out it means that more sequences would be required to obtain the full diversity within the sample (Schloss & Handelsman, 2004). From the rarefaction curves generated, analyses suggest that sequencing could be expanded in order to capture the full diversity of the bacteria in these hot springs. Table 5 summarizes the amount of OTU's and estimated diversity detected at the various distance levels as determined with the Shannon-Chao1 index. The number of OTU's is an estimation of the total bacterial diversity at various distance levels where the Shannon-Chao1 index estimates the upper and lower limit of the total estimation at these distances with a 95% confidence level. These distances once again represent the taxonomic level of where it is able to differentiate - in other words a distance level of 0.03 is able to differentiate at species level, where a distance level of 0.05 is able to differentiate at genus level and a distance level of 0.10 is able to differentiate at family or class level. A distance of 0.00 is where every sequence is seen as a unique sequence.

Table 3: Percentage of the total sequences that was classified into the different taxonomic ranks

Sample	% classified to Phyla level		% classified to Class level		% classified to Order level		% classified to Family level		% classified to Genera level	
	V1-3	V4-7	V1-3	V4-7	V1-3	V4-7	V1-3	V4-7	V1-3	V4-7
Die Eiland	100	93.47	100	91.67	85.71	80.56	85.71	55.28	42.86	41.86
Mphephu	87.20	89.88	78.33	79.75	69.83	74.76	57.56	66.44	35.27	44.80
Sagole	52.58	71.66	39.67	66.50	22.47	33.25	20.94	28.97	13.00	20.65
Siloam	74.33	84.51	66.25	75.00	57.50	62.85	55.68	59.68	42.27	47.01
Souting	69.06	89.39	56.95	81.82	45.96	72.73	44.84	70.45	31.61	51.52
Tshipise	50.98	85.00	44.14	80.00	16.99	51.67	23.83	49.17	20.70	47.50
Mean % classified	72.36	85.65	64.22	79.12	49.74	62.64	48.09	55.00	30.95	42.24

Table 4: Three most abundant genera at the different springs as determined by the V1-3 and V4-7 variable regions for the different springs

Hotspring	Three most abundant Genera	
	V1-3	V4-7
Die Eiland	*Stenotrophomonas* *Hydrogenophaga* *Mycobacterium*	*Stenotrophomonas* *Hydrogenophaga* *None other*
Mphephu	*Flectobacillus* *Stenotrophomonas* Alkanindiges	*Flectobacillus* *Limnobacter* *Stenotrophomonas*
Sagole	*Rheinhemera* *Alishewanella* *Vogesella*	*Rheinhemera* *Vogesella* *Azonexus*
Siloam	*Stenotrophomonas* *Zavarzinella* *Aquasprillum*	*Stenotrophomonas* *Aquasprillum* *Zavarzinella*
Souting	*Rheinhemera* *Thiovigra* *Stenotrophomonas*	*Pseudomonas* *Stenotrophomonas* *Rheinhemera*
Tshipise	*Rheinhemera* *Tepidomonas* *Flavobacterium*	*Chloroplast GpIIb* *Rheinhemera* *Stenotrophomonas*

4. DISCUSSION

The number of reads obtained for the various samples and variable regions was quite varied. Different hypervariable regions have been found to have different efficacies with respect to species calls in different genera (Sundquist et al., 2007; Petrosino et al., 2009). Although a fair amount of diversity was detected, more sequences would have to be evaluated in order to determine the overall bacterial population of the samples.

The bacterial distribution when comparing the results of the two variable regions were very similar; however, variable region V4-7 was able to classify a greater percentage of the total number of sequences up to a lower taxonomic rank and sequences on all taxonomic ranks. On average, between the various taxonomic ranks, region V4-7 was able to classify approximately 10% more sequences than region V1-3 (65.95% versus 54.35% respectively). When only comparing Phyla and Classes, the bacterial distributions of the six hot springs appear very similar; however, it is at the lower taxonomic ranks where the differences were seen. There are some subtle differences in the Genera found between the two variable regions. It seems that *Rheinheimera* and *Stenotrophomonas species* are the most abundant species found in hot springs. *Stenotrophomonas* is a bacterium that is commonly found in the environment as well as an opportunistic pathogen in hospitals (Hauben et al., 1999). The *Rheinheimera* genus was first described by Brettar et al., (2002) (Merchant et al., 2007). Brettar et al., 2002 claimed that *Rheinheimera* was able to grow at temperatures between 4 - 30^{o}C (Brettar et al., 2002). However, in this study *Rheinheimera* was found to be very abundant in three of the hot springs studied (Sagole, Souting, and Tshipise). These hot springs have temperatures of more than 30^{o}C. *Rheinheimera* is, therefore, very diverse in terms of temperatures in which it is able to grow.

Table 5: The number of Operational Taxonomic Units (OTUs) and estimated diversity detected at different confidence limits for the different samples.

Hotspring	V- region	Distance level	OTUs	Chao	LCI95	UCI95
Die Eiland	V1-3	0.00	6	11	7	38
		0.03	6	11	7	38
		0.05	6	5	4	12
		0.10	4	5	4	12
Mphephu		0.00	775	4209	3341	5368
		0.03	224	317	278	383
		0.05	155	238	198	317
		0.10	87	158	114	270
Sagole		0.00	718	4283	3339	5566
		0.03	303	487	423	585
		0.05	221	297	266	550
		0.10	129	150	139	176
Siloam		0.00	643	3492	2722	4546
		0.03	251	392	340	476
		0.05	199	260	234	306
		0.10	134	153	142	176
Souting		0.00	291	1138	847	1584
		0.03	105	198	150	295
		0.05	81	129	102	194
		0.10	52	65	56	91
Tshipise		0.00	295	1826	1234	2793
		0.03	101	155	127	216
		0.05	72	104	84	152
		0.10	42	45	43	58
Die Eiland		0.00	513	2901	2188	3917
		0.03	111	188	148	268
		0.05	68	109	84	172
		0.10	35	61	42	128
Mphephu		0.00	557	3309	2508	4440
		0.03	158	280	260	375
		0.05	115	159	137	203
		0.10	66	80	71	106
Sagole		0.00	666	3889	3025	5069
		0.03	254	488	403	621
		0.05	201	363	298	472
		0.10	123	184	153	247
Siloam		0.00	491	3371	2464	4694
		0.03	191	336	278	433
		0.05	157	234	200	295
		0.10	116	163	139	214
Souting		0.00	118	748	429	1393
		0.03	60	119	85	196
		0.05	47	70	56	109
		0.10	30	41	33	74
Tshipise		0.00	109	445	279	771
		0.03	39	90	56	189
		0.05	29	49	35	95
		0.10	20	43	25	117

The extent of the bacterial diversity detected is not surprising, as the bacteria found in these hot springs occur commonly in the environment and have been described in many different environments studied elsewhere. Groups important to a particular environment are generally known to be enriched and also to be in correlation to the hydrogeochemistry of the area (Edwards et al., 2006; Meyer-Dombard et al., 2005; Whitaker et al., 2003). The possible contamination of the hot water spring by normal surface water, soil and spores cannot be excluded. Nevertheless, it can be concluded that the bacterial phylotypes detected in these hot springs can possibly proliferate in these thermophilic environments.

In conclusion this study showed that a considerable diversity of microbial communities can be revealed by metagenomics using 454 pyrosequencing of the V1-3 andV4-7 variable regions, and gives insight into microbial genetic diversity, community composition, distribution and abundance.

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